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# Polyamines in *Helianthus annuus* L. during Germination under Salt Stress

M. P. Benavides,* G. Aizencang, and M. L. Tomaro

Cátedra de Química Biológica Vegetal, Departamento de Química Biológica, Facultad de Farmacia y Bioquímica. Universidad de Buenos Aires, Junín 956 (1113) Buenos Aires, Argentina

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**Abstract.** The level of the three main polyamines putrescine, spermidine, and spermine and the biosynthetic enzyme arginine decarboxylase (ADC) decreased in *Helianthus annuus* L. seedlings subjected to increasing (50, 100, and 150 mM) NaCl concentrations. The pattern of polyamines in control plants increased during the initial 72 h and then reached a plateau. The putrescine level showed an increase of 370% after 72 h of development. The lower salt treatment slightly diminished the overall polyamine content. The highest NaCl concentration (150 mM) induced a strong putrescine diminution (from 381 to 78.9 nmol $g^{-1}$ FW) at 72 h whereas a small decrease in ADC activity was detected. ODC was detected in neither control nor treated plantlets during the experimental period. The level of spermidine also decreased, but the magnitude of the decay was less pronounced than putrescine. The fact that ODC was not detected and ADC activity followed a pattern similar to that of putrescine led us to suppose that the variation in putrescine content could be attributed entirely to the decrease in ADC activity. α-Difluoromethylarginine and α-difluoromethylornithine (ADC and ODC inhibitor, respectively) did not inhibit but delayed the onset of germination of sunflower seeds, and α-difluoromethylornithine increased the content of spermidine and spermine. The present data suggest that polyamines could be involved in the germination process of *H. annuus* seeds and in response to salt stress.

**Key Words.** Polyamines—Salt stress—ADC—ODC—Polyamine biosynthesis inhibitors—*Helianthus annuus* L.

**Abbreviations:** Put, putrescine; Spd, spermidine; Spm, spermine; PMSF, phenylmethylsulfonyl fluoride; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; FW, fresh weight; TCA, trichloroacetic acid.

*Author for correspondence.

Polyamines are biologically active compounds involved in various physiological processes. They are cationic molecules, positively charged under intracellular pH, which are essential for plant growth and differentiation, related to aging and senescence, and usually involved in plant responses to stress (Flores and Galston 1982, Friedman et al. 1989). The most common polyamines studied in plants are the diamine putrescine (Put), the triamine spermidine (Spd), the tetramine spermine (Spm). The fact that polyamines can activate protein synthesis (Serafini-Fracassini 1991) suggest that they are activators of this process early in germination. Bagni (1970) studied the correlation among the levels of polyamines, protein, and RNA during germination in *Phaseolus vulgaris* and found that Spd and Spm, together with proteins, increased during germination. The same relationship was found in *Phaseolus mungo*, *Pisum sativum*, and *Zea mays* (Villanueva et al. 1978).

It has been suggested that changes in polyamine metabolism under stress may be part of an integrated plant response. Polyamines seem to be involved in salt, osmotic, drought, and oxidative responses in plants (Flores 1991). Put is known to accumulate from micromolar to millimolar concentrations under potassium deficiency, low pH, or herbicide treatment (Di Tomaso et al. 1988, Evans and Malmberg 1989). However, it has been reported that Spm and Spd levels remained unchanged under the same conditions. The Put level declined in *Capsicum annuum* and *Datura stramonium* leaves subjected to osmotic stress, whereas Spd and Spm titers increased (Tiburcio et al. 1986). Protective roles have been postulated for polyamines under adverse environmental conditions. Exogenously added polyamines were effective in preventing chlorophyll loss (Cohen et al. 1984) or inhibiting RNase and protease activities (Kaur-Sawhney and Galston 1982).

Salinity is a stress factor that causes reduction in the water potential of the external medium. Although salinity and osmotic stress may affect polyamine metabolism in different manners (Erdei et al. 1991, 1996, Reggiani et

al. 1994, Zhou et al. 1995), no distinct separation between salt and osmotic stress could be made in some cases (Friedman et al. 1989). The Put titer decreased in seedlings of *Brassica campestris* treated with 100 mM NaCl (Das et al. 1995) but increased twofold in sensitive coleoptiles of rice cultivars (Basu and Ghosh 1991).

In various experimental systems, the variation in polyamine levels is often accompanied by an increase in the activity of arginine decarboxylase (ADC), whereas ornithine decarboxylase (ODC) activity is apparently unaffected (Flores et al. 1989). Polyamine accumulation and activation of both ADC and ODC were found in mung bean plants in response to salt stress (Friedman et al. 1989) and also in cultured tobacco cells and leaves of several plants (Das et al. 1995, Erdei et al. 1996, Reggiani et al. 1994). In all cases the increase in Put appears to originate from the ODC pathway (Kramer and Wang 1990) and/or from induction of ADC activity (Smith 1985). However, contradictory data have been reported on polyamine accumulation as a response to salt stress.

In the present article, the effect of NaCl application on polyamine levels and on ADC and ODC activities during germination were examined. The effect of DFMA and DFMO, irreversible inhibitors of ADC and ODC, respectively, were tested under the same conditions.

## Materials and Methods

### *Germination*

Sunflower (*Helianthus annuus* L) cv. Dekasol seeds were surface sterilized with $HClO_4$ (8% active $Cl_2$) at 50% for 10 min and then rinsed thoroughly with distilled water. They were germinated in Petri dishes at 26/20°C (day and night), with a 16-h photoperiod under fluorescent white light (175 $\mu mol/m^2 \cdot s^{-1}$) in a controlled environmental growth chamber, either on deionized distilled water or with different NaCl solutions (50, 100, and 150 mM). The samples were collected randomly at 24, 48, 72, 96, and 120 h and extracted for polyamine and enzyme determinations. To test the effect of DFMA, DFMO, and exogenously added polyamines, the seeds were first soaked for 6 h in distilled water containing the inhibitors (1 mM) and/or the polyamines (0.5 mM) and then placed on moistened paper prepared with the same solutions.

The irreversible polyamine biosynthesis inhibitors were gifts from Dr. Peter McCann (Merrel Dow Research Center, Cincinnati, OH).

### *Analysis of Polyamines*

Plant material (usually 400 mg FW) was homogenized with 5% perchloric acid, kept for 1 h on ice, and centrifuged at 5000 rpm for 10 min. The supernatants were derivatized using the dansylation method described by Smith and Davies (1985), and dibencyl Put was used as internal standard. Dansylated samples were stored at −20°C and were stable for at least 1 month under these conditions. TLC was performed on high resolution silica gel plates (JT Baker, silica gel plates IB 2-F) with an *n*-hexane:ethyl acetate (2:1) solvent system. They were observed under UV light (254 nm), and their fluorescence was measured at 365 nm excitation and 510 nm emission in a spectrofluorometer (Aminco Bowmann). Concentration curves of standard Put, Spm, and Spd were run simultaneously in the same conditions.

### *Determination of ADC and ODC Activities*

All assays were performed on fresh extracts according to the method described by Flores and Galston (1984). The samples (500 mg FW) were homogenized in a chilled mortar with 5 volumes of 50 mM phosphate buffer (pH 7.8) containing 0.5 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF, 1 mM pyridoxal phosphate, and 1% polyethylene glycol (buffer A). They were centrifuged at 20,000 × *g* for 20 min, and the supernatants were used immediately for enzyme assays.

The incubation mixture for ADC consisted of 100 μL of the crude extract, 70 μL of buffer A, 10 mM pyridoxal phosphate, 25 mM dithiothreitol, and a 0.1 mM concentration of the substrate [1-$^{14}$C]arginine (325 mCi/mmol, DuPont-New England Nuclear) diluted with cold arginine to give a final concentration of 20 mM, in a final reaction volume of 200 μL.

ODC was assayed in a similar way using [1-$^{14}$C]ornithine (54.3 mCi/mmol, DuPont-New England Nuclear) diluted with cold ornithine to give a final concentration of 20 mM. Specific activities of the enzymes are expressed as pmol of $^{14}CO_2 h^{-1}\ g^{-1}$ FW.

Reaction mixtures were incubated for 60 min at 37°C under continuous shaking, the reaction was stopped by adding 100 μL of 20% TCA, and the incubation continued for 45 min. For blanks, TCA was added at zero time. The $^{14}CO_2$ released in the reaction was trapped in Whatman filter papers moistened with Protosol (DuPont-New England Nuclear) and placed above the reaction mixture in glass tubes similar to the plastic wells of Kontes. Blanks with denatured enzymes were used as controls. When the reaction was finished, the filter papers were put in a scintillation solution (2,5-diphenyloxazole plus 1,4-bis[2-(5-phenyloxazolyl)]benzene) in glass vials, and the radioactivity was measured in a Beckman LS 1801 scintillator counter.

### *Statistical Analysis*

All data presented are the mean values of three independent sets of experiments with a minimum of 10 seedlings/treatment. Statistical assays were carried out by one-way ANOVA, and values lower than 0.05% were considered to be significant, according to Tukey's multiple range test.

## Results

### *Germination and Plant Growth under NaCl Stress*

The influence of increasing NaCl concentrations (50, 100, and 150 mM) on the germination and growth of the plants during the initial 120 h was evaluated. All saline treatments affected seedling growth (Table 1). Germination (emergency of the radicle) occurred at 12 h in control and 50 mM NaCl-treated seeds, whereas at higher salt levels (100 and 150 mM) germination occurred at 26 and 36 h, respectively. Plants grown under 50 and 100 mM NaCl developed in a way similar to that of control plants. At 150 mM NaCl all of the seeds germinated, but seedling development was inhibited after germination (data not shown).

To know if growth could be restored in the 150 mM

**Table 1.** FW of sunflower seedlings germinated in distilled water or with exogenously added Put, Spd, and/or Spm (0.5 mM). S, 150 mM NaCl treatment. Samples were taken at 72 h. S.E., standard error. Data are mean values of two independent experiments ±S.E. Each point represents 12 ± 2 replicates. Different letters mean significant differences ($p < 0.05$) according to Tukey's multiple range test.

| Treatment | Fresh weight (g) | S.E. |
|---|---|---|
| Control | 1.5854 | 0.025 a |
| 50 mM NaCl | 1.3112 | 0.045 b |
| 100 mM NaCl | 1.0975 | 0.014 c |
| 150 mM NaCl | 0.6574 | 0.058 d |
| 3 polyamines | 0.9865 | 0.084 e |
| S + Put | 0.9665 | 0.084 e |
| S + Spd | 1.1434 | 0.072 f |
| S + Spm | 1.1665 | 0.018 f |
| S + 3 polyamines | 0.8219 | 0.065 g |

NaCl-treated plants by the addition of exogenous polyamines, Put, Spd, and/or Spm (0.5 mM each) was added to the germination solution. FW was analyzed at 72 h. The addition of exogenous polyamines partially restored growth in the 150 mM salt-treated seedlings (Table 1). In this case, the plantlets reached FW values similar to that of the 100 mM salt treatment. The three polyamines alone were inhibitory, and the addition of three polyamines simultaneously did not improve the recovery of the 150 mM NaCl-treated seeds with respect to the other treatments (Table 1). Spd and Spm showed similar effects on growth restoration and were more effective than Put, although small differences were observed among treatments.

### *Changes in Polyamines and Biosynthetic Enzyme Activities during Germination under NaCl Stress*

The distribution of the main three polyamines Put, Spd, and Spm during the initial 120 h after germination was analyzed (Fig. 1). During the initial 72 h, a rapid increase in polyamine level was observed in control seedlings germinated in distilled water. Put was the polyamine that reached the highest concentration and Spm the lowest. The amount of Put increased 370% during the first 72 h of plant development, reaching a plateau after this time. Similar patterns were obtained with Spd (345%) and Spm (185%), even though the maximum concentrations for Spd (225 nmol $g^{-1}$ FW) and Spm (114.3 nmol $g^{-1}$ FW) were considerable lower than for Put (380.5 nmol $g^{-1}$ FW).

The level of the endogenous polyamines was affected by salt treatment over 72 h. The lower NaCl concentration decreased the overall amount of polyamines slightly compared with the control (Fig. 2). Put decreased 21% and Spd 18%, whereas the Spm level did not show significant differences with respect to the control. Putrescine was the polyamine most affected by salt stress at the higher saline concentrations tested (100 and 150 mM), diminishing from 380.5 to 78.9 nmol $g^{-1}$ FW at 72

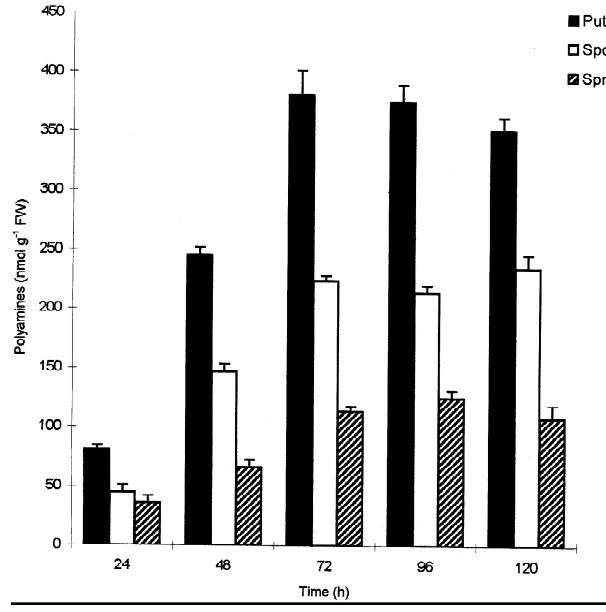


**Fig. 1.** Polyamine changes during seedling development in sunflower. Polyamines are expressed as nmol $g^{-1}$ FW. Data are mean values of three independent experiments ± S.E. Each point represents 10 ± 2 replicates. *Vertical lines* in each point show the S.E.

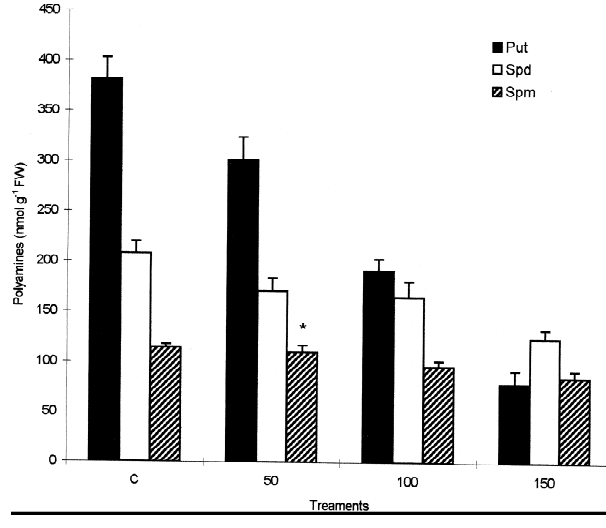


**Fig. 2.** Effect of increasing NaCl concentrations on polyamine content of sunflower seedlings after the initial 72 h of the experiment. Polyamines are expressed as nmol $g^{-1}$ FW. Data are mean values of two independent experiments ± S.E. Each point represents six replicates. * indicates no significant differences ($p > 0.05$) according to Tukey's multiple range test. *Vertical lines* in each point show the S.E.

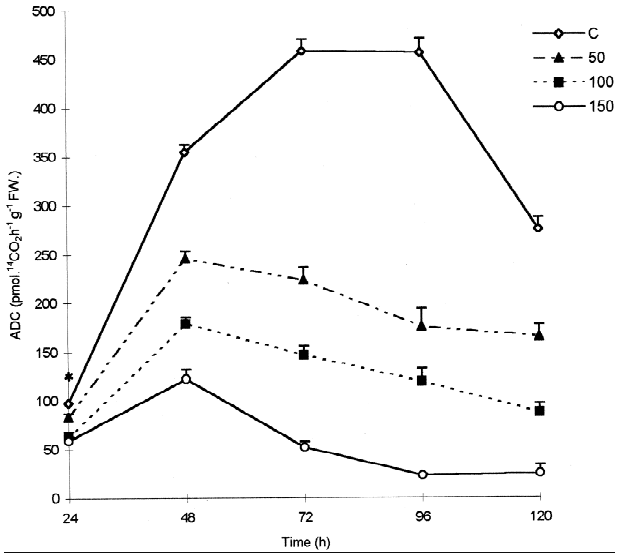


**Fig. 3.** Time course effect of increasing NaCl concentrations on ADC activity in sunflower seedlings. ADC activity is expressed as pmol of $^{14}CO_2$ h$^{-1}$ g$^{-1}$ FW. Data are the mean values of three independent experiments ± S.E. Each point represents six replicates. * indicates no significant differences ($p > 0.05$) according to Tukey's multiple range test. *Vertical lines* in each point show the S.E.

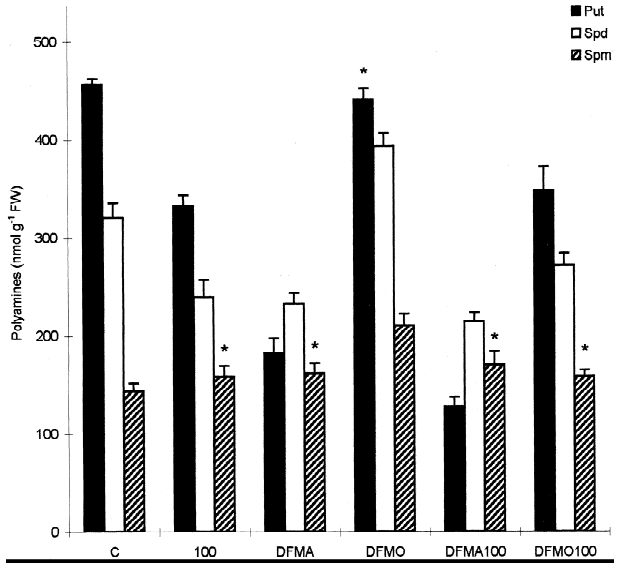


**Fig. 4.** Effect of the addition of polyamine biosynthesis irreversible inhibitors DFMA and DFMO (1 mM), with or without 100 mM NaCl added, on polyamine levels in sunflower seedlings at 72 h of treatment. Polyamines are expressed as nmol g$^{-1}$ FW. Data are the mean values of three independent experiments ± S.E. Each point represents six replicates. * indicates no significant differences ($p > 0.05$) according to Tukey's multiple range test. *Vertical lines* in each point show the S.E.

h under 150 mM, NaCl. The Spm level remained almost unaffected under all of the NaCl concentrations tested, except for the 150 mM NaCl treatment, where a slight decrease was observed; the Spd level dropped to nearly half of the initial value (208.4 to 124 nmol g$^{-1}$ FW) at 150 mM NaCl. The pattern of polyamines changed at 150 mM NaCl, and Spd was the most abundant polyamine under this salt treatment (Fig. 2).

In control plants, ADC increased from 97.4 pmol h$^{-1}$ g$^{-1}$ FW (24 h) to 458 pmol h$^{-1}$ g$^{-1}$ FW at 72 h (370% increment) (Fig. 3). By this time, the activity of this enzyme reached a plateau and then decreased. ADC activity followed a close relationship with Put content in control plants (Fig. 1). ODC activity was not detected in either control or in treated plants.

A general decrease in ADC activity was observed under salt stress. The decay in the enzyme activity at 72 h was more pronounced (89%) at 150 mM NaCl than in plantlets treated with 50 or 100 mM NaCl (45% and 68%, respectively). Although in control plants ADC activity increased between 48 and 72 h and then remained constant up to 96 h, in salt-treated seedlings the enzymatic activity began to decrease after 48 h of salt exposure.

### *Effects of Polyamine Biosynthesis Inhibitors on Germination, Polyamine Levels, and Related Enzymes*

The effects of the polyamine biosynthesis inhibitors DFMA and DFMO on germination, the relative amount of polyamine endogenous content, and ADC and ODC activities were measured in sunflower plants grown at 100 mM NaCl for 72 h. Neither DFMO nor DFMA had an effect on the germination percentage of sunflower seeds, with or without salt in the germination solutions. However, the presence of the inhibitors and the 100 mM NaCl treatment delayed the onset of germination, which occurred at 12 h in controls, at 21 h in DFMO/DFMA-, and at 36 h in 100 mM NaCl-treated seeds (data not shown). Seeds germinated under 100 mM NaCl added with the inhibitors did not experience significant differences in germination percentage or in the onset of germination respect to the 100 mM NaCl-treated seeds.

As shown in Fig. 4, Put decreased nearly 27% at 100 mM NaCl, 59% in DFMA-treated plants, and its level was reduced at 72% with DFMA plus 100 mM NaCl. Spd was enhanced 19% with DFMO, decreased nearly 28% under DFMA or 100 mM NaCl, and was reduced slightly more (38%) with DFMA + 100 mM salt. Spm did not show a significant variation in treatments except with DFMO.

Even though ADC activity was inhibited by DFMA, ODC activity remained undetected in salt-treated and untreated plants throughout the experimental period. ADC activity reached similar values in control and DFMO-treated plants (Fig. 5). A significant decrease in ADC activity was detected after 72 h of development, when seedlings were grown under salt (65%), DFMA (70%), or DFMA + 100 mM NaCl (87%).

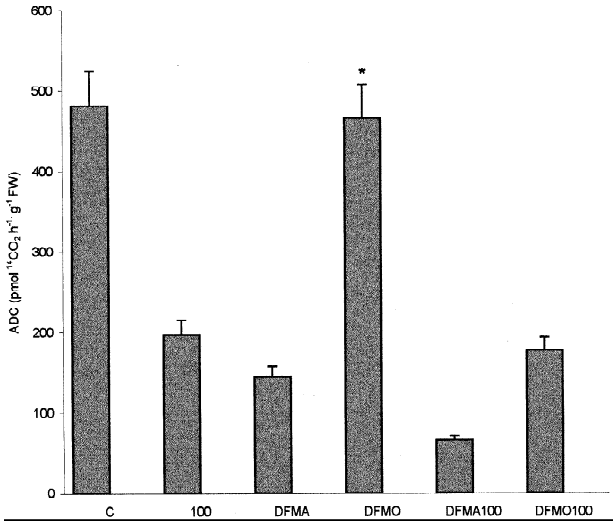


**Fig. 5.** Effect of the addition of polyamine biosynthesis irreversible inhibitors DFMA and DFMO (1 mM), with or without 100 mM NaCl added, on ADC activity in sunflower seedlings at 72 h of treatment. ADC activity is expressed as pmol of $^{14}CO_2$ $h^{-1}$ $g^{-1}$ FW. Data are mean values of three independent experiments ± S.E. Each point represents six replicates. * indicates no significant differences ($p > 0.05$) according to Tukey's multiple range test. *Vertical lines* in each point show the S.E.

## Discussion

In the present study we analyzed the effect of NaCl stress on polyamine content and on the activity of the biosynthetic enzymes ADC and ODC in sunflower plants during germination.

In agreement with other reports (Kingsbury et al. 1984, Mozafar and Goodin 1986), the development of sunflower seedlings was inhibited at variable degrees by increasing the salt concentrations during the first 120 h of development. Reggiani et al. (1994) reported a progressive inhibition of growth by increasing NaCl concentrations in three wheat cultivars differing in salt tolerance. The mechanism of growth inhibition produced by salt is still not clear, although it has been postulated that alteration in the nitrogen metabolism of the seed embryo axis may occur (Prisco 1971, Prisco and O'Leary 1970). Previous reports have demonstrated that exogenous application of Put appeared to counteract the effect of salt in early seedling growth of rice (Prakash and Prathapasenan 1988), and foliar application of the diamine improved growth under saline conditions (Krishnamurty 1991). In agreement, when exogenous Put, Spd, and Spm were added to the germination medium, growth was recovered in the 150 mM NaCl-treated plants, reaching FW values similar to the 100 mM NaCl-treated plants. The addition of the three polyamines together at 150 mM NaCl treatment did not improve the effect exerted by Put, Spm, or Spd added separately on germination. Roberts et al. (1986) demonstrated that polyamines make membrane surfaces rigid. This fact strengthens the possibility that some proportion of polyamines added exogenously induces a physiological effect by acting nonspecifically on the plasmalemma. The mitigating effect of polyamines can presumably be directly attributed to their ability to make membrane surfaces rigid, which may in turn retard membrane deterioration. Salinity may affect the ion balance of plant cells, and polyamines could therefore play an important role in ion balancing (Kingsbury et al. 1984). The deleterious effect of 150 mM NaCl on the plasma membrane could have been prevented by polyamines by improving germination and growth in sunflower seedlings under high NaCl concentrations possibly through their association with the plasma membranes. The beneficial effect of Spd and Spm, which contained three and four amine groups, respectively, is greater than that of Put (Table 1).

The variation in polyamine levels has been analyzed under different stress situations such as osmotic, acid, or water stress, potassium deficiency, or low temperature. In contrast with many of these reports (Aziz and Lahrer 1995, Flores and Galston 1982, Smith 1985, Tiburcio et al. 1994), ADC activity and Put content decreased in salt-treated plants, and this diminution was more pronounced at higher saline concentrations (Figs. 2 and 3). Also, Reggiani et al. (1994) found that the presence of NaCl decreased the level of Put but increased Spd and Spm levels. The stress-induced increase in the diamine has been considered an adaptative response of certain plants to adverse environmental conditions (Slocum et al. 1984), but the excessive accumulation of Put is toxic to certain cells (Guarino and Cohen 1979). Considering that salt did not increase the total level of polyamines, no role in osmoregulation could be attributed to polyamines in sunflower seedlings.

In contrast to many other reports, in salt-treated sunflower seedlings a general decrease in the three main polyamines was observed. The decreased level of Put was related directly to the decrease in ADC activity. Spd and Spm (to a much lesser extent) also decreased with salt treatment, although the diminution was less evident than in the case of Put. This result would indicate that Spd and Spm biosynthesis could be closely related to Put.

The increase in Put during germination in control plants and the decrease in the diamine in seedlings under salt stress are caused exclusively by the ADC pathway inasmuch as ODC was never detected in stressed or nonstressed conditions during the experimental time. ODC was not detected in rice seeds during germination (Bonneau et al. 1994). The inhibition of ADC activity by exogenously added DFMA did not induce an activation of the ODC pathway throughout the experiment. Neither of the salt treatments induced ODC, confirming that ADC and not ODC is more commonly related to stress

situations. DFMO induced an increase in the level of Spd and Spm without affecting either ADC activity or Put content.

The responses associated with the salt treatment might be related to an NaCl effect on enzymes involved in polyamine metabolism. One can propose that a stimulatory effect on polyamine oxidase or diamine oxidase, together with the decreased ADC activity, could be responsible for Put and Spd depletion under salt treatment. Possibly during the metabolic period of adaptation, polyamines stabilize membranes, thus protecting cells against the toxic effect of salt. Increased levels of Spd and Spm have been reported in NaCl-resistant cultivars of rice (Krishnamurty and Bhagwat 1989). The role of polyamines in maintaining membrane integrity and selectivity under salt and osmotic adjustments seems to be obvious, although recent findings indicate that polyamines do not play an essential role in the mechanism of channel regulation (Schroeder 1995). Erdei et al. (1996) proposed that the accumulation of polyamines needs an osmotic signal. If a permeable ion such as sodium is present, salt accumulation can contribute to osmotic adjustment, and the onset of polyamine biosynthesis may be delayed or may not take place.

Our present findings in sunflower plants provide new evidence supporting the assumption that the germination process and polyamine biosynthesis are affected by salt stress.

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