Phytochemistry, 1977, Vol. 16, pp. 841–843. Pergamon Press. Printed in England.

POLYAMINES IN BARLEY SEEDLINGS

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(*Revised received* 6 *January* 1977)

Key Word Index—*Hordeum vulgare*; Gramineae; barley seedlings; putrescine; spermidine; spermine; dansylation.

Abstract—Polyamine levels in barley seedlings grown in the dark or in diurnal illumination have been determined, by direct dansylation, 3, 6 and 12 days after germination. The mean recovery of putrescine, spermidine and spermine added to barley leaf material was 94, 96 and 92% respectively. The effect of illumination was not significant 3 days after germination. By the 12th day in both shoots and roots the polyamine level and especially the putrescine level was higher in the seedlings grown in the light on a fr. wt basis. The mean putrescine level was higher in the leaf tip (× 3.8) than in the stem base. In 12-day-old seedlings grown in the light spermidine was higher in the base of the shoots (× 4) than in the leaf tip. The mean ratio of putrescine/spermidine/spermine in the tops was 1:0.50:0.07 and in the roots 1:0.23:0.006.

INTRODUCTION

The diamine putrescine and the polyamines, spermidine and spermine, have been implicated in the control of nucleic acid metabolism, protein synthesis and growth [1–3]. These amines may function by interacting with organic polyanions such as the phospholipids in membranes or with nucleic acids, due to their basicity. Although putrescine may be concerned in the control of cell pH [4], the precise role of the polyamines in cell metabolism is still unknown. To elucidate their function, many studies have recently been made of the changes in polyamine levels in rapidly growing animal, bacterial and plant systems. In plants, changes in the polyamine levels during the growth of *Lathyrus*, *Phaseolus* and *Pisum* (Leguminosae) seedlings have been established in earlier work [5–7]. In the present study, amine levels of barley (*Hordeum*; Gramineae) seedlings have been determined 3, 6 and 12 days after germination. For this purpose the method of direct dansylation [8] of the tissue extracts was applied for the first time to plants.

RESULTS AND DISCUSSION

Recovery of polyamines in the dansylation procedure

In the dansylation technique used in earlier work [7, 9, 10] an ion exchange step on Dowex 50 resin was used to isolate and concentrate a fraction containing the amines. Using this method recoveries of 2.5 μmol of putrescine, spermidine and spermine in the absence of plant material were 92, 88 and 83% respectively. In the presence of the extract of 5 g of barley leaves, the recovery was 84, 80 and 77% resp. (6 estimations of each, SEM 3%). Recovery of 1 μmol of putrescine, spermidine and spermine added *after* the resin procedure to the amine fraction of 10 g fr. wt of pea leaves was 98, 94 and 95% resp. The losses in this method may therefore be attributed to the resin procedure.

Since recovery of the amines, and especially spermine using this technique was not satisfactory, a method was developed based on the procedure of Seiler and Wiechmann [8] in which the ion exchange step was omitted (direct dansylation, see Experimental). Extracts prepared in 5% TCA were sampled for dansylation after removal of the TCA by ether extraction which otherwise interferes in the derivatization. Duplicate recoveries for 0.5 μmol amine added per g fr. wt barley leaf material were 88 and 86% for putrescine, 94 and 93% for spermidine and 86 and 93% for spermine. For 5 μmol amine/g fr. wt of barley leaf material, recoveries were 97 and 103% for putrescine, 94 and 101% for spermidine and 92 and 97% for spermine. These recoveries were considerably better than those found in the earlier method using the resin step.

Omitting the proline prior to extraction of the dansylated amines into toluene gave a blue halo around the origin of the dansylated amines on TLC, though the separation was otherwise normal. If toluene (0.5 ml) was added to the dansylation mixture without prior evaporation, a single phase was formed on mixing due to the acetone added with the dansyl chloride. The dansylation mixture was therefore evaporated to dryness and taken up again in water before extracting into the toluene. On standing in direct sunlight for 60 min the dansyl amines were completely decomposed. In subdued laboratory light, loss of fluorescence is reduced to *ca* 50% in one week. Derivatives were therefore dansylated in the dark at room temp. and stored in the dark at 4°.

Interference of polyamine estimation by other amines occurring in barley

Besides putrescine, spermidine and spermine, several other amines capable of forming dansyl derivatives have been found in barley at various stages of growth. In the present study, a survey of these has been made to establish the degree of interference which they might contribute to the di and polyamine estimations. Some of these are listed in Table 1.

Dans-adenine is extracted into toluene and gave a bright blue fluorescent spot at R_{am} 0.21 in solvent 1.

Table 1. Concentrations of various amino compounds found in barley plants and the colour and R_f values of their dansyl derivatives on chromatography in cyclohexane–ethyl acetate (5:4) (solvent 1) on Silica gel G

Amine	Concentration nmol/g fr. wt	Tissue	Age/ (days)	Ref.	R_{am} on TLC	Colour of fluorescence	Ref.
Diaminopropane*	(5% of putrescine)	leaves	8–10	[11]	ca. 0.8	green	[9]
Putrescine	see text				0.8	green	
Cadaverine*	187	embryo	0	[7]	0.9	green	
	50	endosperm	0	[7]			
	<1	leaves	42	[9]			
Spermidine	see text				0.6	green	
Spermine	see text				0.4	green	
3-Aminopropyl-pyrroline*				[11]	n.d.		[9]
Agmatine*	260	leaves	16	[10]	0.0	green	[9, 13]
Hordatines*	380	leaves		[12]	—		
4-Aminobutyric acid (butyrolactam)*	13			[14]	0.75	orange	[15]
Homoserine lactone*					ca. 0.68	yellow	[7]
Tryptamine*	3.9	shoots	9	[16]	1.1		[13]
Aminomethylindole*	290	shoots	4	[16]	not known	not known	
Methylaminomethyl-indole*	880	shoots	4–6	[16]	not known	not known	
Gramine	3600	shoots	9	[16]	1.3	green	
5-Hydroxytrypt-amine*	6.4	roots	26	[16]	0.6		[13]
	2	shoots	26	[16]			
N-Methyl-5-hydroxytryptamine*	2	roots	26	[16]	not known	not known	
Tyramine	6900†	roots	3	[17]	1.0	pink	[9]
N-Methyltyramine*	11600†	roots	9	[17]	not known	not known	
Hordenine*	17900†	roots	9	[17]	0		[13]

*Not detected in the present study. †per g dry wt. n.d. = not derivatized.

Dans-thymine and -uracil showed a very weak blue fluorescent spot at R_{am} 0.98 and 0.72 respectively. Dans-cytosine and dans-guanine gave no spot on TLC. Only dans-adenine could be detected in the barley extracts with certainty in the present work. Dans-ethanolamine (R_{am} 0.32) was present in all barley material, and may be formed as an artefact by the decomposition of phospholipids [18]. Older barley leaf extracts gave a dansyl spot with R_f slightly greater than that of dans-ammonia. MS in the present study suggested that this was due to 5-aminovaleraldehyde, formed on dansylation of lysine [19, 20]. Free pyrrolidine and 4-hydroxybenzylamine have been found in barley seed and malt [21, 22], though these could not be identified in the present work. In addition, methylamine, dimethylamine, ethylamine, butylamine, amylamine and piperidine have been detected in distillates of barley grain and seedlings [23, 24]. The smaller aliphatic amines in general have $R_{am} > 1$ in cyclohexane–ethylacetate [13]. Several dansyl spots were found above dans-ammonia and some of these may be due to the aliphatic monoamines, though none would be expected to interfere with polyamine estimation.

2D TLC of the dansylated amines of the 6-day-old barley seedlings in solvents 1 and 2 showed no interference of dans-spermidine and dans-spermine by other amines in solvent 1. Dans-putrescine, however, was almost coincident with an unidentified spot in solvent 1 having R_{am} 0.88 in solvent 2 at *ca* 10 to 20% of the intensity of dans-putrescine. This spot occurred mainly in extracts of shoots grown in the light.

Amine levels in barley seedlings

After logarithmic transformation, the standard errors for the differences of the means of the amine concentrations in the 3, 6 and 12-day-old barley seedlings were respectively 0.067 (12 degrees of freedom, d.f.), 0.061 (24 d.f.) and 0.097 (28 d.f.).

The polyamine levels in the barley seedlings grown in the light for 3 days (Table 2) did not differ significantly from those of the 3-day-old seedlings grown in the dark (Table 3). After 3 day's germination there was very little spermine in the roots and the ratio of putrescine/spermidine was considerably lower in the shoot than in the root.

The spermine in the roots of the 6- and 12-day-old seedlings grown in both light and dark remained low and was almost undetectable. In the shoots of 6-day-old barley seedlings grown in both light and dark all amines increased towards the top (distal) end, but in 12-day seedlings only putrescine showed this gradient. Sinclair [25] similarly found that the putrescine level in potassium-deficient mature barley leaves increased towards the top, in inverse proportion to leaf potassium. In the 12-day-old shoots grown in the light, spermidine showed the

Table 2. Polyamine content of barley seedlings grown in a 16 hr day

Age	Tissue	nmol/g fr. wt Putrescine	Spermidine	Spermine	units/g fr. wt*
3 days	Shoot	1020	811	68	92
	Root	586	228	5	n.d.
6 days	Shoot–upper	1330	492	84	17
	mid	691	404	82	57
	lower	484	421	28	25
	Root	694	145	<5	17
12 days	Leaves–				
	upper	2980	158	81	15
	mid	807	202	131	30
	lower	505	320	96	39
	Stem	707	627	113	25
	Root	1700	217	<5	n.d.

*number of seedling units (shoots, roots, etc.) per g fr. wt. n.d. = not determined.

Table 3. Polyamine content of barley seedlings grown in the dark

Age	Tissue	nmol/g fr. wt Putrescine	Spermidine	Spermine	units/g fr. wt*
3 days	Shoot	1180	941	58	92
	Root	571	213	5	n.d.
6 days	Shoot—				
	upper	1310	780	43	30
	mid	691	374	12	28
	lower	297	364	24	20
	Root	763	238	<5	16
12 days	Leaves—				
	upper	842	231	54	20
	lower	338	218	65	27
	Stem—				
	upper	199	218	28	30
	lower	209	189	9	20
	Root	648	117	<5	n.d.

*number of seedling units (shoots, roots, etc.) per g fr. wt. n.d. = not determined.

opposite trend. In both the light and dark, the putrescine/spermidine ratio in the lower part of the shoot was *ca* 1.

In earlier work, the putrescine content of 15, 16, 28, 28, 42 and 49-day-old barley leaves was 870, 700, 157, 280, 292 and 1480 nmol/g fr. wt resp. [9, 10]. It therefore appears that after the initial rise, the putrescine concentration declines to a minimum after about 4 weeks and then rises again. Since mineral nutrition [10] and mildew infection [25] are important factors in determining putrescine level, these long-term trends should at present be considered to be only tentative. Compared with analyses published in refs. [9] and [10] spermidine seems to decline as barley leaves age, from 811 to *ca* 120 nmol/g fr. wt at 6 weeks after germination. However, spermine remains unchanged over this period.

On a per unit basis, putrescine in the tops increased × 26 in the light, and × 5 in the dark from day 3 to day 12 after germination. Spermidine increased × 5 in the light and × 3.6 in the dark in this period. Spermine increased × 10 in the light and × 5 in the dark. Illumination accelerates protein synthesis, but has no effect on nucleic acid accumulation in germinating barley seedlings [26]. The increases in polyamines, especially of putrescine, may therefore be related to enhanced protein synthesis in the light.

EXPERIMENTAL

Barley (Hordeum vulgare L. cv. Zephyr). Seedlings were grown in sand in polythene pots in the dark at 21°, or in a 16 hr day (10000 lx) with the day temp. 24° and the night temp. 19°. The pots were watered daily with a nutrient medium containing (with concentrations in meq/l.) K_2SO_4 (4), $MgSO_4$ (3), $CaCl_2$ (8), Na_2HPO_4 (1), $NaNO_3$ (4), $(NH_4)_2SO_4$ (8) with FeEDTA and micronutrients. The shoots of the 6-day-old plants were cut at the coleoptile tip and the remaining lamina was divided in two (shoot; -lower, -mid and -upper). The 12-day-old plants grown in the light were cut just below the angle of the first leaf. The lower portion (stem) includes leaf sheaths. The laminae of all leaves (mainly 1st and 2nd) were cut into 3 equal parts (upper, mid and lower). The 12-day-old plants grown in the dark were divided similarly, the 'stem' and laminae each being divided into two equal parts. The roots were washed to remove sand and blotted dry, and all samples were extracted in a pestle and mortar with 3 vol. of 5% TCA.

Direct dansylation. The TCA extract was centrifuged and filtered and a sample of the filtrate (normally 1 ml) was extracted with 2 × 5 ml of Et_2O on a vortex mixer. After separation by centrifugation the Et_2O containing the TCA was discarded. To 0.1 ml of the aq. phase in a 5 ml glass stoppered tube was added 50 mg $NaHCO_3$ and 0.2 ml of dansylchloride (300 mg in 10 ml Me_2CO). The tubes were stored in the dark for 16 hr at room temp. and 15 mg of proline in 0.1 ml of H_2O was then added to destroy excess dansyl chloride. After 30 min the sample was dried at 65° under a stream of air. H_2O (0.1 ml) was added and the dansyl amines extracted into toluene (0.5 ml) by vortex mixing. After centrifugation, samples (40 μl) of the toluene layer were applied to each origin on a TLC plate of Si gel G which had been freshly activated (1 hr at 100°). The dansyl amines were separated in cyclohexane–EtOAc (5:4) (solvent 1) with a paper pad attached to the upper edge of the plate [7]. This solvent successfully separates dans-cadaverine–dans-ammonia and dans-ethanolamine–dans-spermine, pairs which were sometimes difficult to separate in earlier work [7, 10]. On removal from the tank the plate was treated as described in ref. [9]. For 2D TLC following separation in solvent 1, the plate was run in the 2nd dimension with $CHCl_3$–NEt_3 (5:1) (solvent 2). The R_fs of the dans-putrescine, -spermidine and -spermine in solvent 2 were 0.55, 0.73 and 0.86 resp. R_fs of other dansyl amines in solvent 2 are given in ref. [9]. Dans-5-aminovaleraldehyde had R_{am} 1.3 in cyclohexane–EtOAc, 3:2 identical with the derivative formed on dansylation of lysine. MS at 180° showed *m/e* 171 (100), 316 (37), 170 (69), 168 (18), 281 (12), M^+ 334 (3). The peak at *m/e* 316 is due to dans-piperideine, derived from dans-5-aminovaleraldehyde [19].

Acknowledgements—The authors are grateful to Miss Gillian Arnold for the statistical analysis, and to Mrs. Bridget Roberts for the MS.

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