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Cost assessment of the production of pyrrolizidine alkaloids in ragwort (*Senecio jacobaea* L.)

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Abstract Costs of pyrrolizidine alkaloid (Pa) production in vegetative ragwort (*Senecio jacobaea*) were examined under conditions in which plant growth was limited by light, nitrogen and phosphorus. Measurable costs of Pa production were demonstrated under light-limiting conditions. Plants with higher Pa concentrations grew more slowly than those with lower Pa concentration. Under nitrogen- and phosphorus-limited conditions no trade-off between Pa production and growth was observed.

Key words *Senecio jacobaea* · Pyrrolizidine alkaloids Cost of secondary metabolites · Chemical defence Hydroculture

Introduction

A number of theories have been developed to explain patterns and amounts of antiherbivore substances (secondary metabolites) in plants. The most important of these are the "apparency theory" (Feeny 1976; Rhoades and Cates 1976) and the "resource limitation theory" (Coley et al. 1985). An understanding of the balance between benefits and costs of secondary metabolites is central to both. Many studies have demonstrated benefits of secondary compounds (reduced herbivory), but only a few have considered the costs (Berenbaum et al. 1986; Coley 1986; Brown 1988; Østrem 1988; Kakes 1989; Baldwin et al. 1990; Briggs and Schultz 1990). Costs, if demonstrated, are usually estimated in terms of trade-offs between secondary metabolite concentration and plant growth or seed production. It is usually argued that if plants have limited supplies of energy or nutrients, the allocation of these to growth or defence by production of secondary metabolites constitutes an optimality problem for the plant. Hence, increased secondary metabolite production is expected to lead to a decrease in growth, especially if resource availability is low. However, if this is not the case a lack of correlation, or even a positive correlation between growth and secondary metabolite production is possible (van Noordwijk and de Jong 1986; Simms 1992).

Most studies of trade-offs between growth and secondary metabolite concentration have not controlled the availability of resources to individual plants, thus possible trade-offs between these are obscured. Our primary objective in this study was to conduct an experiment using hydroculture in which resource availability can be controlled.

Theoretical calculations of the energetic costs of secondary metabolite production based on the biochemistry of their synthesis indicate similar expenditures as that for primary metabolites [e.g. 1.9–3.5 g glucose/g secondary compound synthesis compared to 0.9–3.0 g glucose/g compound for primary metabolites (Lambers and Rychter 1989)]. Since within plant tissues the concentration of many secondary metabolites (e.g. alkaloids, terpenes) is less than 2%, attendant construction costs would be expected to decrease growth or seed production to a corresponding extent. However, transport (Deus-Neumann and Zenk 1986; Ehmke et al. 1988), storage, self-detoxification mechanisms (Robinson 1974; Twardowski and Majchrzak-Kuczyńska 1989) and turnover (Robinson 1974) of the secondary metabolites will also contribute significantly to the whole process of defence, thus suggesting a greater potential reduction in growth or seed production. The production of nitrogen-containing secondary metabolites has frequently been considered to be limited by nitrogen availability (Bryant et al. 1983; Coley et al. 1985; Augner et al. 1991). In contrast with energy-limited situations, however, there is little or no additional nitrogen needed for transport, storage or self-detoxification as these processes (mostly) require energy. In general, we

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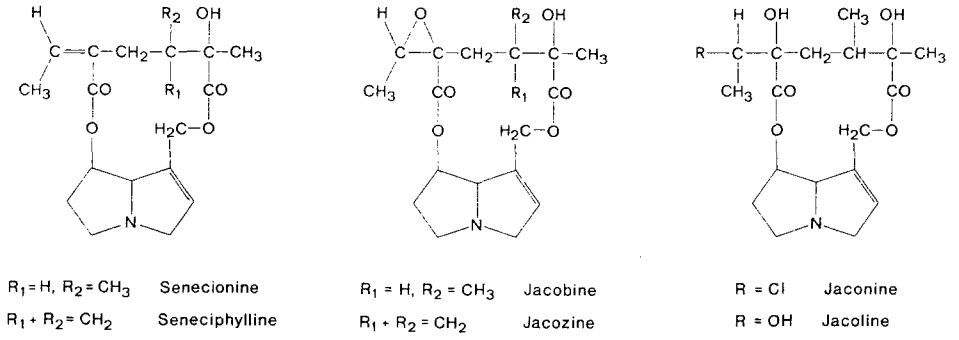

Fig. 1 Structural formulas of the pyrrolizidine alkaloids senecionine, seneciphylline, jacobine, jacozine, jacoline and jaconine. Integerimine (not shown) is a geometric stereoisomer of senecionine

therefore expect that energy, rather than nitrogen, is limiting the production of nitrogen-containing secondary metabolites.

We use *Senecio jacobaea* L. to study the effect of light and nutrient availability on pyrrolizidine alkaloid (Pa) production and growth. The hypothesises explored are that: (1) Under light- and nitrogen limiting conditions, there should be a negative correlation between growth and Pa concentration (since carbon and nitrogen are incorporated in Pas). (2) A negative correlation between growth and Pa concentration will be stronger under light-limited than N-limited conditions. (3) There should be no distinct relationship between growth and Pa concentration under P-limited conditions, since phosphorus is not incorporated as such into Pas.

Materials and methods

Ragwort, *Senecio jacobaea*, is an abundant monocarpic perennial plant. It occurs frequently on dunes in northwestern Europe. *S. jacobaea* contains at least seven Pas (Aplin et al. 1968; Pieters et al. 1989; Witte et al. 1992) (Fig. 1), and the concentration of Pas is genetically determined (Vrieling et al. 1993). It is presently unknown where and how Pas are produced, transported and stored in *S. jacobaea*. In the closely related *Senecio vulgaris* L., Pas are synthesized and transported as N-oxides and stored in the vacuoles (Ehmke et al. 1988; Hartmann and Toppel 1987; Hartmann et al. 1989). There is very little or no turnover of Pas in *S. vulgaris* (Hartmann et al. 1989) and elevation of Pa concentration in *S. jacobaea* is not induced by artificial damage to the plant (Vrieling and Bruin 1987).

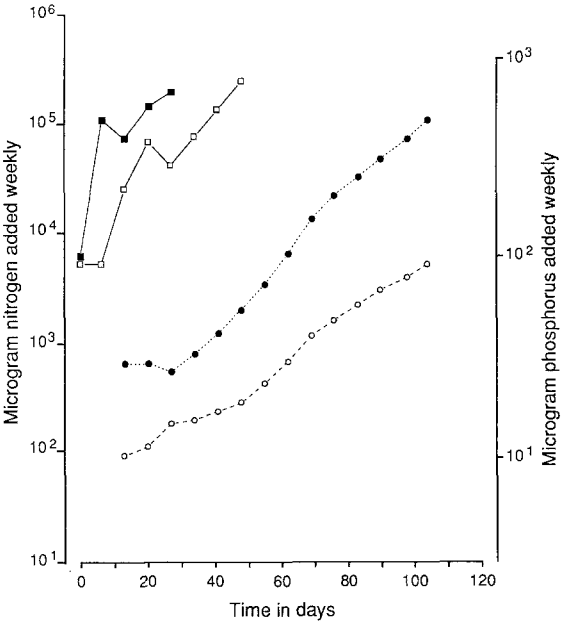

Fig. 2 Weekly nutrient supply (expressed as micrograms of nitrogen or micrograms of phosphorus added) of *Senecio jacobaea* in hydroculture in experiment 1 (three treatments) and experiment 2. The nutrient supply of the phosphorus-limited treatment is expressed as micrograms of phosphorus added (*right axis*), other treatments and experiments are expressed as micrograms of nitrogen added (*left axis*). □—□ control exp. 1, ○···○ nitrogen-limited treatment exp. 1, ●···● phosphorus-limited treatment exp. 1, ■—■ exp 2

Experiment 1

Two *S. jacobaea* plants were selected at random from the Meijendel dunes (near The Hague, The Netherlands), crossed, and 60 of the resulting seeds were germinated in petri-dishes on moist filter paper (97% germination). Seedlings were transplanted into pots containing dune sand and supplied with ample water and small quantities of nutrient solution. After 2 weeks, 45 apparently healthy and equal-sized juvenile plants were selected. The sand was removed from the roots of these and the plants were then placed in 1.2-l containers with Steiner nutrient solution (Steiner 1968) (macronutrients: N 167 mg/l, P 31 mg/l, K 282 mg/l, S 111 mg/l, Ca 180 mg/l, Mg 49 mg/l) and micronutrients according to Arnon and Hoagland (1940). The containers were aerated and kept in a controlled environment chamber [18L/6D, 18° C/12 ° C, RH 70%, 38 W/m^2 (waveband 400-700 nm) at plant level]. The plants were randomly allocated to one of three treatments: control, nitrogen- and phosphorus-limited (NL, PL respectively). We added exponentially increasing amounts of nutrients to plants (Fig. 2) (Ingestad 1982; Waring et al. 1985; Freijsen and Otten 1987). With this technique, nutrient concentration in the plant tissue remains constant during growth (Ingestad 1982). In the NL and PL treatments, nutrients containing nitrogen and phosphorus respectively were omitted from the nutrient solution. $Ca(NO_3)_2$ and KNO_3 were replaced by $CaCl_2$ in the NL treatment. The culture solution was renewed weekly. In the NL and PL treatments, nitrogen and phosphorus were supplied three times a week, in small but exponentially increasing amounts (Fig. 2). Each week, the fresh weight of each individual plant was recorded after the plants were taken out of the container and the roots dried with tissue paper. The control plants were harvested after 63 days and the plants of the NL and PL treatments were harvested after 117 days and fresh weight of roots and shoots were recorded. Plant material was dried (50° C, 72 h) and weighed. Pa and nitrogen concentration were analysed in both shoots and roots.

Experiment 2

To check that plant growth in the control was indeed limited by light, the control was duplicated at a higher light intensity. Eight flowering plants collected from one population in Meijendel were brought into the laboratory and crossed with each other. Seeds were germinated on moist filter paper in petri-dishes, resulting in 79 plants. Plants were grown in the same way as in experiment 1 (Fig. 2). Light intensity in the growth cabinet was however 45 W/m^2 (waveband 400–700 nm) at plant level and temperature during the dark period was 18° C. The fresh weight of plants was recorded weekly and the shoots were harvested after 35 days and Pa and nitrogen concentrations were measured.

Pa determination

The dried plant material was ground and Pas extracted according to a procedure described previously by Vrieling et al. (1991). Total Pa content was determined spectrophotometrically as described by Mattocks (1967). Monocrotaline (purity 99%) was used as a reference.

Nitrogen determination

Total nitrogen was determined by a modification of the Kjeldahl method as outlined by van der Meijden et al. (1984).

Statistical analyses

Relative growth rate (RGR) was estimated by the slope of the linear regression of ln fresh weight of individual plants plotted against time. To test whether slopes (RGR) differed, within treatments, an analysis of variance was performed following Sokal and Rohlf (1981).

Prior to statistical analyses, Pa concentrations were log-transformed to obtain a normal distribution (Østrem 1987).

Results

To check that light, and not nitrogen, was limiting plant growth in the control series the highest growth increment of an individual plant was compared with the amount of nitrogen available in the culture solution during that period. In no case could nitrogen have limited the growth of plants: on average, at least twice the amount needed (assumed to be circa 5% nitrogen dry weight) had been available to the plants. Because the RGRs of plants in experiment 2 were greater than the control in experiment 1 we conclude that the growth of control plants in experiment 1 was indeed limited by light.

Experiment 1

As expected, plants in all treatments grew exponentially (Fig. 3). Ln fresh weight of individual plants increased linearly with time (control $r^2 = 0.972$–0.994, nitrogen-limited $r^2 = 0.984$–0.998, phosphorus-limited $r^2 = 0.848$–0.982, $P < 0.001$ in all cases). RGRs of the plants in the control were significantly higher than the RGRs of plants in the NL and PL treatments (Table 1). Within treatments individual plants differed in RGR (Table 2).

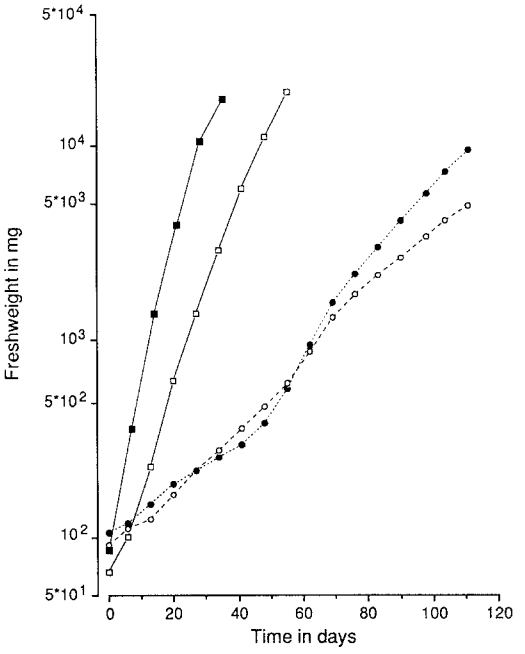

Fig. 3 Ln weight of *S. jacobaea* against time. Plants were grown in hydroculture on a control, nitrogen- and phosphorus-limited nutrient solutions (exp. 1) and a control nutrient solution (exp. 2). □—□ Control exp. 1, ○···○ nitrogen limited treatment exp. 1, ●···● phosphorus limited treatment exp. 1, ■—■ exp. 2

Pa concentrations did not differ significantly between treatments (Table 1). Total plant Pa concentration was negatively correlated with fresh weight in the control. This indicates a trade-off between growth and Pa production (Table 3). RGR was not correlated with total plant Pa concentrations in the control (Table 3). No significant negative correlation was observed between Pa concentration and fresh weight in the NL treatment: Pa production was not limited by nitrogen availability. This agrees with the expectation that virtually no nitrogen (other than the nitrogen in the Pa molecule itself) is needed for Pa production.

Correlations between RGR and Pa concentration are not meaningful in the N- and P-limited series, because of the experimental design. If two plants allocate a fixed (but different) percentage of a limited substance (the supply of which was increased exponentially over time) to the production of a secondary metabolite, these two plants can still have an equal RGR. Total fresh weight, however, was in this case different due to the allocation of the limiting nutrient to secondary metabolite production at the moment when nutrients became limiting. In the control series, correlations with RGR are valid because a plant can adapt the amount of foliage and thereby change its light harvesting strategy. In contrast, in the N- and P-limited series, plants cannot adopt a strategy by which they can obtain more of the limited nutrient.

The proportion of the plant's total amount of nitrogen built into Pas in the NL treatment (mg Pa/mg N, Table 1) differed significantly from the control and the

Table 1 Average values of plant characters of *Senecio jacobaea* reared in hydroculture in three nutrient treatments in two experiments. Different *letters* following averages indicate significant differences (ANOVA, unplanned comparisons). (*Pa* pyrrolizidine alkaloids, *RGR* relative growth rate, *NL* nitrogen limited, *PL* phosphorus limited, *NS* not significant, – not determined, n = number of observation)

	Exp. 1					Exp. 2
	Control	NL	PL	*F*	*P*	
RGR g/g/day[a]	0.105a	0.039b	0.044b	73.98	***	0.168
%Pa shoot fr. wt.[a]	0.019	0.028	0.021	1.44		0.031
%Pa root fr. wt.[a]	0.012	0.015	0.011	1.58		–
%Pa total fr. wt.[a]	0.016	0.020	0.016	2.39		–
%N shoot fr. wt.	0.530a	0.284b	0.516a	100.57	***	0.507
%N root fr. wt.	0.289a	0.119b	0.262c	132.67	***	–
%N total fr. wt.	0.417a	0.180b	0.362c	193.10	***	–
mgPa/mgN shoot[b]	0.035a	0.107b	0.041a	15.20	***	0.061
mgPa/mgN root[b]	0.043a	0.137b	0.045a	21.23	***	–
mgPa/mgN total[b]	0.038a	0.117b	0.043a	38.00	***	–
n	15	15	15			79

*** $P<0.001$

[a] Data were log transformed before statistical analysis

[b] Data were arcsine-root transformed before statistical analysis

Table 2 ANOVA of regression coefficients (RGR) of *Senecio jacobaea* plants within treatments in experiment 1

	SS	*df*	MS	*F*	*P*
Control					
Variation among RGRs	2.603	14	0.186	4.15	***
Average variation within RGRs	4.034	90	0.045		
Nitrogen-limited treatment (NL)					
Variation among RGRs	8.001	14	0.572	30.09	***
Average variation within RGRs	4.278	225	0.019		
Phosphorus-limited treatment (PL)					
Variation among RGRs	22.556	14	1.611	8.58	***
Average variation within RGRs	42.227	225	0.188		

*** $P<0.001$

Table 3 Correlation coefficients (within three nutrient treatments) of experiment 1 of *Senecio jacobaea* plants grown on hydroculture. Pa concentration and RGR were log transformed before correlation. (*NL* nitrogen limited plants, *PL* phosphorus limited plants, *n* number of observations, – not determined

		Control	NL	PL
RGR	– %Pa fr. wt.	–0.22	–	–
Fr. wt.	– %Pa fr. wt.	–0.57*	–0.09	–0.32
%N	– %Pa fr. wt.	0.30	–0.17	0.28
n		15	15	15

* $P<0.05$

PL treatment. A larger percentage of the plant's total amount of nitrogen was used for the production of Pas in nitrogen limited environments compared to the control. This again indicated that Pa production in *S. jacobaea* was not limited by nitrogen. The amount of Pas made per unit fresh weight was not affected by nitrogen limitation, and even increased (Table 1).

In the phosphorus limited treatment, neither a significant negative correlation between Pa concentration and fresh weight was found (Table 3) nor is the proportion of nitrogen used for the production of Pas different from the control (Table 1).

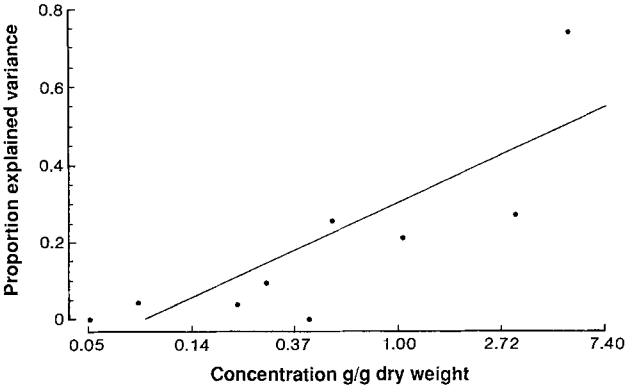

Fig. 4 Relationship between log average concentration of secondary metabolites and the squared correlation coefficient (explained variance) of the correlation between concentration of the secondary metabolite and growth/seed production (see Table 4 and text). $y=0.12 \ln x+0.27$, $r=0.81$, $n=9$, $P<0.01$

Experiment 2

Plants in this experiment all grew exponentially (Fig. 3) and ln fresh weight of individual plants increased linearly with time ($r^2=0.978$–0.998, $P<0.001$ in all cases). Fresh weight was negatively correlated with Pa concentration in the shoot ($r=-0.23$, $P<0.05$, $n=79$). Again this indicates a trade-off between Pa production and biomass production. There is no significant correlation between Pa concentration and RGR ($r=-0.02$, $n=79$). Average Pa concentration in experiment 2 was higher than in the control of experiment 1 (ANOVA, $F=9.61$, $df=1, 92$, $P<0.01$).

Table 4 Summary of data from studies of costs of secondary metabolites. The table shows compounds, average concentrations, range in concentration of the different secondary compounds and the proportion of variance explained in the correlation between concentration of the secondary compound and growth/seed production. (*Alk* alkaloids, *Fu* furanocoumarins, *Tan* tannins, *Pro* proteinase inhibitors, *Cya* cyanogenic glucosides, *n* number of plants in experiment)

Plant species	Compound	*n*	Conc. g/g dry wt.	Range in conc.	Explained variance
Nicotiana tabacum	Alk	10	5.21%	6.03%	74.0%[a]
Pastinaca sativa	Fu	143	0.21%	0.52%	4.1%[b,c]
Cecropia peltata	Tan	45	3.14%	4.70%	27.0%[d]
Phalaris arundinacea	Alk	1430	0.08%	0.72%	4.4%[e,f]
Nicotiana sylvestris	Alk	20	0.53%	0.94%	25.4%[g]
Senecio jacobaea	Alk	15/77	0.28%	0.52%	8.3%[h]
Lycopersicon esculentum	Pro	99	0.05%	0.10%	0.0%[i]
Trifolium repens	Cya	80	1.05%	2.10%	17.0%[j,k]
Lotus corniculatis	Cya	157	0.42%	–[l]	0.0%[m]

[a] Vandenberg and Matzinger (1970)
[b] As Berenbaum et al. (1986) give no actual data, concentration of furanocoumarins are from Berenbaum et al. (1989)
[c] Berenbaum et al. (1986) (total furanocoumarins in leaf against plant weight)
[d] Coley (1986)
[e] Østrem (1987)
[f] Østrem (1988)
[g] Baldwin et al. (1990)
[h] This study (weighted average)
[i] Brown (1988)
[j] Variance between groups was calculated and used as an estimate of explained variance
[k] Kakes (1989)
[l] Range in concentration could not be determined from data
[m] Briggs and Schultz (1991)

Discussion

A trade-off between plant growth and Pa concentration may be expected under conditions in which both are dependent on the same resource. Such a trade-off appears to occur in the (light-limited) control series. If light is limiting Pa production, plants reared at a higher light intensity (more energy available) would be expected to have a higher Pa concentration. This is supported by the higher mean Pa concentration observed in plants in experiment 2 compared to the control plants of experiment 1. The proportion of explained variance of the correlation between fresh weight and Pa concentation in both experiments is rather low, on (weighted) average about 8% (Table 4) suggesting that the costs of Pas are relatively small. Pas contributed about 0.5% to total dry weight in the light-limited series. If the costs of Pas are equal to the costs of the same amount of biomass (Lambers and Rychter 1989) the explained variance should be less than 0.5%. Either the estimate is too low or other costs (e.g. transport or storage) contribute substantially to the total costs of Pas.

The lack of relationship between RGR and Pa concentration in the light-limited plants is probably caused by measurement errors in the RGRs. Because the correlation between fresh weight and Pa concentration is, in both experiments, significant at the 5% level, a greater measurement error in the RGR will quickly obscure a correlation between RGR and Pa concentration.

The higher proportion of the plant's total nitrogen incorporated into Pas in the NL treatment was unexpected, suggesting that the total amount of nitrogen used for the production of Pas was not changed in the NL treatment compared to the control. In the NL treatment on average 0.44% of the total nitrogen present in the plants was incorporated in Pas. This means that total fresh weight was reduced by about 0.5%. Since this seems negligible, we conclude that nitrogen was not limiting Pa production.

In our study area nitrogen (or phosphorus) limitation is an important influence on plant growth. Indeed, in a field study no costs associated with Pas could be detected (Vrieling 1991). Costs of Pas for *S. jacobaea* are therefore expected to be absent in its natural environment.

This study has shown that there are (small) costs associated with the concentration of chemical defence substances in plants. This is in agreement with the findings of a number of previous studies (Table 4). These studies also showed that as the average concentration of the secondary metabolite increased, more of the variance was explained by the correlation between growth or seed production and secondary metabolite concentration (Fig. 4). The available information suggests that costs of secondary metabolites increase with their concentration. The combined results of different experiments, different secondary plant substances and different plant species indicate that secondary plant metabolites are costly. These results have been obtained in greenhouse studies or experimental gardens; it remains unclear whether this is still true in the field.

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