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ORIGINAL PAPER

Sorbitol and NaCl stresses affect free, microsome-associated and thylakoid-associated polyamine content in *Zea mays* and *Phaseolus vulgaris*

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Abstract Most of commercially important crops, including maize and common bean, are sensitive to water deficit and salinity. Polyamines are considered to be osmotic and salt tolerance modulators and biochemical indicators of these stresses. In the present study, we measured organ-specific changes in levels of free, microsome- and thylakoid-associated polyamines in leaves and roots of maize and common bean plants exposed for 24 h to osmotic and saline stresses. Putrescine levels were generally higher in the studied organs of both species and under both stresses; only in the roots of salt-treated bean it considerably decreased. In both species, salt stress (200 mM NaCl) induced a significant decrease in free spermidine in roots. We observed a significant decrease in the contents of all polyamines associated with the microsomes isolated from the roots of maize and bean growing in sorbitol and salt conditions. Also the microsomes isolated from the leaves of stressed plants were characterized by the lower contents of polyamines. Our data showed a reduction of putrescine content, with significantly decreased spermidine and spermine levels in thylakoids isolated from the chloroplasts of maize and bean plants growing under both stresses. The results indicate that the studied maize and bean cultivars are rather drought-sensitive. Additionally, microsome- and thylakoid-associated polyamines seem to be good markers of plant stress tolerance.

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Introduction

Salinity and water deficit are some of the major abiotic stresses affecting agriculture worldwide. Most of commercially important crops are sensitive to both the stresses. Polyamines (PAs) are organic polycations belonging to a specific group of cell growth and development regulators. They are preferentially detected in actively growing tissues and under stress conditions. Plant PA metabolism is extremely sensitive to adverse environmental conditions, so PAs are considered as stress markers in plants (Kumar et al. 1993; Bouchereau et al. 1999; Liu et al. 2007; Kusano et al. 2008; Takahashi and Kakehi 2010). The function of PAs is presumed to be protective, as they act as free radical scavengers, stabilize cellular membranes, and maintain cellular balance (Bors et al. 1989; Besford et al. 1993). However, the precise role and mode of action of PAs in plant stress have long been a matter of debate. Changes in the levels of PAs under stress cannot be predicted and may be affected by several factors, such as plant species or cultivar, duration of stress treatment, developmental stage of tissues, and stress intensity. In addition, cultivars of the same species but differing in stress sensitivity might also show different changes in the pattern of PAs under stress. In nine rice cultivars that differed in salt sensitivity, salt-tolerant cultivars accumulated high concentrations of Spd and Spm, while the salt-sensitive ones accumulated extensive Put and lower levels of Spd and Spm (Krishnamurthy and Bhagwat 1989). Stress-tolerant plants generally have a large capacity to enhance PA biosynthesis in response to abiotic

stresses, compared with intolerant plants (Kusano et al. 2008; Gill and Tuteja 2010). In *Lupinus luteus* (a drought-tolerant species), Put and Spd were accumulated in leaves in osmotic and salt-stress conditions (Legocka and Kluk 2005). Zapata et al. (2004) studied the effect of salinity on plant growth, ethylene production, and PA level in *Spinacia oleracea, Lactuca sativa, Cucumis melo, Capsicum annum, Brassica oleracea, Beta vulgaris,* and *Lycopersicon esculentum.* They found that levels of PAs changed with salinity and in most cases putrescine (Put) content decreased, while spermidine (Spd) and/or spermine (Spm) content increased. On the other hand, mutant *Arabidopsis thaliana* with a lower Put level was more sensitive to salt stress (Urano et al. 2004). Increased tolerance to salt stress has been reported in many transgenic plants with over-expression of arginine decarboxylase (ADC) and/or ornithine decarboxylase (ODC) (Liu et al. 2007). There have been several reports on creating transgenic plants with over-expression of PA biosynthetic genes in an attempt to enhance stress tolerance (Roy and Wu 2001; Kasukabe et al. 2004; Wi et al. 2006; Alcazar et al. 2006; Wen et al. 2008; Wang et al. 2011). Generally attention has been focused on the regulation of free PAs. Most of the studies omitted conjugated and bound forms of PAs. However, these forms appear to be very important for plant development and stress response. In nature, PAs often occur as free molecular bases, but they can also be associated with small molecules like phenolic acids (soluble conjugated forms) and interact more freely by electrostatic linkages with anionic molecules, such as DNA, RNA, chromatin, proteins, and membrane lipids preventing these macromolecules and the membrane system from denaturing under stress conditions (Tabor and Tabor 1984; Feurestein et al. 1990; Schruber 1989). The involvement of PAs in stress resistance and their association with thylakoid membranes indicate that PAs are likely to interact with photosystems (Kotzabasis et al. 1993; Hamdani et al. 2011).

Our study was aimed to analyze the subcellular distribution of free, microsome- and thylakoid-associated PAs in the roots and leaves of two species: maize (*Zea mays* L. 'Kometa') and common bean (*Phaseolus vulgaris* L. 'Sisal') growing under osmotic and salt stresses. The mechanism leading to salinity tolerance in commercial crops is still unclear, and this study may help us understand better the role of various forms of PAs under non-ionic and ionic stresses in plants that differ in tolerance to drought.

Materials and methods

Plant material

Seeds of maize (*Zea mays* L. 'Kometa') and common bean (*Phaseolus vulgaris* L. 'Sisal') were surface-disinfected with 1% sodium hypochloride and then were germinated on several layers of moistened tissue paper in the dark at 23°C. Three-day-old seedlings were transferred to boxes with Hoagland's solution in which they grew for 8 days under continuous light (130 μM m^{-2} s^{-1}) at 23°C in 50% humidity. After this time, isoosmotic concentrations of sorbitol (360 mM) and NaCl (200 mM) were applied for 24 h in Hoaglands's solution. Next the plants were harvested, divided into appropriate organs, weighed, and further processed for polyamine determination. The concentrations of sorbitol and NaCl used for the study were chosen after preliminary experiments with different concentrations of NaCl (100, 200, 300, 400 and 500 mM) and sorbitol (180, 360, 540 and 720 mM). The visual plant conditions and determination of PA levels were taken into account.

Isolation of microsomes

The microsomes were obtained after modification of the procedure described by Tassoni et al. (1996). The plant material (60–80 g of roots or leaves) was homogenized in a mortar by using two volumes of buffer containing 50 mM Hepes, pH 7.2, 2 mM EDTA, 0.4 M sucrose, 0.1 mM DTT and 1 mM polyvinylpyrrolidone (PVP)-10. The homogenate was filtered through four layers of cheesecloth and centrifuged at 9,000*g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 100,000*g* for 60 min to obtain microsomes. The microsome fraction was weighed and used for polyamine analysis.

Isolation of thylakoids

Thylakoid membranes were isolated according to a modified procedure of Hilditch (1986). Leaves were homogenized in a mortar in a buffer for chloroplast isolation. The homogenate was filtered through eight layers of gauze and centrifuged for 10 min at 15,000*g*. The chloroplast pellet was suspended in the isolation buffer and layered on 3 ml of 65% sucrose in isolation buffer and centrifuged as previously. Chloroplasts were collected from the top of the sucrose layer and centrifuged as previously. Next they were osmotically shocked in the isolation buffer without sucrose. Chloroplast membranes were collected after centrifugation for 15 min at 12,000*g*. The membrane pellet was washed twice in the isolation buffer without sucrose and centrifuged as above. The last centrifugation was performed in weighed centrifuge tubes.

PA quantification

Free, microsome-, and thylakoid-associated PAs were determined according to Smith and Best (1977). The tissue

was extracted for 1 h in 5% perchloric acid (PCA) (100 mg fresh weight/ml PCA). The extract was centrifuged for 20 min at 12,000*g*, and the supernatant fraction was used directly for analysis of free PAs. PAs associated with microsomes and thylakoids were released by hydrolysis in 6 N HCl for 18 h at 110°C. Further analysis was carried out as for free PAs. PAs were dansylated and separated on TLC plates (Whatman silica gel 60A LK 6D) using cyclohexane:ethyl acetate (5:4, v/v). PAs were detected by UV lamp and identified by RF values of PA standards. Last, the spots were scraped into test tubes, eluted in ethyl acetate, and quantified using the spectrophotofluorometer (L-2000 Hitachi) with excitation of 350 nm and emission at 445 nm. PA content was expressed per unit fresh weight of leaves and/or roots and weight of isolated microsomes and thylakoids.

Statistical analyzes

Differences in measured parameters were analyzed for statistical significance using Student's *t* test. Means were regarded as significantly different at $P < 0.05$. Each data point represents a mean of three replicates obtained from three independent experiments. The statistical analysis was performed using Standard Method STATISTICA (Stat Soft Inc., Tulsa, OK, USA).

Results

Free, microsome- and thylakoid-associated PAs were determined in leaves and roots of maize and bean growing in osmotic and saline stresses for 24 h.

Effect of sorbitol and salt stresses on free PAs

In maize leaves and roots, Put was a dominant PA. In plants treated with sorbitol or NaCl, Put significantly increased in leaves by about 24 and 29%, respectively, and in the roots of sorbitol-treated plants, by about 24% in comparison with the controls (Fig. 1a, b). In leaves, Spd content only slightly decreased, whereas the level of Spm was lowered significantly (by about 43%) in both stresses as compared with the control (Fig. 1a). The levels of Spd were about 35% lower in the roots of stressed plants than in the control (Fig. 1b).

In bean leaves, Spd was the dominant PA. In the leaves of plants growing under saline stress the level of Put increased significantly, whereas under both stresses, a 50% decrease in Spm was observed (Fig. 2a). In the roots of plants growing under osmotic stress, the PA level slightly increased, whereas in plants treated with NaCl, a high reduction in PA concentration was observed (Fig. 2b).

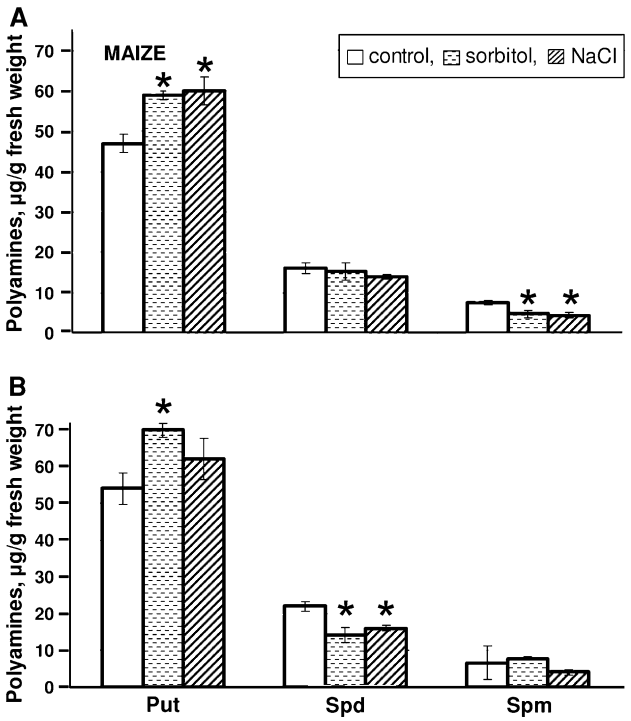

Fig. 1 Levels of free putrescine, spermidine, and spermine in leaves (**a**) and roots (**b**) of 12-day-old maize treated with isoosmotic concentrations of sorbitol (360 mM) and NaCl (200 mM) for 24 h. The asterisks indicate the data for which control/salt and osmotic stress differences are significant. The values are means ± SD of three independent experiments

The levels of Put, Spd, and Spm decreased by about 43, 80, and 36%, respectively, as compared with that of control.

Effect of sorbitol and salt stresses on microsome- and chloroplast-associated PAs

The level of microsome-associated PAs isolated from leaves and roots of maize treated with sorbitol and NaCl was examined. Put, Spd, and Spm were detected in the isolated microsome fraction. The levels of Put and Spd associated with microsomes derived from maize leaves growing under both stress conditions were slightly lower than in the control, whereas the level of Spm significantly decreased (Fig. 3a). In the roots, the decrease in microsome-associated PAs was much higher than in the leaves, particularly in the sorbitol-treated plants, in which the levels of microsome-associated Put, Spd, and Spm were about 48, 33, and 50% lower, respectively, as compared with the control (Fig. 3b).

In the thylakoid fraction isolated from maize leaves, Put, Spd, and Spm were detected. Under the saline stress, the level of thylakoid-associated Put, Spd, and Spm was reduced by about 28, 38, and 40%, respectively, as compared with the control (Fig. 3c). In sorbitol-treated plants,

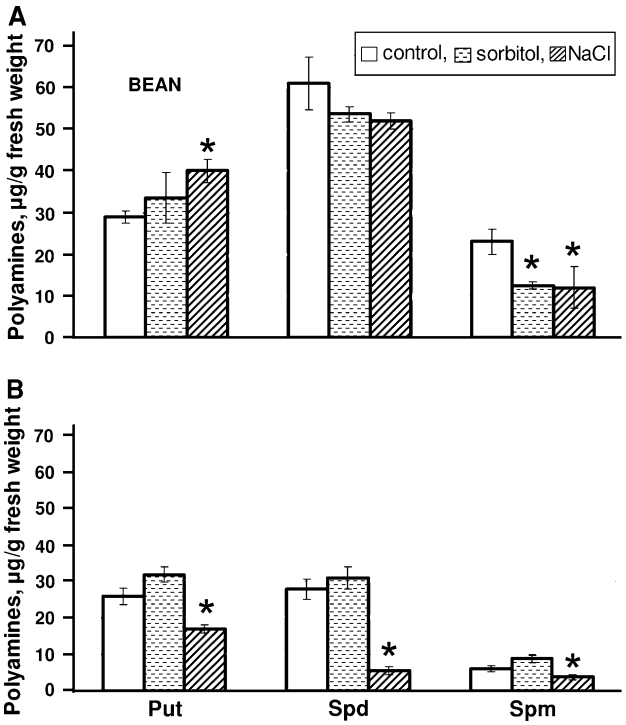

Fig. 2 Levels of free putrescine, spermidine, and spermine in leaves (**a**) and roots (**b**) of 12-day-old common bean treated with isoosmotic concentrations of sorbitol (360 mM) and NaCl (200 mM) for 24 h. The asterisks indicate the data for which control/salt and osmotic stresses differences are significant. The values are means ± SD of three independent experiments

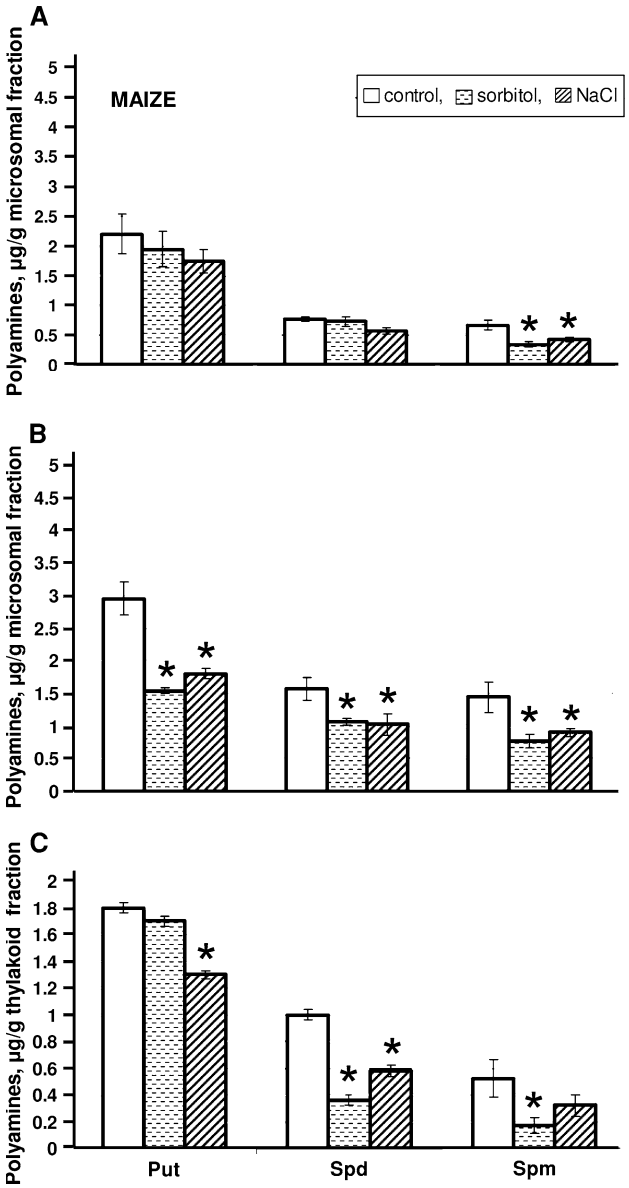

Fig. 3 Levels of putrescine, spermidine, and spermine associated with microsomes isolated from leaves (**a**), roots (**b**) and thylakoids (**c**) of 12-day-old maize treated with isoosmotic concentrations of sorbitol (360 mM) and NaCl (200 mM) for 24 h. The asterisks indicate the data for which control/salt and osmotic stresses differences are significant. The values are means ± SD of three independent experiments

an about 65% decrease in thylakoid-associated Spd and Spm was detected (Fig. 3c).

Spermidine was the dominant PA in the microsome fraction isolated from bean leaves and roots (Fig. 4). After 24 h of salt stress, microsomes isolated from leaves were characterized by about 44 and 50% decrease in Put and Spd content, respectively, as compared with the control (Fig. 4a). The level of microsome-associated Spd and Spm significantly decreased, from 30 to 70%, in roots exposed to both stresses (Fig. 4b).

Thylakoids isolated from chloroplasts of bean plants growing in the presence of sorbitol or salt for 24 h contained significantly lower levels of Put, Spd, and Spm, by about 27, 31, and 33%, respectively, in comparison with the control (Fig. 4c).

Effect of sorbitol and salt stresses on the (Spd + Spm)/Put ratio in free and microsome- and chloroplast-associated PAs

In maize plants, the (Spd + Spm)/Put ratio of free PAs strongly decreased, as compared with the control. Particularly in the roots of plants incubated with NaCl the ratio dropped by 43% as compared with the control (Table 1). The (Spd + Spm)/Put ratio of microsome- and thylakoid-associated PAs in maize plants was slightly lower under both stresses than in the control, with the exception of root microsomes isolated from plants treated with NaCl, in which it was at the control level. In the roots of bean plants treated with NaCl, the (Spd + Spm)/Put ratio of free PAs represented 42% of the control, and in the microsome fraction the ratio constituted 62% of the control (Table 1).

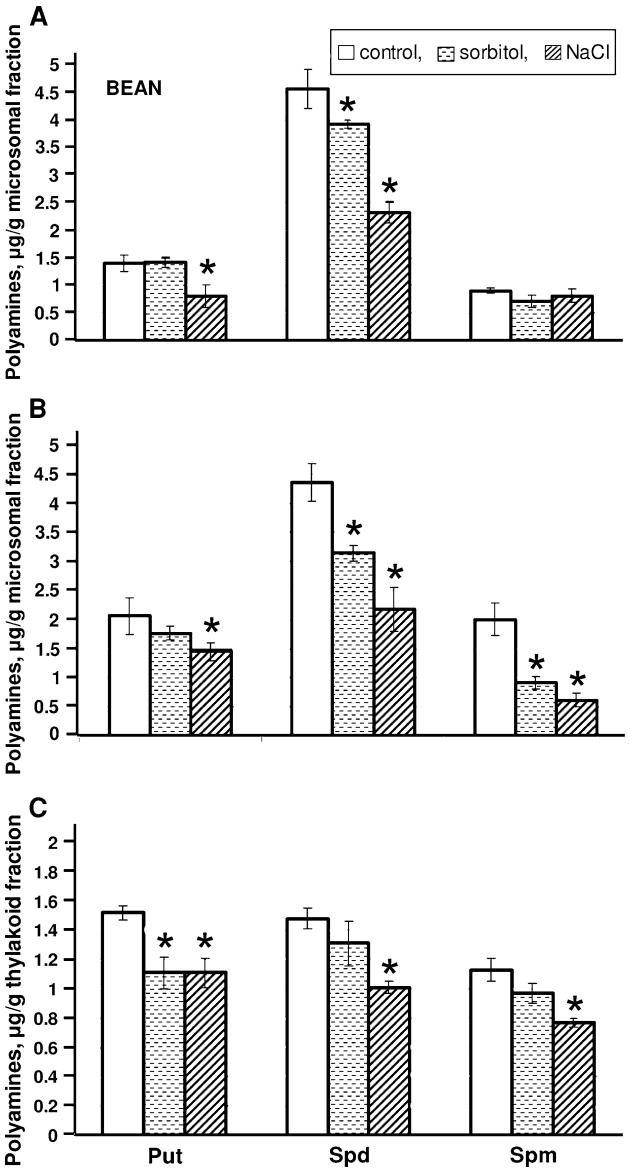

Fig. 4 Levels of putrescine, spermidine, and spermine associated to microsomes isolated from leaves (**a**), roots (**b**) and thylakoids (**c**) of 12-day-old common bean treated with isoosmotic concentrations of sorbitol (360 mM) and NaCl (200 mM) for 24 h. The asterisks indicate the data for which control/salt and osmotic stresses differences are significant. The values are means ± SD of three independent experiments

Discussion

There is an extensive literature describing the correlations of changes in PA levels and physiological perturbations on the protective effect of PAs during environmental stresses (Bouchereau et al. 1999; Alcazar et al. 2006; Groppa and Benavides 2008; Liu et al. 2007; Gill and Tuteja 2010). However, it should be known that under the stress different plant species vary in their response in terms of polyamine fluctuations.

Table 1 The ratio of (Spd + Spm)/Put in leaves, roots, leaf and root microsomes, and thylakoids of maize and bean growing for 24 h in 360 mM sorbitol and 200 mM NaCl expressed as percentage of the control

Organs and subcellular fractions	% of control (Spd + Spm)/Put			
	Maize		Bean	
	NaCl	Sorbitol	NaCl	Sorbitol
Leaves	62	72	93.5	81
Roots	57	70	42	100
Leaf microsomes	88	96	96	64
Root microsomes	103	75	62	80
Thylakoids	82	77	93	108

Zea mays L. 'Kometa' and *Phaseolus vulgaris* L. 'Sisal' were studied to examine the effect of osmotic and saline stresses on the accumulation of free, microsome-, and thylakoid-associated PAs. Osmotic and ionic treatments usually induced an increase in the level of Put in leaves and roots of maize and bean, and a decrease in Spd and Spm content (Figs. 1a, b; 2a, b). Only in bean roots of sorbitol-treated plants were the levels of PAs slightly higher than in the control (Fig. 2a). Our results are not consistent with the data obtained by Jimenez-Bremont et al. (2007), who found in *Zea mays* 'Cafime' plants after one day under salt stress (150 mM NaCl) a significant increase in Put level and a slight increase in Spd and Spm. Longer (7 days) incubation of maize in salt conditions caused a reduction in Spd and Spm levels (Jimenez-Bremont et al. 2007). Two bean cultivars differing in their drought tolerance were studied by Hernandez-Lucero et al. (2008). After the first day of salt treatment, the levels of all PAs increased in the tolerant cultivar, while the sensitive cultivar showed a decrease in PA contents in comparison with the control. It means that the studied bean cultivar belongs to drought-sensitive plants. In another study, after 24 h of 200 mM NaCl treatment (like in our study), the levels of PAs in cucumber roots decreased (Janicka-Russak et al. 2010). The cited authors suggested that PA levels decreased when the concentration of Na^+ increased, so the action of PAs contributed to ionic equilibrium. A salt-tolerant barley cultivar accumulated in the roots much higher levels of Spm and Spm and a lower Put content than the salt-sensitive cultivar under salt stress (Liu et al. 2006). It has often been suggested that Spd and Spm may contribute to stress tolerance, while Put has a lower or no effect on it (Bouchereau et al. 1999; Liu et al. 2007). However, recent findings suggest a direct protective role of Put in abiotic stress tolerance (Takahashi and Kakehi 2010). In a mutant of *A. thaliana* with reduced levels of Put (but not Spd and Spm), stress tolerance was restored by exogenously applied Put (Urano et al. 2004). The reduced levels of PAs in soybean,

especially Put and Spd, were related to higher stress injury and decreased water content (Nayyar et al. 2005).

In many cases, the relationship of plant tolerance was noted with the production of covalently and non-covalently conjugated PAs. The interaction of PAs with subcellular structures is the main mechanism in which these polycations influence their function. It is well known that desiccation results in a loss of membrane integrity and that PAs may interact with membranes to preserve them from degradation (Bouchereau et al. 1999; Kusano et al. 2008). In our study we analyzed the level of microsome- and thylakoid-associated Put, Spd, and Spm. The results showed a decrease in the concentrations of PAs in microsomes isolated from roots and leaves of maize and bean growing for 24 h in osmotic and salt stress (Figs. 3a, b; 4a, b). The root microsomes isolated from maize growing under both stresses were characterized by a significant reduction of PA levels (Fig. 3a) Also root microsomes isolated from salt-treated bean had very low levels of all PAs (Fig. 4b). PAs play an important role in maintaining membrane integrity under most stress conditions. As mentioned earlier, PAs bound to membranes prevent lipid peroxidation and quenching of free radicals needed for conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene (Drolet et al. 1986; Bors et al. 1989). The surface-rigidifying effect on liposomes can be interpreted as reflecting an association with a negatively charged head group of phospholipids (Tassoni et al. 1996). Liu et al. (2004) showed that after 7 days of polyethylene glycol treatment of wheat, the levels of Put and Spm conjugated to root tonoplast vesicles were correlated with maintenance of H^+-ATPase and H^+-PPAse activities and enhanced osmotic tolerance in a drought-tolerant cultivar. This suggests that the loss of PAs in microsome membranes in maize and bean growing under stress conditions may indicate indirectly a loss of function of these membranes. Thus the cultivars of studied species seem to be drought-sensitive plants.

In thylakoids isolated from chloroplasts of maize and bean plants treated with sorbitol or NaCl, the level of PAs decreased (Figs. 3c, 4c). The covalent and non-covalent conjugation of PAs with light-harvesting complex (LHC) polypeptides provide some stability against various stresses and consequently helps in maintaining the photosynthetic activity (Hamdani et al. 2011). Kotzabasis et al. (1993) and Del Duca et al. (1994) reported that the main PAs (Put, Spd and Spm) are associated with the LHC and photosystem II (PSII) of spinach and *Helianthus tuberosus,* while subcomplex of PSII (PSII reaction center) contained only Spm (Kotzabasis et al. 1993). Both the outer and inner surface of thylakoid membranes are negatively charged (Barbier 1982) and, therefore, the strongly cationic PAs may aid in preserving thylakoid morphology (Tiburcio et al. 1994). Additionally, their covalent binding to thylakoid proteins by transglutaminases may play a significant role in the post-translational modification and stabilization of structural proteins in chloroplasts (Sobieszczuk-Nowicka et al. 2007; Sobieszczuk-Nowicka et al. 2008; Serafini-Fracassini and Del Duca 2008). The importance of chloroplast PAs in the function of photosynthetic membranes in tobacco leaves recently has been described by Ioannidis and Kotzabasis (2007). Also exogenous Spd was effective in stabilization of thylakoid membranes under stress conditions and during senescence (Besford et al. 1993; Legocka and Zajchert 1999).

It is also postulated that the (Spd + Spm)/Put ratio increases with salinity in all species with enhanced salinity tolerance (Bouchereau et al. 1999; Zapata et al. 2004; Liu et al. 2007, Gill and Tuteja 2010). Our results showed the decrease in the free and microsome- and thylakoid-associated (Spd + Spm)/Put ratio in maize and bean growing under both stresses (Table 1).

In conclusion, the decrease in the level of microsome- and thylakoid-associated PAs, observed in *Zea mays* 'Kometa' and *Phaseolus vulgaris* 'Sisal' growing in osmotic and saline stresses, suggests that these cultivars are drought-sensitive plants. Additionally, because of the functions of PAs in plant cells, the PAs associated with these cellular structures seem to be good markers of plant stress tolerance.

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