Seasonal variation in defence compounds: a case study on pyrrolizidine alkaloids of clones of Jacobaea vulgaris, Jacobaea aquatica and their hybrids

Wei, X.; Klinkhamer, P.G.L.; Mulder, P.P.J.; Veen, C.A.M. van der; Vrieling, K.

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Seasonal variation in defence compounds: A case study on pyrrolizidine alkaloids of clones of *Jacobaea vulgaris*, *Jacobaea aquatica* and their hybrids

Xianqin Wei [a,b,*], Peter G.L. Klinkhamer [b], Patrick P.J. Mulder [c], Karin van der Veen-van Wijk [b], Klaas Vrieling [b]

[a] College of Life Sciences, Nankai University, 94 Weijin Road, Tianjin, 300071, China
[b] Plant Cluster, Institute of Biology, Leiden University, Sylviusweg 72, P. O. Box 9505, 2300 RA, Leiden, the Netherlands
[c] Wageningen Food Safety Research-Wageningen University & Research, Akkermaalsbos 2, P.O. Box 230, 6700 AE, Wageningen, the Netherlands

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ABSTRACT

Concentration of plant secondary metabolites (SMs) show seasonal variations. However, it is still not well understood how these abiotic and biotic factors influence the seasonal variations of SMs. In addition, it is of interest to know if and how SMs are reallocated to the different plant organs, in particular whether SMs are reallocated to the remaining tissues when biomass is lost, e.g., during winter. Here we used *Jacobaea vulgaris*, *Jacobaea aquatica*, two F1 and four F2 hybrids that differed in their pyrrolizidine alkaloids (PAs) bouquet as a study system. A series of clones of these genotypes were investigated during their vegetative stage spanning 14 months in a semi-natural environment. We found that the total PA concentration in roots and shoots showed a gradual increase until the spring of the second year, whereafter it dropped substantially in shoots. The variation in PA composition due to seasonal changes was significant but relatively small. Senecionine-like PAs were the dominant PAs in roots, while jacobine-/erucifoline-like PAs were dominant in shoots. The variation of PA concentration was significantly correlated with temperature, day length, and plant age. A correlation analysis showed that PAs were not reallocated when biomass was lost in winter. Overall, our study showed that PA composition of each genotype changed over seasons in a different manner but seasonal variation did not overrule the differences in PA composition among genotypes.

1. Introduction

Plants produce a staggering diversity of secondary metabolites (SMs) [1,2]. SMs are important to plants for coping with biotic and abiotic stressors [3–5]. SMs are generally thought to play an important role in the protection of plants against herbivores and pathogens, and they support plant growth and primary metabolites [6,7]. For a single group of SMs, the concentration and composition may change between developmental stages, tissues and organs, seasons and years [8–11]. Higher concentrations of SMs can result in more resistant plants, however, the production of SMs is thought to be costly [12]. Such costs can reduce plant growth and reproduction [13,14]. Hence plants face a dilemma: to grow or to defend. On the one hand, they must grow fast enough to compete with neighbouring plants, and on the other hand, they must produce enough SMs to combat potential herbivores and pathogens [15,16]. Additionally, by producing higher concentrations of SMs a plant can become more attractive to specialist herbivores that can cope with these SMs and often use SMs as feeding stimulants (Generalist - Specialist dilemma) [17,18]. As a consequence, considerable genotypic variation is observed in composition and concentration of SMs between genotypes within a plant species [19–21].

While the production of most SMs is at least in part constitutive, concentration and composition often depend on many abiotic factors, such as temperature, humidity, light and drought [22,23]. High temperature or drought conditions can cause a significant increase in the accumulation of SMs such as flavonoids and quinolizidine alkaloids [24,25]. However, Bhatia, et al. [26] found that a low temperature can induce flavonol synthesis and lead to enhanced flavonoid accumulation in *Arabidopsis thaliana*. Additionally, UV-B light can increase the concentration of glucosinolates and phenolic compounds [27,28]. Besides abiotic factors SM concentration and composition are known to be influenced by biotic stresses. For instance, attack by the specialist herbivore *Manduca sexta* lead to a reduced nicotine accumulation in *Nicotiana attenuata* [29]. Herbivore damage to glucosinolate-containing

* Corresponding author at: College of Life Sciences, Nankai University, 94 Weijin Road, Tianjin, 300071, China.
E-mail address: weixq@nankai.edu.cn (X. Wei).

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plants led even to a 20-fold increased accumulation of indole glucosinolates [30]. Incubation with bacteria and fungi under field conditions led to qualitative and quantitative modifications of SMs in maize roots [31]. Therefore, both the biotic and abiotic factors influence metabolic activity of plants and play an important role in the variation of SMs [32]. Due to the seasonality of abiotic and biotic factors, the type and level of plant defences are expected to depend on the seasons as well [33–35]. Unfortunately, in many studies the concentration and composition of SMs are often only measured once and only for one season. Moreover, it is still unclear how PAs respond to the complex environmental factors in nature and how the PAs vary with the seasons.

As plants develop from seeds to seedlings, juveniles and mature stages, their ontogeny can affect the interaction with herbivores, resource allocation and plant defence [36–38]. Different ontogenetic stages usually occur in a specific season, so plant ontogeny to some extent can reflect the level of plant defences [39]. In winter, low temperatures decrease the biomass of stems and leaves and increase root biomass [40]. Therefore, the shoot/root ratio is expected to decrease in winter for the biannual and perennial plants. To avoid the simultaneous loss of SMs with loss of biomass, the plants are assumed to reallocate SMs and prevent their loss during winter [12,41]. Unfortunately, there is little experimental evidence to conclude whether the SMs disappear together with the decaying tissues, or that they are transported to the remaining tissues.

To study the genotype dependent change of SMs over seasons we used pyrrolizidine alkaloids (PAs) of *Jacobaea* plants as a study system. PAs are present in several plant families, such as the Apocynaceae, Asteraceae, Boraginaceae, Convolvulaceae, Leguminosae and Orchidaceae [42–47]. In *Jacobaea* (Asteraceae) species, they are synthesized in the roots, and translocated as N-oxides from the roots to the shoots, where further PA diversification takes place [48]. The shoots are especially essential for PA diversification of jacobine-like PAs [49]. PA concentration and composition showed a high heritability [50]. Cheng, et al. [51] found that PA concentration and composition were strongly genotype-dependent under climate room conditions in six-week-old plants (humidity 70 %; light 16 h at 20 °C; dark 8 h at 20 °C). However, it is unclear whether the genotype-dependent effect of PAs is still present in a complex natural environment over seasons.

In this study, we studied clones of two *Jacobaea* species, as well as clones from two F1 and four F2 hybrid genotypes resulting from a cross between *Jacobaea vulgaris* and *J. aquatica*. *Jacobaea vulgaris* is a monocarpic perennial [52,53]. 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 [54,55]. *Jacobaea aquatica* is a biannual that is phylogenetically closely related to *J. vulgaris* but ecologically and chemically the two species show distinct differences [51,56]. *Jacobaea vulgaris* has a higher proportion of jacobine-like PAs and grows in dry, sandy soils while *J. aquatica* has a higher proportion of senecionine-like PAs and grows in marsh environments [51]. These two species are phylogenetically close and populations of natural hybrids do occur [56,57]. These genotypes used here cloned to produce genetically identical individuals for each genotype. Therefore, the distinct PA composition among genotypes and identical genetic background among the clones within each genotype provide an opportunity to evaluate the relative contribution of environment and genetics to the PA variations over seasons. We tested whether the seasonal variations in PA concentration and composition can override the genetic differences among genotypes.

PAs can be present as tertiary amine (free base) or as N-oxide. Most PAs predominantly occur as N-oxides but the jacobine-like PAs can also be present in large quantities as free bases. 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 (Fig. S1) [51]. Polyphagous herbivores are generally deterred by the different groups of PAs while the specialist herbivores are attracted by the different groups of PAs [58–61]. The occurrence of different groups of PAs facilitated us to study the variation of PA compositions.

In this study we tracked PA variation over the course of the vegetative growth (14 months) of *Jacobaea* species and their hybrids. 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. We addressed the following questions: 1) Do the seasonal dynamics of PA concentration and composition differ among the eight genotypes? 2) Are differences in PA concentration and composition among genotypes maintained over the seasons? 3) Does the division of PA concentration and composition of roots and shoots differ over the seasons? 4) Are the abiotic factors, such as temperature, precipitation and day length, related to the seasonal variation of PA concentration and composition? 5) Are PAs reallocated to the remaining tissues when the biomass is lost?

2. Materials and methods

2.1. Study system

Jacobaea vulgaris subs. *dunensis* was derived from a seed collected at the Meijendel Nature Reserve (52°7′54″N, 4°19′46″E, The Netherlands), and *J. aquatica* subs. *aquatica* was derived from a seed collected at the Zwanenwater Reserve (52°48′38″N, 4°41′7″E, The Netherlands). The two parents, two F1 and four F2 hybrids of these two species are maintained in our lab in tissue culture and can thus easily be proliferated to produce replicates of the same genotype. The cross was documented in detail by Cheng, et al. [51]. 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 crossed with each other to produce the F2 offspring.

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), 2 F1 hybrids (F1A, F1B) and 4 F2 hybrids (F2A, F2B, F2C, F2D). We chose this system because the concentration of jacobine-like PAs had clear differences between genotypes. The four F2 genotypes represented three levels of jacobine-like PAs in the shoots: F2A and F2B (medium), F2C (low) and F2D (high).

2.2. 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 with soil 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, the weeds and larvae of the cinnabar moths were removed manually. Each plant was numbered and they were randomly planted with a distance of 30 cm from each other. Plants were watered only during planting.

2.3. Sample collection

We started to harvest plants on May 10, 2012. Two randomly chosen plants per genotype were harvested every two weeks from May to September and every three weeks from October to June of the next year. Only the living tissues were harvested and the shed or decaying leaves were not included. Because of a period with frost resulting in a frozen soil, no plants were harvested on March 14, 2013 and due to a broken freeze-dryer, we have no data for the harvest of April 5, 2013. Each

harvest included 16 plants (2 individuals × 8 genotypes). The diameter of the shoot was measured by a ruler. Then 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 an ice box. Plants were gently washed with autoclaved water, separated into roots and shoots with scissors and carefully dried with paper tissues. After measuring the fresh weight of the plants, samples were subsequently freeze dried for four 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 for each plant were measured. In total on 20 occasions 607 samples were prepared, including 304 root samples and 303 shoot samples (one shoot sample of JA harvested on January 8, 2013 was lost). Due to the fact that most plants of genotype F2C started flowering from August 2, 2012, and no rosette plants were left after November 20, 2012, there are only 12 harvests for this genotype. To account for potential diurnal variations in PA accumulation plants were harvested in the morning between 9 and 11 AM.

2.4. Pyrrolizidine alkaloid extraction and analysis

PA analyses were carried out using liquid chromatography-tandem mass spectrometry (LC–MS/MS). The protocol was described in detail by Cheng, et al. [51]. 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). Chromatographic separation was achieved on a Waters Acquity BEH C18 150 × 2.1 mm, 1.7 μm UPLC column, run with a water/acetonitrile linear gradient containing 6.5 mM ammonia at a flow of 0.4 mL min^{-1}. The linear gradient started at 0% acetonitrile and in 12 min the percentage was raised to 50 %. The column was kept at 50 °C and the injection volume was 5 μL. The MS system was operated in positive electrospray mode. Data were recorded in multiple monitoring mode (MRM) using two selected precursor ions to product ion transitions per compound. All the PAs identified in this study were listed in Table S1. Cone energy was 40 V and collision energy settings were optimized for the individual compounds as presented in Table S1.

Data were processed in Masslynx 4.1 software (Waters Corporation, Milford, MA, USA). No data deconvolution was applied nor a threshold for minimum peak area was defined, however data were smoothed (SG, 2 × 1) and checked for peak integration and r.t. compliance (±0.03 min). PAs were qualified against a set of calibration samples of PA reference standards (corresponding to a concentration range of 0–200 ng/mL in plant extracts). PAs for which no authentic standard was available were (semi)quantified using an isomeric reference standard as indicated in Table S1. Calculations on relative concentrations were made in Excel.

2.5. Abiotic parameters

A dataset of the daily temperature, precipitation, and day length (the hours between sunrise and sunset) from the most nearby weather station (Valkenburg Naval Air Base) was obtained from the website of the Royal Netherlands Meteorological Institute (KNMI). The weather station is located at circa 14 km from the experimental field. Temperature, precipitation and day length used in our analysis was calculated by averaging the daily data of 14 days prior to the harvest day (Fig. S2).

2.6. Statistical analysis

The parameters used are calculated as follows.

$$PA\ amount = (total\ PA\ concentration) * (dry\ mass) \quad (1)$$

$$The\ relative\ concentration\ of\ a\ particular\ group\ of\ PAs\ (\%) = \frac{PA\ concentration\ of\ a\ particular\ group}{the\ total\ PA\ concentration\ in\ the\ plant} * 100\% \quad (2)$$

$$SR\ ratio = \frac{the\ dry\ mass\ of\ shoots}{the\ dry\ mass\ of\ roots} \quad (3)$$

The PA amounts referred to individual plants. Two-way ANOVAs were performed to evaluate the effects of genotype (random factor) and harvest dates (fixed factor) on total PA concentration, PA amount, dry mass and SR ratio. The PA concentration, PA amount and dry mass were log transformed to achieve a normal distribution. SR ratio was arcsine square root transformed. All analysis were also carried out on the four groups of PAs: senecionine-, jacobine-, erucifoline- and otosenine-like PAs [51,62].

To test the effects of genotypes on the variation of PAs, principal component analysis (PCA) was conducted in SIMCA 14.1. The relative concentration of four groups of PAs were set as primary variable.

Dry mass, fresh weight and the diameter of shoots were used in a factor reduction analysis in SPSS 24.0. The first component representing 83.4 % of the total variation was used as a measure of plant size. To obtain positive values, the values of the first axis were transformed as $(\text{plant size}+1)^2$ and used in a linear regression analysis. The number of days after planting was used as a measure of plant age, and the first planting day on April 12, 2012 was set as day 1. General linear models were performed to test the relationship between PA concentration and temperature, day length, precipitation, plant age and plant size.

To test if there were PA reallocations from the lost tissue to the remaining tissue, we conducted linear regressions with the total amount of PAs of the whole plant at time T divided by the total amount of PAs of the whole plant at time T-1 (PA_T/PA_{T-1}) as dependent variable and the dry mass of whole plant at time T divided the dry mass of the whole plant at time T-1 (DM_T/DM_{T-1}) as independent variable. Dry mass and PA amount were averaged at each harvest for each genotype. DM_T/DM_{T-1} and PA_T/PA_{T-1} were Napierian logarithm (ln) transformed to obtain equal scales for the ratios above and below 1. If points are above $y = x$ and $DM_T/DM_{T-1}<1$, it indicates that with decreasing biomass from T-1 to T, the total amount of PAs decreased less than biomass, which suggests that PAs are reallocated.

Except for PCA, all the other analyses were performed in SPSS 24.0.

3. Results

3.1. Genotypic dry mass and PA variation between genotypes

3.1.1. Dry mass of the plants and shoot to root ratio

Dry mass differed among genotypes and harvest dates (Table 1). Average shoot dry mass increased over the season and peaked in early October, then decreased until February and increased again during the next spring (Fig. 1a). 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 and February, almost 70 % of the dry mass of shoots and roots was lost. The pattern of seasonal variation was similar between genotypes (Fig. S3).

The mean SR ratio of all genotypes was the highest in mid-summer and then decreased gradually until mid-winter, after which in spring it started to increase again (Fig. S4). Although the variation in SR ratio among harvests was the strongest in the F2 hybrids (Fig. S4), all genotypes showed a 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 and four groups of PAs as dependent variables and with harvest dates and genotypes as factors.

			Root		Shoot	
Dependent variables	Source of 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
Senecionine-like PAs	Date	19	5.399	<0.001	21.226	<0.001
	Genotype	7	20.285	<0.001	48.245	<0.001
	Date*Genotype	125	2.701	<0.001	1.930	<0.001
Jacobine-like PAs	Date	19	9.697	<0.001	3.951	<0.001
	Genotype	7	249.037	<0.001	412.167	<0.001
	Date*Genotype	125	1.428	0.018	1.909	<0.001
Erucifoline-like PAs	Date	19	8.237	<0.001	26.309	<0.001
	Genotype	7	18.006	<0.001	16.455	<0.001
	Date*Genotype	125	1.702	0.001	1.949	<0.001
Otosenine-like PAs	Date	19	7.782	<0.001	12.047	<0.001
	Genotype	7	116.382	<0.001	264.639	<0.001
	Date*Genotype	125	1.369	0.032	1.314	<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 obviously cannot be calculated separately for root and shoot.

3.1.2. PA variation between genotypes

Genotypes differed significantly in total PA amount, total PA concentration, PA concentration of each group of PAs and free base/N-oxide ratio. Except for the free base/N-oxide ratio of shoots all these variables significantly differed over the seasons and genotypes (Table 1).

The first two components of the PCA explained approximately 70 % of the relative concentrations of four groups of PAs in both roots and shoots (Fig. 2). It also showed that the most genotypes clearly differ from each other in PA composition during the seasons in both shoots and roots (Fig. 2). JA and F2C separated from the other genotypes based on the PA composition of the shoots (Fig. 2b). The PCA result was consistent with our initial selection for the eight genotypes. Although there were small fluctuations during the vegetative stage, the dominant PA in each genotype was constant (Fig. S5). Genotypes high in jacobine-like PAs always maintained a higher concentration than genotypes low in jacobine-like PAs during the 14 month period of vegetative growth in the field (Fig. S5).

3.2. PA variations over seasons

3.2.1. PA concentration

The pattern of seasonal variation in total PA concentration was similar for all genotypes (Fig. S5). We used the mean PA concentration over all genotypes to show the seasonal pattern. The mean total PA concentration increased gradually in both shoots and roots until the spring of the second year (just before the initiation of flowering), when it dropped abruptly by 53 % in the shoots (Post Hoc Tests, Tukey HSD, $P < 0.001$) and by 19 % in roots (Post Hoc Tests, Tukey HSD, $P > 0.05$) (Fig. 3a). The total PA concentration had a peak for all genotypes in winter, especially in shoots (Fig. 3a). For instance, the total PA concentration in winter (31 January) was approximately eight times higher than that in summer (5 July) in the shoots of JA (Fig. S5).

PA concentrations in roots were higher than that in shoots (Fig. 3b). The ratio of PA concentration of roots and shoots peaked in July with total PA concentration being almost seven times higher in roots than in shoots (Fig. 3b) while the ratio dropped to almost one during winter (Fig. 3b). The variation in the ratio of PA concentration of roots and shoots were similar for all genotypes (Fig. S6).

The concentration of senecionine-like PAs in shoots started to increase from October onwards and reached its peak in winter, but decreased to its initial level again in the next spring (Fig. S7a). The senecionine-like PA concentration in roots was constant over all seasons and was about 1.5–7 times higher than in the shoots. The concentration of jacobine-like PAs in shoots increased until August and then remained at similar levels until the end of the vegetative stage. In the roots it increased gradually over the seasons. At the start the shoots contained a higher concentration of jacobine-like PAs than the roots, but over the seasons the roots gradually accumulated a higher concentration (Fig. S7b). The concentrations of erucifoline-like PAs were approximately 2.6 times higher in the shoots than in the roots in spring, then the ratio increased to approximately 20-fold in winter. The concentration of erucifoline-like PAs for both shoots and roots peaked in mid-winter and then started to drop in the next spring (Fig. S7c). The concentrations of otosenine-like PAs in general were slightly higher in the roots than in the shoots. Concentrations in the shoots peaked in April and then started to decrease, while it remained constant in the roots (Fig. S7d).

3.2.2. PA composition

For the eight genotypes combined, in roots the highest relative concentration was that of the senecionine-like PAs. In the roots, over the course of time, the relative concentration of senecionine-like PAs gradually decreased while that of jacobine- and otosenine-like PAs increased (Fig. 4a). In shoots jacobine-like PAs were the dominant PAs, except in winter, when the relative concentration of senecionine-like PAs increased (Fig. 4b). The relative concentration of erucifoline-like PAs was very low in roots while it was around 20 % in shoots except for the second spring when its share decreased to 10 % (Fig. 4).

In the roots, the relative concentration of senecionine-like PAs decreased while that of jacobine-like PAs increased gradually over the seasons except in genotypes JA and F2C (Fig. S8), where senecionine-

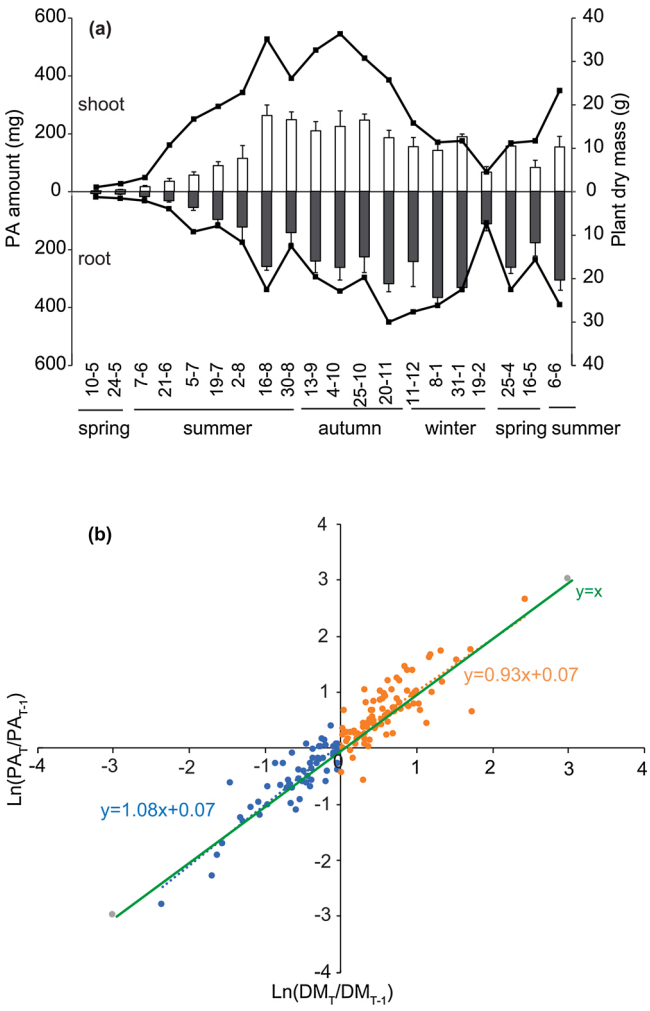

Fig. 1. (a) The mean pyrrolizidine alkaloid (PA) amount (±SE, mg) and plant dry mass (g) of eight *Jacobaea* genotypes at different harvest dates. The line and bar graphs above x-axis represent the shoot data, and those below x-axis represent the root data. The square dots in the lines represent the dry mass averaged over 16 plants of eight genotypes and bar graphs represent the PA amount averaged over 16 plants of eight genotypes. (b) The linear regression between ln (PA_T/PA_{T-1}) and ln (DM_T/DM_{T-1}) at the whole plant level (n = 144). The dependent variable ln (PA_T/PA_{T-1}) represent the amount of PAs at time T relative to that time T-1, and the independent variable ln (DM_T/DM_{T-1}) represent the dry mass at time T relative to that of time T-1. The blue dots (ln (DM_T/DM_{T-1})<0) indicate a decrease in plant dry mass ($r = 0.604$, $n = 67$, $P < 0.01$) and the orange dots (ln (DM_T/DM_{T-1})>0) indicate an increase in plant dry mass ($r = 0.879$, $n = 77$, $P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

like PAs comprised over 80 % of the total content during the whole study. The roots of genotypes F2A and F2B, were relatively rich in otosenine-like PAs and their proportion increased gradually over time (Fig. S8). In the shoots, jacobine-like PAs were the major type of PAs except in genotypes 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 proportion was smallest in winter, favouring senecionine- or erucifoline-like PAs (Fig. S8).

3.3. Abiotic factors affecting PA concentration

The mean of the total PA concentration in roots and shoots averaged over all eight genotypes was negatively correlated with temperature (Fig. 5a) and day length (Fig. 5b) for both shoots and roots. The effect of temperature was stronger for shoots than for roots (General linear model, $P = 0.031$) while there was no significant difference between roots and shoots in the effect of day length (General linear model, $P = 0.074$). With the increase of plant age (days after planting), the mean total PA concentration increased significantly in roots and shoots (Fig. 5c). No significant correlation was found between precipitation and the mean of the total PA concentration in both roots and shoots (Fig. 5d). Likewise, no significant correlation was found between plant size and the mean of the total PA concentration in roots and shoots (Fig. 5e).

3.4. Reallocation of PAs

Averaged over all eight genotypes the total amount of PAs in the plants increased until late summer (August) (Fig. 1a). In the shoots the PA content remained constant until November, and then started to decrease during the winter period (Fig. 1a). By February 70 % of the PAs stored in the shoots had been lost while 80 % of the dry mass was lost. In the roots the total amount of PAs remained rather constant until the end of January, but a sharp decline occurred in February when there was also a sharp loss of root biomass. In total, plants lost approximately 70 % of stored PAs in roots during the winter period and 76 % of the dry mass was lost (Fig. 1a). The most dramatic loss of PAs occurred in genotypes of JV, JA and F1B (Fig. S9).

In Fig. 1b, the 88 orange dots (ln $DM_T/DM_{T-1}>1$) indicated an increase of biomass while the 56 blue dots (ln $DM_T/DM_{T-1}<1$) indicated a decrease of biomass from time T-1 to time T. The ratio DM_T/DM_{T-1} increased with PA_T/PA_{T-1} for both growing plants (Linear regression, $y = 0.93x+0.07$, $R^2 = 0.63$) and plants that decreased in dry mass (Linear regression, $y = 1.08x+0.07$, $R^2 = 0.78$) (Fig. 1b). To check if there is PA reallocation when biomass is lost, attention should be paid to the blue dots. For plants that decreased in biomass (ln $DM_T/DM_{T-1}<1$), the number of points above the line (30) was not significantly different from that below the line (26) (Chi-square = 0.023, df = 1, $P > 0.05$), indicating that the loss in dry mass was approximately equivalent to the loss of PA amount in that time interval. This suggests that PAs were lost when the leaves and roots were shed, and that no PA reallocation occurred.

4. Discussion

In this study, we investigated the seasonal variations of PAs of eight genotypes of *Jacobaea* plants with 20 harvests spanning 14 months, and found that the differences between genotypes are maintained during the vegetative stage while within each genotype there was seasonal fluctuation of PAs.

4.1. Genotypic PA variation

The accumulation of SMs is affected by the abiotic environment [63, 64]. Many studies focused on the effect of environmental factors, such as light [65], temperature [24], water and salinity [23,66]. It is also known that the expression of SMs is controlled by specific genes [67]. A series of genotypes in our study system provided a good opportunity to compare the two factors. In a study encompassing 14 months, we found that there was seasonal fluctuation within a genotype, whereas the differences in PA composition between genotypes were still maintained. For instance, during the whole period of vegetative growth in *J. vulgaris* jacobine-like PAs are dominant and in *J. aquatica* are senecionine-like PAs (Fig. S5). Therefore, it can be concluded that the environmental factors had a significant effect on PA, but still genetic difference of PAs were maintained.

4.2. PA variation over seasons

Several studies document that the accumulation of plant SMs varies substantially across seasons [68–71]. However, different defense

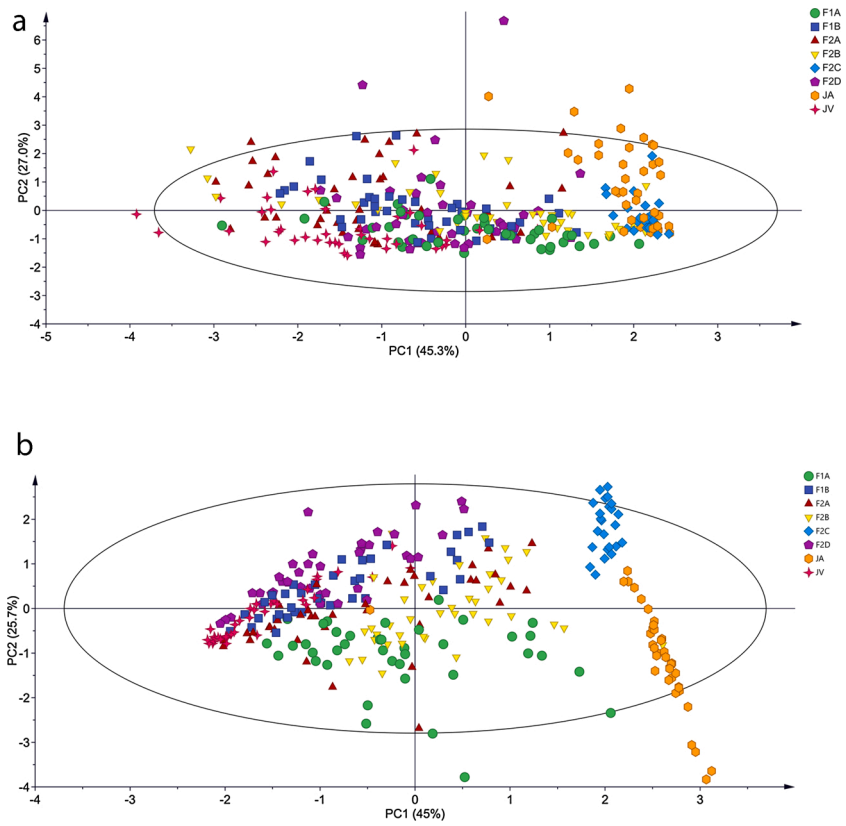

Fig. 2. Principle component analysis (PCA) based on the relative concentration of four groups of PAs in roots (a) (n = 304) and shoots (b) (n = 303) of eight genotypes sampled over 14 months growing period in vegetative plants of *Jacobaea*. JA = *Jacobaea aquatica*, JV = *Jacobaea vulgaris*, F1A-B = first generation offspring of JV and JA, F2A-D = F2 hybrids of JV and JA.

metabolites display different seasonal patterns. For example, Gols, et al. [34] showed that aliphatic glucosinolates in the cabbage leaves gradually increased over the growing season, while indole glucosinolates rapidly increased until mid-summer and then decreased or stabilized. Solar, et al. [72] reported that the concentration of flavonoids in the shoot of common walnut increased from the spring to the summer, while phenolic acids showed an opposite pattern, with highest concentrations in spring and lowest concentrations in the summer. In walnut the maximum amount of vasicine was found in August in both leaves and roots whereas deoxyvasicinone reached its peak in December and January in roots and in November in the leaves [73]. In our study, the PA concentration increased gradually until winter and decreased in the second spring. Hussain, et al. [74] also found that the winter is the best season for polyphenol and flavonoid content. The occurrence of SM peak thus might depend on plant character (woody/herbaceous) or life history of plants (annual/biennial/perennial), or environmental factors as well.

Hama and Strobel [75] investigated the PA variation in *J. vulgaris* whole plants, collected from a field in Denmark and found that plant tissues had around 1000 times higher PA concentrations during mid-summer compared with winter. The high amounts of PA content is explained by flowering of the plants. Flowerheads do contain much higher concentrations than leaves. This increase in summer reflects the different phenological stage and therefore cannot directly be compared with our study that compares vegetative plants of the same genotypes only.

Flade, et al. [76] investigated the occurrence of PAs depending on the developmental stage and seasons in *S. vulgaris* and found the total PA concentrations remained nearly unchanged. To be noted that only nine PAs were detected in their study, and all of them belong to the senecionine-like PA group. Thus the total PA concentration of their study is equal to the concentration of senecionine-like PAs of this study. Additionally, we obtained more than 40 PAs in *Jacobaea* plants. Moreover, in *J. vulgaris* the dominant PAs are jacobine-like PAs, while it was not reported in their study.

4.3. Variation of PA groups over seasons

Senecionine-, jacobine- and erucifoline-like PAs were the main three groups making up approximately 90 % of the total PAs. Over the seasons the senecionine-like PAs were the dominant PAs for all eight genotypes. This is in line with the fact that senecionine N-oxide is the primary product of PA biosynthesis in *Jacobaea* and *Senecio* species [77]. Senecionine N-oxides are then converted to other PA types in the shoots by dehydrogenation, epoxidation, hydroxylation, acetylation or cis/trans isomerization [62,78]. Regardless of the concentration (Fig. S5) and the relative concentration (Fig. S8), senecionine-like PAs increased in winter in both roots and shoots.

Except for JA, the dominant PAs in shoots were jacobine- or erucifoline-like PAs, which have been suggested as the most toxic PAs to generalist herbivores [60,79,80]. The plants with high jacobine-like PAs are generally favored by specialist herbivores. For instance, *T. jacobaeae* laid more eggs on the plants that contained higher concentrations of the free base form of jacobine-like PAs [58]. Genotypes of *Jacobaea* species with higher jacobine concentration were more severely attacked by specialist herbivores [81]. However, several lines of evidence suggest that *Jacobaea* plants increase their erucifoline concentration after attack by generalist herbivores.

Hol, et al. [82] showed that the relative concentration of erucifoline increased while that of jacobine decreased in shoots after *J. vulgaris* shoots were damaged by *Mamestra brassicae*. Treatment of *Jacobaea* plants with MeJA led to an increase of the erucifoline concentration and a decrease of the senecionine concentration [62]. On one hand such an increase in erucifoline is surprising considering the fact that a number of

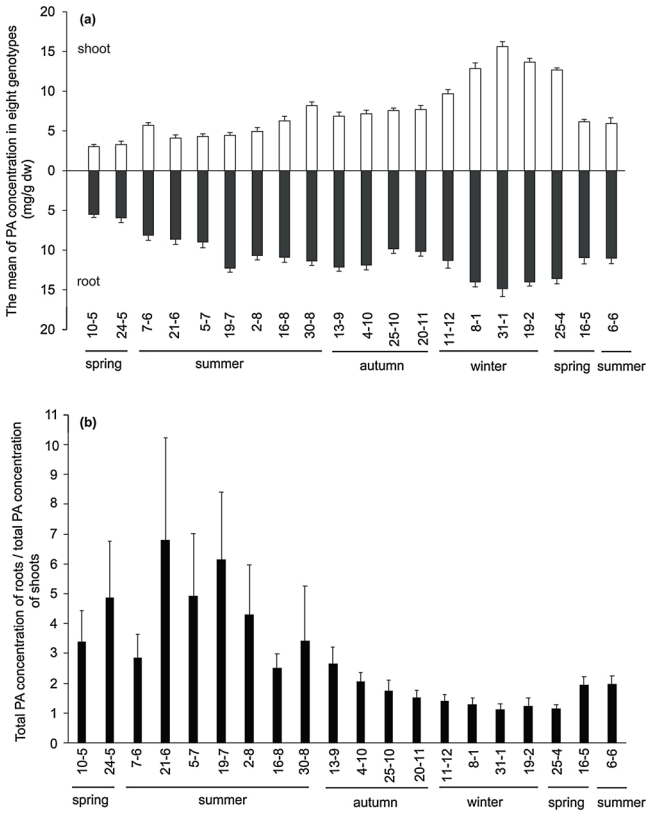

Fig. 3. (a) The mean pyrrolizidine alkaloid (PA) concentration (±SE, mg/g dw) of eight *Jacobaea* genotypes at different harvest dates for shoots (n = 303) and roots (n = 304). (b) The mean of ratio of pyrrolizidine alkaloid (PA) concentration of roots and shoots (±SE, mg/g dw) of eight *Jacobaea* genotypes at different harvest dates. Bars indicate the standard errors. n = 303.

studies suggested that jacobine-like PAs were more effective in protecting the plant against generalist herbivore [83]. 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 [81]. Consistent with their finding, we also found that the erucifoline-like PAs increased during summer and autumn, the period the specialist herbivore *T. jacobaeae* is most active [84]. It suggests that the plants increased the erucifoline-like PAs to prevent damage by specialist herbivores. Among the four groups of PAs, the jacobine-like PAs maintained a relative constant and high level over all seasons (Fig. S7).

4.4. Abiotic factors affecting PA variation

Kumar, et al. [85] showed that seasonal temperature changes played a key role in secondary metabolite variation. Different SMs display different patterns in relation to temperature. In the root of *Taraxacum officinale*, the concentration of two 4-hydroxyphenylacetic acid side groups (Di-PIEs), phenolic inositol esters with three 4-hydroxyphenylacetic acid side groups (Tri-PIEs) and the sesquiterpene lactone taraxinic acid β-D glucopyranosyl ester (TA-G) were positively correlated with temperature [32]. A significant negative correlation between the essential oil concentration and temperature was found in *Thymus pulegioides* [86]. In our study, the total PA concentration was also negatively correlated with temperature. The relatively low concentrations in *Jacobaea* in summer are therefore rather surprising because at that period most herbivores are present. However, if specialist herbivores are attracted to PAs lowering PA concentration might be a strategy to escape by the specialist while at the same time becoming more vulnerable to generalist herbivores namely the “Generalist-specialist Dilemma” [18].

We did not find a significant correlation between the precipitation and the total PA concentration. Similar to our study, Huang, et al. [32] did not find significant correlations either between total metabolites, metabolite classes (Di-PIEs, Tri-PIEs and TA-G) and precipitation in the roots of *Taraxacum officinale*. However, it was documented that drought can induce the increase of SMs, such as in *Glycine max* L. [87], *Zea mays* [88] and *Brassica oleracea* L. [89]. We compared the precipitation between summer and winter in our investigated period, and there was no significant difference (Mann-Whitney test, $P > 0.05$). Additionally, due to the interactive effect of precipitation and temperature [90], here we cannot give a firm conclusion about the effect of precipitation on PA variation.

4.5. PA variation and plant ontogeny

In the last four harvests, there was a decline in the PA concentration, which might mark the onset of flowering. After the last vegetative harvest, plants will reach the reproductive age, flowers and fruits can demand resources for their production and for their defence that were previously stored or might otherwise be allocated to the production of shoot or root biomass. Such a decline was also found in the common dandelion *T.officinale* [32]. Diezel, et al. [91] found that jasmonate and ethylene bursts induced by oral secretion decline with the initiation of flowering in *N. attenuata* plants, and that this loss can rapidly be reset within one day by removing the inflorescence of the plant. Since jasmonate and ethylene pathways are involved in the production of SMs, the flowering in *N. attenuata* decreased the production of SMs, indicating that ontogenetic transition may affect the resource allocation.

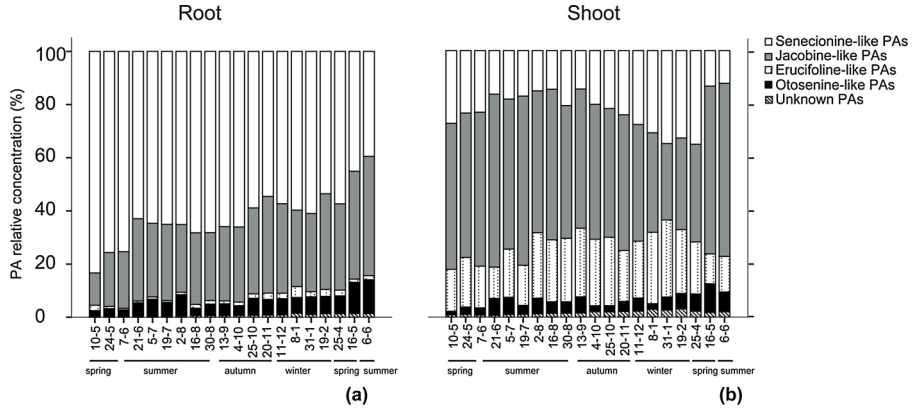

Fig. 4. The relative concentration of different groups of pyrrolizidine alkaloids (PAs, %) mean for eight *Jacobaea* genotypes at different harvest dates in roots (a) (n = 304) and shoots (b) (n = 303).

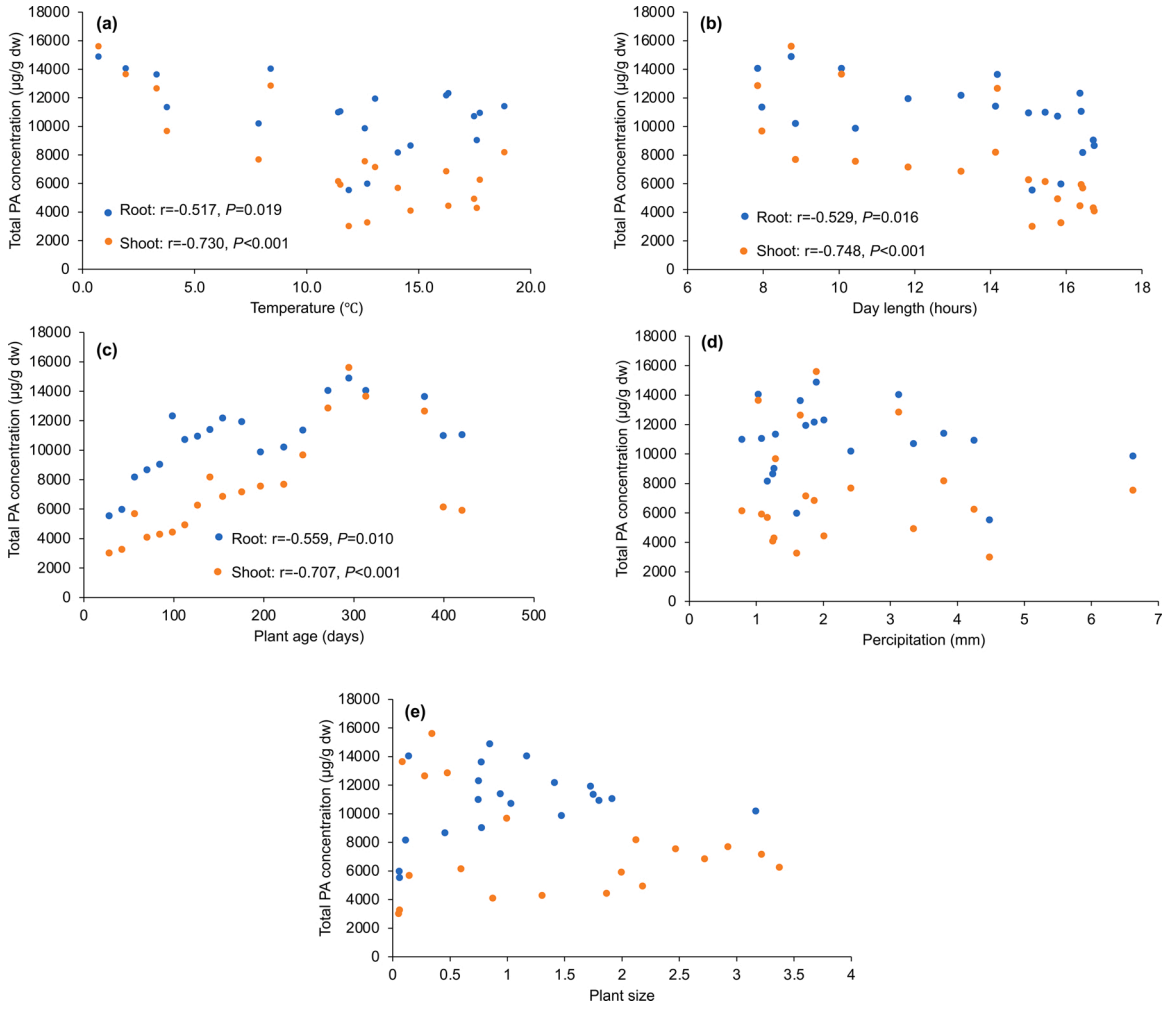

Fig. 5. Mean total pyrrolizidine alkaloid (PA) concentration (mg/g dw) of eight *Jacobaea* genotypes plotted against different abiotic factors in roots and shoots. (a) Temperature (°C), (b) Day length (hours), (c) Plant age (days), (d) Precipitation (mm), (e) An estimate for plant size. The temperature, day length and precipitation are the mean of the data of 14 days previous to the harvest day. Plant age was obtained by counting the days after plantation in the field. Plant size including dry mass, fresh weight and diameter were analysed by PCA. PC1 explained 83.4 % of the total variation and PC1 was used in this analysis as an estimator of plant size. Blue dots indicate root samples (n = 20), and orange dots indicate shoot samples (n = 20). One dot represents the mean of total PA concentration of eight genotypes for each harvest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4.6. Reallocation of PAs

Hartmann and Dierich [78] found that the turnover of PAs is very low or absent in *Senecio vernalis*. Based on the correlation analysis, we found that PAs might be directly lost when plant biomass decreased in *Jacobaea* plants, instead of reallocation to the remaining tissues (Fig. 1b). Interestingly the PA concentration reached its maximum during winter and the main group of PAs contributing to the increase were senecionine-like PAs (Fig. S5), indicating *de novo* synthesis of PAs [77]. Exogenous application of MeJA significantly improved plant freezing tolerance in *A. thaliana*, while blocking JA biosynthesis and signaling pathways resulted in hypersensitivity to freezing stress [92]. JA-mediated regulation of cold-stress and PA production in *Jacobaea* plants might interact with each other as found in *Arabidopsis*. The low temperature during the winter might activate the JA pathway, which helps the plant to better tolerate cold stress and simultaneously produce more PAs. Therefore, the increase of PAs in winter might be derived from *de novo* synthesis based on the JA pathway. It needs further transcriptome data or molecular experiments to confirm this.

5. Conclusions

In summary, we tracked and analysed the whole vegetative growth and their SMs in eight clonal genotypes of *Jacobaea* plants. PA concentration and composition showed similar seasonal variations among genotypes in the vegetative stage, which were affected by climatic factors and plant ontogeny. However, the effects of environmental factors on seasonal variation of PAs did not override the initial differences of PAs among genotypes. This indicates that the PA variation between genotypes was largely genetically determined. When the plant biomass decreased, PAs were lost simultaneously, indicating that no PA reallocation occurred. For further confirmation of reallocation of PAs transcriptome or physiological experiments are needed. It also remains unclear if the soil rhizosphere microbiota plays a role in affecting the seasonal variation of PAs. Despite all this, our study clearly demonstrated that in nature PAs of *Jacobaea* plants showed seasonal variations associated with environmental and ontogenic factors.

Author contributions

Klaas Vrieling, Peter Klinkhamer and Xianqin Wei conceived and designed the research. Xianqin Wei and Karin van der Veen-van Wijk

conducted the field works and lab experiments. Patrick Mulder did the chemical analysis. All the authors participated in the data analysis and discussions. Xianqin Wei wrote the manuscript. Klaas Vrieling, Peter Klinkhamer and Patrick Mulder revised the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.111067.

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