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Genetic and environmental-based variability in secondary metabolite leaf content of *Adenostyles alliariae* and *A. alpina* (Asteraceae). A test of the resource availability hypothesis

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To test the resource availability hypothesis we compared the leaf content in carbon- and nitrogen-based allelochemicals between heavily and lightly shaded plants of *Adenostyles alliariae* and *A. alpina* (Asteraceae). Both species contain pyrrolizidine alkaloids (PAs) but only *A. alpina* also contains sesquiterpenes in its leaves.
In *A. alliariae* we found no difference in leaf alkaloid content between the two treatments. In *A. alpina* alkaloid content tended to be higher in the heavily shaded treatment. One sesquiterpene, cacalol-trimer, was present in higher concentrations in the heavily shaded leaves, whereas concentrations of the other sesquiterpene, cacalol, were reduced. Under light-(carbon)limiting conditions the resource availability hypothesis predicts an increase in nitrogen-based defenses and a decrease in carbon-based defenses; these predictions were met in *A. alpina* with the exception of the carbon-based cacalol-trimer. Leaf nitrogen content was strongly increased in heavily shaded plants of both species.
We found significant genetic variation in PA content in one out of four populations of *A. alliariae*. For PA content in *A. alpina* we found significant genetic variation in two out of four populations and for cacalol content in three populations. We therefore conclude that selection on allelochemical content is possible in some populations, whereas in other populations evolutionary processes must have fixed the level of allelochemical content in the two species.

The resource availability hypothesis of plant antiherbivore defense, as put forward by Coley et al. (1985), predicts patterns of plant defenses to be shaped by light and nutrients, both in an evolutionary and ecological time scale. Two kinds of tests of the hypothesis are therefore possible. At the plant community level one could investigate whether patterns of defenses correspond with plant nutrition, assuming that the present community state has been shaped by the evolutionarily derived characteristics of its individual species (Bryant et al. 1983, 1989, 1994, Coley et al. 1985, Coley 1993, Shure and Wilson 1993). Or, at the level of individual plants, one could manipulate plant nutrition and monitor subsequent changes in defense chemistry (Bryant et al. 1987, Price et al. 1989, Reichardt et al. 1991, Sagers 1992, Ohnmeiss and Baldwin 1994).

Two fundamental assumptions have to be met in order for this hypothesis to be of explanatory value. The first assumption is that one of the primary functions of secondary plant compounds, their 'raison d'être', is to defend plants from herbivory. Although the arguments for and against this case have been made

repeatedly (Stahl 1888, Heikertinger 1914, Fraenkel 1959, Jermy 1984, Haslam 1994/1995), most researchers in this field nowadays agree that at least an important part of their function is for defense (Rausher 1992). The second assumption is that changes in defense chemistry translate from an ecological scale into an evolutionary scale. Although the direct study of the evolution of a plant community under herbivore pressure is almost impossible, it is possible to investigate whether the prerequisites are met for evolutionary change to occur.

Genetically based variation in defense characters is the fundamental prerequisite for evolutionary change to occur in a plant population exposed to herbivore selection pressure. Although it seems a truism that plants vary in all kinds of characters, variation has to be confirmed in all characters of interest, since one would expect fixation, and hence loss of genetic variation, for the most important fitness characters, unless opposing selection pressures or trade-offs maintain variation therein (Berenbaum and Zangerl 1992).

Large amounts of phenotypic variation in plant secondary compounds have been described to exist in natural populations (Berenbaum et al. 1986, Hartmann and Zimmer 1986, Cates and Redak 1988, von Borstel et al. 1989), and at least part of this variation has also been found to be additive genetic variation (Berenbaum et al. 1986, Østrem 1987, Zangerl and Berenbaum 1990, Vrieling et al. 1993, van Dam and Vrieling 1994).

The aim of our study was twofold. First we wanted to test the predictions of the resource availability hypothesis by manipulating the light (carbon) supply of the plants. Second we wanted to establish whether there is genetic variability present for the expression of secondary compounds in different plant populations. So we designed an experiment in which we planted clones of genets from different populations of two congeneric plant species in a common garden and exposed them to two different light treatments. Under light-(carbon)limiting conditions, the resource availability hypothesis predicts the expression of higher levels of nitrogen-based defenses (e.g. alkaloids) and lower levels of carbon-based defenses (e.g. sesquiterpenes).

Our experimental species *Adenostyles alliariae* (Gouan) A. Kerner and *A. alpina* (L.) Bluff & Fingerh. (Asteraceae, Senecioneae) are herbaceous alpine perennial plants that often occur sympatrically, with *A. alpina* reaching higher altitudes than *A. alliariae*. They grow typically in moist, nutrient-rich habitats at forest edges or as understory plants in mixed forest stands. Both species contain pyrrolizidine alkaloids and sesquiterpenes of the furoeremophilane type, both of which are characteristic for the plant tribe, to which these plants belong (Toman et al. 1968, Seaman 1982). However, only in *A. alpina* have sesquiterpenes been found in the leaves (Pavlík et al. 1995). Thus leaves of *A. alpina* contain two kinds of secondary compounds, alkaloids which are nitrogen-based and sesquiterpenes which are exclusively carbon-based, and therefore provide us with the ideal opportunity to test the resource availability hypothesis in a plant system where both types of defenses are present simultaneously.

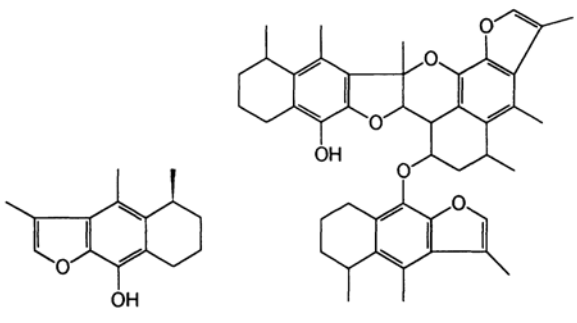
Material and methods

We collected entire plants of *A. alliariae* and *A. alpina* during the growing season in 1993. *A. alliariae* plants came from Col du Lautaret (French Alps near Briançon, 2000 m), Kandersteg (Swiss Alps, Bern 1300 m), Le Hohwald (France, Alsace 600 m), Tschiertschen (Swiss Alps, Graubünden 1800 m), and Weissenstein (Swiss Jura, Solothurn 1200 m). *A. alpina* plants came from Brülisau (Swiss Alps, Appenzell 1100 m), Col du Lautaret, Tschiertschen, Kandersteg, and Wasserfallen (Swiss Jura, Baselland 1200 m). After collection all plants were planted in pots and overwintered in the garden of the Zoological Institut in Basel. In spring 1994 we transferred the pots to the Botanical Garden Brüglingen (Basel). There we partitioned the rhizome of each plant (genet) into four parts, and planted every part (clone) into a plastic pot of 29 cm diameter. As planting substrate we used homogenized natural topsoil from a sheep pasture, which was provided by the Botanical Garden. Although this soil has not been examined for its mineral content, it is safe to assume that there was no shortage of any mineral (L. Dischler pers. comm.). From all five populations of the two plant species we took two clones of five genets and arranged them in a 10×10 square. In this way we prepared two squares arranging all pots randomly within the squares. To reduce edge effects we surrounded the squares by an additional row of potted plants. Then we shaded the squares using green shading nets that provided 35% and 85% shade, respectively. The nets were supported by a wooden scaffold at 1 m height and extended to the ground at the edges of the squares. The squares were situated next to each other on an open field at the botanical garden. We measured the light irradiation on a sunny day with a Minolta Illuminance meter T1. Temperature and relative air humidity were recorded by a Grant squirrel data logger every hour for one week (see Appendix 1).

Seven weeks after shading, we harvested one leaf from each plant. We packed each leaf into a paper bag and transported the bags in cooled boxes to the laboratory where they were frozen, freeze-dried and subsequently stored at room temperature. Then we powdered the leaves with a centrifuge mill (Fritsch pulverisette, 1 mm mesh size) and stored the powder in plastic bags until extraction.

We quantified the sesquiterpenes (STs) cacalol (Cac) and the newly discovered cacalol-trimer (C-Tri, Fig. 1) from the leaf powder. For the extraction of the leaf

powder we weighted 20 mg powder into a centrifuge glass tube and added 5 ml of n-hexane. Then we closed the tube and put it into an ultrasonic bath for 10 min, centrifuged the suspension and filtered the supernatant (Whatman Nr. 1 paper filter on a Büchner funnel, vacuum filtration). After flushing with 2 ml n-hexane the filter was put into the glass tube and the extraction was repeated twice. Then we transferred the combined supernatants into a smaller glass tube and dried them with a vacuum centrifuge (Savant speed-vac concentrator). We stoppered the glass vial and stored it in the refrigerator at 4°C until analysis. For the high performance liquid chromatography (HPLC) analysis we added 200 µl gradient grade MeOH to the vial and put it into an ultrasonic bath for 5 min. Then we filtered the solution with a 0.2 µm nylon membrane filter (Nalgene® No. 176). For compound separation we used a reversed phase column (Tessek, Separon™ SGX C_{18} 7 µm, 4 × 250 mm) on a Varian HPLC system. The mobile phase was acetonitrile (ACN) and water. The elution gradient was: 0–11 min ACN 58%, 11–14 min ACN 58–100% (linear), 14–24 min ACN 100%; flow rate 1 ml/min. 20 µl of solution was injected by the Varian 9100 autosampler and the peak area of Cac was determined from the 215 nm chromatogram with the Varian Star Chromatography (Version 4.0) software. The Cac peak was identified by its retention time and its UV-spectrum. By multiplying the detected absorption units with a calibration factor we calculated the amount of Cac of the sample. We established the calibration line by analyzing known amounts of Cac standard (provided by J. Harmatha, Prague) and regressing the absorption units (AU) against the amount of Cac analyzed ($AU_{Cac} = 644388 \times \mu g$ Cac, $r^2 = 0.998$). We identified C-Tri in the same way as Cac and quantified it from the chromatogram recorded at 285 nm ($AU_{C\text{-}Tri} = 351627 \times \mu g$ C-Tri, $r^2 = 0.997$; standard provided by J. Harmatha, Prague). We verified the integration as well as the UV-spectrum of every chromatogram to ensure the correct quantification and identification of the compounds.



Cacalol Cacalol - Trimer

Fig. 1. Structural formulae of cacalol and cacalol-trimer (J. Harmatha unpubl.), two compounds of *Adenostyles alpina*.

For the pyrrolizidine alkaloid (PA) extraction we weighted 100 mg of leaf powder into glass vials. Then we added 2 ml of 0.5 M H_2SO_4 and gently stirred the suspension with magnetic stirring bars. After 1 h we added excess zinc powder and continued stirring for another 2 h. The extract was then filtered (Whatman No. 1 paper filter on a Büchner funnel, vacuum filtration) into a glass vial. The extraction vial and the Büchner funnel were then flushed with 1.2 ml H_2O. We basified the extract (pH 11) with 0.4 ml 25% ammonium hydroxide. We then transferred the extract on an Extrelut™ column (Merck) and after 20 min we recovered the alkaloids with 2 × 30 ml dichloromethane. We evaporated the dichloromethane and transferred the residues into a 2-ml sample vial, dried the sample again and stored it until analysis. For analysis we redissolved the sample in 1.5 ml methanol. As an internal standard we pipetted 10 µl of a retrorsine (Sigma R-0382) solution (1 g/l) into an eppendorf vial, and after evaporation of the solvent 10 µl of the sample solution was added. For analysis we injected 1 µl into a Perkin-Elmer 8500 gas chromatograph which was equipped with a Supelco SPB-5 (0.25 µm, 0.32 mm, 30 m) column. The temperature gradient was 200–230°C at 1.5°/min and 230–280°C at 8°/min. The PAs in reference samples of *A. alliariae* and *A. alpina* were identified by C. Theuring, Braunschweig. Quantification was done by dividing the detection units of known alkaloids by the units of the internal standard. Detection units of each PA were multiplied by a correction factor, calculated as the quotient of the molecular mass of the PA divided by 12 times the number of carbon atoms of the molecule.

The nitrogen (N) and carbon (C) content of the leaf powder was determined by mass spectrometry with a Carlo-Erba CHN EA1108-Elementaryanalyser.

Data analysis

To achieve normality of our data we transformed values of PAs and STs by log $(x \cdot 10^4 + 1)$, and values of N and C by log x (Sokal and Rohlf 1995). Differences in PA and ST content between treatments (light), populations and genets were analyzed by three-way repeated measurement ANOVA taking individual PAs or STs as the repeated measure, treatment as a fixed effect and genet as nested within population. Differences in N and C content between treatments, populations and genets were analyzed independently with three-way ANOVAs, taking treatment as a fixed effect and genet as nested within population.

We calculated broad sense heritabilities of PAs, STs, N and C content for every population as $h^2 = V_{genet}/V_{genet} + V_{error}$ (Falconer 1984). We estimated the variance components of the random factors (V_{genet}, V_{error}) using the restricted maximum likelihood method

Table 1. Repeated measurement ANOVA on the effects of light, population and genet on the leaf content of pyrrolizidine alkaloids (PAs) in *A. alliariae* and *A. alpina*. PAs in *A. alliariae* were senecionine, seneciphylline, spartioidine and acetyl-seneciphylline; in *A. alpina* the PAs were senecionine, seneciphylline and spartioidine. Light was treated as a fixed treatment effect and genet was nested within population.

Source of variation	df	Mean squares	*F*	*p*
Adenostyles alliariae				
light	1	1.5824	0.524	0.5213
population	3	2.3815	1.313	0.3045
genet (pop.)	16	1.8126	4.338	0.0001
PAs	3	350.169	838.044	0.0001
light × population	3	3.0164	7.219	0.0001
light × genet (pop.)	16	0.8553	2.047	0.0135
light × PAs	3	6.3409	15.175	0.0001
population × PAs	9	5.9991	14.357	0.0001
genet (pop.) × PAs	48	1.7583	4.208	0.0001
light × pop. × PAs	9	1.7805	4.261	0.0001
light × genet(pop) × PAs	48	0.6044	1.446	0.0483
error	152	0.4178		
Adenostyles alpina				
light	1	3.8183	9.537	0.0538
population	3	4.1511	2.686	0.0814
genet (pop.)	16	1.5454	3.977	0.0001
PAs	2	202.409	520.974	0.0001
light × population	3	0.4003	1.030	0.3819
light × genet (pop.)	16	0.4552	1.171	0.3007
light × PAs	2	0.0714	0.184	0.8832
population × PAs	6	16.1724	41.625	0.0001
genet (pop.) × PAs	32	0.6921	1.781	0.0140
light × pop. × PAs	6	1.6686	4.295	0.0006
light × genet(pop) × PAs	32	0.4462	1.148	0.2916
error	117	0.3885		

(REML) of the SAS® VARCOMP procedure (Shaw 1987). For the heritability estimates of the Kandersteg population we had additional genets from the bordering plants at our disposition. So we calculated ANOVAs including those additional genets and added the factor "position" to the model (Appendices 2, 3). Standard errors of heritabilities were estimated as the square root of $(2(1-t)^2(1+(k-1)t)^2/k(k-1)(S-1))$, where t is the intraclass correlation, k the number of individuals within genets and S the number of genets (Becker 1984, Falconer 1984). Significant differences of heritabilities from zero were determined by t tests. Because of multiple testing of the same genets of a population, we adjusted the p-values within each population by the Bonferroni method (Sokal and Rohlf 1995). The "Col du Lautaret" population was omitted from all analyses, since most of the plants died during the experiment. Analyses were computed using StatView™ and SuperAnova™ by Abacus Concepts (1992, 1989) on a Macintosh computer and using SAS® (SAS Institute Inc. 1990) on a VAX 7620 workstation.

Results

Effects of light on pyrrolizidine alkaloids (PAs)

The light treatment had no effect on PAs in *A. alliariae* (Table 1). In *A. alpina*, however, there was a tendency for shade plants to have more PAs in their leaves than the light plants (Table 1, mean PAs ± se: shade 1.83 ± 0.563, "light" 0.64 ± 0.136 [mg/g dry weight]).

The overall PA content was not different between populations of *A. alliariae* and *A. alpina* (Table 1). The PA content between genets, however, differed significantly in both species (Table 1, Fig. 2). Not surprisingly in both species the different PAs were present in different amounts within the leaves (Table 1). The PA content per g dry weight in leaves of *A. alliariae* was (mean ± se): senecionine 4.22 ± 0.749 mg, seneciphylline 30.76 ± 2.75 mg, spartioidine 0.081 ± 0.023 mg and acetyl-seneciphylline 2.09 ± 0.331 mg. In *A. alpina* the PA content was consistently 1–2 orders of magnitude smaller than in *A. alliariae*: senecionine 0.21 ± 0.037 mg, seneciphylline 3.42 ± 0.813 mg and spartioidine 0.06 ± 0.014 mg. We could not detect acetyl-seneciphylline in our samples of *A. alpina*.

In *A. alliariae* all interactions with light were significant (Table 1, Fig. 3) This is mainly the effect of acetyl-seneciphylline and spartioidine, which both vary between populations (Table 2, Fig. 3), treatments (Fig. 3) and genets (Fig. 2).

The interactions PAs × populations and PAs × genets were significant in both species (Tables 1, 2, Figs. 2, 3). This is mainly due to the different concentrations of spartioidine and acetyl-seneciphylline between populations and genets, since seneciphylline and senecionine are always present in about equal proportions (Table 2, Fig. 3).

The three-way interaction light × population × PAs was significant in both species (Table 1, Fig. 3). In *A. alliariae* this is due to the fluctuation of acetyl-seneciphylline and spartioidine between populations and treatments (Fig. 3). In *A. alpina* this result reflects the uniquely high spartioidine content in the Kandersteg population (Table 2, Fig. 3), which was also responsible for the significant population × PAs interaction.

Effects of light on sesquiterpenes (STs) in *A. alpina*

We did not detect any effects of light on the overall content in STs of leaves of *A. alpina* (Table 3). For the different populations we found a tendency of being different in their overall ST content (Table 2) which was: Appenzell 453 ± 36 µg, Kandersteg 489 ± 108 µg, Tschiertschen 266 ± 46 µg and Wasserfallen 158 ± 36 µg (mean ± se/g dry weight). Genets were remarkably different in their overall ST content (Table 3, Fig. 4). The STs themselves were present in different amounts but within equal orders of magnitude; average ST ± se: cacalol 144 ± 18.2 µg and cacalol-trimer 198 ± 21.2 µg.

The effect of light was different between genets and populations (Table 3). The significant light × population effect is however the result of the pooled values for Cac and C-Tri, since when those are decoupled in the light × population × STs effect, the significance vanishes (Fig. 5). Averaged over populations, light has a different effect on the expression of Cac and C-Tri in the leaves (Table 3, Fig. 5). Whereas in the light both STs are present in equal amounts, in the shade the amount of Cac is lower and that of C-Tri higher (Fig. 5).

The interaction STs × population was significant (Tables 2, 3, Fig. 5), but when again the effect of light is also considered the significance vanishes (Table 3, Fig. 5). The interaction effect STs × genet (Fig. 4) is also significant and when the effect of light is considered simultaneously (interaction light × genet(pop) × STs) it remains significant (Table 3).

Effects of light on nitrogen and carbon content

The experimental treatment had a significant effect on the leaf nitrogen (N) and carbon (C) content of both species (Table 4). N content was always higher in the

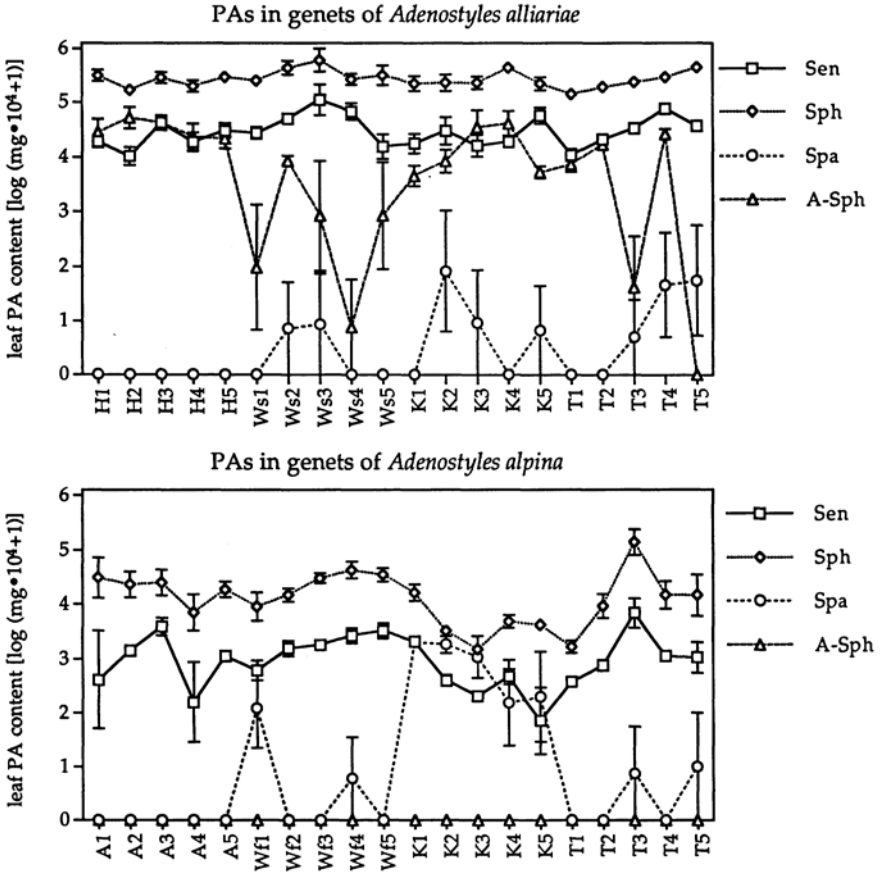


Fig. 2. Mean leaf pyrrolizidine alkaloid (PA) content of all genets of *A. alliariae* and *A. alpina*. PAs are: senecionine (Sen), seneciphylline (Sph), spartioidine (Spa) and acetyl-seneciphylline (A-Sph). Populations and genets are: Appenzell (A1–5), Hohwald (H1–5), Kandersteg (K1–5), Wasserfallen (Wf1–5), Weissenstein (Ws1–5) and Tschiertschen (T1–5).

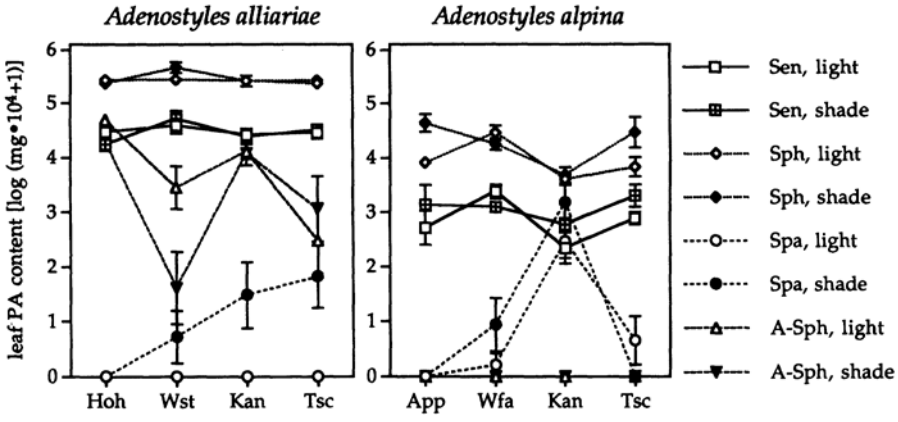


Fig. 3. Mean leaf pyrrolizidine alkaloid (PA) content in plants from populations of *A. alliariae* and *A. alpina* under the experimental light and shade treatment. PAs are: senecionine (Sen), seneciphylline (Sph), spartioidine (Spa) and acetyl-seneciphylline (A-Sph). Populations are: Appenzell (App), Hohwald (Hoh), Kandersteg (Kan), Wasserfallen (Wfa), Weissenstein (Wst) and Tschiertschen (Tsc).

shaded plants (*A. alliariae*: light $2.18 \pm 0.077\%$, shade $3.76 \pm 0.085\%$; *A. alpina*: light $1.95 \pm 0.043\%$, shade $3.70 \pm 0.091\%$; all values are % of leaf dry weight). C content in shade plants was always lower than in light plants (*A. alliariae*: light $40.3 \pm 0.16\%$, shade $38.5 \pm 0.19\%$; *A. alpina*: light $40.2 \pm 0.14\%$, shade $38.3 \pm 0.27\%$; all values are % of dry weight).

Populations of *A. alliariae* were different in their overall N content (Table 4, Fig. 6). Genets of *A. alliariae* and *A. alpina* were also different in their overall N content (Table 4). A significant treatment × population effect was apparent in *A. alliariae* for leaf N content (Table 4, Fig. 6).

Heritabilities of PAs, STs, N and C

Adenostyles alliariae

Heritabilities for different PAs in *A. alliariae* varied considerably between the different populations (Table 5). For seneciphylline, the most prominent PA (around 80% of total PA content), senecionine, and acetyl-seneciphylline we detected very high genetic variation in the Tschiertschen population. In all other populations we could not detect any significant genetic variation for leaf PA content. Heritabilities for leaf nitrogen and carbon content were never significantly different from zero, which indicates that most of the observed phenotypic variation was based on environmental factors.

Adenostyles alpina

In *A. alpina* the heritabilities for different PAs varied widely between populations (Table 5). For seneciphylline content we found about the same amount of genetic variation in the populations from Kandersteg and Tschiertschen. In the populations from Wasserfallen and Appenzell we could not detect any genetic variation for leaf seneciphylline content. For senecionine content genetic variation was highest in the Tschiertschen population and lower in the Kandersteg population; no genetic variation was detectable in the Appenzell and Wasserfallen populations. For spartioidine content we could not detect any significant genetic variation.

For nitrogen and carbon content we could not detect any significant heritabilities in populations of *A. alpina* (Table 5).

Heritability for the ST cacalol was high in the Tschiertschen, Wasserfallen and Kandersteg populations but no significant genetic variation could be detected in the Appenzell population. In no population was the heritability of cacalol-trimer significantly different from zero (Table 5).

Discussion

Effects of light on PAs and STs

Although there was no overall effect of light in the expression of PAs in *A. alliariae* a significant light × PAs and light × pop × PAs interaction indicated that PAs were expressed differently under the two light treatments. In all populations except Hohwald spartioidine was detectable only in the shaded plants (Fig. 3). The production of acetyl-seneciphylline varied between populations from being equal in expression between light and shade plants (Hohwald and Kandersteg), to being more expressed in shade plants (Tschiertschen) to being more expressed in light plants (Weissenstein).

In *A. alpina* there was a tendency for all PAs to be more concentrated in shaded plants than in light plants (Table 1). This is true for all PAs since no significant light × PAs interaction could be detected. Plants from the population at Wasserfallen were more advanced in their phenology at the beginning of the experiment. This could explain their apparent deviation from the other populations (Fig. 3) and, indeed, when we calculated the analyses omitting this population, the treatment effect became significant (data not shown).

Table 2. Mean amounts of pyrrolizidine alkaloids and sesquiterpenes in leaves of *A. alliariae* and *A. alpina* of different populations. Values are mg/g dry weight.

Species and population	Seneciphylline		Senecionine		Spartioidine		Acetyl-Seneciphylline		Cacalol		Cacalol-Trimer	
	mean (se)	%	mean (se)	%	mean (se)	%	mean (se)	%	mean (se)	%	mean (se)	%
Adenostyles alliariae												
Hohwald	27.4 (2.71)	79.6	2.8 (0.411)	8.1	0		4.2 (0.72)	12.2				
Weissenstein	46.5 (9.82)	84.5	7.9 (2.949)	14.3	0.04 (0.030)	0.1	0.6 (0.17)	1.1				
Kandersteg	29.8 (3.41)	81.6	3.6 (0.726)	9.8	0.11 (0.057)	0.3	3.0 (1.03)	8.2				
Tschiertschen	27.0 (2.68)	84.7	3.7 (0.571)	11.6	0.06 (0.027)	0.2	1.1 (0.29)	3.5				
Adenostyles alpina												
Appenzell	4.10 (1.45)	85.3	0.25 (0.074)	5.2	0	0			0.199 (0.026)	4.1	0.254 (0.018)	5.3
Wasserfallen	3.14 (0.54)	88.5	0.23 (0.041)	6.5	0.02 (0.013)	0.60			0.056 (0.019)	1.6	0.102 (0.023)	2.9
Kandersteg	0.69 (0.20)	47.3	0.07 (0.017)	4.8	0.21 (0.041)	14.40			0.177 (0.047)	12.1	0.311 (0.068)	21.3
Tschiertschen	6.43 (3.42)	91.4	0.32 (0.147)	4.5	0.02 (0.019)	0.30			0.142 (0.040)	2.0	0.124 (0.016)	1.8

For both STs no general effect of light could be detected (Table 3). There is however a significant light × STs interaction, indicating that the response to shade is different with regard to the two STs. Cacalol tends to decrease in its concentration in the shade, whereas cacalol-trimer concentration increases considerably in the shade (Fig. 5).

The resource availability (RA) hypothesis (Bryant et al. 1983, Coley et al. 1985) predicts lower levels of carbon-based defenses and higher levels of nitrogen-based defenses under carbon limiting (low light) conditions. For the nitrogen-based PAs this prediction was met in *A. alpina* and for the PA spartioidine also in *A. alliariae*.

We found no change in the carbon-based STs between treatments when considering both sesquiterpenes together. More specifically we found a slight decrease in cacalol whereas for cacalol-trimer we actually found increased concentrations in the shaded plants. Thus our results support the RA hypothesis only in the case of cacalol. Reichardt et al. (1991) found only one out of six carbon-based metabolites in *Populus balsamifera* to correspond to the predictions of the (RA) hypothesis. They proposed that "static" compounds with low metabolic turnover would respond according to the (RA) whereas "dynamic" compounds with a high potential of metabolic turnover would not always respond to an altered carbon balance. Since we do not know which of the two compounds might be the more static one (sensu Reichardt et al. 1991), we must restrain ourselves from an interpretation of these results in relation to metabolic turnover. However, since cacalol is probably the monomer of cacalol-trimer it is tempting to assume that it should be metabolically more "dynamic" than cacalol-trimer, which would argue against Reichardt et al.'s (1991) suggestion. Jansen and Stamp (1997) examined the effects of shading in tomato and found increased levels of the phenolics rutin and chlorogenic acid and the alkaloid tomatine in the full sunlight treatment. For the carbon-based phenolics this result would have been predicted by the RA hypothesis, whereas for the nitrogen-based alkaloid their result is against the expectations. Other studies on trees and shrubs found the predicted decreased concentration in carbon-based substances after shading (Larsson et al. 1986, McCloud et al. 1992, Sagers 1992, Rousi et al. 1993). Contrary to the RA hypothesis, however, Collinge and Louda (1988) found no change in isothiocyanate-yielding glucosinolates of the herb *Cardamine cordifolia* (Brassicaceae) when they imposed artificial shade on the plants in a field experiment.

Effects of light on nitrogen and carbon content

Perhaps the strongest effect of the light treatment was the dramatic increase in nitrogen in the shaded plants (Table 4, Fig. 6). Collinge and Louda (1988) also found higher total nitrogen content in artificially shaded plants of *C. cordifolia* and Jansen and Stamp (1997) found higher protein content in shaded tomato plants.

Table 3. Repeated measurement ANOVA on the effects of light, population and genet on the leaf content of sesquiterpenes (STs) in *A. alpina*. STs were cacalol and cacalol-trimer. Light was treated as a fixed treatment effect and genet was nested within population.

Source of variation	df	Mean squares	*F*	*p*
light	1	0.2182	0.667	0.4737
population	3	5.8719	3.178	0.0527
genet (pop.)	16	1.8477	18.599	0.0001
STs	1	2.3958	24.117	0.0001
light × population	3	0.3267	3.288	0.0249
light × genet (pop.)	16	0.5484	5.521	0.0001
light × STs	1	3.5915	36.153	0.0001
population × STs	3	0.7129	7.176	0.0003
genet (pop.) × STs	16	0.3232	3.354	0.0002
light × pop. × STs	3	0.1881	1.893	0.1373
light × genet(pop) × STs	16	0.1982	1.995	0.0232
error	80	0.0993		

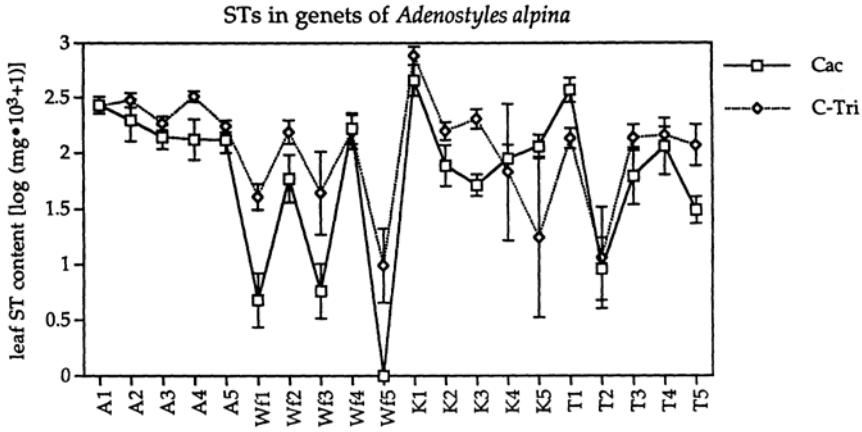


Fig. 4. Mean leaf sesquiterpene (ST) content of all genets of *A. alpina*. STs are: cacalol (Cac) and cacalol-trimer (C-Tri). Populations and genets are: Appenzell (A1–5), Kandersteg (K1–5), Wasserfallen (Wf1–5) and Tschiertschen (T1–5).

In several tree studies which also determined nitrogen content after shading, no difference was found compared to the unshaded plants (Larsson et al. 1986, Sagers 1992), but in the data of Reichardt et al. (1991) at least a trend was visible towards higher leaf nitrogen in experimentally shaded *P. balsamifera*. Whether these findings reflect a fundamental difference between plants of different growth forms, in their ability to respond to environmental stress, is unknown to us.

Although Jansen and Stamp (1997) also report elevated protein levels, most of the nitrogen accumulation in the shaded leaves is probably due to an accumulation of nitrate due to the decreased function of nitrate reductase under low light conditions (Yazawa et al. 1986, Muthuchelian et al. 1989, Merlo et al. 1994, Wu and Wang 1995). Since nitrate is a potential toxin for insects (Camargo and Ward 1992, Lindroth et al. 1993), feeding on shaded plants forces insects not only to ingest higher alkaloid levels, but also to ingest higher quantities of the potentially toxic nitrate ion. Insect feeding on shaded plants could face a delicate choice between gaining more protein at the cost of also ingesting more nitrate and nitrogen-based defense chemicals.

Carbon content in light plants was higher in both species. This is probably a result of phenotypic variation in leaf thickness and toughness, whereby light leaves are generally thicker and more sturdy than shaded leaves.

Heritabilities of PAs, STs, N and C

In *A. alliariae* we found very high and significant heritabilities for the PAs seneciphylline, senecionine, and acetyl-seneciphylline only in the Tschiertschen population. This finding indicates that this population harbors high genetic variability for alkaloids except spartioidine and thus the potential for response to selection in this population should be very high. In *A. alpina* we could detect significant genetic variation for the PAs seneciphylline and senecionine in both the Tschiertschen and Kandersteg population. In both of these populations as well as in the Wasserfallen population genetic variability for the ST cacalol was also large and significant.

The loss of variability for all other characters could indicate fixation of the responsible alleles as a result of

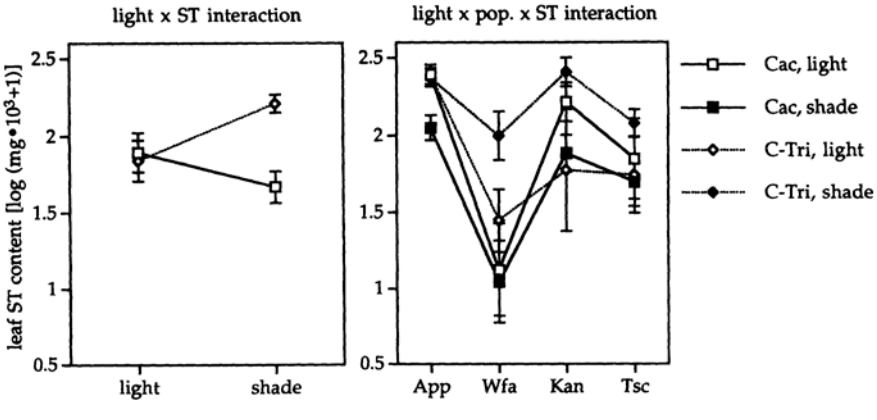


Fig. 5. Mean leaf sesquiterpene (ST) content in plants from populations of *A. alpina* under the experimental light and shade treatment. STs are: cacalol (Cac) and cacalol-trimer (C-Tri). Populations are: Appenzell (App), Kandersteg (Kan), Wasserfallen (Wfa) and Tschiertschen (Tsc).

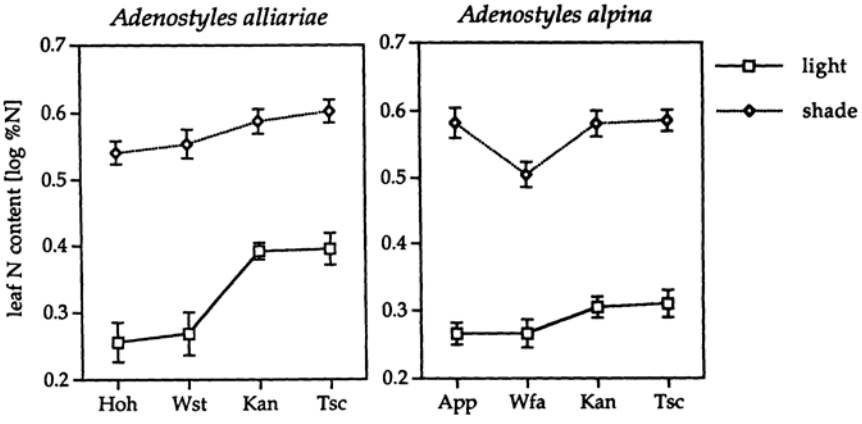


Fig. 6. Mean leaf nitrogen content in plants from populations of *A. alliariae* and *A. alpina* under the experimental light and shade treatment.

past selection pressure or genetic drift. In all other populations there was a considerable amount of genetic variation detectable. An interesting result emerges in the Kandersteg population. Here the leaf concentrations of seneciphylline, senecionine, and spartioidine is remarkably different from all other populations (Table 2), whereas STs and genetic variation for all substances are comparable to other populations. One possible interpretation is that this is a result of past selection for low seneciphylline, senecionine and high spartioidine content.

Apart from gene flow and genetic drift a major force maintaining genetic polymorphism for plant defenses could be differential selection by specialist and generalist herbivores (van der Meijden 1996). In his graphic model van der Meijden (1996) assumes that, if specialists select for low levels of defense and generalists select for high levels, then a polymorphism in levels of defensive compounds would be maintained within the plant community with, on average, low levels of defense in individual plants.

Heritabilities for nitrogen and carbon content were all not significantly different from zero, which means that no genetic variation in nitrogen and carbon content could be detected between genets. All observed differences in the experiment in those traits were therefore environmentally induced.

Table 4. Nested ANOVA on the effects of light, population and genet on the leaf nitrogen and carbon content of *A. alliariae* and *A. alpina*. Light was treated as a fixed treatment effect and genet was nested within population.

Source of variation	df	Mean squares	*F*	*p*
Adenostyles alliariae				
log %N				
light	1	1.1292	89.986	0.0025
population	3	0.0476	5.840	0.0068
genet (pop.)	16	0.0082	2.245	0.0206
light × population	3	0.0125	3.451	0.0259
light × genet (pop.)	16	0.0044	1.222	0.2962
error	38	0.0036		
log %C (1)				
light	1	8.3189	57.653	0.0047
population	3	0.0412	0.231	0.8728
genet (pop.)	16	0.1780	1.257	0.2729
light × population	3	0.1442	1.019	0.3950
light × genet (pop.)	16	0.1411	0.996	0.4801
error	38	0.1415		
Adenostyles alpina				
log %N				
light	1	1.5221	291.929	0.0004
population	3	0.0174	2.393	0.1065
genet (pop.)	16	0.0072	3.342	0.0011
light × population	3	0.0052	2.395	0.0829
light × genet (pop.)	16	0.0028	1.325	0.2308
error	39	0.0021		
log %C (1)				
light	1	7.7981	31.036	0.0114
population	3	0.5873	1.753	0.1965
genet (pop.)	16	0.3349	1.930	0.0473
light × population	3	0.2512	1.448	0.2435
light × genet (pop.)	16	0.1796	1.035	0.4434
error	39	0.1734		

(1) mean square values multiplied by 1000.

Conclusions

Light availability proved to be an important factor, both for the expression of the level of allelochemicals and for the foliar nitrogen content in the species examined. This is not only of importance for the herbivore-plant interaction itself but links the interaction with community ecological dynamics, since regrowth and succession in patches and plant understory are mainly a question of light availability to plants (Coley 1993, Shure and Wilson 1993). Shaded plants, which should also be a more protein-rich resource, are probably well protected from browsing through increased levels of alkaloids. However, this is probably only true for generalist herbivores; specialists may even seek plants containing high alkaloid levels, especially if they also contain higher levels of protein. Plants growing in well-lit environments, on the other hand, are well protected from browsing if they contain carbon-based de-

Table 5. Broad sense heritabilities of pyrrolizidine alkaloid, sesquiterpene, nitrogen and carbon content in leaves of *A. alliariae* and *A. alpina* from different populations. In the population Kandersteg 9 genets of *A. alliariae* and 11 genets of *A. alpina* were included in the analyses (see Appendices 2, 3). Calculations of all other populations are based on 5 genets.

Species and population	Seneci-phylline h^2 (se)	p	Senecionine h^2 (se)	p	Spartioidine h^2 (se)	p	Acetyl-Seneci-phylline h^2 (se)	p	Nitrogen h^2 (se)	p	Carbon h^2 (se)	p	Cacalol h^2 (se)	p	Cacalol-Trimer h^2 (se)	p
Adenostyles alliariae																
Hohwald	0.11 (0.26)	n.s.	0.46 (0.27)	n.s.	0		0	0.31 (0.28)	n.s.	0						
Weissenstein	0		0.33 (0.27)	n.s.	0		0.25 (0.26)	n.s. (0.27)	0.42	n.s. (0.26)	0.47	n.s.				
Kandersteg	0		0.06 (0.16)	n.s.	0.26 (0.19)	n.s.	0.18 (0.18)	n.s.	0		0					
Tschiertschen	0.87 (0.09)	<0.006	0.86 (0.10)	<0.006	0		0.99 (0.01)	<0.006	0		0					
Adenostyles alpina																
Appenzell	0.16 (0.25)	n.s.	0		0			0.61 (0.23)	n.s.	0.26 (0.26)	n.s.	0.12 (0.24)	n.s.	0.48 (0.26)	n.s.	
Wasserfallen	0.37 (0.27)	n.s.	0.56 (0.24)	n.s.	0.45 (0.26)	n.s.		0.03 (0.21)	n.s.	0.49 (0.26)	n.s.	0.89 (0.08)	<0.007	0.63 (0.21)	<0.07	
Kandersteg	0.73 (0.11)	<0.007	0.52 (0.16)	<0.014	0.38 (0.17)	n.s.		0.23 (0.17)	n.s.	0		0.66(0.13)	<0.007	0		
Tschiertschen	0.86 (0.10)	<0.007	0.85 (0.11)	<0.007	0			0.54 (0.25)	n.s.	0.15 (0.26)	n.s.	0.75 (0.17)	<0.007	0.63 (0.22)	n.s.	

fenses. This should be true for generalists, which are deterred by the high levels of carbon-based defenses, as well as for specialists for which, despite being adapted to the defense, the protein-richer and defense-poorer conspecific plants in the shade should be a more attractive resource.

Because we found significant genetic variation for secondary compound content in some populations, response to herbivore selection can be expected. Differences between populations in concentrations of individual compounds (population × compound interactions) and population differences in genetic variation of individual compounds, can be interpreted as a reflection of the differences in the selection history between populations. It is, however, not obvious what the striking differences between populations in their genetic variability means. If interpreted according to the results of Lande and Shannon (1996), this could mean that populations with low genetic variability have experienced either highly constant or completely unpredictable environments, whereas those with high genetic variability have experienced highly predictable but variable environments and thus would have benefited from genetic variation. The Tschiertschen population, for which we observed the highest levels of genetic variability, is certainly the most diverse community with regard to leaf beetles (B. Hägele pers. obs.). This could make the herbivore pressure predictable and variable at the same time, taking population density fluctuations of herbivores into account. It is however not immediately obvious to us which factors would make the Kandersteg population of *A. alliariae* so different from the Tschiertschen population, since the diversity of leaf beetles at Kandersteg is also relatively high. However, our impression about the similarity of the two sites is supported by the close similarity in levels of genetic variability we found in *A. alpina*.

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Appendix 1

Light intensity, soil temperature, air temperature and relative humidity under the two experimental treatments. Light intensity was measured at 17 June, 13.00 in full sunlight. Temperatures and humidity were measured every hour from 17–28 June. All measures are significantly different between treatments ($p < 0.0001$).

	light treatment mean (se)	shade treatment mean (se)	t	N
light intensity [lx]	51590 (1069)	7134 (272)	38.124	10
soil temp. [°C]	20.85 (0.218)	21.31 (0.255)	9.504	263
soil temp. (night) [°C]	18.85 (0.212)	19.07 (0.228)	6.370	99
air temp. [°C]	22.94 (0.435)	22.03 (0.382)	9.701	263
air temp. (night) [°C]	17.21 (0.238)	16.95 (0.282)	3.955	99
rel. humidity [%]	80.4 (1.34)	75.6 (1.38)	12.904	263
rel. humidity (night) [%]	96.6 (0.39)	92.9 (0.75)	8.423	99

Appendix 2

Nested ANOVA of heritability characters of the Kandersteg population of *Adenostyles alliariae*. With the effect "position" genets were distinguished as being placed either randomly in the centre of the squares or within the surrounding row.

Source of variation	df	Mean squares	F	p	Variance component
Seneciphylline					
light	1	0.1191	1.066	0.3360	$V_l = 0.0041$
position	1	2.1705	29.805	0.0009	
genet (position)	7	0.0728	1.163	0.3726	$V_g = 0$
light × position	1	0.1336	2.135	0.1622	
light × genet (pos.)	7	0.1116	1.784	0.1558	
error	17	0.0625			$V_e = 0.0613$
Senecionine					
light	1	0.0137	0.163	0.6983	$V_l = 0.0003$
position	1	2.0469	15.718	0.0054	
genet (position)	7	0.1302	1.101	0.4058	$V_g = 0.0068$
light × position	1	0.0443	0.375	0.5484	
light × genet (pos.)	7	0.0841	0.711	0.6638	
error	17	0.1415			$V_e = 0.1055$
Spartioidine					
light	1	6.4781	4.466	0.0724	$V_l = 0.3575$
position	1	59.5029	16.973	0.0045	
genet (position)	7	3.5055	2.309	0.0752	$V_g = 0.5207$
light × position	1	3.1425	2.070	0.1684	
lightgenet (pos.)	7	1.4503	0.955	0.4923	
error	17	1.5180			$V_e = 1.4867$
Acetyl-Seneciphylline					
light	1	0.3364	0.607	0.4613	$V_l = 0.0333$
position	1	0.1306	0.090	0.7728	
genet (position)	7	1.4493	1.979	0.1185	$V_g = 0.1486$
light × position	1	0.2485	0.339	0.5678	
light × genet (pos.)	7	0.5539	0.756	0.6299	
error	17	0.7322			$V_e = 0.6739$
Nitrogen					
light	1	0.2691	44.192	0.0003	$V_l = 0.0165$
position	1	0.0876	23.308	0.0019	
genet (position)	7	0.0037	1.229	0.3403	$V_g = 0$
light × position	1	0.0024	0.813	0.3798	
light × genet (pos.)	7	0.0061	1.992	0.1164	
error	17	0.0030			$V_e = 0.0030$

Appendix 2 (continued)

Source of variation	df	Mean squares	F	p	Variance component
Carbon (1)					
light	1	3.8544	12.561	0.0094	$V_l = 0.0003$
position	1	4.4219	37.230	0.0005	
genet (position)	7	0.1187	0.783	0.6106	$V_g = 0$
light × position	1	0.0941	0.620	0.4418	
light × genet (pos.)	7	0.3068	2.022	0.1116	
error	17	0.1517			$V_e = 0.0001$

(1) mean square values multiplied by 1000.

Appendix 3

Nested ANOVA of heritability characters of the Kandersteg population of *Adenostyles alpina*. With the effect "position" genets were distinguished as being placed either randomly in the centre of the squares or within the surrounding row.

Source of variation	df	Mean squares	F	p	Variance component
Seneciphylline					
light	1	0.3936	4.909	0.0539	$V_l = 0.0196$
position	1	13.8876	14.250	0.0044	
genet (position)	9	0.9745	11.512	0.0001	$V_g = 0.2228$
light × position	1	0.1293	1.527	0.2295	
light × genet (pos.)	9	0.0801	0.947	0.5065	
error	22	0.0846			$V_e = 0.0833$
Senecionine					
light	1	1.2378	9.655	0.0126	$V_l = 0.0543$
position	1	7.1014	7.971	0.0199	
genet (position)	9	0.8909	4.948	0.0011	$V_g = 0.1821$
light × position	1	0.0840	0.466	0.5015	
light × genet (pos.)	9	0.1282	0.712	0.6923	
error	22	0.1800			$V_e = 0.1624$
Spartioidine					
light	1	0.0163	0.013	0.9117	$V_l = 0.0001$
position	1	47.6606	17.349	0.0024	
genet (position)	9	2.7470	4.510	0.0019	$V_g = 0.3728$
light × position	1	4.7862	7.858	0.0104	
light × genet (pos.)	9	1.2555	2.061	0.0803	
error	22	0.6090			$V_e = 0.6090$
Nitrogen					
light	1	0.7969	322.575	0.0001	$V_l = 0.0363$
position	1	0.0023	0.348	0.5693	
genet (position)	9	0.0066	2.200	0.0634	$V_g = 0.0008$
light × position	1	0.0005	0.158	0.6939	
light × genet (pos.)	9	0.0024	0.823	0.6015	
error	22	0.0030			$V_e = 0.0027$
Carbon (1)					
light	1	1.4823	14.284	0.0044	$V_l = 0.0615$
position	1	2.0065	15.211	0.0036	
genet (position)	9	0.1319	0.965	0.4931	$V_g = 0.0012$
light × position	1	0.5557	4.067	0.0561	
light × genet (pos.)	9	0.1037	0.759	0.6534	
error	22	0.1366			$V_e = 0.1270$
Cacalol					
light	1	1.3959	101.008	0.0001	$V_l = 0.0644$
position	1	6.5582	22.235	0.0011	
genet (position)	9	0.2949	7.057	0.0001	$V_g = 0.0655$
light × position	1	0.0051	0.122	0.7301	
light × genet (pos.)	9	0.0138	0.331	0.9552	
error	22	0.0417			$V_e = 0.0327$
Cacalol-Trimer					
light	1	7.6458	1.971	0.1939	$V_l = 0.2894$
position	1	14.0234	3.670	0.0876	
genet (position)	9	3.8207	3.806	0.0050	$V_g = 0$
light × position	1	10.5120	10.473	0.0038	
light × genet (pos.)	9	3.8790	3.864	0.0046	
error	22	1.0036			$V_e = 1.0036$

(1) mean square values multiplied by 1000.