The ecological relevance of chemical diversity in plants: pyrrolizidine alkaloids in Jacobaea species

Wei, X.

Chapter 2

Seasonal variation in pyrrolizidine alkaloid concentration and composition in clones of *Jacobaea vulgaris*, *Jacobaea aquatica* and their F1and F2 hybrids

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Abstract

Plants produce a variety of secondary metabolites (SMs). The concentration and diversity of SMs are affected by biotic factors, such as herbivores and pathogens, and abiotic factors, such as temperature and light. Because these factors change during the seasons SMs are also expected to show seasonal variation. We used *Jacobaea vulgaris*, *Jacobaea aquatica*, 2 F1 and 4 F2 hybrids, and their pyrrolizidine alkaloids (PAs) as a system to study such seasonal variation. We used a series of clones of genotypes differing in their alkaloid content to explore how PA concentration and composition varied in their vegetative stage during 14 months in a semi-natural environment. Our results showed that after being planted in April shoot and root dry mass showed a steady increase until October-November, but in the winter period approximately 70% of the biomass was lost, before the plants recovered during the next spring. The total PA concentration in roots and shoots showed a gradual increase until the spring of the next year, when as a result of rapid plant growth the PA concentration dropped, most noticeably in the shoots. The variation of the total PA amount was strongly correlated with that of dry mass and together with the loss of dry mass in winter a large part of the alkaloids was lost. Senecionine- and otosenine-like PAs were mainly stored in the roots while the erucifoline- and jacobine-like PAs were mainly stored in shoots. For the 6 genotypes with higher concentrations of jacobine-like PAs, PA composition showed a decrease of this type of PAs during winter, followed by a strong increase in the next spring. In contrast, the PA composition of the roots over the whole period showed a gradual increase of jacobine-like PAs at the expense of senecionine-like PAs. The ratio of total free base and N-oxide PAs was relatively constant over the seasons until the second spring, when it sharply increased in the shoots of some of the jacobine-rich genotypes, most notably in the parent *J. vulgaris*. While the genotypes differed in PA composition their differences in the variation of the seasonal changes were significant but relatively small. As a result the variation among genotypes was maintained across seasons.

Key words

Secondary metabolites, LC-MS/MS, Genotypes, Senecionine-like PAs, Jacobine-like PAs, PA reallocation, Temperature, Day length

Introduction

Plants produce a staggering diversity of secondary metabolites (SMs) (Hartmann et al. 2005). For a single group of SMs, the concentration and composition may change between developmental stages, tissues and organs, seasons and years (Gols et al. 2007; Liu et al. 1998; Qasem and Hill 1995). SMs are important to plants for coping with the biotic and abiotic stressors of their environments (Iriti and Faoro 2009; Walters 2011). SMs are generally thought to play an important role in the protection of plants against herbivores and pathogens (Stout et al. 2006; van Dam et al. 1995; Wink 1988).

Higher concentrations of SMs can result in more resistant plants. At the same time the production of SMs is thought to be costly. Such costs can reduce plant growth and reproduction (Cipollini et al. 2003; Herms and Mattson 1992; Karban and Baldwin 2007). Hence plants face a dilemma: to grow or to defend (Herms and Mattson 1992). On the one hand, they must grow fast enough to compete with neighbouring plants, on the other hand, they must produce enough defence compounds for survival in the presence of herbivores and pathogens (Siemens et al. 2002).

Secondary metabolite production can be constitutive but is often influenced by many environmental factors, such as temperature, humidity, light intensity and drought (reviewed in Akula and Ravishankar 2011). Temperature strongly influences both plant ontology and metabolic activity. For instance, low and high temperatures caused significant reductions in fresh weight, dry weight, total phenolics, flavonoids and total eleutheroside accumulation in *Eleutherococcus senticosus* (Shohael et al. 2006). Drought conditions were reported to decrease the content of saponins in *Chenopodium quinoa* (Solíz-Guerrero et al. 2002). Low light intensity increased methylxanthine content in leaves of *Ilex paraguariensis* (Coelho et al. 2007), while it decreased resin content in leaves of *Grindelia chiloensis* (Zavala and Ravetta 2001). UV-B light induced the accumulation of glucosinolates in broccoli (Mewis et al. 2012) and flavonoids in *Ribes nigrum* (Schreiner et al. 2012).

In addition to abiotic factors SM concentration and composition are known to be influenced by biotic stresses. For instance, herbivore attack of *Spodoptera littoralis* led to the induction of metabolites in the leaves and roots of maize (Marti et al. 2013). Incubation with bacterial genera *Azospirillum* and *Pseudomonas*, mycorrhizal genus *Glomus* under field conditions led to qualitative and quantitative modifications of SMs in maize roots, particularly of benzoxazinoids and diethylphthalate (Walker et al. 2012). Due to the seasonality of pests, pathogens and climate, we expect the type and level of plant defences to depend on the seasons as well (Brooks and Feeny 2004; Kumar et al. 2012; Shiojiri and Karban 2008).

Seasonal fluctuations of SM concentrations were mostly reported for woody plants. The leaf phenolics of *Quercus robur* L. showed a sharp decline early on in the growing season, followed by relatively stable low amounts until autumn (Salminen

et al. 2004; Sommavilla et al. 2012). In contrast, the flavonoid concentration in the mountain birch *Betula pubscens* subsp. *caerepanovii* increased until early summer, dropped during summer and increased again during autumn (Riipi et al. 2002). Some studies reported seasonal variation in herbaceous plants. For instance, Lubbe et al. (2013) found that the concentration of alkaloids decreased in the leaves of *Narcissus pseudonarcissus* during the growing season while the concentration in roots remained constant. In *Senecio madagascariensis* in Brazil the highest PA concentration occurred in spring (Karam et al. 2011) while in *Senecio riddellii* and *Senecio longilobus* leaves this was in summer (Johnson et al. 1985). Although these studies documented seasonal variation of SMs, there are still very few studies that encompass a time series over all seasons for distinct genotypes.

In this study we used pyrrolizidine alkaloids (PAs) as a study system. PAs are present in several plant families, such as the Apocynaceae, Asteraceae, Boraginaceae, Convolvulaceae, Leguminosae and Orchidaceae (Brehm et al. 2007; Flores et al. 2009; Frölich et al. 2006; Hartmann and Witte 1995; Jenett-Siems et al. 2005; Trigo et al. 2003). In *Jacobaea* (Asteraceae) species, they are synthesized in the roots, and translocated as N-oxides from the roots to the shoots, where further PAs diversification takes place (Hartmann et al. 1989). The shoots are especially essential for PA diversification of jacobine-like PAs (Nuringtyas 2013). PA concentration and composition showed a high heritability (Vrieling et al. 1993). In a climate room PA concentration and composition were strongly genotype-dependent (Cheng et al. 2011a). PAs are well known for their interaction with herbivores. Polyphagous herbivores are generally deterred by the different types of PAs while the specialist herbivores are attracted by PAs (Cheng et al. 2011b; Cheng et al. 2013; Macel et al. 2002; Wei et al. 2015).

To investigate the seasonal variation of SM concentration, we set up a series of clones of the same genotype to specifically explore the effects from seasonal variation. We tracked PA variation over the course of vegetative growth for 14 months. Our aim was to get an understanding of how PA concentration and composition vary when plants are exposed to natural environments and varying climatological conditions and whether genotypic differences are maintained over time. This is, as far as we know, the first study to explore the SM variation with the same genotypes over the whole vegetative growth period. We addressed the following questions: 1) How does the total PA amount vary over the seasons? 2) How does the PA concentration and composition vary over the seasons? 3) Are differences in concentration and composition of PAs between genotypes maintained?

Materials and Methods

Study system

Jacobaea vulgaris subs. *dunensis* was derived from seeds collected at the Meijen-

del Nature Reserve (52°7′54″N, 4°19′46″E, The Netherlands), and *J. aquatica* subs. *aquatica* was derived from seeds collected at the Zwanenwater Reserve (52°48′38″N, 4°41′7″E, The Netherlands). The parents, the F1 and F2 hybrids of these two species are maintained in our lab in tissue culture and can thus easily be multiplied to produce replicates of the same genotype. The cross was documented in detail by Cheng et al. (2011a). In brief, seeds of the parental species were grown until blooming. Both species are self-incompatible, and crosses were performed by rubbing flower heads together. Two rayed F1 offspring were selected and reciprocally crossed with each other to produce two sets of F2 offspring.

Jacobaea vulgaris is a monocarpic biannual / perennial (Chater and Walters 1976; van der Meijden and van der Waals-Kooi 1979). In the first year this species forms a rosette, whereas flowering can occur in the second year or later depending on the size of the plant. After flowering, plants die (Harper and Wood 1957; Wesselingh and Klinkhamer 1996). *Jacobaea aquatica* is a biannual that is phylogenetically closely related to *J. vulgaris* but ecologically and chemically the two species show distinct differences (Cheng et al. 2011a; Pelser et al. 2003). *Jacobaea vulgaris* has a higher concentration of jacobine-like PAs and grows in dry, sandy soils while *J. aquatica* has a higher concentration of senecionine-like PAs and grows in marsh environments (Cheng et al. 2011a). Over 60 herbivores were reported to feed on *J. vulgaris*. This species is regularly completely defoliated by its specialist herbivore *Tyria jacobaeae* (van der Meijden et al. 1990). No severe defoliation events have been reported for *J. aquatica*.

Based on the data of previous field work at Lisse (52°25′12″N, 4°54′10″E, The Netherlands), eight genotypes were chosen for this experiment, including the parental plants *J. vulgaris* and *J. aquatica* (JV and JA), two F1 hybrids (F1A, F1B) and four F2 hybrids (F2A, F2B, F2C, F2D). The four F2 genotypes represented different levels of jacobine-like PAs in the shoots: F2A and F2B (medium), F2C (low) and F2D (high).

Plant growth

Each genotype was cloned into 120 replicates in tissue culture. After two weeks, all the 960 cloned individuals were potted in 0.5 L pots. Potting soil was collected from the experimental field at Lisse (52°25′8″N, 4°54′34″E, The Netherlands). Plants were kept in a greenhouse next to the experimental field for 5 weeks and watered three times per week until planting in the field on April 12, 2012. The experimental field is an open area with rich sandy soil where *J. vulgaris* occurs naturally at its borders. The area was cleared before planting. In the process of growing, plants were also kept free of weeds and their specialist herbivore, the cinnabar moth. Each plant was numbered and they were randomly planted with a distance of 30 cm from each other. Plants were watered only during planting.

Sample collection

We started to harvest plants in May 10, 2012. Two randomly chose plants per genotype were harvested every two weeks from May to September and every three weeks from October to the next June. Because of wintery conditions frozen soil, no data could be obtained from the harvest on March 14, 2013 and due to a broken refrigerator we have no data for the harvest on April 5, 2013. Each harvest included 16 plants (2 individuals × 8 genotypes). Plants were gently removed from the soil and put in a plastic bag. In summer, when the temperature was high, the plastic bags with the plants were kept in a box cooled with ice. Plants were washed, with scissors separated into roots and shoots and dried with paper tissues. After measuring the fresh weight of the plants, two leaves and two roots were wrapped in aluminium foil and snap frozen in liquid nitrogen. Samples were subsequently freeze dried for 4 days under vacuum with a collector temperature of -80°C (Cryotheque®, Sniders Scientific Company, Tilburg, The Netherlands). The freeze dried samples were ground into fine powder for PA extraction. The dry mass of shoots and roots were additionally measured. Plants were harvested from May 2012 to June 2013. In total on 20 occasions 608 samples were prepared. Due to the fact that most plants of genotype F2C flowered in summer of 2012, there are only 12 harvests for this genotype.

Pyrrolizidine alkaloid extraction and analysis

PA analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protocol was described in detail by Cheng et al. (2011a). In brief, 10 mg finely ground powder was extracted by 1.0 ml 2% formic acid solution containing heliotrine (1 μg ml^{-1}) as internal standard. After shaking and centrifuging, 25 μl of the extracted supernatant was diluted with 975 μl of 0.1% ammonia hydroxide solution and 10 μl was injected in a Waters Acquity ultra performance liquid chromatographic system (UPLC) coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). The instrumental settings used were the same as described by Cheng et al. (2011a). Data were processed in Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

PAs are present in two forms: tertiary amine (free base) and N-oxide. Based on their chemical characteristics and biosynthetic pathway, PAs are classified into four groups: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Cheng et al. 2011a).

Temperature and day length

A dataset of the daily temperature and day length for the most nearby weather station (Valkenburg Naval Air Base) was obtained from the website of the Royal Netherlands Meteorological Institute (KNMI). Temperature and day length used in our analysis was calculated by averaging the daily temperature and day length data of 14 days previous to the harvest day. The weather station is located at circa 14 km from

the experimental field.

Statistical analysis

The parameters used are calculated by the following equations: shoot/root ratio (SR ratio) = (the mean dry mass of shoots) /(the mean dry mass of roots), PA amount = (total PA concentration) * (dry mass), the relative concentration of each group of PAs (%) = (PA concentration of each group) *100 / (the total PA concentration in the plant), free base/N-oxide ratio (FN ratio) (%) = (the concentration of free base PAs) *100 / (the concentration of N-oxide PAs).

Two-way ANOVAs were performed to evaluate the effects of genotype (random factor) and harvest dates (fixed factor) on dry mass, PA concentration, PA amount, FN ratio and SR ratio. General linear models were performed to test the relationship between SR ratio and PA concentration and temperature.

All analyses were performed in SPSS 21.0 for windows and Microsoft Excel 2010.

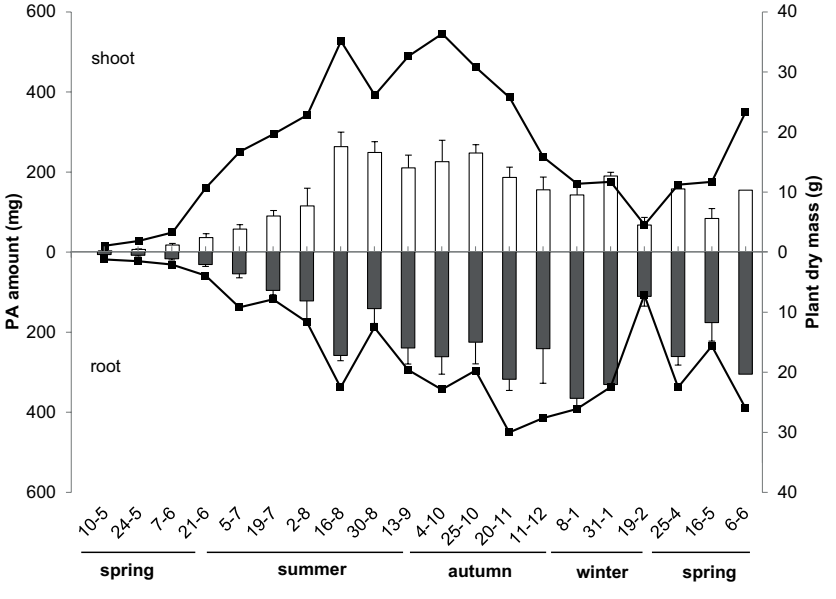


Fig. 1 The mean pyrrolizidine alkaloid (PA) amount (±SE, mg) and plant dry mass (g) of 8 genotypes at different harvest dates. The line charts represent the plant dry mass and bar graphs represent the PA amount.

Results

Variation in plant dry mass

Dry mass was significantly different among genotypes and harvest dates (Table 1), while the pattern of seasonal variation was similar for all genotypes except for genotype F2C (Fig. S1). Therefore dry mass is presented graphically as the overall mean

of 8 genotypes in Fig. 1. Shoot dry mass increased over the season and peaked in early October, then decreased until February and increased again during the next spring (Fig. 1). Root dry mass followed a similar pattern but peaked at the end of November and then declined until February after which it increased again until the last harvest in June. In the winter season, between November 2012 and February 2013, almost 70% of the dry mass of shoots and roots was lost.

Variation in shoot/root ratio

The SR ratio was positively correlated with temperature and day length (Fig. S2). The mean SR ratio of the 8 genotypes was the highest in mid-summer and then decreased gradually until mid-winter, after which in spring it started to increase again (Fig. 2).

Although the variation among harvests was the strongest in the F2 hybrids (Fig. S3), all genotypes showed a relatively similar pattern of variation in SR ratio across harvest dates even though differences in this pattern between genotypes were significant (Table 1).

Table 1 Two-way ANOVAs with plant dry mass, total pyrrolizidine alkaloid (PA) amount, total PA concentration, free base/N-oxide (FN) ratio, shoot/root (SR) ratio as dependent variables and with harvest dates and genotypes as independent variable

	Source of		Root		Shoot	
Dependent variables	variation	DF	F	P	F	P
Dry mass	Date	19	3.391	<0.001	3.369	<0.001
	Genotype	7	12.648	<0.001	7.488	<0.001
	Date*Genotype	125	1.871	<0.001	2.738	<0.001
Total PA concentration	Date	19	7.138	<0.001	18.581	<0.001
	Genotype	7	37.197	<0.001	21.868	<0.001
	Date*Genotype	125	1.633	0.002	2.119	<0.001
Total PA amount	Date	19	3.112	<0.001	2.511	0.001
	Genotype	7	13.224	<0.001	8.598	<0.001
	Date*Genotype	125	1.677	0.001	2.932	<0.001
Free base/N-oxide ratio	Date	19	2.864	<0.001	1.550	0.08
	Genotype	7	9.605	<0.001	4.934	<0.001
	Date*Genotype	125	2.307	<0.001	2.703	<0.001
SR ratio*	Date	19	4.191	<0.001		
	Genotype	7	6.211	<0.001		
	Date*Genotype	125	1.119	0.253		

*SR ratio is for the whole plant.

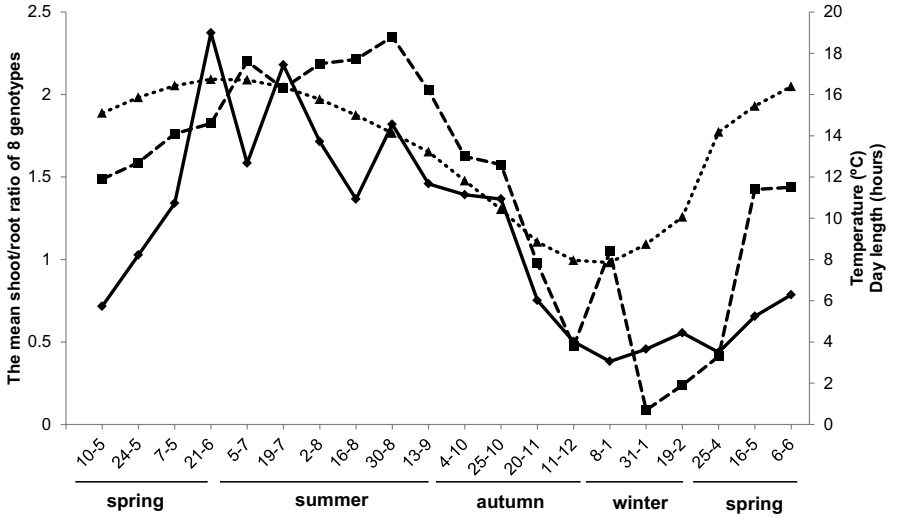


Fig. 2 The mean shoot-root dry mass ratio (solid line) of 8 genotypes, temperature (dashed line) and day length (dot line) at different harvest dates. Temperature and day length are the average of 14 days previous to the harvest day.

Variation in total PA content

Averaged over all 8 genotypes the total amount of PAs stored in the plants increased until late summer (August) (Fig. 1). In the shoots the PA content remained constant until November, and then started to decrease during the winter period. By February 70% of the PAs stored in the shoots had been lost. In the roots the amount of PAs remained rather constant until the end of January, but a sharp decline occurred in February. In total, plants lost approximately 60% of stored PAs during the winter period. The seasonal variation of PA amounts can largely be explained by the seasonal variation in plant dry mass. (Table 2, Fig. 1)

Across harvest dates the variation between genotypes was largely maintained, although the pattern of variation over the seasons differed among genotypes (Table 1, Fig. S4). F2 hybrids contained a smaller total PA amount than the parents and F1 hybrids (Fig. S4). This could largely be explained by smaller amount of dry mass produced by the F2 hybrids compared to the parents and F1 hybrids. Each genotype contained a larger amount of PAs in roots than shoots (Fig. S4).

Roots were relatively rich in senecionine- and otosenine-like PAs while the shoots contained more erucifoline- and jacobine-like PAs (Fig. S5). In the shoots the amount of senecionine-like PAs showed a steady increase until the end of the winter and then decreased dramatically in the next spring (Fig. S5A). The amount of jacobine-like PAs increased until August, remained at similar levels until the end of October and then decreased in winter. The amount of jacobine-like PAs increased again in the next spring (Fig. S5B). The amount of erucifoline-like PAs showed an

increase until August after which it remained at a similar level until the end of winter. In the second spring the amount of erucifoline-like PAs was low (Fig. S5C). The amount of otosenine-like PAs present in the shoots was relatively small, but showed a gradual increase continuing over the whole period until the next spring (Fig. S5D).

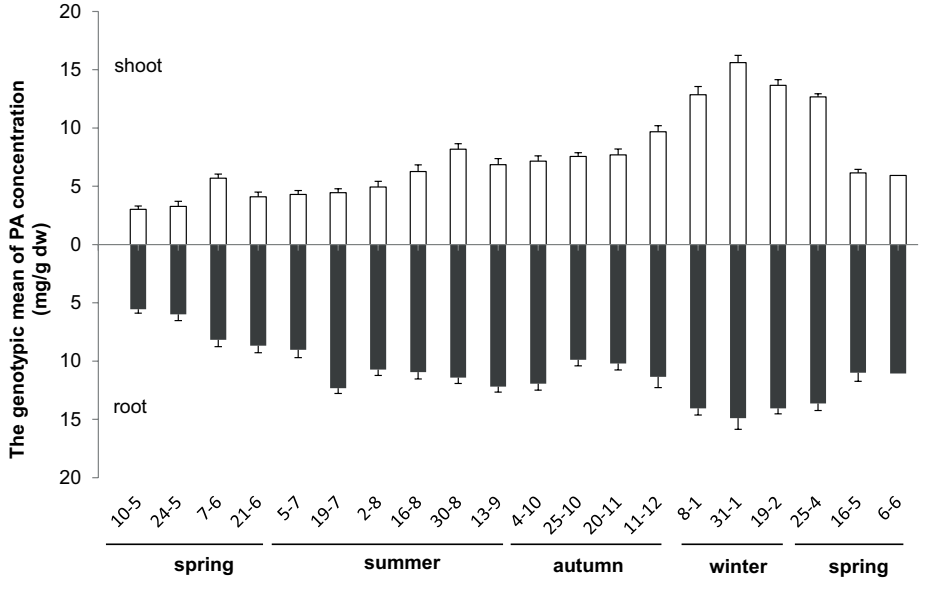


Fig. 3 The mean pyrrolizidine alkaloid (PA) concentration (±SE, mg/g dw) of 8 genotypes at different harvest dates.

Variation in PA concentration

The PA concentration averaged over the 8 genotypes increased gradually over the seasons in both shoots and roots (Fig. 3). However, during the spring of the next year PA concentrations dropped significantly in the shoots (Fig. 3). The PA concentration in roots was on average twice in shoots. In the second spring the PA concentration in shoots and roots were 1.5-2 times higher compared to the first spring. The total PA concentration was different among genotypes (Table 1). Differences in the pattern of seasonal variation were relatively small although significant among genotypes (Table 1). Except JA, all genotypes had higher PA concentrations in roots than in shoots (Fig. S6).

The mean PA concentration in roots and shoots averaged over all 8 genotypes was negatively correlated with temperature (Fig. S7A and B) and day length (Fig. S7C and D).

The concentration of senecionine-like PAs in shoots started to increase from October onwards (Fig. S8A) and reached its peak in winter, but decreased to its initial level in the next spring. Roots had about 1.5-7 times higher senecionine-like PA concentrations than shoots (Fig. S8A). The concentration of jacobine-like PAs in

roots increased gradually over the seasons while in shoots it increased until August and then remained at similar levels until the end of the study (Fig. S8B). Concentrations of erucifoline-like PAs were much higher in the shoots than in the roots. The concentration of erucifoline-like PAs peaked in mid-winter and then started to drop in the next spring (Fig. S8C). The concentrations of otosenine-like PAs in general were slightly higher in roots than in shoots. Concentrations in shoots peaked in April and then started to decrease in spring, while remaining constant in the roots (Fig. S8D).

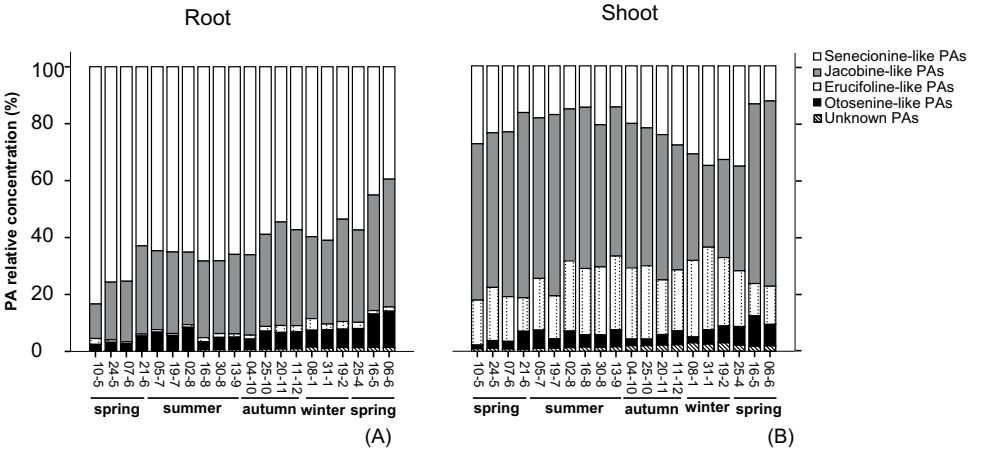


Fig. 4 The relative concentration of different types of pyrrolizidine alkaloids (PAs, %) for 8 genotypes at different harvest dates.

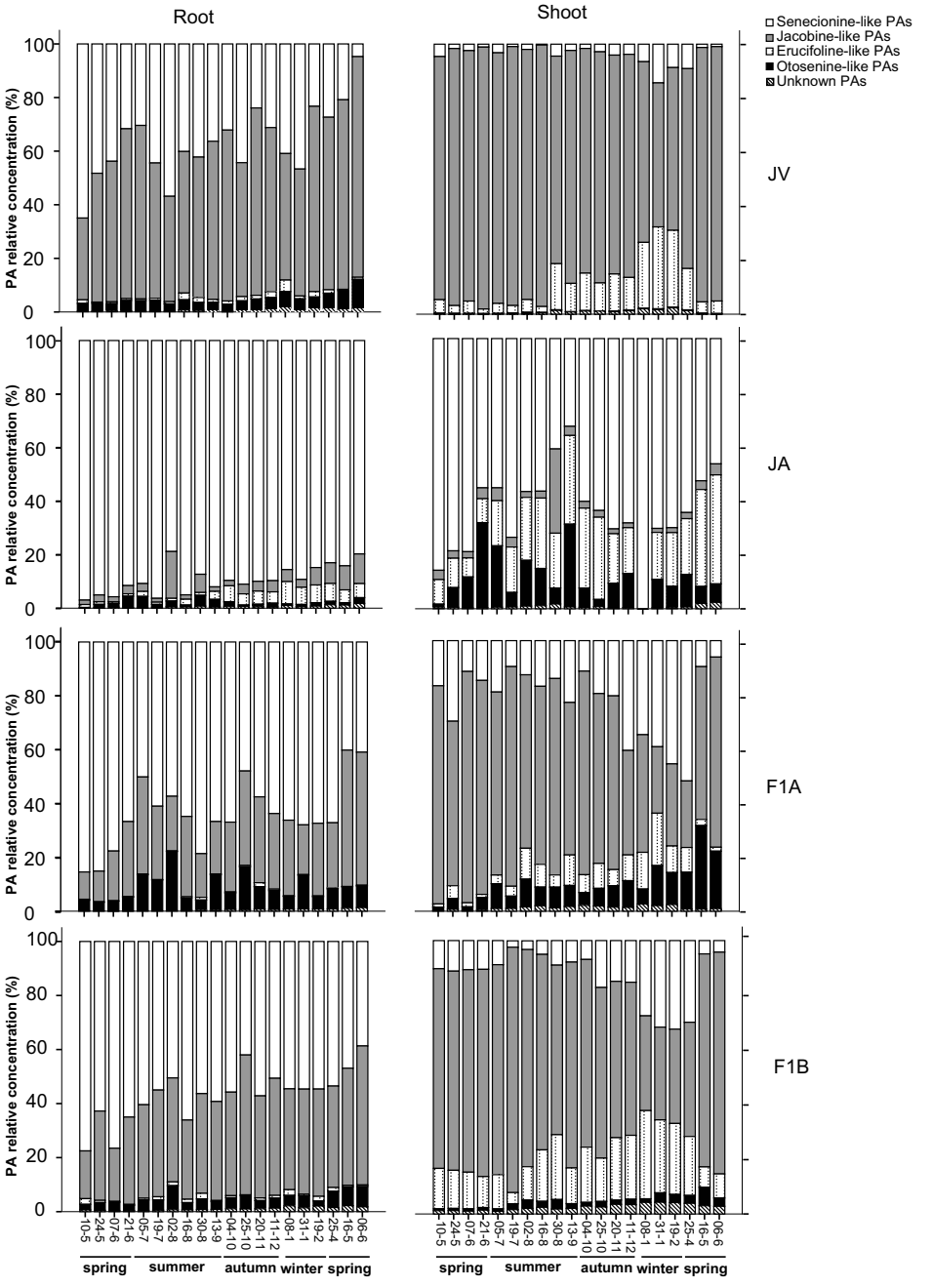
Variation in PA composition

Variation of the relative concentration of four structurally related PA types

For the 8 genotypes combined, in roots senecionine-like PAs were the dominate PA type. However, in the course of time, the relative concentration of senecionine-like PAs gradually decreased at the expense of jacobine- and otosenine-like PAs increased (Fig. 4A). In shoots jacobine-like PAs were the most abundant type of PAs except in winter when the relative concentration of senecionine-like PAs increased (Fig. 4B). The relative concentration of erucifoline-like PAs was close to zero in roots while it was around 20% in shoots except for the second spring when its share decreased to 10% (Fig. 4).

The variation of PA composition differed among genotypes. In the roots, the relative concentration of senecionine-like PAs decreased while that of jacobine-like PAs increased gradually over the seasons except in JA and F2C (Fig. 5), where senecionine-like PAs comprised over 80% of the total content during the whole study. The roots of F2A and F2B, were relatively rich in otosenine-like PAs and their shares increased gradually over time (Fig. 5). In the shoots, jacobine-like PAs were major

type of PAs except in JA and F2C, in which they were practically absent. In the other genotypes the share of jacobine-like PAs showed a similar pattern of variation: the relative contribution was smallest in winter, at the expense of senecionine- or erucifoline-like PAs (Fig. 5).



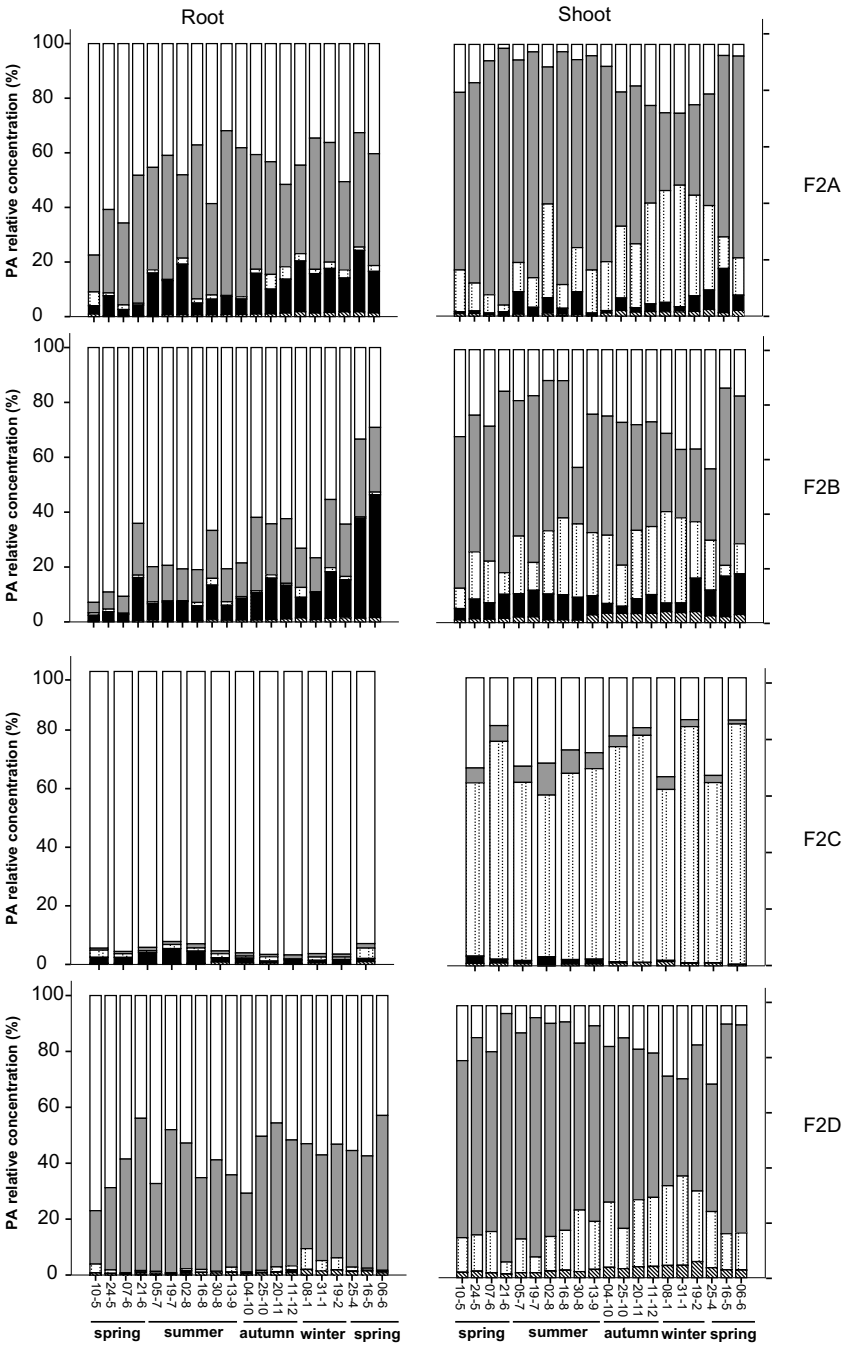


Fig. 5 The relative concentration of pyrrolizidine alkaloids (PAs, %) in roots and shoots in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 and 4 F2 hybrids at different harvest dates.

Variation in free base/N-oxide ratio

The free base/N-oxide (FN) ratio remained relatively constant over time until April 2013 (Fig. 6A). It then increased in several genotypes, but the increase was most notable in the shoots of the parent JV (Fig. S9C). The change in overall FN ratio strongly related to the change in FN ratio of the jacobine-like PAs (Fig. 6B). The FN ratio was much higher in shoots than in roots (Fig. S9).

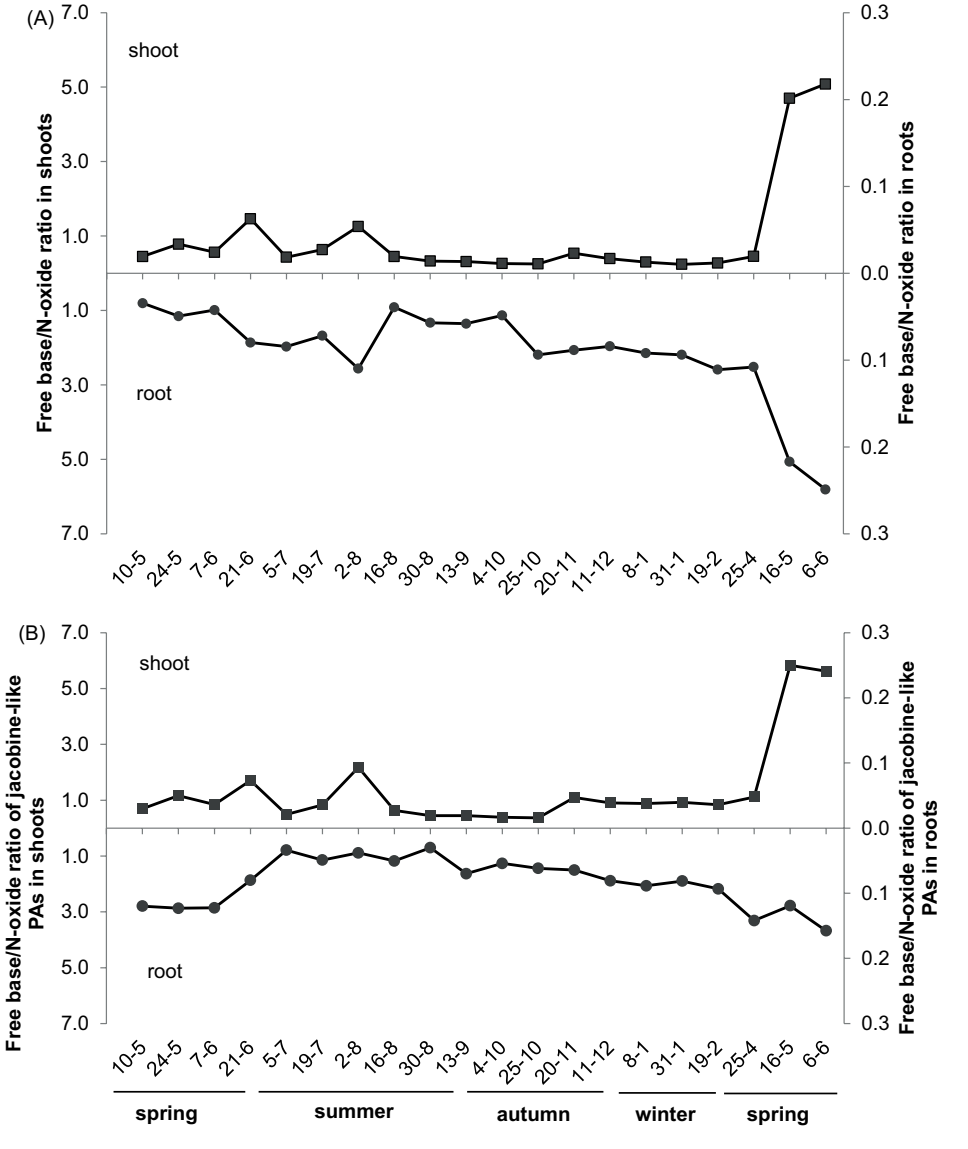


Fig. 6 The total free base/N-oxide ratio averaged over all 8 genotypes (A) and within jacobine-like PAs (B) in roots and shoots at different harvest dates.

Discussion

Seasonal impact on the total PA amount

We found for both roots and shoots a sharp decrease of dry mass and total PA amount in winter. For instance, for shoots over the period from October to February the average dry mass decreased from 35 to 5g and the calculated PA amount decreased from 210 to 70 mg. Interestingly, there was a stronger decrease of the dry mass than of the total PA amount, resulting in an increase of the total PA concentration in winter. Although the total PA concentration peaked in January, the total amount of PAs averaged over the 8 genotypes peaked in August and remained rather stable until November in both shoots and roots. After November, the PA amount in shoots decreased while it remained constant in the roots, indicating that the PAs that were lost in the shoots were not reallocated to the roots. The surprising conclusion is that plants do not seem to invest in the reallocation of PAs from decaying tissues during winter.

PAs are exclusively synthesized in the roots, and there is no substantial degradation of PAs in living tissue (Hartmann and Dierich 1998). Senecionine-like PA N-oxides are the primary products of biosynthetic pathway (Hartmann and Toppel 1987). In this study senecionine-like PAs are mainly stored in the roots. In contrast, the majority of erucifoline-like PAs were present in the shoots and only in very low amounts in the roots. This indicates that the senecionine-like PAs are first transferred from roots to shoots before conversion to erucifoline-like PAs occurs in shoots.

Seasonal impact on the PA concentration

The PA concentration in the roots and shoots gradually increased as the plants grew older. The PA concentration reached its maximum during winter, when the loss of dry mass was stronger than the loss of PAs. The increase in PA concentration was however relatively small given the large decrease in plant dry mass. In spring the increase in dry mass was more significant than the increase in total PA amount resulting in a drop of the PA concentration.

Seasonal impact on PA composition

In the roots the patterns of variation in PA composition were relatively straight-forward. When plant grew older the relative concentration of senecionine like PAs declined while, depending on the genotype, that of jacobine-like PAs increased. In the shoots the patterns were more complex. In general the relative concentration of senecionine-like PAs decreased during the growing season but increased again when plants stopped growing in autumn and during winter. In spring when plants started to grow again the relative concentrations sharply decreased. In contrast the relative concentrations of jacobine-like PAs reached their minimum in winter and increased sharply when plants started growing again. The relative concentration of erucifoline-like PAs was highest in the winter season. In *J. vulgaris* the increase of the relative concentration of jacobine-like PAs after winter was at the cost of

both senecionine- and erucifoline-like PAs. In *J. aquatica* which hardly produces jacobine-like PAs the relative concentration of erucifoline-like PAs increased after winter while that of senecionine-like PAs decreased.

One of the most interesting changes was the increase in the F/N ratio in both shoots and roots during the second spring. The increase was most pronounced in JV shoots, but was also seen in the shoots of F1A, F2A and F2B. In roots the largest increase was seen for F2B. This increase was not only caused by the fact that the relative concentration of jacobine-like PAs increased but was also found within the group of jacobine-like PAs. It is hard to judge at this point whether or not these changes are adaptive to the environment. With respect to generalist insect herbivores it has been shown that in general free base PAs are more toxic than their corresponding N-oxides (van Dam et al. 1995). It has also been shown that jacobine- and erucifoline-like PAs are more toxic than senecionine (Nuringtyas et al. 2014). On the other hand, Macel and Klinkhamer (2010) found that at Meijendel the jacobine chemotype of *J. vulgaris* was more severely attacked in May and June by the specialist herbivores *Tyria jacobaeae* and *Longitarsus jacobaeae* and by *Puccinia dioicae* (rust fungus) than erucifoline and mixed chemotype. Cheng et al. (2013) found that *T. jacobaeae* laid more eggs on the plants with higher concentrations of the free base of jacobine-like PAs. It is therefore difficult to understand why in spring *J. vulgaris* plants increase the relative concentration of this type of PAs, because this is the period when *T. jacobaeae* lays its eggs.

Several lines of evidence suggest that after attack by herbivores *Jacobaea* plants increase their erucifoline concentration. Kostenko et al. (2013) found that root herbivory by wireworms (*Agriotes lineatus*) lead to an increase in the concentration of erucifoline-like PAs. Hol et al. (2004) showed that the relative concentration of erucifoline increased while that of jacobine decreased in shoots after *J. vulgaris* shoots were damaged by *Mamestra brassicae*. In addition treatment with methyl jasmonate led to an increase of the erucifoline concentration at the cost of a decrease of the senecionine concentration (see chapter 5 of this thesis). On the one hand such an increase in erucifoline is surprising considering the fact that a number of studies suggested that jacobine-like PAs were more effective in protecting the plant against generalist (Cheng et al. 2011b; Leiss et al. 2009) but on the other hand it is in line with the observation that erucifoline chemotype plants showed less damage when there was a strong pressure of specialist herbivores (Macel and Klinkhamer 2010). Perhaps the increase of erucifoline-like PAs is a compromise between the effects on specialist and generalist herbivores.

Seasonal impact on differences in PA concentration and composition between genotypes

Although PA composition considerably changed over the seasons, generally the changes found in the genotypes were in a similar manner. Compared to the effects of

genotype and harvest date, the interaction between the two variables explained only a small amount of variation. For instance, at each harvest point there was a clear distinction between the 6 genotypes (JV, F1A, F1B, F2A, F2B and F2D) that produced higher levels of jacobine-like PAs and the two genotypes (JA and F2C) that produced much lower levels of these PAs. Therefore, although PA composition changed over seasons within each genotype in a significantly different manner, these differences were not strong enough to overrule the initial genetically derived variation among the genotypes.

Acknowledgements

We thank Karin van der Veen-van Wijk, Henk Nell, Kees Koops, Rita Rakhmawati, Yan Yan, Tiantian Lin, Xiaojie Liu, Ke Ma, Changsheng Wu and Zhiyi Yu for their help in field work. We thank Dandan Cheng for her suggestions in choosing hybrid genotypes. Xianqin Wei was supported financially by China Scholarship Council.

References

Akula R, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 6:1720-1731

Brehm G, Hartmann T, Willmott K (2007) Pyrrolizidine alkaloids and pharmacophagous Lepidoptera visitors of *Prestonia amabilis* (apocynaceae) in a montane rainforest in Ecuador. Ann Missouri Bot Gard 94:463-473

Brooks JS, Feeny P (2004) Seasonal variation in *Daucus carota* leaf-surface and leaf-tissue chemical profiles. Biochem Syst Ecol 32:769-782

Chater A, Walters S (1976) *Senecio* L. Flora europaea 4:191-205

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL (2011a) Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Cheng D, Kirk H, Vrieling K, Mulder PPJ, Klinkhamer PGL (2011b) The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 37:1071-1080

Cheng D, van der Meijden E, Mulder PPJ, Vrieling K, Klinkhamer PGL (2013) Pyrrolizidine alkaloid composition influences cinnabar moth oviposition preferences in *Jacobaea* Hybrids. J Chem Ecol 39:430-437

Cipollini D, Purrington CB, Bergelson J (2003) Costs of induced responses in plants. Basic Appl Ecol 4:79-89

Coelho GC, Rachwal MF, Dedecek RA, Curcio GR, Nietsche K, Schenkel EP (2007) Effect of light intensity on methylxanthine contents of *Ilex paraguariensis* A. St. Hil. Biochem Syst Ecol 35:75-80

Flores AS, Tozzi AMGdA, Trigo JR (2009) Pyrrolizidine alkaloid profiles in *Crotalaria* species from Brazil: Chemotaxonomic significance. Biochem Syst Ecol 37:459-469

Frölich C, Hartmann T, Ober D (2006) Tissue distribution and biosynthesis of 1, 2-saturated pyrrolizidine alkaloids in *Phalaenopsis* hybrids (Orchidaceae). Phytochemistry 67:1493-1502

Gols R, Raaijmakers C, van Dam N, Dicke M, Bukovinszky T, Harvey J (2007) Temporal changes affect plant chemistry and tritrophic interactions. Basic Appl Ecol 8:421-433

Harper JL, Wood W (1957) *Senecio Jacobaea* L. J Ecol 45:617-637

Hartmann T, Dierich B (1998) Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: biological need or coincidence? Planta 206:443-451

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C (1989) Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris* L. Planta 177:98-107

Hartmann T, Kutchan TM, Strack D (2005) Evolution of metabolic diversity. Phytochemistry 66:1198-1199

Hartmann T, Toppel G (1987) Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. Phytochemistry 26:1639-1643

Hartmann T, Witte L (1995) Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. Alkaloids: chemical and biological perspectives 9:155-233

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol:283-335

Hol WG, Macel M, van Veen JA, van der Meijden E (2004) Root damage and aboveground herbivory change concentration and composition of pyrrolizidine alkaloids of *Senecio jacobaea*. Basic Appl Ecol 5:253-260

Iriti M, Faoro F (2009) Chemical diversity and defence metabolism: how plants cope with pathogens and ozone pollution. Int J Mol Sci10:3371-3399

Jenett-Siems K, Ott SC, Schimming T, Siems K, Müller F, Hilker M, Witte L, Hartmann T, Austin DF, Eich E (2005) Ipangulines and minalobines, chemotaxonomic markers of the infrageneric *Ipomoea* taxon subgenus Quamoclit, section Mina. Phytochemistry 66:223-231

Johnson AE, Molyneux RJ, Merrill GB (1985) Chemistry of toxic range plants. Variation in pyrrolizidine alkaloid content of *Senecio*, *Amsinckia*, and *Crotalaria* species. J Agric Food Chem 33:50-55

Karam F, Haraguchi M, Gardner D (2011) Seasonal variation in pyrrolizidine alkaloid concentration and plant development in *Senecio madagascariensis* Poir.(Asteraceae) in Brazil. Poisoning by Plants, Mycotoxins and related Toxins:179-185

Karban R, Baldwin IT. 2007. Induced responses to herbivory, University of Chicago Press, Chicago

Kostenko O, Mulder PPJ, Bezemer TM (2013) Effects of root herbivory on pyrrolizidine alkaloid content and aboveground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. J Chem Ecol 39:109-119

Kumar A, Abrol E, Koul S, Vyas D (2012) Seasonal low temperature plays an important role in increasing metabolic content of secondary metabolites in *Withania somnifera* (L.) Dunal and affects the time of harvesting. Acta Physiol Plant 34:2027-2031

Leiss KA, Choi YH, Abdel-Farid IB, Verpoorte R, Klinkhamer PGL (2009) NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Liu Z, Carpenter SB, Bourgeois WJ, Yu Y, Constantin RJ, Falcon MJ, Adams JC (1998) Variations in the secondary metabolite camptothecin in relation to

tissue age and season in *Camptotheca acuminata*. Tree Physiol 18:265-270
Lubbe A, Gude H, Verpoorte R, Choi YH (2013) Seasonal accumulation of major alkaloids in organs of pharmaceutical crop *Narcissus Carlton*. Phytochemistry 88:43-53
Macel M, Klinkhamer PGL (2010) Chemotype of *Senecio jacobaea* affects damage by pathogens and insect herbivores in the field. Evol Ecol 24:237-250
Macel M, Klinkhamer PGL, Vrieling K, van der Meijden E (2002) Diversity of pyrrolizidine alkaloids in *Senecio* species does not affect the specialist herbivore *Tyria jacobaeae*. Oecologia 133:541-550
Marti G, Erb M, Boccard J, Glauser G, Doyen GR, Villard N, Robert CAM, Turlings TC, Rudaz S, Wolfender JL (2013) Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. Plant, Cell Environ 36:621-639
Mewis I, Schreiner M, Nguyen CN, Krumbein A, Ulrichs C, Lohse M, Zrenner R (2012) UV-B irradiation changes specifically the secondary metabolite profile in broccoli sprouts: induced signaling overlaps with defense response to biotic stressors. Plant Cell Physiol 53:1546-1560
Nuringtyas TR (2013) Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. Dissertation, Section Plant Ecology and Phytochemistry, Institute of Biology Leiden, Faculty of Science, Leiden University
Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA (2014) Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616
Pelser PB, Gravendeel B, van der Meijden R (2003) Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. Mol Phylogen Evol 29:613-628
Qasem J, Hill T (1995) Growth, development and nutrient accumulation in *Senecio vulgaris* L. and *Chenopodium album* L. Weed Res 35:187-196
Riipi M, Ossipov V, Lempa K, Haukioja E, Koricheva J, Ossipova S, Pihlaja K (2002) Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia 130:380-390
Salminen J-P, Roslin T, Karonen M, Sinkkonen J, Pihlaja K, Pulkkinen P (2004) Seasonal variation in the content of hydrolyzable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. J Chem Ecol 30:1693-1711
Schreiner M, Mewis I, Huyskens-Keil S, Jansen M, Zrenner R, Winkler J, O'Brien N, Krumbein A (2012) UV-B-induced secondary plant metabolites-potential benefits for plant and human health. Crit Rev Plant Sci 31:229-240
Shiojiri K, Karban R (2008) Seasonality of herbivory and communication between individuals of sagebrush. Arthropod Plant Interact 2:87-92

Shohael AM, Ali MB, Yu K-W, Hahn E-J, Paek K-Y (2006) Effect of temperature on secondary metabolites production and antioxidant enzyme activities in *Eleutherococcus senticosus* somatic embryos. Plant Cell Tiss Org Cult 85:219-228

Siemens DH, Garner SH, Mitchell-Olds T, Callaway RM (2002) Cost of defense in the context of plant competition: *Brassica rapa* may grow and defend. Ecology 83:505-517

Solíz-Guerrero JB, de Rodriguez DJ, Rodríguez-García R, Angulo-Sánchez JL, Méndez-Padilla G (2002) Quinoa saponins: concentration and composition analysis. Trends in new crops and new uses. ASHS Press, Alexandria:110-114

Sommavilla V, Haidacher-Gasser D, Sgarbossa M, Zidorn C (2012) Seasonal variation in phenolics in leaves of *Celtis australis* (Cannabaceae). Biochem Syst Ecol 41:110-114

Stout MJ, Thaler JS, Thomma BP (2006) Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. Annu Rev Entomol 51:663-689

Trigo JR, Leal IR, Matzenbacher NI, Lewinsohn TM (2003) Chemotaxonomic value of pyrrolizidine alkaloids in southern Brazil *Senecio* (Senecioneae: Asteraceae). Biochem Syst Ecol 31:1011-1022

van Dam NM, Vuister LW, Bergshoeff C, de Vos H, van der Meijden E (1995) The "Raison D'être" of pyrrolizidine alkaloids in *Cynoglossum officinale*: Deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

van der Meijden E, van der Waals-Kooi R (1979) The population ecology of *Senecio jacobaea* in a sand dune system: I. Reproductive strategy and the biennial habit. J Ecol:131-153

van der Meijden E, van Wijk CA, Kooi RE (1990) Population dynamics of the cinnabar moth (*Tyria jacobaeae*): oscillations due to food limitation and local extinction risks. Neth J Zool 41:158-173

Vrieling K, de Vos H, van Wijk CA (1993) Genetic analysis of the concentrations of pyrrolizidine alkaloids in *Senecio jacobaea*. Phytochemistry 32:1141-1144

Walker V, Couillerot O, Von Felten A, Bellvert F, Jansa J, Maurhofer M, Bally R, Moënne-Loccoz Y, Comte G (2012) Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum*, *Pseudomonas* and *Glomus* consortium under field conditions. Plant Soil 356:151-163

Walters D (2011) Plant defense: warding off attack by pathogens, herbivores and parasitic plants, John Wiley & Sons, New York

Wei X, Vrieling K, Mulder PPJ, Klinkhamer PGL (2015) Testing the Generalist-Specialist Dilemma: the role of pyrrolizidine alkaloids in resistance to inverte-

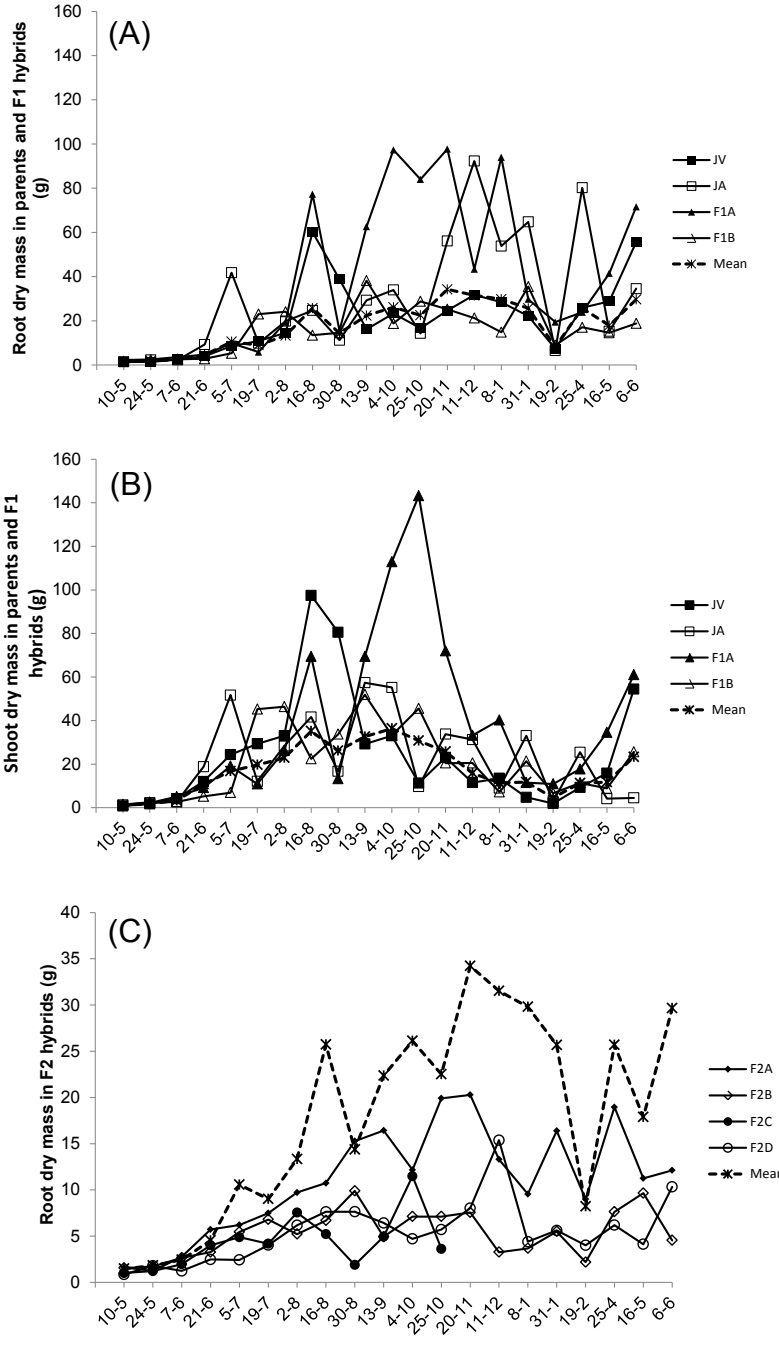
brate herbivores in *Jacobaea* Species. J Chem Ecol 41:159-167

Wesselingh R, Klinkhamer PGL (1996) Threshold size for vernalization in *Senecio jacobaea*: genetic variation and response to artificial selection. Funct Ecol 10:281-288

Wink M (1988) Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet 75:225-233

Zavala J, Ravetta D (2001) Allocation of photoassimilates to biomass, resin and carbohydrates in *Grindelia chiloensis* as affected by light intensity. Field Crops Res 69:143-149

Supplementary Materials:



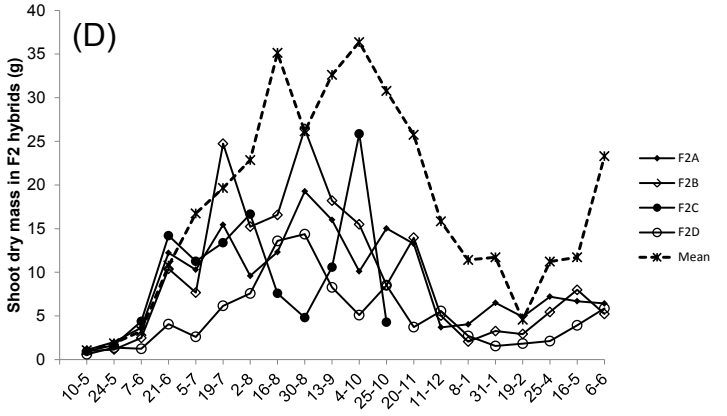


Fig. S1 The dry mass variation in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. The dash line represents the mean dry mass over all 8 genotypes in roots or shoots.

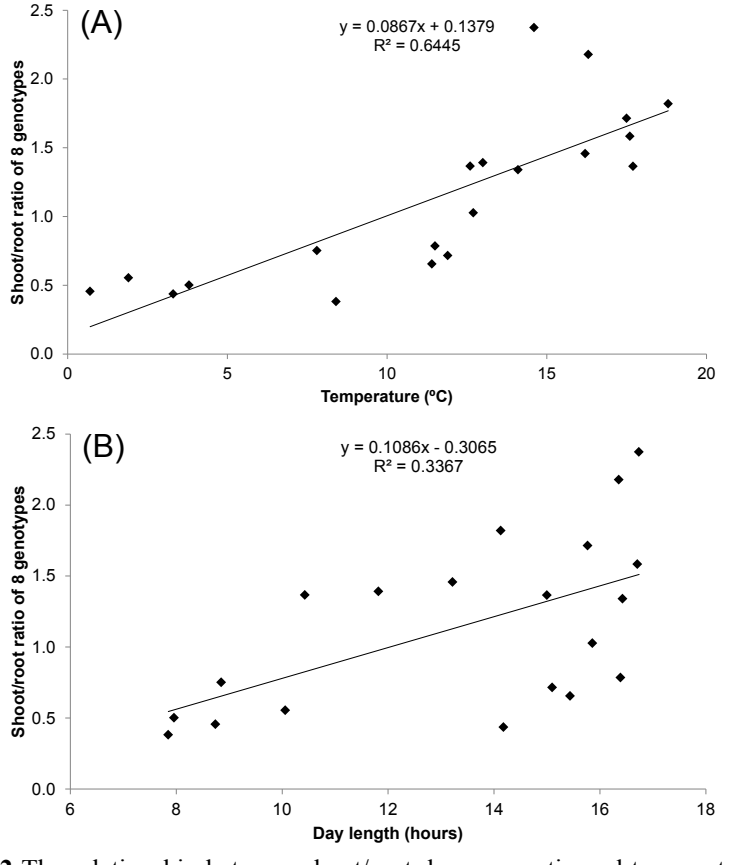


Fig. S2 The relationship between shoot/root dry mass ratio and temperature, day length at different harvest dates. The temperature and day length are the mean of data of 14 days previous to the harvest day.

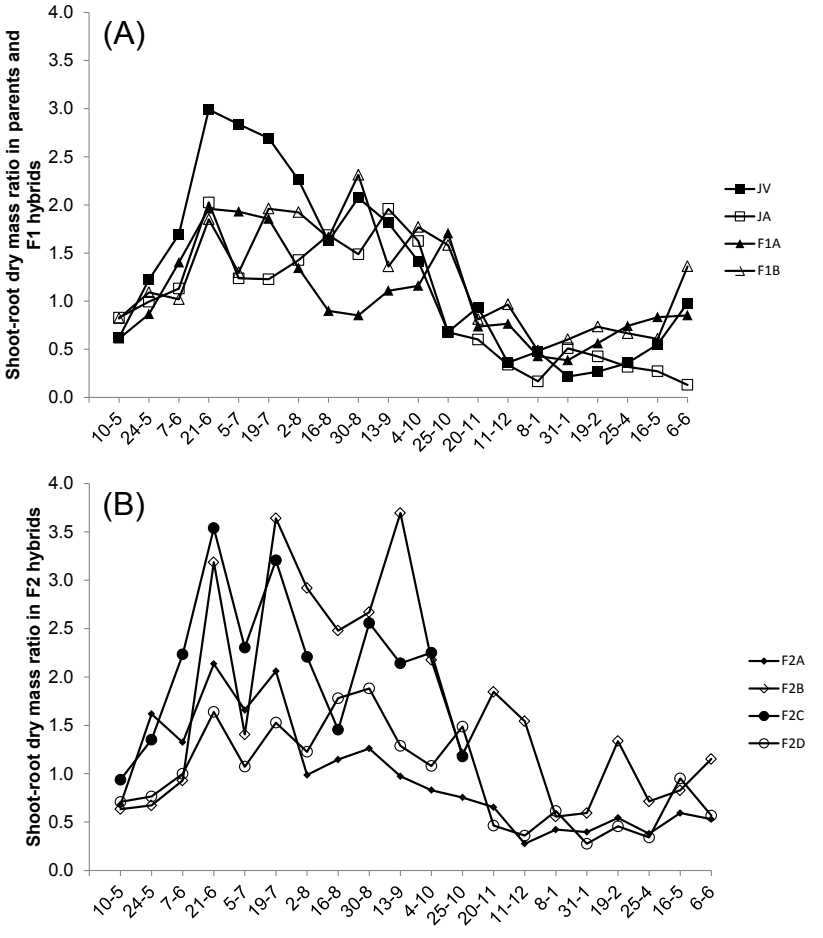
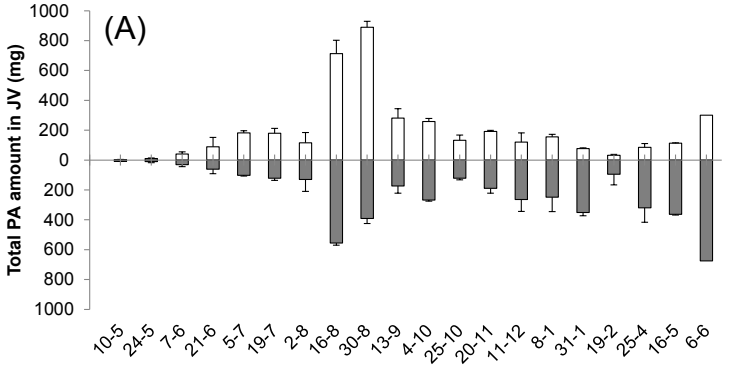
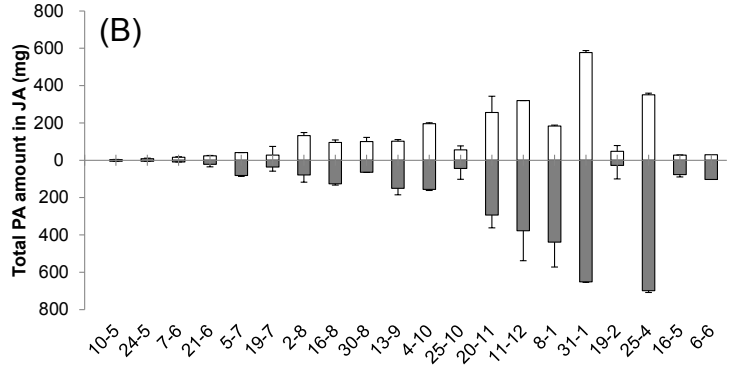
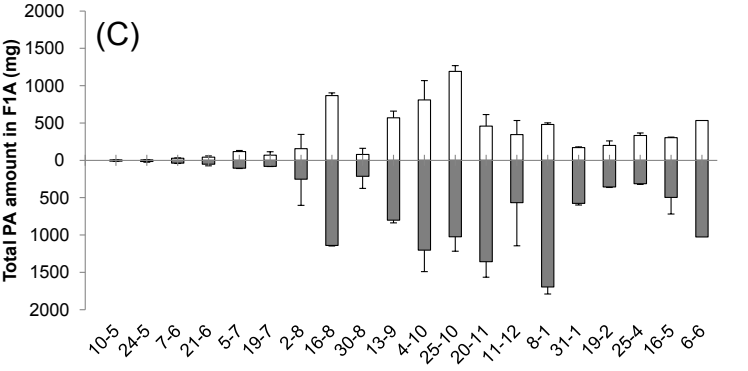
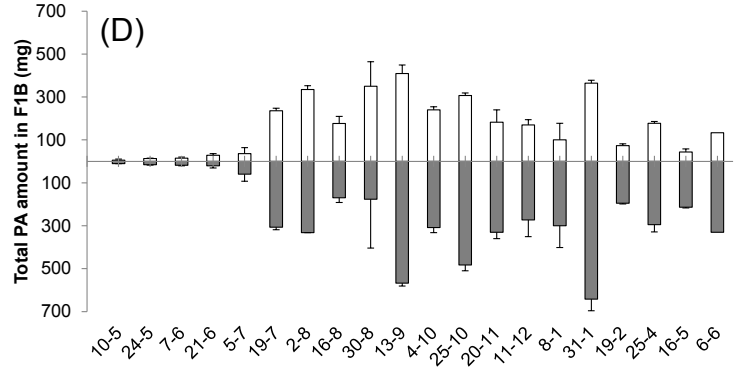


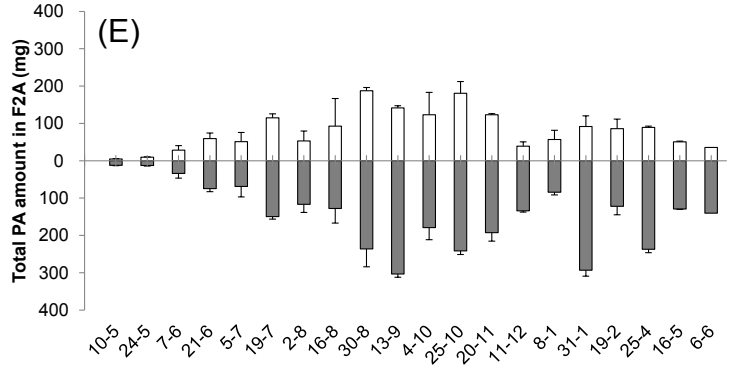
Fig. S3 The shoot/root ratio in *Jacobaea vulgaris*, *Jacobaea aquatica*, 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates.




(B)
Total PA amount in JA (mg)
800
600
400
200
0
200
400
600
800
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6


(C)
Total PA amount in F1A (mg)
2000
1500
1000
500
0
500
1000
1500
2000
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6


(D)
Total PA amount in F1B (mg)
700
500
300
100
100
300
500
700
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6


(E)
Total PA amount in F2A (mg)
400
300
200
100
0
100
200
300
400
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
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8-1
31-1
19-2
25-4
16-5
6-6

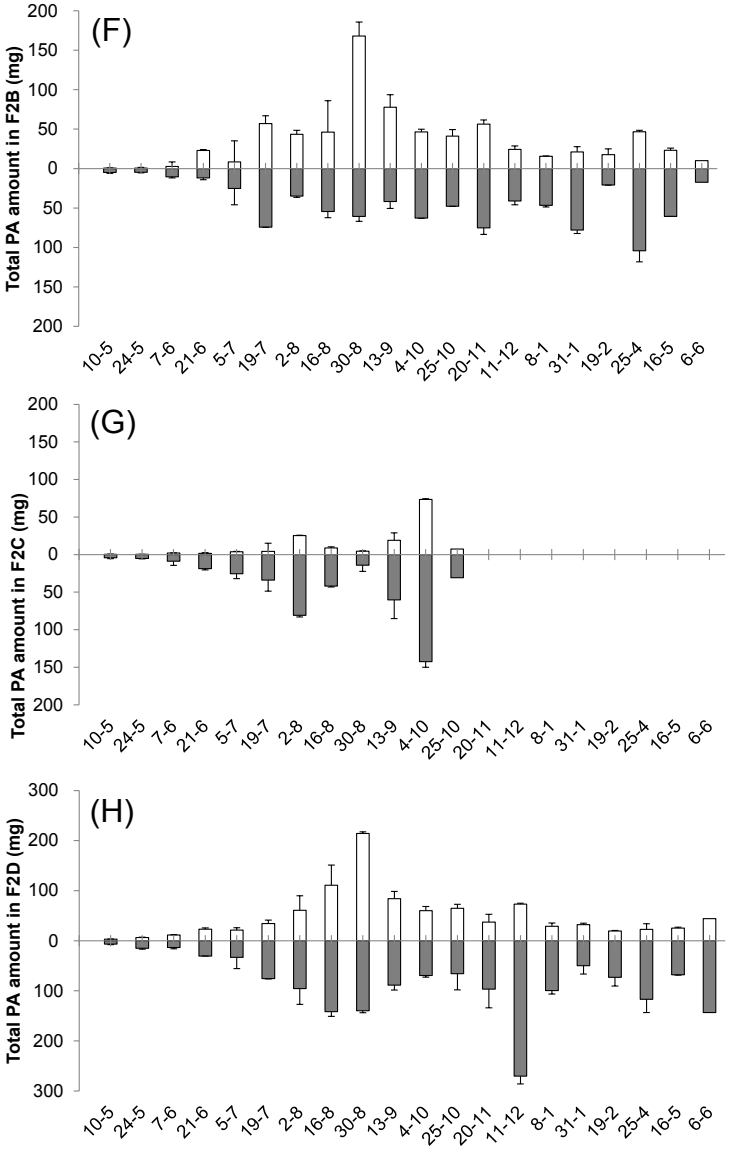
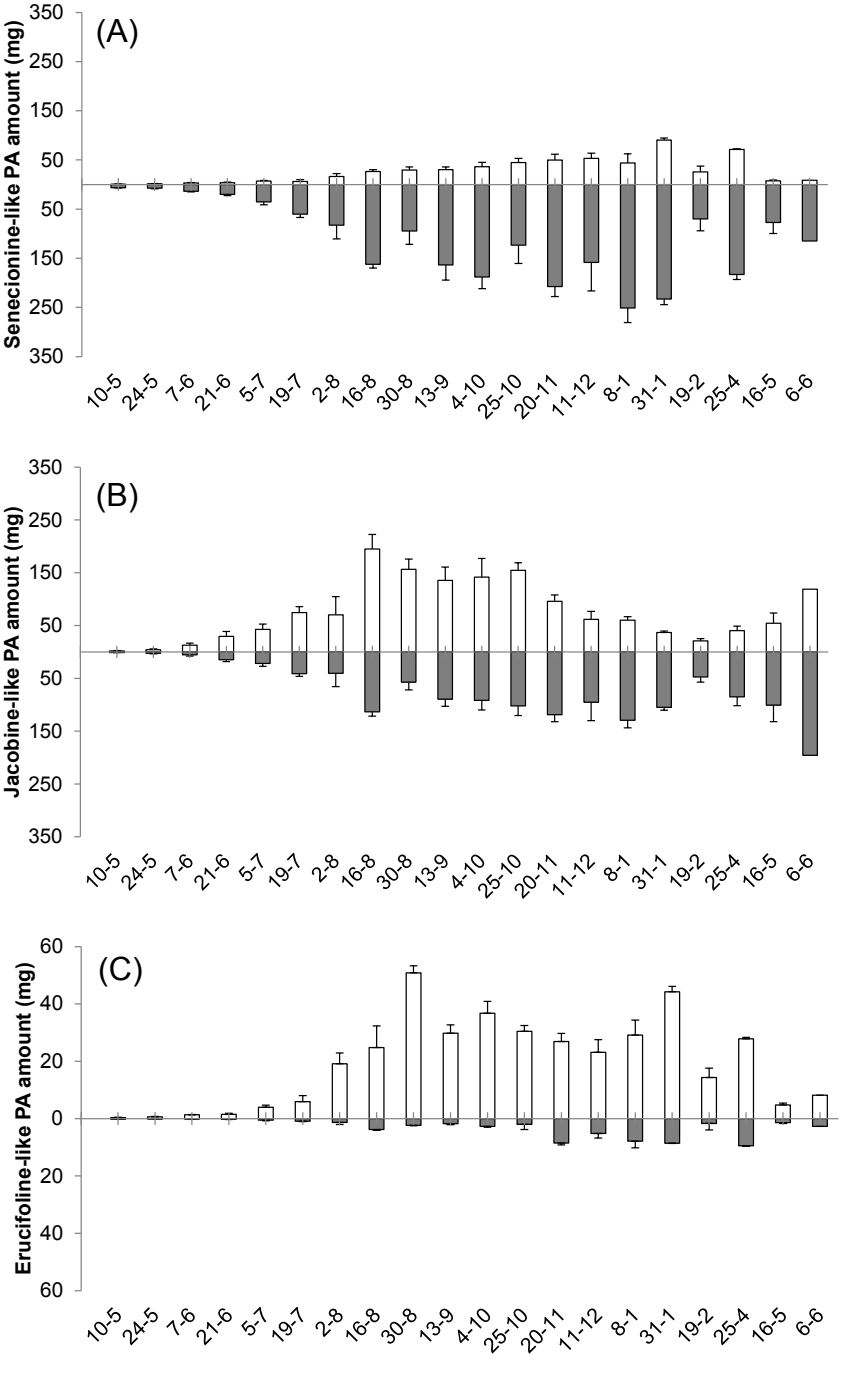


Fig. S4 The pyrrolizidine alkaloid (PA) amount (±SE, mg) in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. White bars represent shoots and grey bars represent roots.


(A)
Senecionine-like PA amount (mg)
350
250
150
50
50
150
250
350
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6
(B)
Jacobine-like PA amount (mg)
(C)
Erucifoline-like PA amount (mg)
60
40
20
0
20
40
60

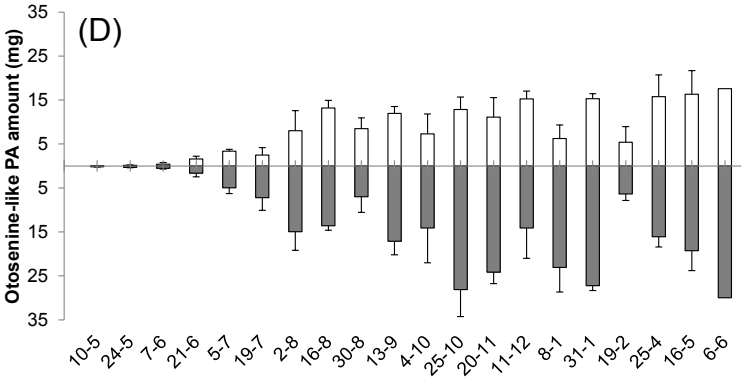
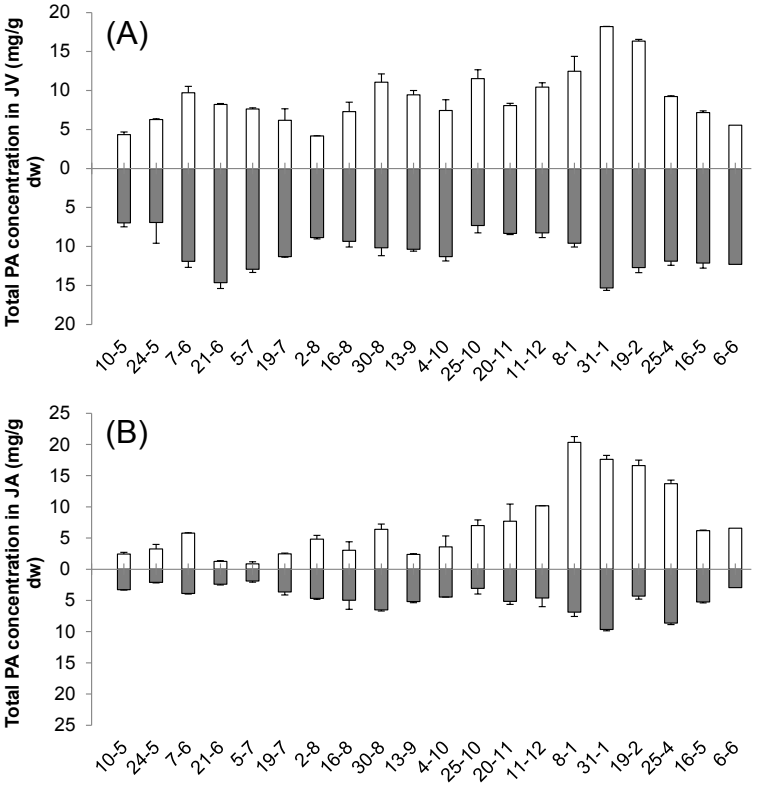
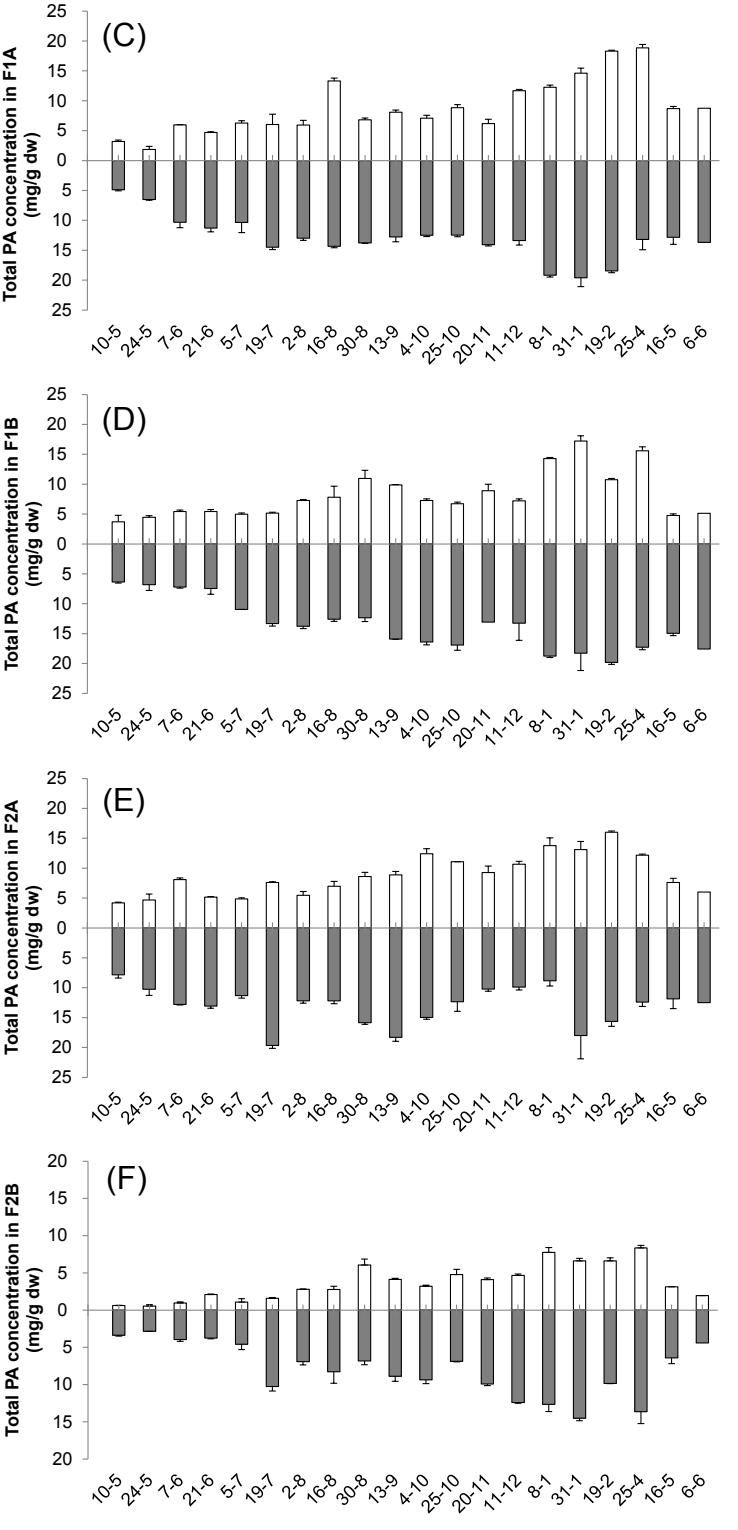


Fig. S5 The mean pyrrolizidine alkaloid (PA) amount (±SE, mg) of four structurally related PA groups averaged over all the 8 genotypes at different harvest dates. White bars represent shoots and grey bars represent roots.




(C)
Total PA concentration in F1A (mg/g dw)
(D)
Total PA concentration in F1B (mg/g dw)
(E)
Total PA concentration in F2A (mg/g dw)
(F)
Total PA concentration in F2B (mg/g dw)
10-5 24-5 7-6 21-6 5-7 19-7 2-8 16-8 30-8 13-9 4-10 25-10 20-11 11-12 8-1 31-1 19-2 25-4 16-5 6-6

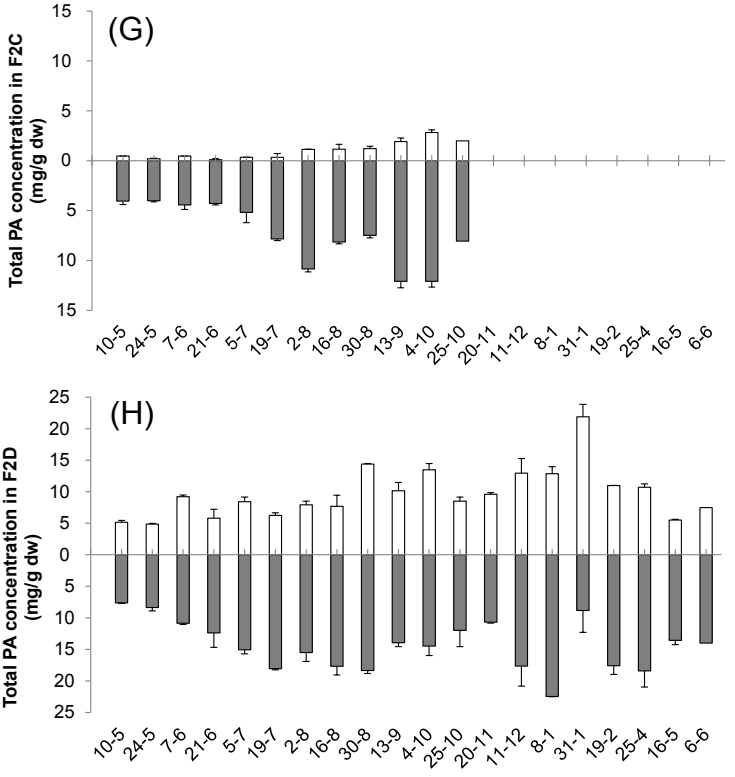
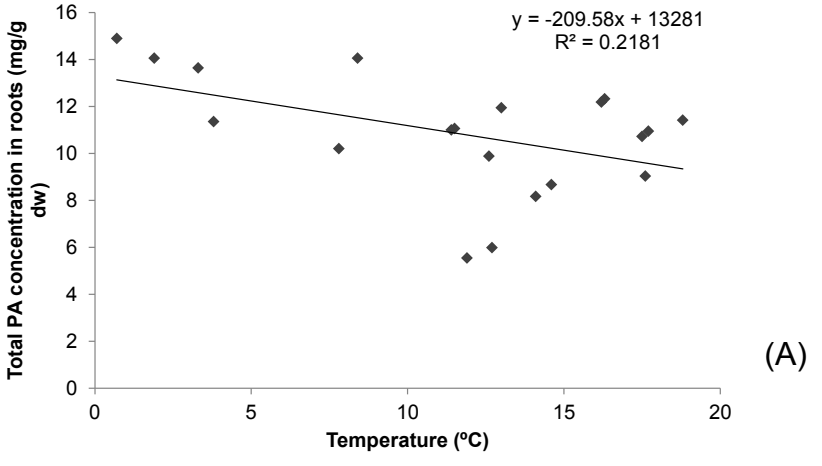


Fig. S6 The mean pyrrolizidine alkaloid (PA) concentration (±SE, mg/g dw) in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. White bars represent shoot s and grey bars represent roots.



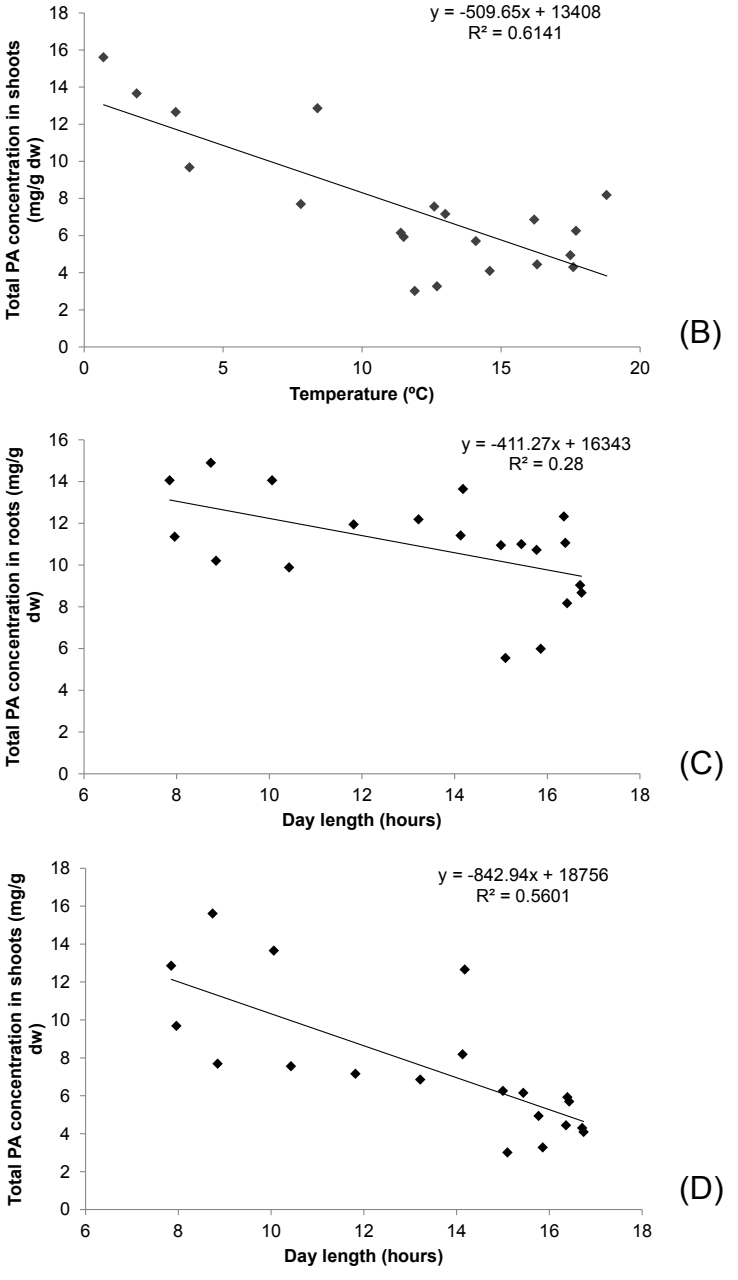
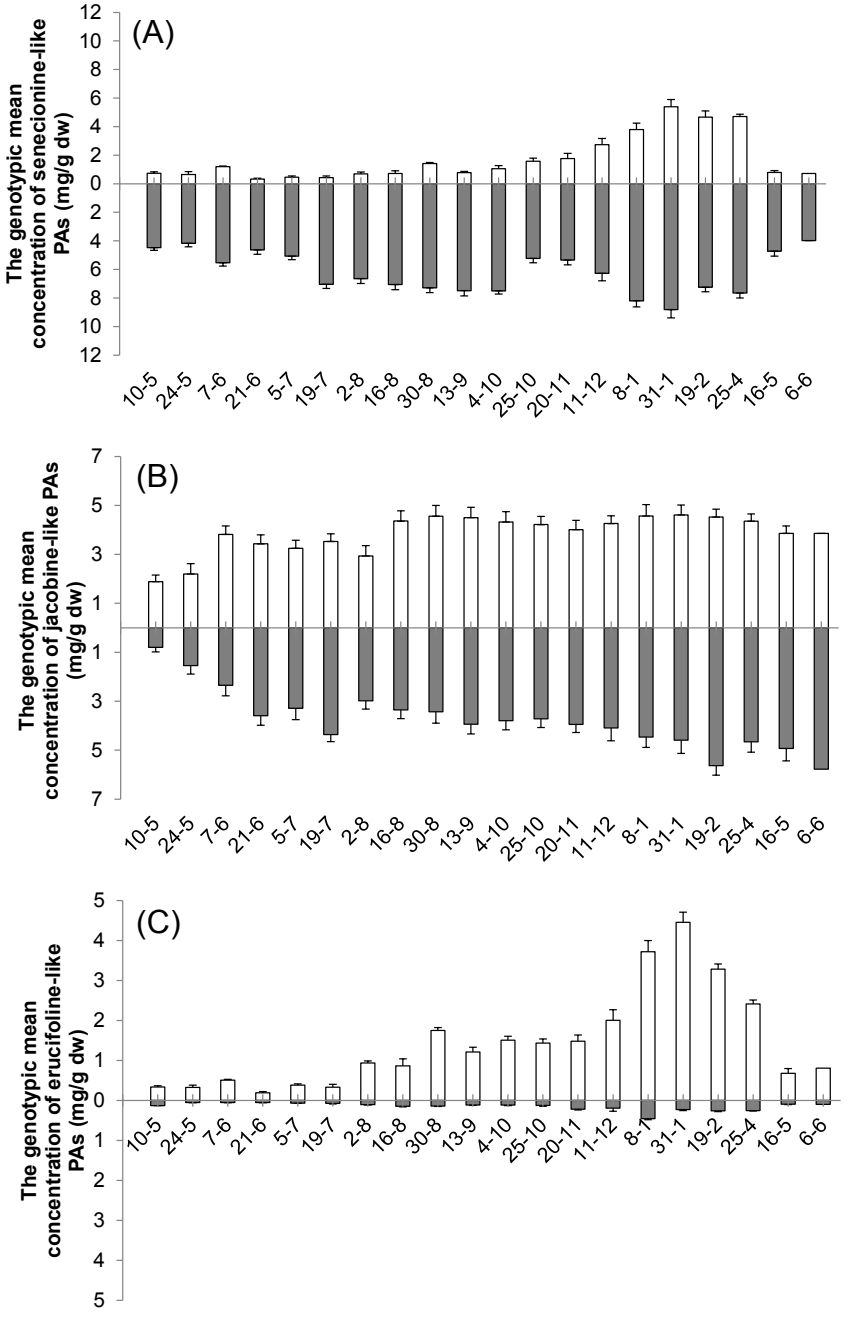


Fig. S7 Relationship between the mean pyrrolizidine alkaloid (PA) concentration (mg/g dw) of 8 genotypes and temperature and day length at different harvest dates in roots and shoots . The temperature and day length are the mean of the data of 14 days previous to the harvest day.


(A)
The genotypic mean concentration of senecionine-like PAs (mg/g dw)
12
10
8
6
4
2
0
2
4
6
8
10
12
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6
(B)
The genotypic mean concentration of jacobine-like PAs (mg/g dw)
7
5
3
1
1
3
5
7
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6
(C)
The genotypic mean concentration of erucifoline-like PAs (mg/g dw)
5
4
3
2
1
0
1
2
3
4
5
10-5
24-5
7-6
21-6
5-7
19-7
2-8
16-8
30-8
13-9
4-10
25-10
20-11
11-12
8-1
31-1
19-2
25-4
16-5
6-6

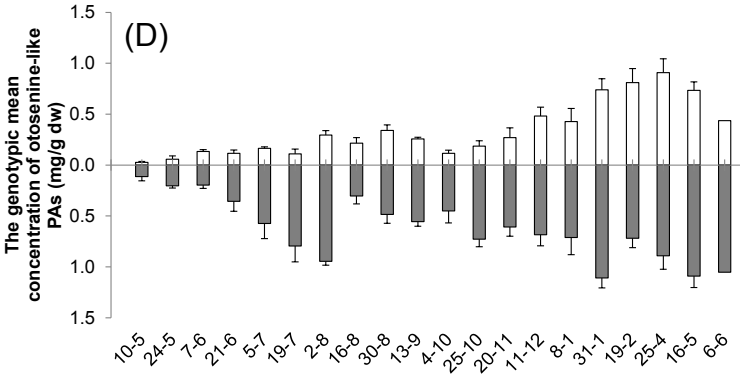
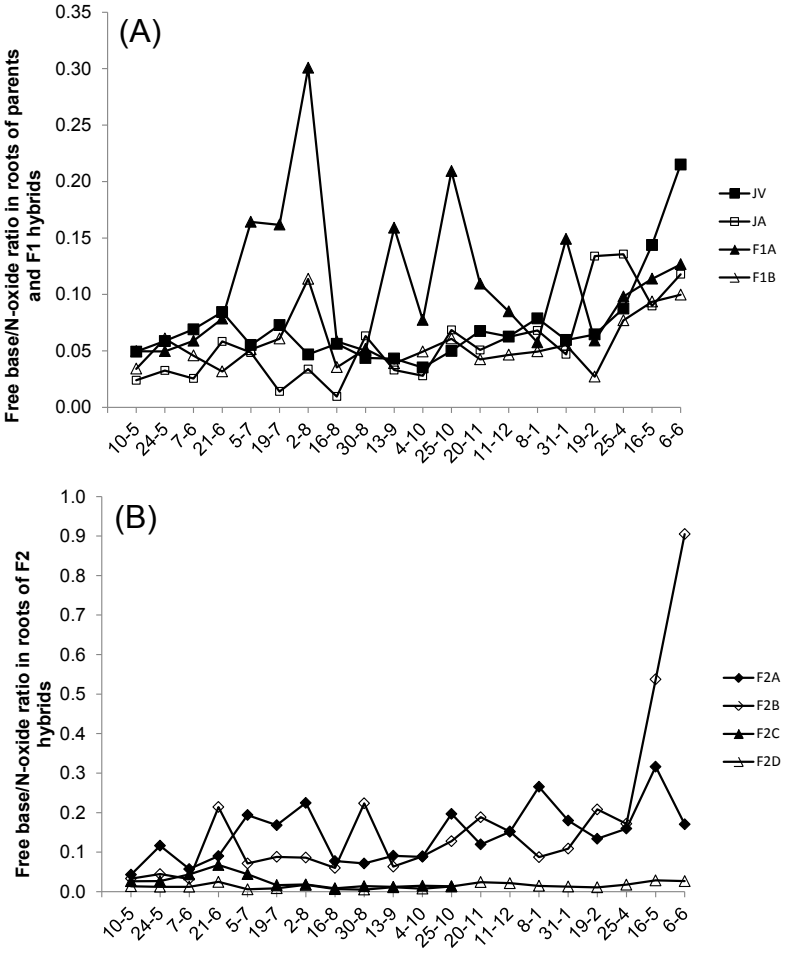


Fig. S8 The mean pyrrolizidine alkaloid (PA) concentration (±SE, mg/g dw) of four structurally related PA groups over all 8 genotypes at different harvest dates. White bars represent shoots and grey bars represent roots.



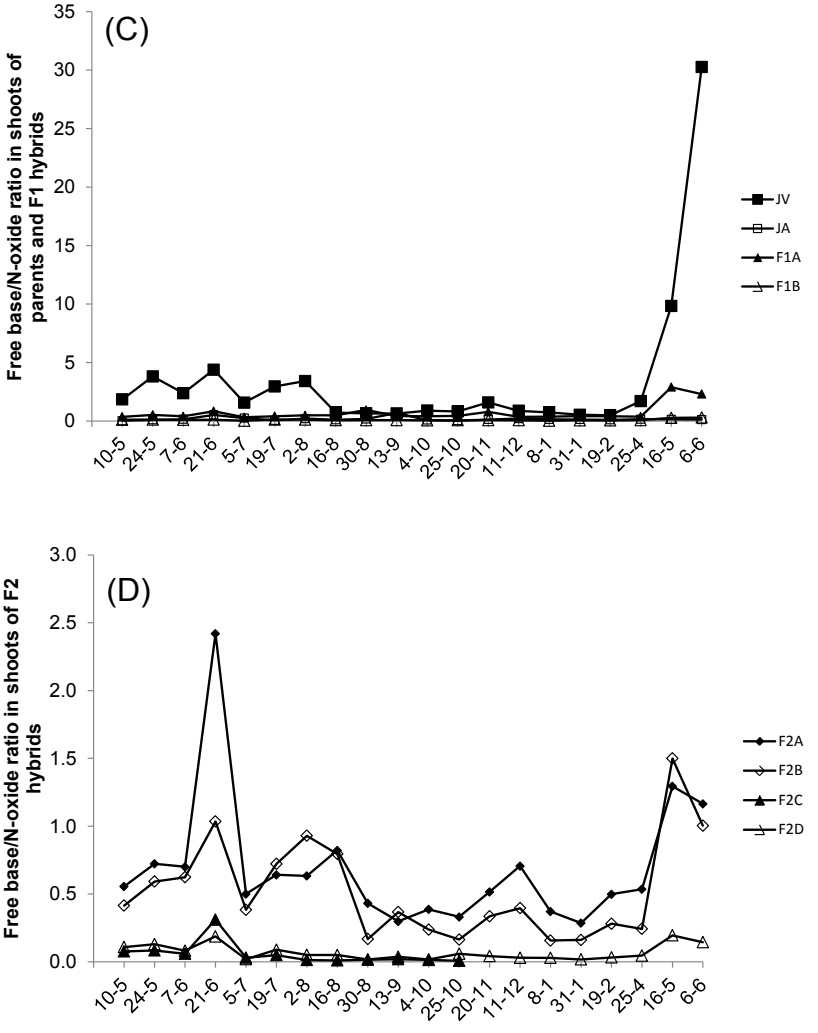


Fig. S9 The free base/N-oxide ratio in roots and shoots in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates.