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EFFECT OF DROUGHT AND WOUNDING STRESS ON INDOLE ALKALOID FORMATION IN *CATHARANTHUS ROSEUS*

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Key Word Index *Catharanthus roseus*; Apocynaceae; stress physiology; developmental physiology; chemical defence; indole alkaloids.

Abstract In *Catharanthus roseus* indole alkaloid formation was monitored under drought and wounding stress (caused by cutting off plant parts) with the purpose of characterizing chemical defence strategy under these conditions. Drought stress effects a decrease of the relative (% dry wt) alkaloid content, but concentration in the living tissue differs little from non-stressed plants. The effectiveness of chemical defence is therefore not affected. In growing tissue wounding stress leads to an increase (up to 100%) in alkaloid accumulation, but under very severe stress conditions (when more than 50% of the plant biomass was detached), alkaloid formation is greatly reduced during a period of recovery time. In non-growing tissue alkaloid accumulation is not enhanced by wounding. Variation of the alkaloid content of stress-exposed plants decreases, indicating a very economic allocation of energy and/or nutrients.

INTRODUCTION

Catharanthus roseus is a source of pharmaceutically important indole alkaloids. Great efforts are being made to improve their production in cell culture systems. Since many secondary plant compounds may protect plants against the physical and biotic environment their formation is greatly influenced by external factors. We have it in mind to take advantage of these ecological connexions with regard to production of *Catharanthus* alkaloids. Plants living under stress, for instance in unfavourable climatic conditions, with a nutrient deficit or in competition with other plants, are preferentially attacked by predators. These observations are explained by a weakened chemical defence system [1, 2]. However, several reports exist on more or less unchanged [3, 4] or even increased levels of chemical defence compounds under environmental stress conditions [5–9]. Additionally, in cell culture systems the application of stress factors may promote secondary product formation [10, 11]. A stress-induced increase of allelochemicals is interpreted as a shift from effective but costly defence systems to less costly but less specific defence systems [2]. We assume that the response in a particular plant organ is the result of the stress intensity and of the value of that organ for the plant.

The two stress factors applied in these studies were drought stress effected by limited water supply and wounding stress effected by cutting off plant parts. Although no reports exist on the allelochemical properties of the *Catharanthus* alkaloids general deterrent activities against herbivores may be assumed. In field experiments we observed a molluscicidal effect. The clinical studies demonstrating pharmaceutical activities for a number of alkaloids [12] indicate their potency as defence compounds against mammalian herbivores.

RESULTS AND DISCUSSION

Indole alkaloid content (% dry wt) in the green part of *C. roseus* increases during plant development from 0.24% (S.D. ± 0.04) in young 2-month-old plants with six leaf pairs to 0.47% (S.D. ± 0.058) in flowering 6-month-old plants. An analogous increase of the alkaloid content (serpentine and ajmalicine) was observed in roots during the same developmental period [13]. Shoot tips, i.e. leaflets of less than *ca* 50 mg fr. wt, have an alkaloid content of 1–1.3%, a value which is independent of the developmental stage of the plant (Fig. 1). From the top to

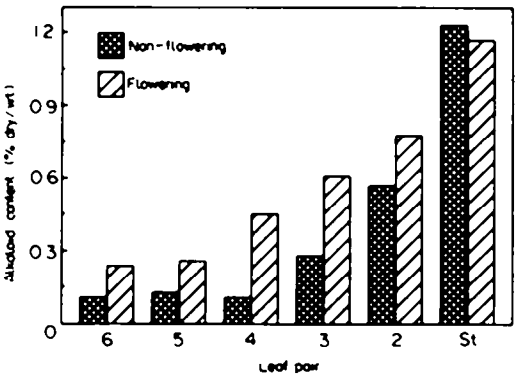

Fig. 1. Alkaloid content (% dry wt) of the shoot tip (St) and the five consecutive leaf pairs of 2-month-old non-flowering plants with a total of six leaf pairs and of 6-month-old flowering plants. Bars of the non-flowering plants are the mean of six plants analysed individually, bars of the flowering plants are the mean of three plants analysed at a time.

the base the alkaloid content of consecutive leaf pairs decreases. In the flowering plants this alkaloid gradient is less pronounced that in the non-flowering plants. Thus, fully developed leaves of flowering plants have a two to three times higher alkaloid content which leads to the higher average alkaloid content mentioned above. In the very youngest parts of the shoot tips (fr. wt < 10 mg) an alkaloid content of more than 3% was found. Very similarly, in *Coffea arabica* a purine alkaloid content of *ca* 4% has been determined in emerging leaflets [14]. Coefficients of variation of the alkaloid content of the leaf pairs (30–70, calculated for the non-flowering plants) are considerably higher than those of the whole plant.

The alkaloid decrease during leaf development may partly be explained by dilution as a result of growth, but since the absolute alkaloid amount per leaf pair starts to decrease already in the second youngest leaf pair, other physiological and/or metabolic processes are involved, e.g. alkaloid transport, increased catabolism, lowered synthesis. The alkaloid pattern also changes during plant development (Table 1). Mainly in older leaves, however, coefficients of variation of the relative alkaloid parts are high (up to 100, calculated for non-flowering plants). The relative part of catharanthine tends to increase with plant age and that of vindoline decreases with leaf age.

Based on dry wt the alkaloid content of water-stressed plants is lower than that of non-stressed plants. There is a positive correlation between the alkaloid and the water content of the tissue (Fig. 2). Based on fr. wt, however, the alkaloid content and alkaloid pattern of well-irrigated plants is not significantly different from plants with reduced water supply (Fig. 3). During the experiment the alkaloid content increases slightly probably due to plant development. Since for a predator only the living tissue concentration of a toxic compound is of importance, effectiveness of chemical defence was not changed by the different water conditions investigated in the present experiment. It is most remarkable that under stress variation of the alkaloid accumulation decreases considerably. The coefficient of variation ranged between 5 and 10 compared to 15–20 in control experiments.

To test the effect of wounding stress of low intensity (loss of biomass < 1%) halves of young expanding leaves and of fully developed leaves of flowering plants (*ca* 6 months old) were cut off and analysed; after 11 days the second halves of the leaves were analysed (always without the middle vein). Assuming that mainly the damaged organ exhibits a stress reaction, leaf halves of the opposite leaf of a pair were used as a control to monitor developmental changes. In damaged leaves a compensatory

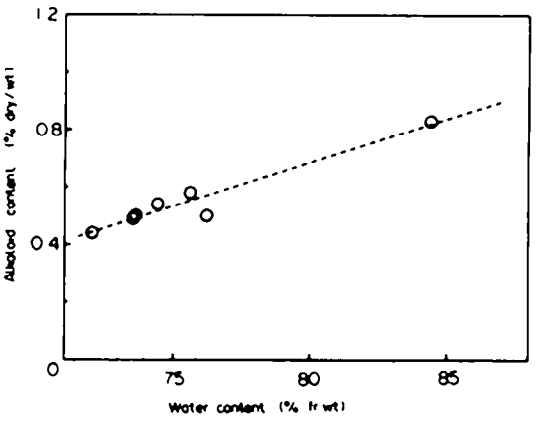

Fig. 2. Correlation between the water content (100 – [dry wt/fr. wt] 100) and the alkaloid content (% dry wt) of 6-month-old plants. Coefficient of determination: 0.94. Each point represents the mean of six analyses.

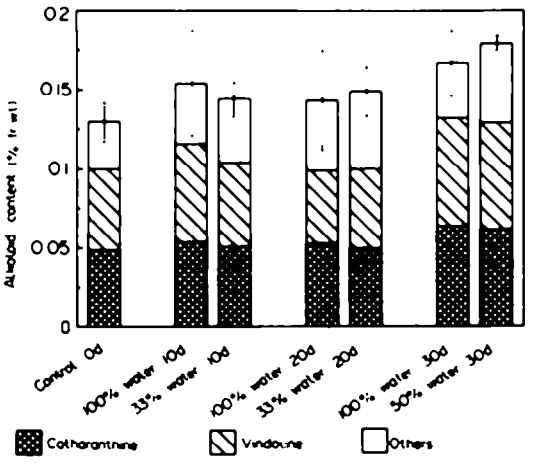

Fig. 3. Alkaloid content (% fr. wt) of 6-month-old plants under different water conditions. Plants with 100% water were kept in permanently water saturated soil (control), the analyses (mean of six plants) were carried out after 10 days, 20 days (33% of the water of the control) and 30 days (50% of the water of the control).

Table 1. Indole alkaloid pattern in leaves of non-flowering (nfl) 2-month-old plants and flowering (fl) 6-month-old plants. Mean of six plants (nfl) and three plants (fl), respectively

	Alkaloid composition (%)					
	Catharanthine		Vindoline		Others	
Leaf pair	nfl	fl	nfl	fl	nfl	fl
Shoot tip	27	48	48	40	25	12
1. Leaf pair	38	49	38	37	24	14
2. Leaf pair	33	51	43	32	24	17
3. Leaf pair	9	50	36	30	55	20
4. Leaf pair	25	45	17	27	56	28
5. Leaf pair	33	28	0	23	67	39

growth of *ca* 10–20% was observed (Table 2). The fr./dry wt ratio was not affected. In fully developed leaves alkaloid metabolism seems not to be changed by the experimental treatment. According to leaf ageing absolute alkaloid content decreases within 11 days from 69 to 56 μg in damaged and in control leaves. Due to compensatory growth the relative alkaloid content is 20% lower in the damaged leaves. In expanding leaves alkaloid formation is stimulated by leaf injury, the absolute alkaloid amount is 60% higher compared to the control leaves. In a second experiment damaged leaf halves were analysed after 4 days, but within this time no significant change of alkaloid content was observed.

Wounding stress of high intensity (loss of biomass > 50%) was applied by cutting off stems and five leaf pairs of plants with six leaf pairs. The plants regenerated two lateral shoots, one being considerably more vigorous than the other. The dominating shoots were analysed after 15 and 30 days, respectively, having developed two and five leaf pairs, respectively. Fresh and dry wt were *ca* 10% lower in the regenerated leaf pairs than in the corresponding original leaf pairs. The dry wt to fr. wt ratio was also 15–20% lower in the newly developed leaves; this means that lamina rigidity is reduced. Alkaloid formation (Fig. 4) is strongly inhibited at the beginning of the regeneration process: after 15 days, when two leaf pairs were regenerated alkaloid content (% dry wt) is 50–70% lower compared to corresponding original leaf pairs, but after a period of recovery of 30 days the alkaloid content of the regenerated leaf pairs exceeds that of the corresponding original leaf pairs by 100% at the most. The degree increases with leaf age. Therefore, the stress effects a slower decrease of the alkaloid content during leaf ageing. The alkaloid pattern is not significantly different from the control. Comparing alkaloid variation after the recovery time of 30 days the coefficient of variation is considerably lower in the stressed leaf pairs (10–23) than in the non-stressed leaf pairs (30–70).

In conclusion, the results allow us to postulate hypotheses concerning strategies of optimizing chemical defence: (1) Variation of alkaloid accumulation in leaves decreases during stress. Whereas under non-stress conditions alkaloid levels in *C. roseus* may fluctuate to a high degree, severe stress conditions impose a very economic allocation of energy and/or nutrients and therefore reduc the degree of metabolic 'variations'. (2) Commitment to chemical defence is increased in leaves which are growing at the moment of stress (tissue of high value) and unchanged in leaves fully developed (tissue of low value). (3) Very vigorous stress conditions exhaust the energy and/or nutritional reserves of a plant and may greatly reduce the chemical potential.

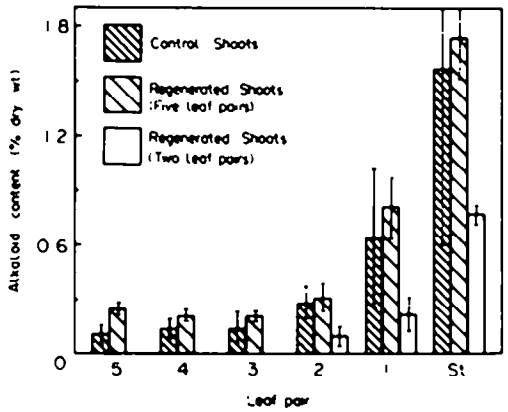

Fig. 4. Alkaloid content (% dry wt, mean of six analyses) of shoot tips (St) and five consecutive leaf pairs of plants with six leaf pairs compared with regenerated leaf pairs of plants of which the shoot and five leaf pairs were cut off (mean of three analyses).

EXPERIMENTAL

Plant material and cultivation conditions. *C. roseus* (L.) G. Don with red eyed or pink corolla (drought stress experiment) grown from seeds (Altorfer Samen, Zurich, Switzerland) were cultivated in the greenhouse with day/night temperatures of 28–30°/21–23° and 70–80% humidity. Plants were supplied with nutrient soln once a week (Wuxal 0.3%, Maag, Dielsdorf, Switzerland). For the drought stress expt plants were transferred to a controlled-environment chamber and cultivated under the following conditions: 12-hr light period, photosynthetically active radiation at the top of the plants of 400–500 μmol/m²/sec, day/night temp. 24/18° and day/night H_2O vapour deficits of 6.0/3.0 mg/l. After 1 month of acclimatization plants were cultivated under three different H_2O conditions: the first group was kept in permanently H_2O satd soil, the second group was cultivated with 50% and the third group with 33% of the H_2O of the first group. Before irrigation (every 2nd day) the stressed plants looked wilted, but leaf H_2O potential increased only slightly from −10 bar to −12 bar (50% H_2O) after 30 days and to −13 bar (33% H_2O) after 20 days, respectively.

Table 2. Effect of wounding stress. One half of a young expanding leaf (leaf pair no. 1) and of a fully developed leaf (leaf pair no. 4) were cut off and analysed (first half); after 11 days the second halves of the leaves were analysed and compared with leaf halves of the opposite leaf of a pair (control). All values are mean of three analyses

Leaf pair no., Pos.	Dry wt: abs. (mg, ± s.d.)	rel. (%)	Alkaloid content (μg)	(%)	(% dry wt, ± s.d.)
1. First half	4.6 ± 1.5	100	80	100	1.8 ± 0.21
1. Second half	6.8 ± 2.7	148	130	163	2.0 ± 0.55
1. Control	6.3 ± 3.7	137	84	105	1.4 ± 0.43
4. First half	8.7 ± 3.4	100	69	100	0.81 ± 0.04
4. Second half	9.8 ± 4.6	112	56	81	0.58 ± 0.11
4. Control	8.0 ± 2.9	92	56	81	0.74 ± 0.24

Dry wt determination. Since alkaloid analysis was carried out with fresh plant material, dry wt of control plants was determined. Multiplication of the fr. wt of the experimental plants with dry wt/fr. wt ratio of the control plants gave the dry wt of the experimental plants.

Alkaloid determination. Fresh plant material was homogenized immediately after fr. wt determination with 0.1 M HCl/MeOH (1:1) and extracted at 65° for 20 min (50–150 mg plant material/ml). After cooling an aliquot was made alkaline to pH 9.6 and purified on a Extrelut column (Merck, Darmstadt, West Germany). Separation and quantitation were performed with a HPLC system equipped with a photodiode array detector and a data processing unit which allows integration of the signal at eight different wavelengths and an automatic purity check. For peak identification UV-spectra were recorded from 240 to 400 nm with a band width of 2 nm. The column (15 cm × 4.6 mm) and the pre-column (3 cm × 4.6 mm) were packed with 5 μm Supelcosil LC-18-DB (Supelco Inc., Bellefonte, U.S.A.) which is especially suitable for the sepn of basic compounds. As mobile phase MeCN aq. 0.05 M KH_2PO_4 buffer (pH 5.6) was used at a flow rate of 0.9 ml/min in the following proportions (% MeCN): 0–1 min isocratic 35.5 %, 1–30 min linear gradient 35.5–54 %. The sepn was carried out at a column temp. of 50°. Under these chromatographic conditions the following k' values were obtained for our ref. substances: serpentine 2.1, catharanthine 3.2, ajmalicine 4.1, vincristine 4.5, vinblastine 6.2, vindoline 6.8, tetrahydroalstonine 11.9, tabersonine 15.9. Vindoline and catharanthine were obtained from Ely Lilly & Co (Indianapolis, U.S.A.), tabersonine was a gift from Mr. A. Guggisberg (Organic Chemistry Institute of the University of Zurich, Switzerland), all other reference substances were purchased from Roth GmbH (Karlsruhe, West Germany).

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