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Induced responses in three alkaloid-containing plant species

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Abstract. In this paper we test three plant species for the inducibility of their alkaloid production. The plants were heavily damaged by cutting off 50% of their leaf surface using a pair of scissors. The cut-off leaf tips were used as controls for possible diurnal fluctuations. After 3, 6, 12, 24 and 48 h, respectively, the leaf bases of the damaged plants were harvested and the alkaloid concentration was measured. In *Senecio jacobaea* the pyrrolizidine alkaloid (Pa) concentration in damaged plants decreased within 6–12 h after damage. Within 24 h after damage the Pa concentration of *Cynoglossum officinale* doubled compared to control values. Indole alkaloid production in *Catharanthus roseus* was found not to be induced in this experiment. The responses are discussed in a functional context. We hypothesize that the nature of the response is not a feature of the type of secondary metabolite, but is related to whether the plants are damaged mainly by generalist or by specialist herbivores.

Key words: *Senecio jacobaea* – *Cynoglossum officinale* – *Catharanthus roseus* – Alkaloids – Induced responses

Induced defences in plants have received much attention lately. It is thought that induced defences enable plants to defend themselves at lower costs and to avoid auto-intoxication by defence chemicals (Karban and Myers 1989). Although there has been a boom in papers on induced defences during the past 10–15 years, it still remains an open question how the mechanism of induced defences can be understood in an ecological and evolutionary context. This is mainly due to the fact that the selection of the species studied is biased towards economically important agricultural crops (Baldwin 1991; Green and Ryan 1972; Brown 1988; Broadway et al. 1986) or trees (Edwards and Wratten 1983; Neuvonen and Haukioja 1984; Neuvonen et al. 1988; Gibberd et al. 1988; Clausen et al. 1991). Trees are relatively long-lived

plants in which both short- and long-term induced defences can play a role (Clausen et al. 1991). Research on induced defences in trees therefore encounters some specific problems (Neuvonen and Haukioja 1985). It also remains questionable whether the results obtained in trees are generally applicable to herbaceous plants. To fully understand how induced defences evolved in nature, it is necessary to measure the effects on fitness. Costs and benefits of induced defences in relation to fitness can be most easily measured on small, short-lived plants (Karban and Myers 1989).

A logical first step in the study of induced plant defences is to test if a certain plant species shows an induced response. An induced response can be generally defined as a change of plant quality after damage (Karban and Myers 1989). This definition thus includes changes in defence chemicals as well as in nutrient levels (Neuvonen and Haukioja 1984). In our study we focus on changes in levels of alkaloids.

Alkaloids are generally known as compounds biologically active against pathogens and herbivores (Hartmann 1991). There are only a few well-studied examples of induction of alkaloids. Provided the roots were not restricted in growth, removing 40% of the shoot mass of wild tobacco (*Nicotiana glauca*) resulted in a fourfold increase in total alkaloids 8 days after damage (Baldwin 1991). In *Atropa acuminata* repeated artificial damage resulted in maximal induction of alkaloids after 22 days (Bashir-Kahn and Harborne 1990). Moreover the response depended on the amount of damage: a minimum of 9% of the leaf area had to be removed before alkaloid levels increased significantly (Bashir-Kahn and Harborne 1991).

These examples indicate that the amount of damage and the timing can be important factors when measuring an induced response. Additionally the natural variation in quantity of plant chemicals, like diurnal and seasonal fluctuations, and differences between genotypes may lead to varying responses (Coleman and Jones 1991).

In this paper we study the inducibility of alkaloids in three plant species: *Senecio jacobaea* L., *Cynoglossum*

officinale L. and *Catharanthus roseus* (L.) G. Don. In these species we expect an inducible response within a short period of time (hours to days), because these species are relatively small and short-lived and because alkaloids are considered as mobile defences (*sensu* Coley et al. 1985).

Senecio jacobaea L. (Compositae) and *Cynoglossum officinale* L. (Boraginaceae) are common biennial plants in the coastal dunes of western Europe (Harper and Wood 1957; De Jong et al. 1990). Both species contain pyrrolizidine alkaloids (Pa's) and have been under investigation in our laboratories for several years (Van der Meijden 1979; Prins et al. 1989; Vrieling et al. 1991a, b). Pa's are composed of a pyrrolizidine nucleus, with one or two ester linkages. *S. jacobaea* contains mainly closed macrocyclic diesters, like senecionine and jacobine, while *Cynoglossum officinale* contains "open" molecules like heliosupine (Hegnauer 1964, 1989). Pa's are known to be deterrent to generalist herbivores (Vrieling et al. 1991a, b) and toxic to vertebrates (Anonymous 1988). In a bio-assay, larvae of *Ethmia bipunctella* (Lepidoptera; Ethmiidae) preferred leaf discs from undamaged *Cynoglossum officinale* plants over discs from plants that were damaged 24 h before. There was, however, no negative effect on the growth rate and fecundity of *E. bipunctella* caterpillars raised on leaves of damaged plants (Prins et al. 1987). Until now these results have not been related to chemical data of the plant.

As well as Pa's, there are other secondary metabolites present that may have defence properties. *S. jacobaea* contains sesquiterpenes and *C. officinale* contains phenolics and phenolic acids (Hegnauer 1964, 1989). Although sesquiterpenes as well as phenolics can act as chemical defences (e.g. Rosenthal and Berenbaum 1991), their role in these plants species is still unknown.

Catharanthus roseus (L.) G. Don (Apocynaceae) is a perennial sub-shrub, originating from Madagascar, but nowadays found in tropical areas around the world (Markgraf 1976). Leaves of *C. roseus* plants contain monomeric indole alkaloids, e.g. vindoline and catharanthine, as well as dimeric ones like 3',4'-anhydrovinblastine (Van der Heijden et al. 1989; Naaranlahti et al. 1991). Indole alkaloids are potent anti-feedants. Tested in a bio-assay using a 0.04% solution, vinblastine and catharanthine appeared to be the most deterrent alkaloids against the polyphagous *Spodoptera* larvae (Meisner et al. 1981).

Materials and methods

Plant material

Senecio jacobaea plants were half-sibs, grown from a cross with three fathers, originating from a natural population in the dunes of Meijndel near The Hague (The Netherlands). Seeds were soaked for 10 days in petri-dishes with wet filterpaper (16 h L/20° C, 8 h D/10° C). The seedlings were planted in a 1:1 mixture of dune sand and compost and placed in a greenhouse (min. 20° C, 16 h L/8 h D). Adequate nutrient solution (Steiner 1968) was supplied three times a week. The plants used in this experiment were 12 weeks old.

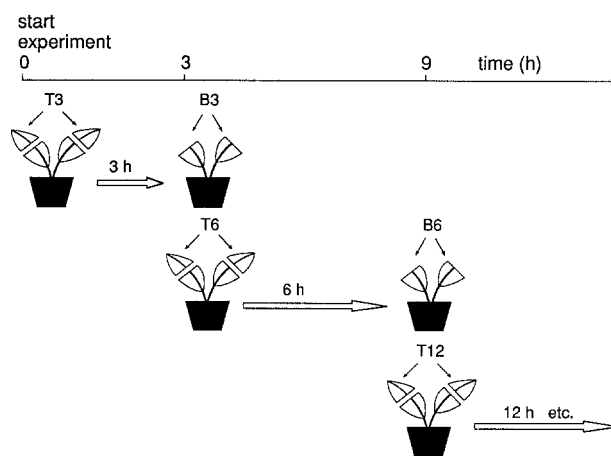


Fig. 1. Procedure of the experiment. Plants were damaged and harvested in an estafette. Leaf tips of the next group thus served as control values for naturally occurring fluctuations in time

Cynoglossum officinale plants were half-sibs, grown from seeds of one plant collected in the dunes of Meijndel. The seeds were scarified and soaked for 10 days in petri-dishes with wet filterpaper (16 h L/20° C, 8 h D/10° C). The seedlings were grown as above. The *C. officinale* plants used for experimentation were 8 weeks old.

Catharanthus roseus seeds were obtained from plants bred at the Division of Pharmacognosy, University of Leiden, since 1988. In every cross-pollination at least three plants were used as parents. The plants were grown in 100% compost in a growth chamber (mean temperature 25° C, 12 h L/12 h D, relative humidity 60%). The plants used for this experiment were 20 weeks old.

Leaf damage experiment

We damaged the plants by removing 50% of the leaf area with a pair of scissors. This was done to avoid problems with damage thresholds (e.g. Bashir-Kahn and Harborne 1991) and reproducibility of the damage. Moreover the cut leaf tips could be used as controls for diurnal fluctuations.

Because leaf tips were used as controls, it was necessary to test for possible natural differences in alkaloid concentration between leaf tips and bases. The leaves of nine *S. jacobaea*, nine *Cynoglossum officinale* and five *Catharanthus roseus* plants were cut in half, freeze-dried and extracted.

For the damage experiment 120 plants of each species were randomly divided up into five groups of 24 plants. To control for possible diurnal fluctuations, the different time groups were damaged and harvested in an estafette (Fig. 1). At time 0 the first group of plants was damaged by clipping off the leaf tips (50% biomass loss) with a pair of scissors. The leaf tips (code T3) for each plant were collected and stored at -20° C. Three hours later, the leaf bases of these plants were harvested (code B3). At the same time the leaf tips of the second group were collected (T6). Six hours later, 9 h after the experiment started, the bases of the second group were harvested (B6) and the next group was damaged (T12), and so on. The leaf tips thus served as controls for possible diurnal fluctuations (comparing T3, T6, T12 and so on), as well as for measuring changes in the plant caused by the damage (comparing B3, B6, B12 and so on). Times between damage and harvesting were 3, 6, 12, 24 and 48 h. The samples were freeze-dried and ground using a Waring Blender. To limit the number of samples for alkaloid determination, equal amounts of six individuals were mixed to obtain four samples per group.

Alkaloid determination

For Pa determination 100 mg dry weight (DW) was extracted with 4.5 ml 0.5 M sulphuric acid. After 1 h of extraction excess zinc powder was added to reduce the alkaloid N-oxides. After another 2 h the extract was filtered over a Büchner funnel supplied with a paper filter and made alkaline (pH > 10) with 0.5 ml 25% ammoniumhydroxide. The extract was brought upon a Extrelut column (Merck) and after 20 min the Pa's were recovered with 2 times 15 ml dichloromethane. This is a modification of a method described by Hartmann and Zimmer (1986).

After evaporation of the dichloromethane the residue was re-dissolved in 5.0 ml methanol. Total Pa concentration was spectrophotometrically determined in duplicate after a colour reaction (Mattocks 1967). Monocrotaline (Sigma, 99% pur.) was used as a reference.

Indole alkaloids were extracted from 50 mg DW plant material in 2 ml methanol: 0.1 M hydrochloric acid = 1:1, containing 54 µg dihydroquinine as internal standard. The extract was mixed for 30 s with an Ultra-thurax. After centrifugation, the supernatant was collected and the pellet was extracted once more with 2 ml methanol: 0.1 M hydrochloric acid = 1:1. The samples were left for 30 min in an ultrasonic bath. After another centrifugation, the combined supernatants were freeze-dried. After resuspension in 0.5 ml of HPLC eluent (15% acetonitril), the samples were analysed on HPLC. The system existed of a Waters Bondapak Phenyl column (300 * 3.9 mm i.d.), eluted with a phosphate buffer - acetonitril 15-50% gradient. The alkaloids were identified by their UV spectrum and by comparing retention times of reference samples.

Statistical analyses

Differences in alkaloid levels between leaf tips and leaf bases of the same plant were analysed by Wilcoxon's signed-ranks matched-pairs test. Diurnal fluctuations of alkaloid levels were tested for by analysing the measured alkaloid levels in the leaf tips in time by ANOVA (Statgraphics, version 5.0, Statistical Graphics Corporation, 1991). To test for changes in alkaloid concentration as a result of damage, the alkaloid levels measured in the damaged leaf bases were also analysed by ANOVA.

Because of the diurnal fluctuations in *Senecio jacobaea* the induction ratio had to be calculated from two means. The variance was thus estimated as

$$\text{var} \frac{\bar{x}}{\bar{y}} = \frac{\bar{x}^2}{\bar{y}^2} \left(\frac{\sigma_x^2}{\bar{x}^2} + \frac{\sigma_y^2}{\bar{y}^2} \right)$$

These ratios were analysed by the following procedure: (1) sum the differences of the ratios with the initial value: $\Sigma(\text{diff})$. (2) Calculate test statistic $Z = \Sigma(\text{diff}) / \sqrt{\Sigma(\text{var})}$. The value of P corresponding to Z is extracted from a normal distribution table.

For all statistical tests mentioned above α was set as 0.05.

Results

Senecio jacobaea

No significant difference was found between the Pa concentration of undamaged leaf tips and leaf bases of *S. jacobaea* plants (Table 1; Wilcoxon's signed-ranks matched-pairs test; $n=9$; $P=0.81$). The Pa concentration of the leaf tips varied significantly during the experiment (ANOVA; F -ratio = 7.143; $P=0.002$), which indicates that the alkaloid concentration in the undamaged plants

Table 1. Initial concentration of alkaloids in bases and tips of leaves of *Senecio jacobaea* ($n=9$), *Cynoglossum officinale* ($n=9$) and *Catharanthus roseus* ($n=5$)

	Base mean conc. mg/g DW	Tip mean conc. mg/g DW	Ratio base/tip pairwise
<i>Senecio jacobaea</i>			
Total Pa	1.08 (0.12)	1.08 (0.11)	1.05 (0.13)ns
<i>Cynoglossum officinale</i>			
Total Pa	1.33 (0.15)	0.91 (0.10)	1.54 (0.17)*
<i>Catharanthus roseus</i>			
Total indole alkaloids	19.88 (1.46)	16.12 (0.77)	1.23 (0.06)*
Vindoline	3.01 (0.44)	2.15 (0.26)	1.41 (0.16)ns
Catharanthine	4.83 (0.82)	2.99 (0.30)	1.59 (0.18)*
Serpentine	0.81 (0.06)	0.63 (0.08)	1.34 (0.13)*
Vindoline	3.03 (0.67)	1.89 (0.21)	1.56 (0.20)*
AVBL	8.19 (0.43)	8.45 (0.30)	0.97 (0.03)ns

Figures in parentheses are SEMs

Abbreviations: Pa = pyrrolizidine alkaloids; AVBL = 3', 4'-anhydrovinblastine

Last column: ratio of alkaloid concentrations in leaf bases by leaf tips, pairwise per plant

Statistical analysis with Wilcoxon's signed-ranks matched-pairs test
n.s. = not significant (i.e. not different from 1); * = $P < 0.05$

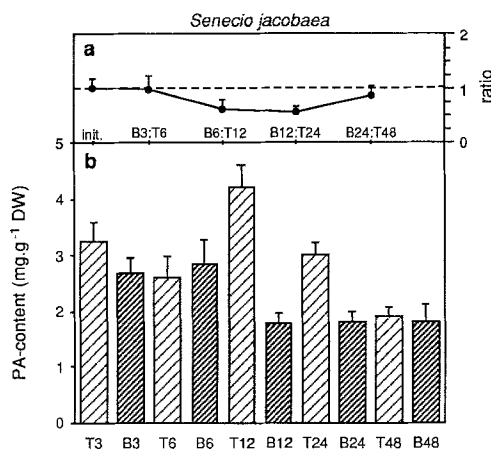


Fig. 2. a Ratio of mean Pa concentration of induced leaf bases against mean Pa-concentration of the control leaf tips of the next group, harvested at the same time. *Init.* = initial ratio in undamaged plants. This value is also indicated by the dashed line. b Pa-concentration (+ SEM; $n=4$ per group) of leaf tips (light shading) and damaged leaf bases (heavy shading) of *Senecio jacobaea*, measured at different time intervals after damage

is not constant in time (Fig. 2b). The Pa concentration of the damaged leaf bases also differed over time (ANOVA; F -ratio = 3.419; $P=0.036$). To correct for fluctuations over time, we calculated an induction ratio to express the changes in Pa levels caused by the damage. For this ratio the mean Pa concentration of the induced leaf bases was divided by the mean Pa concentration of the leaf tips of the next group, which were harvested at the same time (e.g. B6/T12; Fig. 2a). Statistical analysis of these ratios was performed as described in Materials and methods. In *S. jacobaea* the decrease in Pa concentration as a result of damage appeared to be statisti-

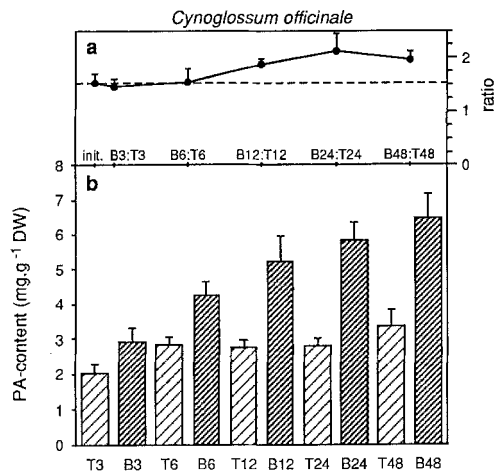


Fig. 3. **a** Ratio of mean Pa concentration of induced leaf bases against mean Pa concentration of the control leaf tips of the same plants. *Init.* = initial ratio in undamaged plants. This value is also indicated by the *dashed line*. **b** Pa-concentration (+ SEM; $n=4$ per group) of leaf tips (*light shading*) and damaged leaf bases (*heavy shading*) of *Cynoglossum officinale*, measured at different time intervals after damage

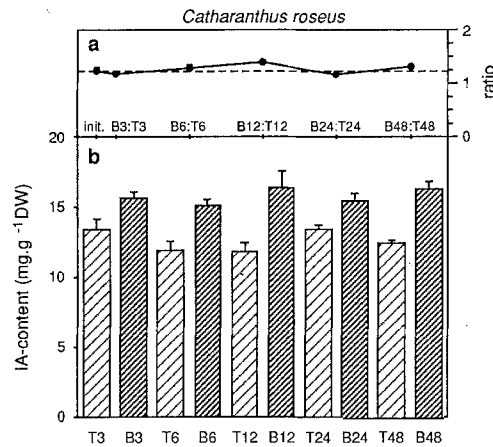


Fig. 4. **a** Ratio of mean indole alkaloid (IA) concentration of induced leaf bases by mean indole alkaloid concentration of the control leaf tips of the same plants. *Init.* = initial ratio in undamaged plants. This value is also indicated by the *dashed line*. **b** Indole alkaloid concentration (+SEM; $n=4$ per group) of leaf tips (*light shading*) and damaged leaf bases (*heavy shading*) of *Catharanthus roseus*, measured at different time intervals after damage

cally significant ($Z=2.56$; $P=0.01$). The largest decrease was observed at 12 h after damage. For plants harvested 24 h after damage the induction ratio was almost back to the initial value.

Cynoglossum officinale

In *C. officinale* the leaf bases in undamaged plants already contained 1.54 times more Pa's than the leaf tips (Table 1; Wilcoxon's signed-ranks matched-pairs test; $n=9$; $P=0.02$).

During the damage experiment the Pa concentration in the leaf tips did not significantly change in time (Fig. 3b; ANOVA, F -ratio = 2.629; $P=0.08$). This indicates that Pa concentrations in *C. officinale* plants do not show diurnal fluctuations. These results are confirmed by another experiment, in which ten plants were harvested at 4-h intervals over 24 h (ANOVA; $P=0.61$; N.M. Van Dam and T. Schijvens, unpublished). It is thus not necessary to correct for diurnal fluctuations as in *S. jacobaea*. The induction ratios were calculated as mean Pa concentration of the induced leaf bases by the undamaged leaf tips of the same plants (e.g. B3/T3).

The Pa concentration in leaf bases of the clipped plants however, differed significantly with time after damage (ANOVA; F -ratio = 6.062; $P=0.004$). Despite the natural difference in Pa concentration between leaf tips and bases, it is very clear that the Pa concentration in the damaged leaf bases steadily increased with time after damage (Fig. 3a).

Catharanthus roseus

Leaf bases of *C. roseus* plants contained 1.23 times more indole alkaloids than leaf tips (Table 1; Wilcoxon's

signed-ranks matched-pairs; $n=5$; $P=0.03$). The concentrations of most indole alkaloids were higher in the bases than in the tips, except for 3',4'-anhydrovinblastine (AVBL). Because it turned out that during the experiment the changes in all individual alkaloid levels were of the same order of magnitude, the total amount of alkaloids was taken as a measure for the induced response.

The indole alkaloid concentration in the leaf tips showed no significant diurnal fluctuations (Fig. 4b; ANOVA; F -ratio = 1.896; $P=0.16$), so induction ratios were calculated as for *C. officinale* (Fig. 4a). The indole alkaloid concentration in the damaged leaf bases also remained constant in time (ANOVA; F -ratio = 0.726; $P=0.59$). Damage did thus not change the alkaloid concentration of *C. roseus* plants.

Discussion

The three plant species studied all reacted differently to severe mechanical damage. Alkaloid levels decreased in *S. jacobaea*, they increased in *Cynoglossum officinale* and no response was observed in *Catharanthus roseus*. We hypothesize that these differences in response are adaptations related to the amount and type of herbivory the plant species normally suffers in the field. Assuming that defences are costly and induced responses are a way to optimize the cost-benefit balance in the plant (Karban and Myers 1989), we make some predictions about the occurrence of different types of defences in plants.

Constitutive defences will be an advantageous strategy if there is a high risk of being attacked by generalist herbivores. Positive induction of defence chemicals will be most advantageous in plants that suffer from low and unpredictable generalist herbivore pressure. In plants that are mainly attacked by specialist herbivores (i.e.

herbivores that have defeated the chemical defences of the host plant), the amount of damage will hardly be decreased by producing more defence chemicals. If herbivore attack is limited to a period that is considerably shorter than the growing season of the plant, a regrowth mechanism may provide a successful alternative (Van der Meijden et al. 1988; Van der Meijden 1990).

For *S. jacobaea* the results indicate a decrease in Pa concentration after damage (Vrieling and Bruin 1987; this paper). We observe a tendency in the literature to consider only increases in levels of secondary metabolites as induced defences. But there is no *a priori* reason why a decrease in secondary metabolites could not be seen as a functional response. The disappearance of Pa's from the leaves can be the result of reallocation or of catabolism. The latter is not very probable: tracer studies showed that Pa's in *S. jacobaea* are stable products with no significant degradation (Sander and Hartmann 1989). On the other hand, compared to other biennials *S. jacobaea* has a great capacity for regrowth after severe leaf damage (Prins et al. 1989). In the field *S. jacobaea* is completely defoliated once every 2 years by the larvae of the specialist *Tyria jacobaeae* (Lepidoptera) (Van der Meijden et al. 1988). Herbivory is limited to approximately 1 month only, so the natural conditions favour selection for regrowth after severe damage. The decrease of Pa's in the leaves might reflect reallocation of resources for future regrowth.

Cynoglossum officinale plants are seldom severely damaged (Van der Meijden et al. 1988; Prins and Nell 1990). If *C. officinale* is placed under high herbivory pressure reduction in plant size and reproduction occur (Prins and Nell 1990). Moreover the plants are not able to recover fast after severe damage of the leaves (Prins et al. 1989). Under these circumstances investing in more chemical defences will be more advantageous than in regrowth.

There are hardly any data available on the extent of herbivory suffered by *Catharanthus roseus*, probably because it is hardly eaten at all (Meisner et al. 1981; pers. observations in Kenya, Zambia and Zimbabwe). Generalist herbivores like *Spodoptera* larvae avoid *C. roseus* leaves. In a no-choice situation, they are not able to survive on *C. roseus*, because of the combined effect of a lower ingestion rate and inhibition of digestive enzymes by the indole alkaloids (Meisner et al. 1981; Chockalingam et al. 1989).

The results of the damage experiment are similar to those of earlier studies on the induction of indole alkaloids in *C. roseus* (Frischknecht et al. 1987; Naaranlahti et al. 1991). In contrast to these studies, we did not find a large variation in alkaloid concentrations of the plants. This might be due to the genetic composition of the plants, the rearing conditions or the method of extraction. Our results also demonstrate that there is an initial difference in indole alkaloid content between leaf tips and leaf bases. Using leaf halves without measuring or mentioning the initial differences on an undamaged plant (Naaranlahti et al. 1991) might lead to false conclusions.

A functional explanation for the fact that indole alkaloids in *C. roseus* are not inducible is hard to give, be-

cause ecological data are scarce. The high concentration of indole alkaloids in the leaves (up to 1.5%) and the fact that they are mainly found in latifolers and specialized cells (Yoder and Mahlberg 1976) indicate that the plant stores indole alkaloids constitutively and probably has already evolved mechanisms to overcome auto-intoxication.

The general conclusion of this study is that different plant species, all containing alkaloids, respond quite differently to the same amount of mechanical damage. To be inducible or not is thus not related to the type of secondary metabolite. However, the way the experiment is performed affects the results. Because only artificial damage was applied, it remains unknown how the plants react to natural damage. It is known from the literature that the effects of artificial and natural damage are not always fully comparable (Bashir-Kahn and Harborne 1991). It is also possible that chemical substances we did not measure change in response to the damage. Nevertheless we conclude that *S. jacobaea* and *Cynoglossum officinale* respond to severe mechanical damage by changing their leaf Pa concentration. Further research on these species can now follow according to the scheme proposed by Karban and Myers (1989). According to this scheme the next step will be to assess whether the induced response leads to a decrease of herbivore preference or performance. Finally, before we can truly speak of induced defence, it has to be established that induction really enhances plant fitness.

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