Phytochemistry, Vol. 21, No. 11, pp. 2669–2675, 1982.
Printed in Great Britain.

0031-9422/82/112669-07$03.00/0

ACCUMULATION OF ALKALOIDS AND THEIR NECINES IN *HELIOTROPIUM CURASSAVICUM*, *H. SPATHULATUM* AND *H. INDICUM**

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(*Received* 2 *November* 1981)

Key Word Index—*Heliotropium*; Boraginaceae, pyrrolizidine alkaloids; necines; trachelanthamidine; supinidine; lindelofidine; retronecine; ontogenetic and organ variation.

Abstract—Plants of *Heliotropium curassavicum*, *H. spathulatum* and *H. indicum* grown in the greenhouse between May and September, showed the greatest accumulation of alkaloids after the beginning of flowering, especially during the last three weeks of growth. In all three species the highest alkaloid content was found in the roots and inflorescence. These parts also exhibited the highest relative amounts of *N*-oxides which ranged in the plants from 60 to 90% of the total alkaloid content; no significant age-dependent differences in *N*-oxides were found. Young leaves, young inflorescences, or seedlings showed very high alkaloid levels, reaching 5–6% dry matter in *H. spathulatum*. With ageing the content of alkaloids in leaves decreased 20-fold, apparently due to their efflux from these organs. The three species contained trachelanthamidine, supinidine, and retronecine; an additional necine with a mass spectrum, R_T and R_f of lindelofidine was detected in trace amounts in *H. spathulatum* and *H. indicum*. *H. curassavicum* and *H. spathulatum* contained (−)-trachelanthamidine and (−)-supinidine. In all organs at all developmental stages, supinidine was the minor necine; trachelanthamidine was the dominant base in *H. curassavicum*, whereas retronecine was dominant in the other two species ranging from 93 to 99% of the total in *H. indicum*. The share of retronecine in the roots and generative parts was higher than that in leaves and stems in both *H. curassavicum* and *H. spathulatum*; it ranged from 17 to 65% in the former and from 67 to 74% in the latter.

INTRODUCTION

Information about the developmental changes in the content and composition of pyrrolizidine alkaloids (PA's) in plants are very scarce, particularly in *Heliotropium* species. The reported PA content of the aerial parts of 20 *Heliotropium* examined species, collected in various parts of the world mostly at the stage of flowering, ranged from 0.2 to 4.9% dry matter [2]. Only two of the species were analysed at two to three different development stages, namely *H. arguzioides* from Kazachstan [3] and *H. europeum* from Australia [4, 5]. The PA level in their aerial parts varied from 0.2 to 2.0% and from 0.6 to 3.1%, respectively, being highest at flowering and decreasing towards the end of the growth period.

In 24 previously reported *Heliotropium* species, collected at flowering and/or fruiting in Mexico and adjacent U.S.A., the PA level was extremely low, in most cases ranging from 0.006 to 0.1% [6]. However, when grown in the greenhouse, some species had a much higher PA content than the corresponding field samples.

This study reports developmental changes in the levels of PA's and their necines in three species, grown under greenhouse conditions, i.e. *H. curassavicum* L., *H. spathulatum* Rydb. and H. *indicum* L. The reported qualitative composition of PA's in aerial parts of *H. curassavicum*, collected in Delhi, India [7] was quite different from that found in plants of the same species collected in Madras, India [8]. The former consisted of heliotridine esters, whereas the latter of trachelanthamidine-based PA's; the Madras sample also contained a free aminoalcohol, apparently a pyrrolizidine-2,9-diol, MW 157.

Divergent results were also reported for *H. indicum*. Plants collected in Ghana and Australia showed only retronecine esters [9, 10], whereas those collected in Bangladesh revealed the presence of retronecine, heliotridine and supinidine esters [11].

Plants of *H. spathulatum*, frequently not distinguished at the species level from *H. curassavicum*, were tested only once [6]; they originated from Mexico and contained saturated and unsaturated PA's in their leaves at early flowering.

RESULTS AND DISCUSSION

Of the three species examined, *H. currassavicum* showed the lowest and *H. indicum*, the highest dry wt per plant. However, during the first 54–71 days, all species exhibited very weak growth rates apparently

*Part 2 in the series "Aminoalcohols of Pyrrolizidine Alkaloids in *Heliotropium* Species". For Part 1 see ref. [1].

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due to the relatively low greenhouse temperature in May and June. In the later period all species formed flowering branches of several orders. The final total yields of the plants, especially of *H. indicum*, were somewhat lowered due to losses of ripe fruits falling off the older branches as well as to the abscission of some old leaves.

H. curassavicum

The average PA content of the plants was *ca* 2.5% during the whole vegetation period; thus, the total PA accumulation was correlated with the growth rate (Table 1). However, the distribution of PA's was not uniform. The lowest percentage was found in the leaves, and the highest one was found in the roots and inflorescence including seeds; in the latter the PA's reached 6% dry wt at the early generative stage.

The level of *N*-oxides in the leaves and stems ranged between 65 and 90% and that in the roots and generative organs between 85 and 90%; no significant differences depending on the developmental stage were observed.

The composition of necines was obtained from pooled organs of 54- and 74-day old plants, each organ of 99- and 120-day old plants, and from 135-day old ones sampled for preparative purposes. In all cases three necines were detected after PA hydrolysis. They are (−)-trachelanthamidine, (−)-supinidine and retronecine (free trachelanthamidine and retronecine were detected in relatively small amounts prior to hydrolysis). Neither heliotridine nor any base with a MW of 157 could be detected. Trachelanthamidine was the dominant aminoalcohol in all organs except for the generative ones; supinidine was the minor necine in all cases. The share of retronecine in the vegetative organs ranged from 31 to 45% and from 17 to 29% in 99- and 120-day old plants, respectively; in the generative parts it was 54 and 65%, respectively. Thus, the composition of the necines in *H. curassavicum* plants, grown in the greenhouse from root cuttings of plants which germinated from seeds collected in Baja California Sur, greatly differs from that reported for plants collected either in Delhi or Madras. These differences cannot be ascribed to changes in the qualitative composition during ontogenesis; nor can they be ascribed to differences between organs in this regard.

H. spathulatum

The average PA content of the plants ranged be-

Table 1. Growth and alkaloid content of *Heliotropium curassavicum* plants (per plant)

Days after planting	Organ	Dry wt (g)	Alkaloid content*			Aminoalcohols† (% total)		
			Total		*N*-oxides (% total)	T	S	R
			% dry wt	mg				
54	Leaves	0.08	1.86	1.4	79	—	—	—
	Stems	0.04	2.86	1.3	65	—	—	—
	Roots	0.05	3.50	1.6	87	—	—	—
	Total	0.17	2.53	4.3	—	—	—	—
74	Leaves	0.21	1.72	3.6	80	—	—	—
	Stems	0.19	3.02	5.7	67	—	—	—
	Roots	0.06	3.96	2.4	85	—	—	—
	Total	0.46	2.57	11.7	—	—	—	—
99	Leaves	0.44	1.82	8.1	69	60	8	32
	Stems	0.46	2.93	13.5	72	62	7	31
	Roots	0.13	2.90	3.8	88	53	2	45
	Inflorescence and seeds	0.07	6.10	4.1	90	42	4	54
	Total	1.10	2.68	29.5	—	57	6	37
120	Leaves	0.84	1.90	16.3	70	62	9	29
	Stems	1.00	2.39	23.8	72	70	9	21
	Roots	0.53	3.28	17.4	86	73	10	17
	Inflorescence and seeds	0.30	3.31	9.9	88	30	5	65
	Total	2.67	2.52	67.4	—	60	9	31

*Calculated as monocrotaline.

†(−)-Trachelanthamidine, T; (−)-supinidine, S; retronecine, R.

tween 1.9 and 2.5%, the higher values occurring at the very early and very late stages of growth (Table 2). Like in *H. curassavicum* *ca* 60% of the total PA accumulation took place during the last 21 days of growth, and again, the roots and the generative parts revealed the highest levels of PA's, the roots with PA's at 5% dry matter being the main storage site of these compounds at the end of vegetation. Similar PA accumulation in roots was found also in dormant plants of *H. spathulatum* collected in California in 1980 [2]. As in *H. curassavicum*, roots and generative organs showed the highest levels of *N*-oxides, reaching 82–94% of total PA's.

In all organs analysed at all stages of development four necines were found after PA hydrolysis. They are (−)-trachelanthamidine, (−)-supinidine, retronecine, and traces of an aminoalcohol with a mass spectrum, R_T, and R_f of lindelofidine (free trachelanthamidine and retronecine were detected in small amounts prior to hydrolysis). No other necines could be detected. Retronecine was the dominant necine in all organs at all stages, reaching 67–74% of the total in the roots and generative parts. The highest share of trachelanthamidine, *ca* 41%, was found in leaves of young plants; it decreased towards the end of growth and was accompanied by a significant increase in the share of retronecine.

H. indicum

This species showed the lowest, *ca* 1%, average PA content. The highest level, over 2%, was found in generative parts and the lowest in the leaves (Table 3). Inflorescence and seeds, accumulated *ca* 60% of total PA's towards the end of plant growth. The levels of *N*-oxides ranged between 63 and 90% of total PA's, again being the highest in the roots and inflorescence. No significant development-dependent differences were observed.

Hydrolysates of PA's from pooled organs of 91-day old plants and from each organ of 114- and 136-day old plants revealed the presence of retronecine in amounts up to 93–99% of the total. Three additional necines were also detected; they were identified on the basis of their mass spectrum, R_T and R_f values, but not optical rotation. They are trachelanthamidine (or laburnine), supinidine and lindelofidine, the latter

Table 2. Growth and alkaloid content of *Heliotropium spathulatum* plants (per plant)

Days after planting	Organ	Dry wt (g)	Alkaloid content*			Aminoalcohols† (% total)		
			Total		*N*-oxides (% total)	T	S	R
			% dry wt	mg				
54	Leaves	0.13	2.48	3.3	80	—	—	—
	Stems	0.06	2.45	1.5	69	—	—	—
	Root stock	0.02	3.80	0.5	90	—	—	—
	Roots	0.04	3.28	1.2	94	—	—	—
	Total	0.25	2.65	6.5	—	—	—	—
74	Leaves	1.44	1.88	27.1	70	41	15	44
	Stems	1.58	1.45	22.9	59	32	13	55
	Roots	0.42	2.91	12.2	89	18	12	70
	Inflorescence	0.06	6.54	3.9	82	24	8	68
	Total	3.50	1.89	66.1	—	33	13	54
99	Leaves	3.70	1.68	62.3	65	35	16	49
	Stems	5.27	1.42	74.8	59	36	14	50
	Roots	1.02	4.19	42.7	89	21	10	69
	Inflorescence and seeds	0.73	4.07	29.7	79	17	12	71
	Total	10.72	1.95	209.5	—	30	13	57
120	Leaves	5.14	1.66	85.3	81	33	8	59
	Stems	8.80	1.46	128.5	75	38	9	53
	Roots	3.43	5.11	175.3	91	13	13	74
	Inflorescence and seeds	1.53	5.02	79.5	87	20	13	67
	Total	18.90	2.47	468.6	—	25	11	64

*Calculated as monocrotaline.

†(−)-Trachelanthamidine, T, including traces of a necine with a mass spectrum, R_T and R_f of lindelofidine detected in all organs; (−)-supinidine, S; retronecine, R.

always occurring in trace amounts. No heliotridine cound be detected. Thus, the *H. indicum* plants, originating from plants collected in the hot tropics of Mexico, differ in the qualitative composition of their necines from those collected on other continents. Again, these differences cannot be ascribed either to differences in the developmental stage or organs. Assuming that the botanical identification of *H. curassavicum* and *H. indicum* as well as the chemical identification of their PA's were correct, apparently each population tested represents a different variant with a different PA composition.

It has been previously shown that leaves in *H. spathulatum* are the main organs responsible for necine biosynthesis [1]. In this study the analysed leaves represented a mixture of young, mature and old leaves, whose ratio changed with the developmental stage. Leaves sampled from flowering branches of old *H. spathulatum* and *H. indicum* plants revealed great differences in the PA content depending on their physiological state (Table 4). In very young leaves, one-fifth of their full size, the PA's amounted to 5.3 and 1.6% respectively, whereas old yellowing leaves showed a 20-fold lower content. In *H. spathulatum* leaves changes in the ratios between necines were also found. The observed high PA content of leaves is not exceptional; a PA level of 7.7% dry matter has been reported for leaves of *Trachelanthus hissoricus* Lipsky collected in Tadzhikistan [12]. Significant differences in the PA content depending on leaf age have been found in field-collected *Symphytum* × *Uplandicum* [13, 14].

The decrease in the levels of PA's in leaves with ageing could be due to translocation to other organs and/or to their transformation into non-alkaloidal compounds. When exposed to complete darkness for 6 days, detached mature leaves of *H. indicum* lost 10–20% of their dry matter. However, no decrease in their PA amounts was found (Table 5). Of the three experiments carried out with *H. spathulatum* leaves in darkness, two indicated a slight decrease in the amounts of PA's. However, the relative PA content remained the same or increased. Thus, the observed age-dependent decrease in the content of PA's in attached leaves appears to be mainly due to an efflux of these compounds rather than to their transformations.

Detachment and darkness are known to accelerate senescence in leaves and to cause a rapid breakdown of chlorophyll and proteins [15, 16]. However, in

Table 3. Growth and alkaloid content of *Heliotropium indicum* plants (per plant)

Age of plants (days)	Organ	Dry wt (g)	Alkaloid content*			Aminoalcohols† (% total)		
			Total		*N*-oxides			
			% dry wt	mg	(% total)	T	S	R
71	Leaves	0.34	0.76	2.6	70	—	—	—
	Stems	0.06	1.46	0.9	71	—	—	—
	Roots	0.09	1.45	1.3	85	—	—	—
	Total	0.49	0.98	4.8	—	—	—	—
91	Leaves	2.91	0.62	18.0	65	—	—	—
	Stems	3.65	1.13	41.3	64	—	—	—
	Roots	1.42	1.71	24.3	90	—	—	—
	Inflorescence	0.93	2.69	25.0	76	—	—	—
	Total	8.91	1.22	108.6	—	—	—	—
114	Leaves	10.51	0.42	43.9	59	6	1	93
	Stems	11.70	0.47	54.9	63	< 2	trace	98
	Roots	3.62	1.41	51.0	81	3	2	95
	Inflorescence and seeds	6.60	2.26	149.1	67	5	2	93
	Total	32.43	0.92	298.9	—	—	—	—
136	Leaves	13.77	0.43	49.3	74	4	trace	96
	Stems	32.28	0.35	111.8	72	4	1	95
	Roots	6.87	1.50	103.4	91	trace	trace	99
	Inflorescence and seeds	18.81	2.07	387.3	87	4	2	94
	Total	71.73	0.92	651.8	—	—	—	—

*Calculated as monocrotaline.

†Trachelanthamidine, T, including traces of a necine with a mass spectrum, R_T and R_f of lindelofidine detected in all organs; supinidine, S; retronecine, R.

Table 4. Alkaloid content in leaves of old *H. spathulatum* and *H. indicum* plants

Leaves*	Alkaloid content† (% dry wt)	Aminoalcohols‡ (% total)		
		T	S	R
H. spathulatum				
Young	5.34	60	6	34
Mature	1.05	43	10	47
Old	0.26	35	trace	65
H. indicum				
Young	1.63	6	<1	94
Mature	0.15	8	1	91
Old	0.09			

*The dry matter in young, mature and old leaves of *H. spathulatum* and *H. indicum* was 13.8, 9.2 and 8.2%; and 22.3, 21.3 and 19.0% fr. wt, respectively.

†Calculated as monocrotaline.

‡Trachelanthamidine (including traces of lindelofidine), T; supinidine, S; retronecine, R.

the reported experiments with mature leaves no decrease in chlorophyll could be detected, except for one experiment with *H. spathulatum* when the chlorophyll content of leaves fell by *ca* 30% after 6 days of darkness.

In all three species the PA content of young tissues was very high; the observed levels cannot be fully ascribed to the small cell size as compared with that in expanded organs. A very high PA content was also found at a very early developmental stage, namely in sprouts from seeds of *H. indicum* (Table 6).

In that experiment only 65% of the initial PA amount was recovered after a 15–21 day exposure of seeds in Petri dishes to GA solutions and then to water. However, the PA losses could be due to microbial activities. When PA's extracted from dry seeds were added to GA solution, to which seeds were previously exposed for 7 days, the recovery of

Table 5. Changes in weight and alkaloid content of detached and halved leaves (per half-leaf)*

Heliotropium species	Exposure to	Days	Immediately after detachment					After exposure-II				Alkaloids recovered from water (μg)
			Weight (mg)			Alkaloids of I		Weight (mg)		Alkaloids		
					Dry I	% dry wt	μg	Fresh	Dry	% dry wt	μg	
			Half-leaves floating on water									
spathulatum	Dark	9	35	37	3.9	2.01	77	49	3.0	2.18	66	‡
spathulatum		6	53	54	5.2	1.59	83	66	4.6	1.67	76	4.6
indicum		6	94	89	23.8	0.14	34	118	19.1	0.13	25	8.2
			Half-leaves in a chamber at 100% air humidity									
spathulatum	Light	6	92	90	8.5	0.78	65	94	8.1	0.79	64	
	Dark†	6	94	98	8.5	0.81	68	99	7.8	0.80	63	
indicum	Light	6	144	142	28.1	0.39	109	150	26.5	0.40	107	
	Dark†	6	157	160	31.2	0.36	111	169	28.1	0.39	112	

*One half (I) was sampled immediately; the other one (II) exposed to darkness or light.

†The chlorophyll content of *H. spathulatum* half-leaves immediately after detachment and after 6 days of dark exposure was 0.67 and 0.49 mg/g fr. wt, respectively; and that of *H. indicum* half-leaves was 1.44 and 1.43 mg/g fr. wt, respectively.

‡Great differences between three replicates; the PA's recovered from water ranged from 0.2 to 7 μg/half leaf.

Table 6. Changes in alkaloid content during germination of *H. indicum* seeds (per 1000 seeds)

Specimen	Dry wt (mg)	Alkaloids (mg)	% dry wt
Seeds	1716	10.72	0.63
After germination			
Sprouts	260	4.16	1.60
Seeds, coats, etc.*		2.25	
Aqueous solutions		0.21	
Total	—	6.62	—

*This fraction includes seeds that did not germinate as well as those that developed a radicle only; combined, they amount to 20–25% of the total number of seeds.

the PA's after 3 days was less than 10%. Thus, the 35% loss during germination may not be due substantially to PA metabolism in the plant tissues, assuming that no or insignificant biosynthesis of PA's occurred at this stage. This study supports our previous observation [1] that PA's undergo very slow, if any, metabolic changes. The PA's occur in relatively large amounts in tissues exhibiting a very high as well as a very low physiological activity. What physiological significance do they have?

EXPERIMENTAL

Plant material. In 1979 *H. indicum* L., *H. spathulatum* Rydb. and *H. curassavicum* L. plants were grown in the greenhouse from seeds collected in the hot tropics of Mexico, roots collected in California and seeds collected in Baja California Sur in 1978, respectively [6]. These plants were the source of seeds for *H. indicum* and root cuttings for *H. spathulatum* and *H. curassavicum* plants, grown between May and September 1980 in a rich general purpose soil mixture and fertilized periodically with N, P and K. *H. indicum* seeds required a 7-day treatment with 1% GA (Eastman, 74 50) for germination (many *Heliotropium* species require GA treatment [Frohlich M. W., personal communication]). The plants were sampled ×4 during the vegetation period in 2 or 3 replicates of 2–25 plants each. At the stage of flowering/fruiting large numbers of plants were harvested separately for preparative purposes.

In addition: (A) Young, mature and old leaves were sampled from the same flowering branches of *H. spathulatum* and *H. indicum* plants at the age of 138 and 154 days, respectively. The young leaves were *ca* 1/5 of their fully expanded size, the mature ones were fully expanded and deep green, and the old ones were yellowing, prior to abscission. (B) Mature leaves (15–20 per replicate) of *H. spathulatum* and *H. indicum* plants were detached, divided along the mid-vein into halves; one half was immediately sampled and the other was exposed to continuous darkness or to 12 hr light/12 hr dark periods for 6 or 9 days floating on H_2O in a glass Petri dish or enclosed in a chamber at 100% humidity at room temp. The H_2O on which the leaves were floating was collected, concd and assayed for PA's after exposure to Zn in 2N H_2SO_4. (C) Mature seeds (1000 per replicate in three replicates) from H. *indicum* plants were exposed in Petri dishes to 1% GA (Eastman 7450) in darkness for 7 days; then the soln was replaced by tap H_2O for 7–14 days. On days 15 and 21 sprouts were removed, dried and combined. They represented 70–73% and 5–10%, respectively, of the total number of seeds. Seeds that failed to germinate were combined with all seed coats and also sampled. The GA soln as well as tap H_2O were replaced every 3–4 days, stored combined and analysed for PA's.

All plant samples were oven dried at 70°. When dried material of the three species studied was compared with fresh frozen material, the amount of PA's extracted from the former was sometimes even higher. This was due to an incomplete *N*-oxide reduction in extracts from fresh material; the latter required relatively more Zn (see below) and occasionally even readjustment of the pH.

Analyses. Extraction, *N*-oxide reduction, purification and hydrolysis of the PA's as well as extraction of necines were similar to those described previously [1]. However, no purification of the hydrolysates with Et_2O was performed. Extraction with $CHCl_3$–EtOH (3:1 or 3:2) following $CHCl_3$ extraction was applied to combined organs of each of the species in search for free pyrrolizidine diols after PA reduction.

Conditions of TLC and GC/MS analyses were similar to those described previously. In addition to I_2 and Dragendorff reagent, the Mattock's method [17] for detection of unsatd. necines on TLC plates was also used. In order to increase the sensitivity for detection of heliotridine in the presence of high amounts of retronecine, Si gel strips corresponding to the R_f of heliotridine (0.35) with the uppermost part of the retronecine band (R_f 0.26) were extracted, re-chromotographed, re-extracted and analysed on GC/MS.

The PA's and their necines were quantitatively assayed as previously. The optical rotation of the necines was determined using an automatic polarimeter, Auto Pol II, Ruedolph Research. Chlorophyll content was measured in 80% Me_2CO at 652 nm [18].

Mass spectra. Trachelanthamidine (R_T 2.7 min): M^+ 141(16), 140(24), 124(15), 110(11), 108(8), 84(10), 83(100), 82(63), 80(7), 70(9), 68(11), 55(79) and 53(13). Lindelofidine (R_T 3.1 min): M^+ 141(17), 140(21), 124(15), 110(12), 108(8), 84(10), 83(100), 82(69), 80(11), 70(12), 68(12), 55(74) and 53(14). Supinidine (R_T 3.1 min): M^+ 139(55), 122(32), 120(13), 111(16), 110(20), 108(30), 80(100), 68(25), 67(16), 55(16) and 53(24). Retronecine (R_T 9.3 min): M^+ 155(21), 111(51), 94(20), 93(11), 80(100), 68(25), 67(17), 55(11) and 53(27).

The $[\alpha]_D^{20}$ values for trachelanthamidine, supinidine and retronecine were −13.6°–−13.9° (EtOH; *c* 0.85); −10.4° (EtOH; *c* 0.80) and +50.3° (EtOH; *c* 0.52) respectively.

Acknowledgement— We thank Dr. M. W. Frohlich for the supply of plants needed for propagation.

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