Polyamine Metabolism and Osmotic Stress[1]

I. RELATION TO PROTOPLAST VIABILITY

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ABSTRACT

Cereal leaves subjected to the osmotica routinely used for protoplast isolation show a rapid increase in arginine decarboxylase activity, a massive accumulation of putrescine, and slow conversion of putrescine to the higher polyamines, spermidine, and spermine (HE Flores, AW Galston 1984 Plant Physiol 75: 102). Mesophyll protoplasts from these leaves, which have a high putrescine:polyamine ratio, do not undergo sustained division. By contrast, in *Nicotiana, Capsicum, Datura, Trigonella*, and *Vigna*, dicot genera that readily regenerate plants from mesophyll protoplasts, the response of leaves to osmotic stress is opposite to that in cereals. Putrescine titer as well as arginine and ornithine decarboxylase activities decline in these osmotically stressed dicot leaves, while spermidine and spermine titers increase. Thus, the putrescine:polyamine ratio in *Vigna* protoplasts, which divide readily, is 4-fold lower than in oat protoplasts, which divide poorly. We suggest that this differing response of polyamine metabolism to osmotic stress may account in part for the failure of cereal mesophyll protoplasts to develop readily *in vitro*.

Regeneration of entire plants from mesophyll protoplasts of *Gramineae* is still difficult (4), with only one authenticated report in the literature (21). By contrast, in the Solanaceae (3) or Leguminosae (11, 13, 14), many species of plants have been successfully regenerated from protoplasts.

A decade ago, when we observed that PAs[2] could retard the rapid senescence and lysis of oat protoplasts (1, 8), we initiated a detailed study of the role of PAs in plant cells (16). We were surprised to find that the Put content of mesophyll protoplasts of oat is 5 to 10 times higher than that of the leaves from which they were derived. Later work showed that cereal leaves subjected to 0.4 to 0.6 M sorbitol exhibit a rapid increase in ADC activity, a massive accumulation of Put, and very slow changes in the higher PAs, Spd, and Spm (5, 6), which are normally formed from Put.

In the present investigation, we have studied the effect of osmotic stress in tobacco and other species that can be readily regenerated from protoplasts. The results indicate that tobacco response differs from that observed previously for cereals during osmotic stress (18). To establish whether this osmotic stress response is a general phenomenon, PA titers of two other solan-aceous (*Capsicum* and *Datura*) and legume (*Trigonella* and *Vigna*) genera were analyzed in osmotically stressed leaves. Finally, we compared the Put and PA contents of protoplasts of species yielding poorly dividing protoplasts with those of species yielding readily dividing protoplasts.

MATERIALS AND METHODS

Plant Materials. The following plant species were used: *Nicotiana tabacum* cv Wisconsin 38 (tobacco); *Capsicum annuum* var. California Wonder (pepper); *Datura stramonium* var Chalibea; *Trigonella foenum-graecum* L.; *Vigna aconitifolia* (Jacq.) Marechal var Jadia (moth bean); *Avena sativa* cv Victory (oat); and *Pisum sativum* var Progress (dwarf pea). Seeds were sown in vermiculite in plastic pots which were subirrigated twice daily with a 1.2 g/L solution of Hyponex (7-6-19 by N:P:K: analysis, Hydroponics Chemical Co., Copley, OH) and grown in a controlled chamber under a 16-h light/8-h dark photoperiod (9:1 energy mixture of fluorescent and incandescent light at 17.6 W· m^{-2}) at 24° C.

Osmotic Treatment of the Excised Leaves. Median leaves from about 2-month-old tobacco, pepper and *Datura* or about 1-month-old *Trigonella* or moth bean or the first leaf of 8-d-old oat seedlings were excised. The lower epidermis was peeled off, and leaf discs (2 cm diameter) or segments (5 cm length) were floated in the dark, unless otherwise indicated, at room temperature over 10 ml 1 mM K-phosphate (pH 5.8) contained in a 100 × 15 mm Petri dish, in the absence (control) or presence of osmotica (0.6 M sorbitol) for various incubation times.

Preparation of Protoplasts. Leaf mesophyll protoplasts from seedlings of oat, moth bean, dwarf pea, and tobacco were isolated under nonaseptic conditions. After removal of the lower epidermis, the leaves were floated in the dark at 30° C, stripped side down, over the appropriate digestion medium. Oat leaves were digested for 2 h over 0.5% Cellulysin in 0.5 M mannitol (pH 5.8) (12). Moth bean leaves were digested over 1% purified Driselase in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives (4). Dwarf-pea leaves were digested for 4 h over 0.75% Cellulase (Onozuka R-10, Kinki Yakult Manufacturing Co.) in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives as in moth bean, except for CaCl$_2$, whose final concentration was 2 mM. Tobacco leaves were digested for 5 h over 2% purified Cellulase + 2% unpurified Pectolyase Y-23 in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives as in moth bean, except for CaCl$_2$, whose final concentration was 6.4 mM (N Shekhawat, personal communication). Digestion of the leaves was followed by gentle shaking at room temperature for 15 to 60 min to release the protoplasts. The released protoplasts were collected by centrifugation at 100g for 6 min and rinsed once with the appropriate washing medium. For oat protoplasts the washing medium consisted of B-5 medium plus additives (12). For moth bean, dwarf pea and tobacco protoplasts, the wash

[2] Abbreviations: PA, polyamine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine.

medium was the same as the digestion medium, but without Driselase and with a higher concentration of 6 mM $CaCl_2$ (4). The final pellet was resuspended in 21% sucrose solution (pH 5.8) containing the same salts as the wash medium. The suspension was then covered by a small layer of wash medium and centrifuged again at 100g for 6 min. The intact protoplasts, concentrated at the interface, were taken up gently with a broad-tipped Pasteur pipette and washed once again. For PA determination, the protoplast pellet remaining after the wash with sucrose-containing medium was resuspended in an excess of a salt solution containing 2.5% KCl and 1% $MgSO_4 \cdot 7$ H_2O (12) and centrifuged to remove the sucrose. This is necessary, since dansylated sugars interfere with dansyl-PAs in the TLC separation. The pellet obtained after 6 min centrifugation at 100g was resuspended in a small volume of salt solution and the number of protoplasts determined with a hemocytometer. More salt solution was added to the suspension and the protoplasts were pelleted again by centrifugation. The final pellet was used for analysis of PAs.

Polyamine Analysis. Leaf-samples (previously washed with distilled H_2O and gently blotted on filter paper) were extracted by homogenizing in 5% (v/v) cold PCA at 100 mg fresh weight/ml PCA. Protoplasts from the final pellet were extracted with 5% PCA in a ratio varying according to the species. The homogenates were kept on ice 30 to 60 min before centrifugation at 27,000g for 20 min. The supernatant was set aside and the pellet was resuspended in the original volume of 1 N NaOH by vortexing. Aliquots (200 μl each) of the pellet suspension and the original supernatant were mixed 1:1 (v/v) with 12 N HCl and hydrolyzed as previously described (19). The filtered hydrolysates (19) were resuspended in 200 μl PCA. The nonhydrolyzed PCA supernatant containing the free, soluble PAs (S), the hydrolyzed PCA supernatant (SH), and the hydrolyzed pellet (PH) containing PAs liberated from conjugates were dansylated and chromatographed as previously described (19, 22). Trace amounts of PCA-insoluble conjugated PAs (PH-fraction) were found in all species except *Trigonella*. Therefore, unless otherwise indicated, data on total PA titers represent the sum of the free (S-fraction) and PCA-soluble PAs (SH-fraction). In *Datura* leaves and protoplasts of different species the free PAs were the only fraction analyzed. Dansyl-PAs were separated on high-resolution silica gel TLC plates (Whatman LK6D). For the dicot species either of two solvents, *i.e.* chloroform:triethylamine (4:1, v/v) or cyclohexane:ethylacetate (5:4, v/v) were used (19). For oats either of the two solvents, *i.e.* chloroform:triethylamine (5:1, v/v) (22) or cyclohexane:ethylacetate (5:4, v/v) were used. The former gave better separations of Put and diaminopropane (Dap). The latter gave better separations of Spm from contaminant bands. The PA standards Put, Spd, and Spm were included each time PA levels were analyzed, and in oats, standard Dap was included as well. After development of the plates, bands were scraped into 2 ml of ethyl acetate and the fluorescence at 495 nm determined in an Aminco spectrophotofluorimeter with an activating wavelength of 350 nm.

Protein Analysis. Protein was determined according to the method of Bradford (2) in the insoluble PCA-pellet resuspended in 1 N NaOH. Bovine γ-globulin (Sigma) was used as a standard.

Determination of ADC and ODC Activities. Samples were ground in chilled mortars in a ratio of 100 mg fresh weight/ml of 100 mM K-phosphate (pH 7.5) containing 10 mM DTT, 20 mM sodium ascorbate, 5 mM EDTA, and 1 mM pyridoxal phosphate. Purified insoluble PVP was added to the extract at 0.5 g wet weight/g tissue to absorb phenolics (19). The extract was sonicated as previously described (19) and pelleted for 20 min at 27,000g. The supernatant fraction was made 60% saturated with $(NH_4)_2SO_4$, allowed to stand for 30 min, then centrifuged for 20 min at 27,000g. The resulting pellet was resuspended in the
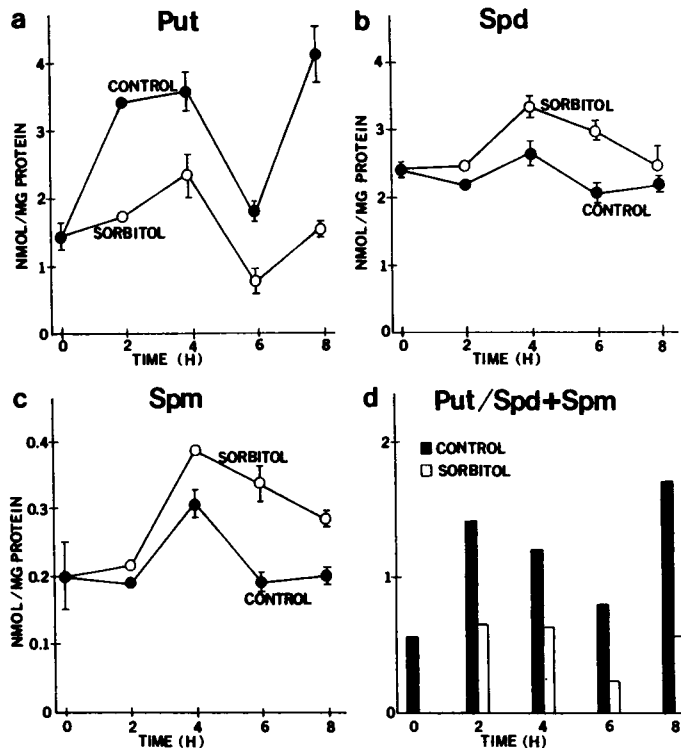

FIG. 1. Osmotic stress and polyamine titers in tobacco leaves. Peeled leaf discs were floated over buffer (●) or buffer + 0.6 M sorbitol (O) in the dark. a, Putrescine; b, spermidine; c, spermine; d, Put/polyamine (Spd + Spm) ratio. Values are the sum of the free and PCA-soluble conjugate polyamines. Bars represent ± SEM. Points without bars are the mean value of two analyses.
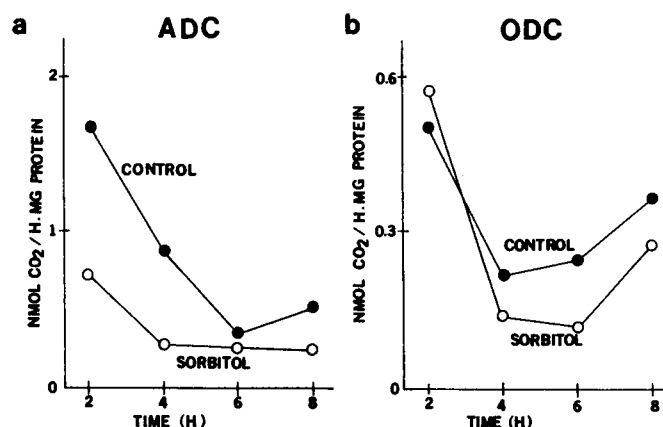

FIG. 2. Osmotic stress and arginine and ornithine decarboxylase activities in tobacco leaves. Samples were taken at the indicated times and assayed for enzyme activity. a, Arginine decarboxylase; b, ornithine decarboxylase. Points represent the mean value of two analyses.

original volume of 100 mM K-phosphate (pH 8.0) containing 1 mM DTT, 0.1 mM EDTA, and 0.05 mM pyridoxal phosphate, and dialyzed against the same buffer for 24 h in the dark (19). The activities of ADC and ODC were determined as described elsewhere (6, 22). For assay of ADC activity, the labeled substrate was 20 μCi/ml L-[U-^{14}C]arginine (276 mCi/mmol; ICN) diluted with unlabeled arginine to give a final concentration of 10 mM. For assay of ODC activity, the labeled substrate consisted of 20 μCi/ml DL-[1-^{14}C]ornithine (58 mCi/mmol; NEN) diluted with unlabeled ornithine to give a final concentration of 50 mM. Reaction mixtures were incubated for 45 min with gentle shaking at 37°C, at which time the reaction was stopped by adding 0.2

Table I. *Polyamine Titers and Osmotic Stress in Pepper Leaves*

Peeled leaf discs were floated over buffer or buffer + 0.6 M sorbitol in the dark and samples were analyzed for PAs after 4 h and 6 h of incubation. Numbers represent means ± SE of three replicates.

Treatment	Time of Incubation	PA Fraction	Put	Spd	Spm
	h			*nmol/mg protein*	
Control[a]	0	S[b]	0.76 ± 0.01	1.75 ± 0.16	ND[c]
		SH	4.10 ± 0.21	0.61 ± 0.02	ND
		Total	4.87 ± 0.09	2.3 ± 0.02	ND
Control	0	S	0.87 ± 0.24	1.25 ± 0.40	0.39 ± 0.10
		SH	3.90 ± 0.5	0.5 ± 0.18	0.22 ± 0.06
		Total	4.75 ± 0.24	1.8 ± 0.13	0.61 ± 0.06
Control	4	S	1.30 ± 0.14	1.10 ± 0.14	0.29 ± 0.12
		SH	2.40 ± 0.2	1.05 ± 0.11	0.16 ± 0.07
		Total	3.65 ± 0.14	2.16 ± 0.11	0.45 ± 0.04
0.6 M Sorbitol	4	S	1.13 ± 0.09	1.78 ± 0.2	0.64 ± 0.15
		SH	2.02 ± 0.10	1.75 ± 0.4	0.56 ± 0.11
		Total	3.15 ± 0.02[d]	3.53 ± 0.38[d]	1.20 ± 0.11[d]
Control	6	S	1.10 ± 0.13	0.85 ± 0.10	0.19 ± 0.02
		SH	2.27 ± 0.24	0.85 ± 0.09	0.15 ± 0.03
		Total	3.36 ± 0.13[d]	1.72 ± 0.09	0.35 ± 0.02[d]
0.6 M Sorbitol	6	S	1.01 ± 0.10	1.40 ± 0.17	0.53 ± 0.11
		SH	1.70 ± 0.15[d]	1.56 ± 0.21[d]	0.41 ± 0.10
		Total	2.7 ± 0.13[e]	3.00 ± 0.17[d]	0.95 ± 0.11

[a] Unpeeled control leaves. [b] S, Free, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis. [c] ND, not determined. [d] Significantly different from 0-h control (peeled leaves) at P < 0.05. [e] Significantly different from 0-h control (peeled leaves) at P < 0.01.

ml of 10% (v/v) PCA. Trapping of the labeled CO_2 onto the KOH-impregnated collection disc continued for 45 min at 37°C. The discs were then removed and immersed in 2 ml of Betafluor (NEN). The radioactivity liberated was determined by counting for 10 min in a Beckman LS 7000 scintillation counter. Enzyme was expressed as nmol $^{14}CO_2$ released/h·mg protein.

RESULTS

Osmotic Stress in Tobacco Leaves. Peeled tobacco leaves were floated over buffer (pH 5.8), in the absence (control) or presence of 0.6 M sorbitol and incubated in the dark for various times. PA titers (free and PCA-soluble or insoluble conjugates) and ADC and ODC activities were determined. In tobacco leaves, Spd and Put are the major PAs, while Spm is found only in small amounts (Fig. 1; Table V). Putrescine and Spd are present mainly in the PCA-soluble conjugate form (about 70% of the total); and Spm is equally distributed into the PCA-soluble fraction (free and bound; S- and SH- fractions). Only trace amounts of PCA-insoluble conjugated (PH-fraction) PAs were found.

Figure 1a shows that Put levels in control tobacco remained higher than in osmotically stressed leaves during the incubation. Put titers increased during the first 4 h, then declined to their lowest values after 6 h, and then increased again after 8 h incubation in both control and osmotically stressed leaves. In contrast, Spd and Spm levels increased during osmotic treatment, reaching their maximum difference with control after 6 h of incubation (Fig. 1, b and c). The changes occurring in Put titers during osmotic stress were produced mainly in the free fraction, while the osmotically induced changes in Spd and Spm titers occurred mainly in the PCA-soluble conjugate fraction (data not shown). Thus, the ratio of Put to Spd + Spm in tobacco leaves decreased during osmotic stress, reaching its lowest value after 6 h (Fig. 1d; Table V). This response is opposite from that observed previously for cereal leaves (5, 6).

In contrast with cereals (5, 6), ADC activity of tobacco leaves

does not increase after exposure to osmotic stress. ADC activity declined after 4 h of osmotic treatment and then remained constant (Fig. 2a). ODC activity also declined after 4 h, but then increased after 8 h in control and osmotic-stressed leaves (Fig. 2b).

Polyamines and Osmotic Stress in Pepper and *Datura* Leaves. Peeling the leaf had no effect on Put content in pepper, but decreased Spd titer by about 20% (Table I). In contrast with tobacco, pepper leaves contained Put as the main PA (see also Table V). Spermine titers were very low and Put was found mainly (about 80% of the total) in the form of PCA-soluble conjugates, as in tobacco leaves. However, Spd and Spm in pepper leaves were present mainly in the free PA fraction (70 and 65% of the total, respectively). Table I shows that, as in tobacco but unlike cereals, Put titers in pepper decreased during osmotic treatment while Spd and Spm titers increased in both fractions analyzed (S- and SH-fractions). As in tobacco, the maximum difference from the control occurred after 6 h of incubation in 0.6 M sorbitol. In this case, the osmotically induced changes on PA titers were produced mainly in the PCA-soluble conjugate fraction (Table I).

In *Datura*, we studied the effect on PA titers of incubating peeled leaves on 0.6 M sorbitol in dark or light. Table II shows that *Datura* leaves contained Put as the main PA, but unlike tobacco and pepper, contained Spm in relatively high quantities, approximately equal to Spd. Light did not affect the decrease in Put and increase in Spd and Spm after 6 h (Table II). Light did reduce somewhat the osmotically induced decrease of Put, and it reversed the relative rise in Spd *versus* Spm.

Polyamines and Osmotic Stress in *Trigonella* and Moth Bean Leaves. The response of PA levels to osmotic stress in two leguminous plants which can be regenerated from protoplasts (13, 14) was investigated. Peeled leaves of *Trigonella* were floated in the dark over buffer in the presence or absence of osmotica and PA titers analyzed after 6 h. Spd was the major PA, followed

Table II. *Polyamine Titers and Osmotic Stress in Datura Leaves*

Peeled leaf discs were floated over buffer or buffer + 0.6 M sorbitol in the dark or under light and samples were analyzed for free PAs (S-fraction) after 6 h of incubation. Numbers without ± SE are the mean value of two analyses.

Treatment	Time of Incubation	Put	Spd	Spm	Put/Spd + Spm
	h		*nmol/mg protein*		
Control	0	1.20 ± 0.06	0.40	0.40 ± 0.03	1.5
Control (dark)	6	2.00 ± 0.50	0.40 ± 0.04	0.30 ± 0.01	2.8
0.6 M Sorbitol	6	1.20	0.45 ± 0.05	0.40 ± 0.05	1.2
Control (light)	6	1.70 ± 0.1[a]	0.50 ± 0.04	0.33 ± 0.03	2.0
0.6 M Sorbitol (light)	6	1.40	0.63 ± 0.10	0.47 ± 0.01	1.2

[a] Significantly different from 0-h control at $P < 0.05$.

by Put and Spm (32 and 22% of the total, respectively) (Table III). Putrescine and Spd were present mainly in the PH-fraction, while Spm was concentrated mainly in the S-fraction. In *Trigonella*, after 6 h of osmotic treatment, Put titers did not increase but Spm titers increase slightly over the 6-h control (Table III).

In moth bean leaves, Spd was also the main PA, followed by Spm and Put (36 and 32% of the total, respectively) (Table IV; Table V). Polyamines were present mainly in the SH-fraction. When osmotically stressed, peeled moth bean leaves did not exhibit increased Put titers, but Spm titers increased as compared with the 6-h control (Table IV). As in *Trigonella*, osmotically stressed moth bean leaves showed an increase of Spm titers of more than 2-fold when compared with 0-h control.

Effect of Osmotic Stress on the Put/Polyamine Ratios. Table V shows the relative distribution of PAs as well as the Put/Spd + Spm ratios in various control or osmotically stressed peeled leaves. After 6 h of incubation in buffer, Put titers increased in tobacco and remained almost unchanged in pepper, *Trigonella*, and moth bean leaves; spermidine titers decreased in tobacco and *Trigonella* and increased in pepper; and Spm titers decreased in pepper and increased in *Trigonella* (Table V). After 6 h of incubation in osmotica, Put titers decreased in all these dicotyledonous leaves; Spd titers (except in *Trigonella*) increased; Spm titers also increased. As a consequence of these changes, the diamine/polyamine (Put/Spd + Spm) ratio decreased in all osmotically stressed dicot leaves. This phenomenon occurred in all the PA fractions analyzed (Tables II and III). Although for

Trigonella and moth bean the differences are small, the ratio does not increase as in oat leaves (Table V).

Polyamine Titers in Oat and Moth Bean Protoplasts. Table VI shows the PA titers of protoplasts isolated in the presence of osmotica from peeled oat and moth bean leaves. In oat protoplasts, Put was the major PA (66% of the total) and Spd and Spm represent 29 and 5% of the total, respectively. In contrast, in moth bean protoplasts Spm was the main PA (about 45% of the total) and Put and Spd represented 32 and 22% of the total, respectively. As a consequence of these differences, the Put/PA ratio in moth bean protoplasts was 4-fold lower than in oat protoplasts (Table VI).

DISCUSSION

We reported previously that mesophyll cells of cereals (oat, barley, corn, and wheat) exposed to the osmotica used routinely in protoplast isolation exhibit a rapid and massive increase in Put, with minor and slow changes in Spd and Spm. The accumulation of Put results from *de novo* synthesis of ADC (5, 7). In this study we report that leaves of tobacco and other dicots, which readily regenerate from leaf mesophyll protoplasts, show no such reaction to osmotic stress. In fact, Put titers declined in osmotically stressed tobacco leaves, while Spd and Spm titers increased. Thus, osmotic stress caused a 4-fold decrease of the Put/polyamine ratio in tobacco leaves in relation to the 6-h control, contrasting with the 6-fold increase of the same ratio in oat (Table V). The activities of the Put biosynthetic enzymes

Table III. *Polyamine Titers and Osmotic Stress in Trigonella foenum-graecum leaves*

Peeled leaf discs were incubated in the dark for 6 h as indicated in Table I.

Treatment	Time of Incubation	PA Fraction	Put	Spd	Spm	Put/Spd + Spm
	h					
Control	0	S[a]	0.32 ± 0.05	1.51 ± 0.03	1.08 ± 0.01	0.4
		SH	0.90 ± 0.11	1.13 ± 0.15	0.30 ± 0.05	0.6
		PH	2.35 ± 0.45	2.34 ± 0.30	0.92 ± 0.04	0.7
		Total	3.57 ± 0.44	5.00 ± 0.04	2.30 ± 0.09	0.5
Control	6	S	0.55 ± 0.06	1.65 ± 0.10[b]	1.05 ± 0.06	0.2
		SH	1.55 ± 0.20	2.25 ± 0.08[b]	1.30 ± 0.03[b]	0.4
		PH	1.85 ± 0.26	0.69 ± 0.10[b]	2.07 ± 0.10[c]	0.7
		Total	4.00 ± 0.17	4.65 ± 0.25[b]	4.50 ± 0.20[c]	0.4
0.6 M Sorbitol	6	S	0.44 ± 0.69	1.00 ± 0.10[b]	1.35 ± 0.20	0.2
		SH	0.69 ± 0.06	1.60 ± 0.25	2.15 ± 0.25[b]	0.2
		PH	1.45 ± 0.10	0.65 ± 0.10[b]	1.91 ± 0.30	0.5
		Total	2.60 ± 0.05	3.30 ± 0.50	5.40 ± 0.11[c]	0.3

[a] S, Free, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis; PH, PCA-insoluble conjugated PAs.   [b] Significantly different from 0-h control at $P < 0.05$.   [c] Significantly different from 0-h control at $P < 0.01$.

Table IV. *Polyamine Titers and Osmotic Stress in Moth Bean Leaves*

Peeled leaf discs were incubated in the dark for 6 h as indicated in Table I.

Treatment	Time of Incubation	PA Fraction	Put	Spd	Spm
	h				
Control	0	S[a]	0.14	0.41	0.35
		SH	0.23	0.71	0.62
		Total	0.57	1.12	0.97
Control	6	S	0.18 ± 0.02	0.55 ± 0.03	0.39 ± 0.04
		SH	0.62 ± 0.03	0.87 ± 0.10	0.80 ± 0.12
		Total	0.80 ± 0.02	1.42 ± 0.20	1.19 ± 0.10
0.6 M Sorbitol	6	S	0.17 ± 0.02	0.63 ± 0.04	0.86 ± 0.05[b]
		SH	0.54 ± 0.12	0.85 ± 0.03	1.44 ± 0.07[b]
		Total	0.71 ± 0.10	1.48 ± 0.06	2.30 ± 0.07[b]

[a] S, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis. [b] Significantly different from 6-h control at $P < 0.01$.

Table V. *Relative Distribution of Polyamines (S + SH Fractions) and Put/Spd + Spm in Various Dicot Leaves, as Compared with Oat as a Representative Monocot*

Peeled leaf discs or segments were incubated in the dark as indicated in Table I.

Peeled Leaf of	Treatment	Put	Spd	Spm	Put/Spd + Spm
		% relative to total Put + PA (100%)			
Tobacco	0-h Control	36	59	5	0.6
	6-h Control	44	51	5	0.8
	6-h 0.6 M Sorbitol	19	73	8	0.2
Pepper	0-h Control	66	25	9	1.9
	6-h Control	62	32	6	1.7
	6-h 0.6 M Sorbitol	41	45	14	0.7
Trigonella	0-h Control	24	50	26	0.3
	6-h Control	25	47	28	0.3
	6-h 0.6 M Sorbitol	16	36	48	0.2
Moth bean	0-h Control	23	41	36	0.3
	6-h Control	24	41	35	0.3
	6-h 0.6 M Sorbitol	16	33	51	0.2
Oat	0-h Control	15	47	38	0.2
	6-h Control	17	46	37	0.2
	6-h 0.6 M Sorbitol	55	27	18	1.2

Table VI. *Polyamine Titers in Freshly Isolated Protoplasts from Peeled Oat and Moth Bean Leaves*

Oat protoplasts were isolated from primary leaves of 7-d-old seedlings. Moth bean protoplasts were isolated from seedlings 8 and 12 d old in experiments 1 and 2, respectively.

Protoplasts of	Experiment No.	Put	Spd	Spm	Put/Spd + Spm
		nmol/mg protein			
Oat	1	17.60 (62)[a]	9.50 (33)	1.42 (5)	1.60
	2	18.60 (70)	6.81 (25)	1.30 (5)	2.30
Moth bean	1	2.79 (32)	1.91 (22)	4.08 (46)	0.46
	2	2.22 (33)	1.44 (22)	3.00 (45)	0.50

[a] Relative distribution of the PAs as % (total Put + PA is 100%).

Spm levels were observed in the dark (Table V). A decreased Put/Spd + Spm ratio occurred in all osmotically stressed dicot leaves.

In oat protoplasts, Put rises after osmotic stress, as does the Put/Spd + Spm ratio. In moth bean protoplasts, Put does not rise after osmotic stress, and the Put/Spd + Spm ratio is several-fold lower than in oat. In addition, the diamine/polyamine ratio in readily dividing tobacco protoplasts is lower than in poorly dividing dwarf pea protoplasts (1 *versus* 1.5, respectively; FM Dumortier, unpublished data).

We have shown that the high Put/polyamine ratio in leaves and protoplasts of species which yield poorly dividing protoplasts contrasts with the low ratio in leaves and protoplasts of species which yield readily dividing protoplasts. This is interesting since many investigations point to an important role of the higher PAs (Spd and Spm) in DNA synthesis and mitosis (review in Heby [10]). On the other hand, high concentrations of exogenously applied or endogenous Put appear toxic for the plant (9, 15–17). Therefore, the results of this investigation suggest that the difference in response to osmotic stress might be related to failure of cereal mesophyll protoplasts to divide *in vitro*. Additional data supporting such a hypothesis are presented in the following paper (20).

(ADC and ODC) also remained higher in control tobacco leaves which also contrasted with the 2-fold increase of ADC induced by osmotic stress in oat leaves (6).

Put accumulation in oat leaves is maximal after 4 h of osmotic treatment (6), while in tobacco and pepper the maximum difference in PA titers between control and stressed tissues occurred after 6 h, which was then used routinely for calculation of the Put/Spd + Spm ratio. Thus, in the solanaceous species (tobacco, pepper, and *Datura*) the osmotically induced increase in higher polyamines was due mainly to Spd, whereas the legumes *Trigonella* and moth bean showed increased Spm levels after osmotic treatment.

The increase of the higher polyamines Spd and Spm by osmotic shock in dicots contrasted with a gradual decline of Spd and Spm titers during the initial 4 h of incubating oat leaves on osmotica (6). The response (as indicated in tobacco and pepper) is rapid and peaks at 4 h of osmotic treatment. An eventual but not significant rise of Spd was observed in osmotically stressed oat leaves after 6 to 10 h under light (6), but decreased Spd and

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