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## Genetic variation in constitutive and inducible pyrrolizidine alkaloid levels in *Cynoglossum officinale* L.

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**Abstract** The constitutive pyrrolizidine alkaloid (PA) concentration of both shoots and roots differed significantly between 17 selfed families. The broad-sense heritability accounted for 33–43% of the variation in PA levels. Families also differed significantly in the amount and the direction of PA induction in both shoots and roots, 24 h after punching 15 holes in the leaves. We found a significantly negative relationship between the changes in PA content of the shoots and changes in PA content of the roots. The total PA content of the plants did not increase. We thus concluded that changes in PA distribution over the plant resulted from transport of PAs within the plant. The direction of transport differed between families: some transported PAs to the shoots, others to the roots. This makes it questionable whether PAs act as damage-induced defences. The effect of damage on the PA concentration is far less than the differences found between families in the constitutive PA concentration. This again strongly suggests that damage-induced defences in *Cynoglossum officinale* do not play an important role. We argue that the general lack of attention that is given to genotype in induction experiments, has led to false conclusions.

**Key words** *Cynoglossum officinale* · Pyrrolizidine alkaloids · Induced defences · Genetic variation · Transport

### Introduction

Pyrrolizidine alkaloids (PAs) in plants are assumed to act as anti-feedants against generalist herbivores (Hartmann 1991). PAs are deterrent for invertebrate herbivores (Bentley et al. 1984; Dreyer et al. 1985; Vrieling et al. 1991b; Speiser et al. 1992), while they are toxic to cattle and humans (Mattocks 1986; Wink 1993).

Nevertheless, not all individual plants of a species contain high levels of PAs. For example, the concentration of PAs in *Cynoglossum officinale* ranges from 0.25 to 2.1% of the dry mass (De Jong et al. 1990). Thus high PA levels may not always be selected for. Firstly, production of PAs may be costly when resources are diverted from other fitness-enhancing functions, for example, growth or reproduction (Rhoades and Cates 1976; Fagerström et al. 1987; Zangerl and Bazzaz 1992). Secondly, alkaloids may be phytotoxic (Wink 1993) or inhibit seed germination (Aerts et al. 1991). Finally, ecological costs may be involved (Vrieling et al. 1991a; Simms 1992). Some herbivores sequester plant-derived PAs for their own defence against predators and parasites (Boppré 1986). Moreover, secondary metabolites that serve as deterrents for generalist herbivores can act as feeding stimulants for adapted specialist herbivores (Berenbaum 1991).

Inducible defences may provide a way to decrease the costs of secondary compounds and avoid problems with auto-toxicity (Karban and Myers 1989). However, species differ considerably in the way they induce their secondary metabolites. After the same amount of severe mechanical damage, the PA level decreases in *Senecio jacobaea* and increases in *C. officinale* (Van Dam et al. 1993). Since variation in inducibility within a single plant species has previously been reported (Zangerl and Berenbaum 1990), it is possible that these opposing trends in induction occur even within the same plant species.

Inducibility of defences should thus not be seen as a constant trait within a species. As noted by Coleman and Jones (1991), most studies on induced defences disregard the variation between genotypes, which can lead to the misinterpretation of results. Therefore, it is important to check for genetic variation in inducibility within a plant species before the results of a few experiments are generalized.

In this paper we examine the variation in constitutive and inducible PA levels within *C. officinale*. Costs of constitutive PA production are estimated by examining

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the relationship between PA concentration and growth. In this design only the physiological costs (sensu Simms 1992) of PAs can be determined.

PA levels in leaves of *C. officinale* increase after different amounts of mechanical damage (Van Dam and Bhairo-Marh e 1992; Van Dam et al. 1993). In our study we consider not only the leaves but also the roots of the plants. There are several ways a plant can increase the concentration of defence chemicals in a specific organ: increased synthesis rate, decreased degradation rate, or transport from one organ to the other (Baldwin 1991). In the first two cases, the damaged plants in our experiment should contain more PAs than the undamaged control plants. Transport of PAs should result in a negative linear relationship (with a slope of -1) between the amount that disappears from the one organ and the amount that appears in the other. Only by making a total PA balance of the whole plant is it possible to study the mechanism of induction.

## Materials and methods

Seeds of 17 selfed greenhouse-grown plants of *C. officinale* were scarified and soaked in water for 2 weeks at 20°C/16 h L, 10°C/8 h D. On 18 June 1992, ten seedlings per family were individually potted in 1 l pots. The pots were filled with a mix of dune sand and 25% compost. The plants were randomly assigned to a place on the bench in a growth room, and grown at 18 h L/29–34°C, 6 h D/17–22°C, relative humidity 60–70%. The amount of photosynthetically active light at plant-level was 44 W/m<sup>2</sup>. The plants were given ample nutrient solution to allow exponential growth (Steiner 1968). On 29 July 1992, five plants per family were damaged using a perforator, 15 holes (3 mm diameter) being punched in an even distribution over the leaves, because PA induction was found to be maximal at this level of damage (Van Dam and Bhairo-Marh e 1992). Twenty four hours after the damage was inflicted, leaves and roots of the damaged and control plants were harvested separately. The plant parts were flash-frozen in liquid nitrogen and freeze-dried. Roots and leaves were weighed to determine total dry weight (DW) and ground with a Waring Blendor before extraction.

### Alkaloid extraction and determination

PAs were extracted from 75 mg of DW as described in Van Dam et al. (1993). A spectrophotometric colour reaction was used to determine PA concentrations (Mattocks 1967). Monocrotaline (99% pure, Sigma) was used as reference compound.

### Statistical analysis

Standard statistical tests were performed with Statgraphics 5.0 (Statgraphics Corporation 1992). Data were tested for normality using the Kolmogorov-Smirnov test for goodness of fit, before further analysis.

As a measure of induction we used induction ratios. To obtain these ratios, the mean PA concentration of the induced plants were divided by the mean PA concentration of the control plants. We thus measured the genotypic values for induction. In order to meet the normality assumption, the data were log transformed. The variances of the ratios (var) were estimated as follows:

$$\log(x/y) = \log x - \log y, \quad (1)$$

thus

$$\text{var}(\log(x/y)) = \text{var}(\log x - \log y) \quad (2)$$

By application of the general rule

$$\text{var}(a-b) = \text{var } a + \text{var } b \quad (3)$$

it follows that:

$$\text{var}(\log x - \log y) = \text{var}(\log x) + \text{var}(\log y) \quad (4)$$

where  $x$  is the mean value of the PA concentration of the damaged plants of a family and  $y$  is the mean value of the PA concentration of the undamaged plants of the same family. Differences between families were tested using an  $F$ -test (Sokal and Rohlf 1981).

The data in Fig. 3 were analysed using principal axes analysis (Sokal and Rohlf 1981).

### Heritabilities

Following Lawrence (1984), the broad-sense heritability for self progenies (assuming no maternal effects) were estimated as:

$$h^2 = 1.5 \frac{s_b^2}{s_b^2 + s_w^2} \quad (5)$$

Where  $s_b^2$  and  $s_w^2$  denote the between-family and the within-family component of variance, respectively.

The 95% confidence intervals of the heritabilities were calculated (Wolff and van Delden 1987) as:

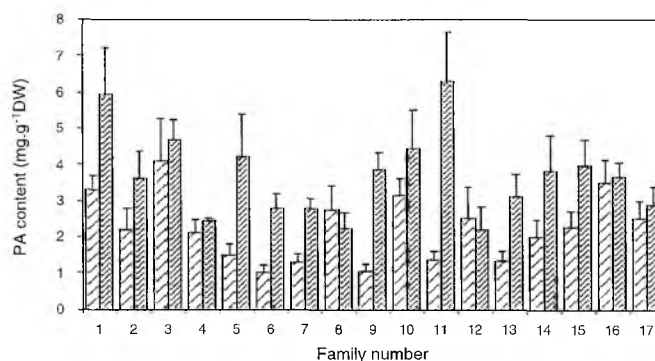
$$\frac{A}{1 + \frac{n^* F_\alpha}{R - F_\alpha}} < h^2 < \frac{A}{1 + \frac{n^* F_{1-\alpha}}{R - F_{1-\alpha}}} \quad (6)$$

In the design used here (selfed plants)  $A=1.5$ ,  $n$  is the number of plants within a family (in this experiment  $n=5$ ),  $R$  is the  $F$ -value of the corresponding ANOVA and  $F_\alpha$  is the critical value of the  $F$ -distribution, with the appropriate degrees of freedom, for  $\alpha=0.025$  (Rohlf and Sokal 1981).

## Results

### Constitutive PA concentration

The 17 full-sib families differed significantly in constitutive PA concentrations in both the shoots and the roots of undamaged plants (Fig. 1). Heritabilities for PAs in shoots, roots, and whole plants were significantly greater than zero and ranged from 0.33 to 0.43 (Table 1). The



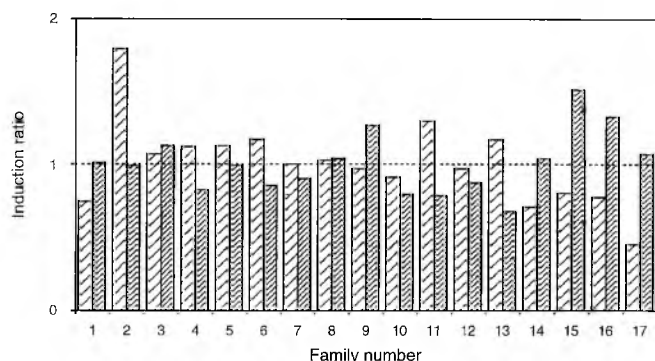
**Fig. 1** Constitutive pyrrolizidine alkaloid (PA) levels in shoots (light shading) and roots (heavy shading) of 17 full-sib families of *Cynoglossum officinale* (+ SE;  $n=5$  per family). ANOVA,  $F=3.00$ ,  $P=0.0008$  for shoots and  $F=2.39$ ,  $P=0.0068$  for roots.

**Table 1** Broad-sense heritabilities of constitutive and inducible PA concentration in shoots, roots, and whole plants of *Cynoglossum officinale*

Organ	$h^2$	(95% confidence limits)
Constitutive PA concentration		
Shoots	0.43	(0.14; 0.75)
Roots	0.33	(0.06; 0.64)
Plants	0.33	(0.13; 0.48)
Inducible PA concentration		
Shoots	0.35	(0.28; 1.11)
Roots	0.36	(0.30; 1.13)
Plants	0.36	(0.32; 1.15)

**Table 2** Means (+ SE) of total dry weight (DW), shoot/root ratio, and total pyrrolizidine alkaloid (PA) content per plant for control and damaged plants; paired  $t$ -test;  $n=17$  ( $ns$  not significant).

	Controls	Damaged	$P$ -value
Total DW (g)	2.02 (0.06)	2.02 (0.06)	0.98 ns
Shoot/root ratio	2.16 (0.13)	2.17 (0.17)	0.93 ns
Total PA (mg/plant)	5.58 (0.46)	5.51 (0.44)	0.76 ns



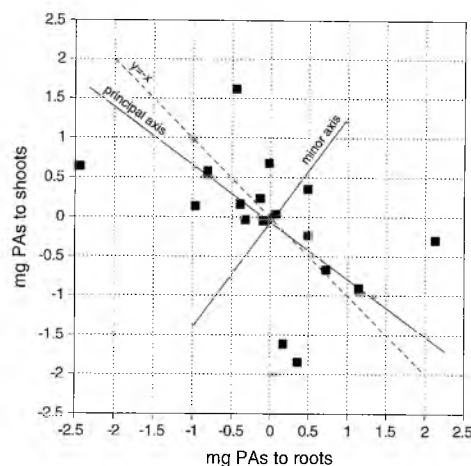
**Fig. 2** Induction ratios in shoots (light shading) and roots (heavy shading) of 17 full-sib families of *Cynoglossum officinale*. Dashed line denotes level of no induction.  $F$ -test on variances of log transformed data:  $F=3.63$ ,  $P < 0.002$  for shoots and  $F=3.75$ ,  $P < 0.0002$  for roots

roots had higher PA concentrations than the shoots (paired  $t$ -test,  $n=85$ ,  $P < 0.001$ ).

To establish whether there was a trade-off between PA concentration and growth, the total plant DW of undamaged plants was correlated to the mean PA concentration of the whole plant. No significant correlation was found ( $n=85$ ,  $r=-0.07$ ,  $P=0.55$ ). Thus no physiological costs of constitutive PA concentration could be shown.

#### Inducibility of PAs

The plants in the control group did not differ significantly from the damaged plants with respect to DW or shoot/root ratios (Table 2). Thus the amount of damage inflicted did not significantly decrease the biomass of the damaged plants.



**Fig. 3** Relation between the amount of pyrrolizidine alkaloids (PAs) gained in roots and in shoots after damage in *Cynoglossum officinale*. A negative value indicates a loss of PAs from an organ. Principal axis:  $Y_1 = -0.071 - 0.741Y_2$  with 95% confidence limits for the slope of  $-2.37$  and  $-0.11$ . Minor axis:  $Y_1 = -0.071 + 1.349Y_2$ . Eigenvalues:  $\lambda_1 = 1.23$  and  $\lambda_2 = 0.44$

Induction ratios for both shoots and roots were calculated (Fig. 2). Families differed significantly in the amount and the direction of PA induction in both shoots and roots (Fig. 2). Some families had a higher induction ratio in the leaves, others in the roots. We did not find any significant relationship between the direction of the induction and the shoot/root ratio or the constitutive PA concentration in shoots and roots. The heritabilities for the inducibility of PAs in shoots, roots, and whole plants were all significantly greater than zero (Table 1).

In contrast, when averaged over all families the total amount of PAs in damaged plants was not significantly different from the amount in undamaged plants (paired  $t$ -test; Table 2).

To acquire some insight into the mechanism of the observed process, we calculated the average amount of PAs that were gained or lost in damaged shoots in comparison with the undamaged shoots of the same family. This was done for the roots as well (Fig. 3).

The slope of the principal axis was significantly negative and the line did not differ significantly from  $y=-x$  (for values of the slope and the 95% confidence intervals see the legend to Fig. 3). The eigenvalue of the principal axis was 2.8 times greater than the eigenvalue of the minor axis. This indicates that the gain of PAs in the shoots and the loss of PAs from the roots were correlated with each other.

On average, the shoots gained or lost 20% and the roots gained or lost up to 25% of the original amount of PAs present in the organ.

#### Discussion

With the exclusion of maternal effects, we may conclude that, under greenhouse conditions, one-third of the varia-

tion in constitutive PA concentrations in *C. officinale* is due to genetic variation. Genetic variation in secondary metabolite concentrations has also been found in other wild plant species; such as *Senecio jacobaea* (Vrieling et al. 1993) and *Pastinaca sativa* (Zangerl and Berenbaum 1990).

Costs and herbivory are thought to be the two main selective forces on the levels of defence compounds in plants (Rhoades and Cates 1976; Fagerström et al. 1987; Simms 1992; Zangerl and Bazzaz 1992). We did not find any physiological costs for PA production in *C. officinale* in terms of decreased biomass. On the one hand, it may be possible that physiological costs were not detectable in our experiment, because the plants were not grown under light-limiting conditions (Vrieling and Van Wijk 1994). On the other hand, physiological costs could have disappeared in the course of evolution. The ability to produce PAs might have been costly when it first arose, but these costs could have been reduced or even eliminated during subsequent selection (Simms 1992).

However, this does not mean that PAs are not costly at all. Ecological costs, for example, whether PAs are attractants to specialist herbivores, were not measured in our experiment. Since many studies have failed to determine physiological costs of secondary metabolites in wild plant species (examples listed in Simms 1992), ecological costs might be the most important determinants of selection.

Although costs of secondary metabolites are hard to determine, benefits of secondary metabolites are more easily assessed. Echinatine-*N*-oxide, one of the major PAs in *C. officinale* (De Jong et al. 1990), is known to reduce feeding by spruce budworm by 35% (Bentley et al. 1984). Spruce budworm, however, is not a natural herbivore of *C. officinale*. Before we can draw conclusions about optimal PA concentrations, we need to know more about the ecological role of *Cynoglossum* PAs as defence chemicals against its natural herbivores.

We found genetic variation not only in constitutive PA levels, but also in the inducibility of PAs. In contrast to *P. sativa* (Zangerl and Berenbaum 1990), the families in our experiment differed significantly in the direction of induction. To our knowledge, this is the first time that such a variability in damage-induced responses has been shown within a single plant species.

Since changes in PA levels in shoots and roots are negatively correlated, they probably result from transport within the plant. Depending on the rate at which synthesis is able to take place, transport may be the fastest way to manipulate the amount of secondary metabolites in an organ. For example, within 3 h of damage, cucurbitacin concentration doubles in zucchini leaves as the result of transport to the damaged leaf (Tallamy 1985). Both roots and shoots of *C. officinale* are able to synthesize PAs from labelled precursors (Van Dam and Hartmann, in preparation), but the rate and efficiency of PA production in *C. officinale* plants is considerably lower than in *Senecio vulgaris* (Hartmann et al. 1989). PAs are stable prod-

ucts with low turnover rates (Hartmann 1991). Thus transport of PAs may be the most plausible way for *C. officinale* plants to change their PA concentration in the short term.

Since we did not find any physiological costs of constitutive PA levels, the inducibility of PAs cannot be a way to reduce these costs of defence, as was postulated by Karban and Myers (1989). Our results also provide food for thought about the relative importance of induced versus constitutive levels of PAs in *C. officinale*. The differences between families in constitutive PA levels are twice as high (factor 4) than the differences caused by induction (factor 2). This suggests that damage-induced defences do not play an important role in *C. officinale*.

The question is whether we observed a defence response evolved against herbivores or not. The observed changes may be a generalized wound response, which herbivores coincidentally cannot tolerate (Jermy 1984). But even then we would expect a uniform reaction in all families. The fact that some families seem to transport PAs to the roots and others to the shoots makes it difficult to think of a functional explanation.

The genetic variation in inducibility may have consequences for the interpretation of results of other induction experiments. Different genotypes may not only show different responses, as in our experiment, but also have different thresholds of damage (Coleman and Jones 1991). Thus the fact that a genotype does not show an induced response does not mean that it will never show any response under any circumstance. Different directions of the responses and different thresholds make it important to control for variation in genotype during induction experiments. This was also noted by Coleman and Jones (1991), who warned that the general lack of attention given to plant genotype in some studies may have resulted in unsubstantiated conclusions.

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