Bound Putrescine, a Distinctive Player under Salt Stress in the Natrophilic Sugar Beet in Contrast to Glycophyte Tobacco

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Abstract

The influence of salinity on the different polyamine fractions (free, conjugated, and bound) was compared in a natrophilic halophyte (*Beta vulgaris* L. cv. IC) and a salt sensitive glycophyte (*Nicotiana rustica* L. cv. Basmas). Low-level salinity (25 mM NaCl) and high salinity (150 and 50 mM NaCl for sugar beet and tobacco, respectively) were supplied in hydroponics. Under low salinity shoot dry weight increased in sugar beet, but decreased in tobacco. Under high salinity growth reduction in sugar beet and tobacco were similar. However, sugar beet accumulated higher Na and Cl in roots and shoots than tobacco. Low salinity caused an increase (22%) in the rate of net CO_2 assimilation in sugar beet. This parameter was depressed in both species under high salinity. Sugar beet had constitutively higher free spermine levels in roots and shoots than tobacco. Spermidine levels were constitutively higher in shoots of sugar beet and in roots of tobacco. Under salt stress tobacco plants tended to increase free polyamine levels. The most important salt–induced rise in polyamine titer, however, was found in roots and shoots of sugar beet. In sugar beet roots the bound putrescine fraction increased 9.3-fold under growth- stimulating salt supply (25mM) and 20-fold under salt stress (150 mM). In tobacco roots this fraction only increased 2.3 and 3.8-fold under mild (25 mM) and high salt stress (50 mM), respectively. Our results provide support to the view that bound putrescine contributes to the protection against salt stress in the natrophilic sugar beet.

Keywords: *Beta vulgaris;* Ion relations; *Nicotiana rustica*; Photosynthesis; Polyamines; Salinity

Introduction

Salt stress is one of the most serious and widespread factors limiting the productivity of agricultural crops [6]. The deleterious effects of salinity on plant growth are associated with the low osmotic potential of the soil

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solution, nutritional imbalance, specific ion effects, or a combination of these factors. All these factors have adverse effects at the molecular, metabolic, and physiological level causing inhibition of plant growth and development [3]. Similar with other abiotic stresses, salinity is known to negatively influence CO_2 assimilation via affecting both stomatal and non-stomatal components of photosynthesis [18].

Accumulation of different organic compounds such as sugars, amino and organic acids, betaines, and polyamines and the expression of different sets of genes have been described as part of the signaling and defense systems of plants against salinity stress [14, 29, 45].

Polyamines, mainly spermidine (Spd), spermine (Spm) and their diamine precursor putrescine (Put), are ubiquitous aliphatic polycations that for long have been recognized as modulators of plant growth and development, especially under stress conditions [8,11]. More recent approaches using model plants either overexpressing enzymes involved in polyamine biosynthesis or loss-of-function mutants also strongly support a role for polyamines in plant stress tolerance [1,2].

Polyamine concentrations significantly change in plants stressed by salinity [37, 39], drought [22] or other abiotic factors [11]. It has been suggested that polyamines may undertake complex functions in relation to adaptation to various abiotic and biotic stress factors as a part of an integrated plant response [28].

Polyamines occur as free bases, but they are often conjugated to small molecules like phenolic acids (acid-soluble conjugated forms) or to macromolecules such as proteins and DNA (acid-insoluble bound forms) [11]. In recent years, conjugated and bound forms of polyamines attracted considerable attention in plant stress defense [32]. It was proposed that these conjugated and bound polyamine compounds can take part in polyamine translocation and have important functions in plant morphogenesis, regulation of the free polyamine-titer and interaction with cell wall components under stress conditions [32]. However, participation of conjugated and bound polyamines in the plant response to abiotic stress is still poorly understood.

Changes in polyamine levels, expression of genes and/or activity of polyamine biosynthetic enzymes have been investigated in many plant species under salt stress [8, 24]. However, reports on the salt stress-mediated changes in polyamine titers are contradictory. Induction of arginine decarboxylase and accumulation of Put was reported under salt shock [9]. Under long-term salinization, in contrast, high titers of Spd and/or Spm, but not Put were demonstrated [20]. The Put level declined in *Capsicum annuum* and *Datura stramonium* leaves subjected to osmotic stress, whereas Spd and Spm titers increased [38]. In seedlings of *Brassica campestris* treated with 100 mM NaCl, the Put titer decreased [10] but increased twofold in coleoptiles of sensitive rice cultivars [5]. Fluctuation of polyamine levels clearly vary among individual polyamine components and plant species depending on stress intensity and duration. Due to this contrasting results reported in the literature, the physiological significance of changes in polyamine levels is still a matter of controversy [1, 4].

An important factor in the conflicting results concerning the role of polyamines in the protection of plants against salt stress could be the fact that most studies do not take into account that under salinity plants exhibit a two-phase response, the osmotic and the ionic phase [26]. The relevance of polyamines as protectors against salt stress may be different during both stress phases, especially when ion toxicity causes enhanced senescence. Studies addressing the role of polyamines in plant responses to salinity by comparing varieties or species differing in salt tolerance should use plants suffering from comparable strain, i.e. similar adverse stress effects.

The present work explores the influence of mild and more severe salt stress on polyamine patterns in two contrasting species, tobacco and sugar beet. Sugar beet (*Beta vulgaris* L.) is a natrophilic species with improved growth under mild salinity due to its ability to replace K by Na and its use in osmotic adjustment, water balance and expansion of leaf cells [13, 19]. In contrast, *Nicotiana* is a glycophyte (natrophobic) plant suffering from osmotic stress even under mild salinity. The possible role of polyamines in positive response of natrophilic plants to mild salinity has not been studied. We hypothesize that in natrophilic sugar beet polyamines may play a distinctive role both in the growth improvement under mild salinity and in their higher tolerance to more severe salinity in comparison to glycophyte species.

The aim of this work was to study the effects of low and high salinity on polyamine titers in two species with contrastive response to salinity. Concentrations of NaCl for the high salinity treatment were 50 mM for tobacco and 150 mM for sugar beet, respectively. These concentrations were chosen based on similar growth reduction and absence of senescence symptoms in both species. Additional measurements on growth, gas exchange and ion relations were performed in order better to characterize the degree of stress suffered by the plants.

Materials and Methods

Plant Cultivation and Treatments

Seeds of sugar beet (*Beta vulgaris* L. cv. IC) and tobacco (*Nicotiana rustica* L. cv. Basmas) plants provided by Agricultural Research Center, Tabriz, Iran, were surface-sterilized using sodium-hypochlorite at 5% and germinated in the dark on filter paper soaked with saturated $CaSO_4$ solution. Young seedlings were pre-cultured in 50% Hoagland nutrient solution [15] for three weeks. Thereafter, salinity was applied at 0 (control), low (25 mM NaCl) and high (150 mM NaCl) levels for sugar beet and at 0 (control), low (25 mM NaCl) and high (50 mM NaCl) levels for tobacco plants. Tobacco plants exposed to 75 mM NaCl and higher died 5 days after treatment, therefore, 50 mM NaCl was set as the maximum salinity stress for tobacco in this work.

Plants were grown under controlled environmental conditions with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and at a photon flux density of about 400 μmol m^{-2}s^{-1}.

Determination of Chlorophyll Fluorescence and Gas Exchange Parameters

Chlorophyll fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK). Measurements were carried out on the second youngest, fully expanded and attached leaves of 4 plants per treatment. An average of 4 records from different parts of each individual leaf was considered for each replicates. Leaves were acclimated to dark for 30 min using leaf clips before taking the measurements for dark-adapted leaves. Maximum quantum yield of PSII (F_v/F_m) was calculated using initial (F_0), maximum (F_m) and variable ($F_v=F_m-F_0$) fluorescence parameters. Calculations for excitation capture efficiency of open PSII (F'_v/F'_m) and effective quantum yield of PSII ($\Phi_{PSII}=F'_m-F_t/F'_m$) were made using initial (F_t), maximum (F'_m) and variable fluorescence ($F'_v=F'_m-F_t$) of light adapted leaves [25].

CO_2 assimilation and transpiration rates were measured in parallel for chlorophyll fluorescence measurements in the same leaf with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 A.M. and 13:00 P.M. at harvest. The measurements were conducted with photosynthetically active radiation (PAR) intensity at the leaf surface of 400 μmol m^{-2} s^{-1}. The net photosynthesis rate by unit of leaf area (*A*, μmol CO_2 m^{-2} s^{-1}), transpiration rate (*E*, mmol H_2O m^{-2} s^{-1}) and the stomatal conductance to water vapor (g_s, mol m^{-2} s^{-1}) were calculated using the values of CO_2 and humidity variation inside the chamber, both measured by the infrared gas analyzer of the portable photosynthesis system.

Plant harvest and Analysis

Two weeks after treatment, plants were harvested. For determination of Na, K, and Ca content, oven-dried samples were weighed and ashed in a muffle furnace at 550º C for 8 h, resolved in HCl, and made up to volume by distilled water. Concentrations of Na, K, and Ca were determined by flame photometry (PFP7, Jenway, Dunmow, Essex, UK). For determination of Cl, the dried plant material was treated with 2% K_2CO_3 prior to ashing at 550º C in a muffle furnace for 60 min. The Cl content of the sample was determined by titration with silver nitrate according to the procedure of Johnson and Ulrich [16].

Leaf concentration of Chl a and b was determined in fresh materials after extraction of pigments in cold acetone and allowing the samples to stand for 24 h in the dark at 4 °C [23].

Extraction and Determination of Polyamines

Extraction and determination of polyamines were performed according to the method described by Li and Burritt [21]. Plant tissue was frozen in liquid nitrogen and ground to a fine powder using a chilled mortar and pestle. The frozen powder was homogenized in cold 5% perchloric acid (PCA) (100 mg ml^{-1} PCA), and the homogenate maintained at 4°C for 60 min, then centrifuged for 20 min at 25000 g. Free and PCA-soluble conjugated polyamines were determined in two separate aliquots of the supernatant. A 0.2 ml aliquot of supernatant was hydrolyzed with 0.2 ml of 12 N HCl for 18 h at 110°C in a reaction vial for analysis of PCA-soluble conjugated polyamines. Insoluble bound polyamines were determined after acid hydrolysis of the pellet derived from the centrifugation. The pellet was washed at least three times with 3 ml of 5% PCA. After centrifugation for 15 min at 4000 g, the supernatant was discarded. The pellet was acid hydrolyzed with 1 ml of 6 N HCl for 18 h at 110°C. The hydrolyzate was centrifuged at 4000 g for 6 min and 0.5 ml of the supernatant was diluted to 2 ml with 5% PCA. Concentration of polyamines in all three fractions was determined after dansylation. A 0.1 ml aliquot of extract was added to 0.1 ml of saturated sodium carbonate and 0.2 ml dansyl chloride dissolved in acetone (7.5 mg ml^{-1}). The mixture was incubated at 60°C for 30 min in
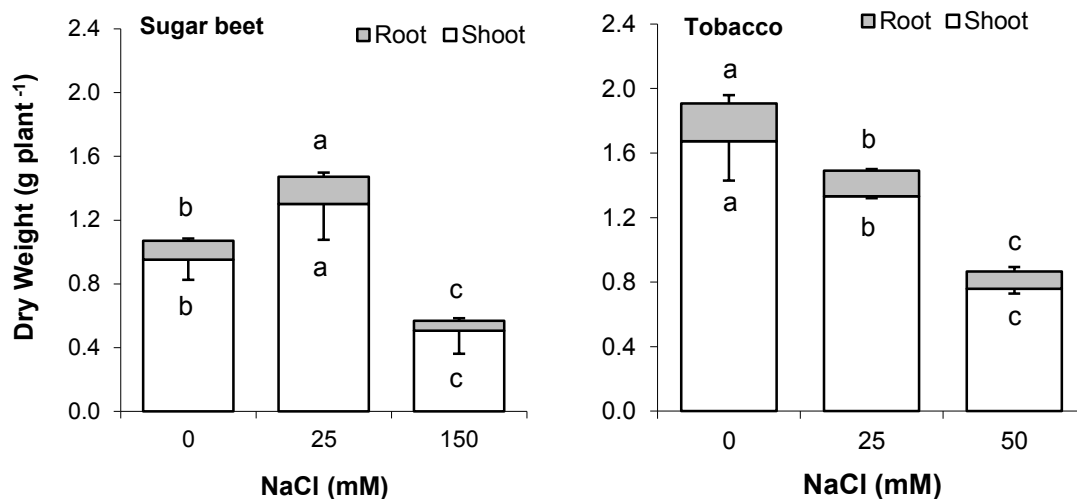
Figure 1. Effect of different levels of salinity on dry weight of shoot and root (g plant^{-1}) in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana rustica* L.) plants. Bars indicated by the same letter are not significantly different (P<0.05).

the dark. Excess dansyl chloride was eliminated with the addition of 0.1 ml of proline (100 mg ml^{-1}), and incubation at room temperature for 15 min in the dark. Dansylated polyamines were extracted with 0.25 ml of benzene by vortexing for 1 min and centrifuging for 1 min at 25 000 g. The benzene phase was removed and the polyamines analyzed by thin layer chromatography. Pre-coated plates of silica gel 60 (Merck) were used and run with ethylacetate:cyclohexane (2:3 v/v) as the eluent. Spots were visualized under UV radiation and those corresponding to Put, Spd, and Spm identified by comparison with dansylated standards. The spots were scraped from the TLC plate, eluted with ethylacetate, and their relative fluorescence measured using a spectrofluorometer (RF-5301PC-Shimadzu, Japan). The soluble conjugated polyamines were estimated as the concentration of polyamines in the hydrolyzate of the supernatant minus that of the free polyamines.

Statistical Analysis

Statistical analysis was performed using Sigma Stat 2.03. Differences between the means were detected using a one-way analysis of variance, in conjunction with the Tukey test (P<0.05).

Results

Effect of Low and High Salinity on Dry Matter Production of Plants

Tobacco and sugar beet clearly differed in their

responses to salinity. While in sugar beet an exposure to 25 mM NaCl increased shoot dry weight (DW) by about 37%, tobacco growth was reduced by up to 20%.

High salinity (150 mM NaCl) reduced shoot DW of sugar beet by 47%. A reduction of 55% of shoot DW was achieved in tobacco by only 50 mM NaCl. Similar trends were observed for root dry weight in both species (Fig. 1).

Effect of Low and High salinity on Chlorophyll Content, Photochemistry and Gas Exchange of Leaves

Low salinity caused a significant increase in Chl a, b and total Chl content of leaves in tobacco, but not in sugar beet. High salinity, in contrast, caused similar reduction of Chl a, b and total Chl in both species. Effects on Chl a and Chl b were similar (Fig. 2).

Photochemical efficiency of PSII (F_v/F_m), excitation capture efficiency of open PSII (F'_v/F'_m) and quantum yield of PSII (Φ_{PSII}) were not affected by salinity in any of the species (Table 1). However, gas exchange parameters were strongly influenced by both salinity levels. In sugar beet, low salinity caused an increase in the rates of net assimilation (*A*) and transpiration (*E*), accompanied by a substantial (91%) rise of stomatal conductance (g_s). Contrastingly, in tobacco low salinity caused a significant reduction of both the net assimilation rate and the stomatal conductance. Transpiration rate was not affected by low salinity in tobacco plants (Table 1).

High salinity, caused reduction in the rates of net assimilation and transpiration, as well as in stomatal
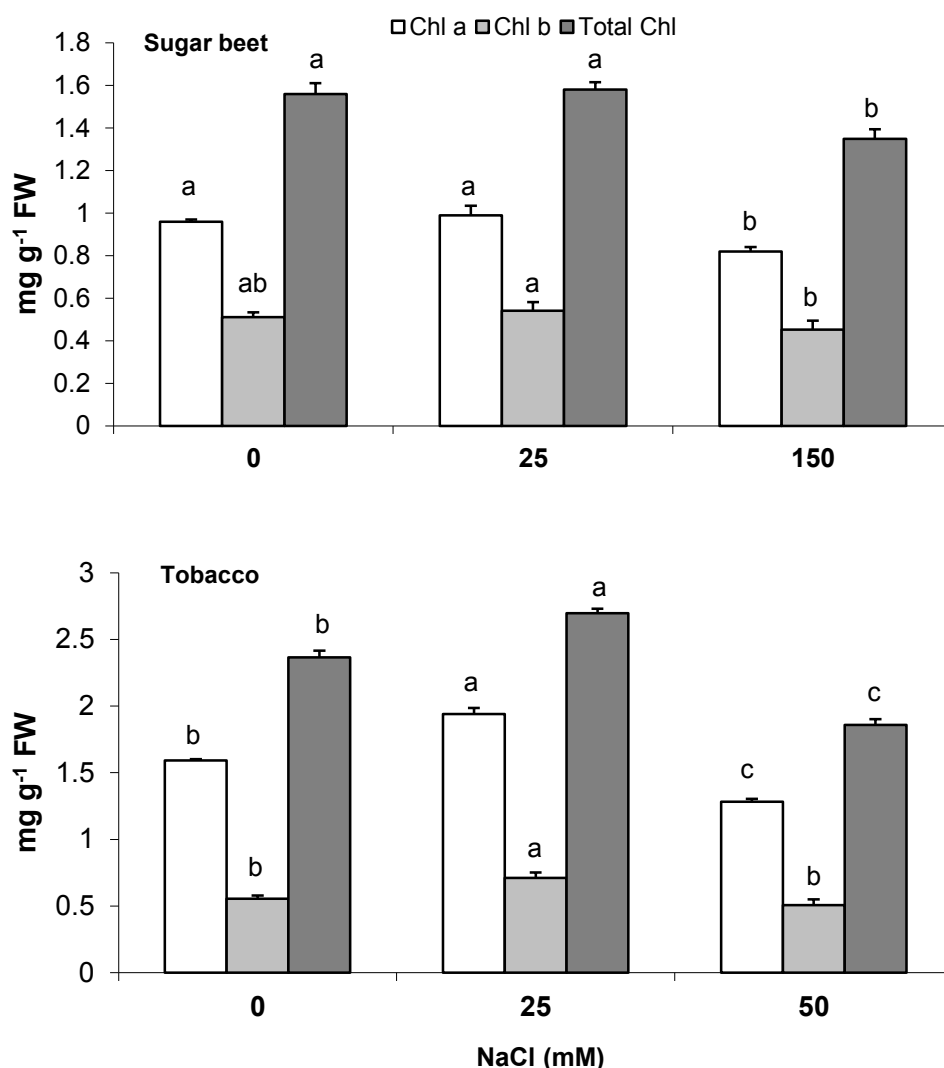
Figure 2. Effect of different levels of salinity on leaf chlorophyll a, b and total chlorophyll content (mg g^{-1} FW) in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana rustica* L.) plants. Bars indicated by the same letter are not significantly different (P<0.05).

conductance of both species. CO_2 assimilation rate was depressed by about 44% in sugar beet under 150 mM NaCl salinity, while this reduction was 57% for tobacco plants exposed to only 50 mM NaCl. Transpiration rate was not affected by high salinity in sugar beet, but decreased up to 49% in tobacco. In both species stomatal conductance was reduced by about 60% under high salinity (Table 1).

Effect of Low and High Salinity on Ion Concentration

As expected, Na concentration increased under saline conditions. In general, Na concentrations in leaves and roots were higher in sugar beet than in

tobacco under both control and saline conditions. Leaf K and Ca concentrations were hardly affected by salt in both species. Contrastingly, salinity treatments caused a significant reduction of root K concentrations. The most intense reduction of root K levels was observed under low salinity in sugar beet (62%) and under higher salinity in tobacco (69%). Under high salinity shoot Ca concentrations increased significantly in sugar beet but not in tobacco. Chlorine concentrations increased in parallel with Na in leaves and roots of both species when exposed to high salinity. Similar to Na, Cl concentrations were higher in sugar beet than in tobacco under both saline and control conditions (Table 2).

Table 1. Effect of different levels of salinity on chlorophyll fluorescence (F_v/F_m: photochemical efficiency of PSII; F'_v/F'_m: excitation capture efficiency of open PSII; Φ_{PSII}: quantum yield of PSII) and gas exchange (A: net photosynthetic rate (μmol m^{-2} s^{-1}); E: transpiration rate (mmol m^{-2} s^{-1}); g_s: stomatal conductance (mol m^{-2} s^{-1})) parameters in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana rustica* L.) plants. Data of each parameter within each species followed by the same letter are not significantly different (P<0.05)

NaCl (mM)	F_v/F_m	F'_v/F'_m	Φ_{PSII}	A	E	g_s
			Sugar beet			
0	0.86±0.03 [a]	0.58±0.04 [a]	0.76±0.01 [a]	7.58±0.09 [b]	3.37±0.81 [b]	1.09±0.11 [b]
25	0.85±0.00 [a]	0.57±0.03 [a]	0.76±0.01 [a]	9.23±0.29 [a]	6.38±0.55 [a]	2.08±0.21 [a]
150	0.84±0.00 [a]	0.56±0.01 [a]	0.76±0.01 [a]	4.23±1.03 [c]	3.85±0.41 [b]	0.39±0.07 [c]
			Tobacco			
0	0.85±0.01 [a]	0.59±0.02 [a]	0.75±0.01 [a]	8.03±0.07 [a]	2.96±0.47 [a]	0.75±0.05 [a]
25	0.84±0.01 [a]	0.58±0.03 [a]	0.76±0.01 [a]	6.18±0.26 [b]	2.64±0.88 [ab]	0.59±0.05 [b]
50	0.84±0.00 [a]	0.58±0.01 [a]	0.75±0.01 [a]	3.48±0.39 [c]	1.52±0.43 [b]	0.29±0.04 [c]

Table 2. Effect of different levels of salinity on the concentration (mg g^{-1} DW) of Na, K, Ca and Cl in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana rustica* L.) plants. Data of each element within each species followed by the same letter are not significantly different (P<0.05)

NaCl (mM)	Shoot				Root			
	Na	K	Ca	Cl	Na	K	Ca	Cl
				Sugar beet				
0	21±6 [c]	287±64 [a]	4±1 [a]	24±4 [b]	26±10 [b]	299±33 [a]	7±1 [b]	124±62 [b]
25	93±17 [b]	221±30 [a]	4±0 [a]	14±4 [b]	37±7 [b]	111±3 [b]	10±2 [ab]	88±26 [b]
150	298±57 [a]	165±84 [a]	5±1 [a]	63±17 [a]	155±48 [a]	124±16 [b]	15±3 [a]	298±99 [a]
				Tobacco				
0	7±0.4 [b]	132±41 [a]	19±3 [a]	13±1 [b]	12±2 [b]	165±30 [a]	8±1 [a]	48±11 [c]
25	36±7 [b]	181±9 [a]	17±1 [a]	17±1 [b]	29±5 [b]	98±6 [b]	9±2 [a]	89±23 [b]
50	152±36 [a]	161±33 [a]	15±3 [a]	31±6 [a]	66±30 [a]	51±21 [c]	10±1 [a]	171±41 [a]

Effect of Low and High Salinity on Polyamine Titers of Leaves and Roots

Putrescine was found in free, bound and conjugated form in shoots and roots of both species. However, only free Spd and Spm were detectable. Considering total concentrations on a fresh weight basis, Put concentrations were strongly increased under both salinity levels. This increase was more prominent in sugar beet than in tobacco. Under low salinity, rise of Put titer reached up to 69% and 55% in shoots and 310% and 145% in roots of sugar beet and tobacco, respectively. However, the responses of the different Put fractions were not uniform. The soluble Put fraction in shoots of sugar beet increased significantly under both low and high salinity up to 33% and 73% respectively. The salinity-induced increase of the conjugated and bound fractions of Put, however, was much more prominent than that of soluble Put. Under high salinity, for instance, conjugated and bound fractions of Put increased up to 3.4 and 3.6 times respectively. In root, bound Put was much more increased under both low and high salinity compared to free and conjugated Put. Put content in the bound fraction increased in root of sugar beet up to 9 and 20 times under low and high salinity conditions, respectively (Table 3).

In tobacco shoots, in contrast, bound Put responded less than free and conjugated Put fractions to salinity.

Table 3. Effect of different levels of salinity on the levels (nmol g^{-1} FW) of free, acid soluble conjugated and acid insoluble bound putresin (Put) and soluble spermidine (Spd) and spermine (Spm) in shoot and root of sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana rustica* L.) plants. Spd and Spm were not found in detectable amounts as conjugated or bound forms in sugar beet and tobacco and Spm in any fraction of root in tobacco plants. Data of each polyamine fractions within each organ and species followed by the same letter are not significantly different (P<0.05)

NaCl (mM)	Put				Spd	Spm
	Free	Conjugated	Bound	Total	Free	Free
	Sugar beet					
	Shoot					
0	158±42 [b]	233±12 [c]	381±69 [c]	771±109 [c]	394±13 [b]	411±8 [b]
25	211±17 [b]	309±14 [b]	780±73 [b]	1300±94 [b]	418±11 [ab]	438±17 [b]
150	274±17 [a]	803±63 [a]	1362±46 [a]	2439±107 [a]	466±43 [a]	510±15 [a]
	Root					
0	143±14 [c]	140±63 [c]	131±22 [c]	413±67 [c]	49±13 [b]	139±17 [b]
25	291±11 [b]	329±61 [b]	1218±169 [b]	1694±462 [b]	82±12 [b]	161±15 [ab]
150	468±50 [a]	472±56 [a]	2603±118 [a]	3543±189 [a]	153±52 [a]	187±2 [a]
	Tobacco					
	Shoot					
0	159±44 [c]	101±15 [c]	453±49 [b]	713±88 [c]	57±11 [c]	154±13 [c]
25	323±43 [b]	201±57 [b]	578±37 [a]	1102±125 [b]	116±21 [b]	182±12 [b]
50	473±40 [a]	367±31 [a]	552±41 [a]	1392±81 [a]	184±46 [a]	204±5 [a]
	Root					
0	148±27 [c]	180±54 [b]	231±27 [c]	559±94 [b]	571±17 [b]	n.d.
25	475±62 [a]	351±20 [a]	544±52 [b]	1370±121 [a]	609±40 [ab]	n.d.
50	377±40 [b]	223±12 [b]	891±25 [a]	1491±61 [a]	651±19 [a]	n.d.

n.d. Not detectable

The salinity-induced increase in free and conjugated Put in tobacco shoot was respectively 46% and 99% for low and 197% and 263% for high salinity. Bound Put only increased a 25% at both low and high salinity levels. In tobacco root, free and bound Put increased considerably under both salinity levels, while this increase was much smaller for conjugated Put under low and, particularly, under high salinity (Table 3).

Spermidine and Spm were detectable only as soluble fraction in shoots and roots of both species. In tobacco, Spm, was not found in roots neither free or in conjugated or bound form. Under low salinity, Spd and Spm concentrations were not significantly affected in sugar beet. In tobacco, Spd concentrations in shoots, but not in roots increased significantly under low salinity. Under high salinity, the rise of leaf Spd titer in tobacco shoots (222%) was much higher than in sugar beet (18%). In roots, however, the opposite was observed. Greater rise in Spd titer was detected in sugar beet (212%) than in tobacco (14%). Changes in the Spm titer due to low and high salinity were similar in both species (Table 3).

Under control conditions no differences in Put levels between the two species were observed. In contrast, constitutive shoot concentrations of Spd and Spm were significantly higher in sugar beet than tobacco. In contrast, tobacco roots exhibited much higher Spm levels than roots of sugar beet (Table 3).

Discussion

Sugar beet and tobacco largely differed in both the external and the internal salt concentrations required for causing a reduction of growth by about 50%. This expected result confirms the large difference in salt tolerance between the glycophytic tobacco and the natrophilic sugar beet. The adverse effects of salt stress on the leaf chlorophyll content and net assimilation rate observed in this work were in agreement with many other reports on various plant species [26, 29]. Under the selected experimental conditions used in this study, salt-induced growth effects were mainly due to the influence of the NaCl treatment on stomatal resistance. Stomatal conductance (g_s) and CO_2 net assimilation rate (A) were enhanced by low salinity in sugar beet. In contrast, the same level of salinity increased stomatal resistance and lowered CO_2 net assimilation rate in tobacco. This different behavior is clearly related to

species differences in the tolerance to osmotic stress, i.e. what is considered the first category of response types to salinity in plants according to Munns and Tester [26]. Possible reasons for growth stimulation by low salinity in sugar beet are osmotic adjustment leading to higher leaf turgor, greater leaf area expansion and higher potential photosynthesizing component on the basis of leaf area or weight, at least during the earlier growth period [13]. It was suggested that, Na can substitute for K not only in vacuolar osmotic adjustment; it appears also to be able to carry out this role for stomata turgor in plant species such as sugar beet [36]. Elevated stomatal conductance under low salinity in sugar beet could be attributed to the replacement of K by Na in guard cells, where Na may act more efficiently than K, resulting in greater osmotic water flow into the guard cells and consequent opening. Influence of salinity-mediated changes in the polyamines content on the leaf pigments and assimilation rate could not be evaluated precisely by our data in this work. In other work, however, we observed a significant ameliorative effect of exogenously applied Put on the leaf chlorophyll content and net assimilation rate of salinized tobacco plants [12]. Similar results have been obtained in cucumber plants [33].

Growth inhibition under high salinity observed in this study can also be attributed mainly to osmotic effects and stomatal limitations of photosynthesis. This is supported by the fact that photochemical parameters remained unaffected in both tobacco and sugar beet despite an about 50% inhibition of growth. Moreover, no senescence symptoms were observed and chlorophyll concentrations were reduced by only 20 and 15% in tobacco and sugar beet, respectively (Figure 1). Senescence symptoms are typically caused by ion specific toxicity in the second phase of response of plants to severe and prolonged salt stress [26]. The lack of salt effects on photochemical parameters in salt sensitive tobacco in this study contrasts with recent observations by others comparing glycophyte *Arabidopsis thaliana* and halophyte *Thelungiella* [35]. In *A. thaliana* salt induced a substantial decrease of electron flow through PSII and an increase of non-photochemical quenching. The difference in response between *Arabidopsis* from Stepien's and Johnson's study and tobacco in the present study are most probably is due to the considerably lower salt concentration used here.

Regardless the salt treatment, tobacco and sugar beet differed in polyamine composition. Sugar beet had high constitutive levels of free Spm in roots and shoots and of free Spd in shoots, while tobacco had higher constitutive Spd levels in roots (Table 3). Whether a

higher proportion of polyamines with a higher number of cations could be important in the tolerance of Na^+ - accumulating halophytes remains obscure. The concentration of these polycations in the range of nM is much lower than the concentration of compatible solutes reported in the order of μM to mM in halophytes. A role for Spm in salt tolerance is supported by observations that spermine synthase deficient mutants of *A. thaliana* are highly salt sensitive and that exogenous supply of Spm has a protective effect [41]. Because of the relatively low tissue concentrations, the role of free polyamine levels in protection of plants against salt stress cannot rely on a direct action as osmotically active compounds or as players in the overall cell cation balance. More feasible is a role for free polyamines in plant responses to abiotic stress through the modulation of ABA biosynthesis and regulation of ion channels [2, 44]. In fact, salt-induced ABA is an important factor in salt–induced inhibition of leaf expansion in glycophytes [34].

In this study, the most conspicuous salt-induced changes in polyamine levels were not observed in the levels of free polyamines, but in the bound fraction of Put (Table 3). In sugar beet the increase of Na^+ concentrations in both roots and shoots was paralleled by a concomitant increase in the bound Put fraction. In tobacco, a glycophyte that restricts Na^+ accumulation in the shoots, such a relation between tissue Na^+ and bound Put fraction was only observed in roots, but not in shoots. Also the salt–induced increase of conjugated Put levels was higher in shoots of sugar beets than in tobacco. This is in line with a distinctive increase in conjugated Put in salt tolerant, but not in salt sensitive rice [27]. A metabolomic study comparing salt-sensitive and salt-tolerant barley varieties has recently questioned a role for Put in salt stress tolerance [40]. According to these authors a rise in putrescine is a sign of salt-induced senescence and not related to tolerance. In contrast to the study presented here, the metabolomic approach only assessed the free, but not the conjugated and bound Put fractions. Also no increase in free Put levels was observed in beet seedlings exposed to salinity [42,43]. These contrasting results reinforce the importance of distinguishing the different fractions of polyamines when investigating their role in plant stress responses.

In our work, the bound Put, as the most abundant fraction of Put, contributed mainly to the rise in the total Put titer under both low and high salinity in sugar beet. The polycationic nature of polyamines facilitates their interaction with nucleic acids and phospolipids and other negatively charged functional groups of membranes and cell wall components and with

enzymatic or structural proteins in the cell [17]. Thus, bound polyamines operate as membrane stabilizers, prevent leakage and protect cells against the toxic effect of salt [4, 7]. Post-translational covalent linkage of polyamines to proteins (bound polyamines) is catalyzed by a class of enzymes known as transglutaminases which can be intra- or extracellular [30]. The rise of bound Put titers that was observed under both low and high salinity in sugar beet suggests that Put contributed to growth promotion and tolerance via a common mechanism. Maintaining cellular structure and membrane integrity is an important aspect in plants response to salinity and may be involved greatly in the mechanisms of plant tolerance to saline conditions.

In the natrophobic tobacco plants, salt-induced changes in the polyamine fractions differed from those in sugar beet. In tobacco leaves, free and conjugated Put responded more than bound Put to salinity. In roots, in contrast, free and bound Put contributed more to the increased total Put titer than did the conjugated Put. Comparison of tobacco and sugar beet in the total Put titer as well as its distribution among different fractions suggests that greater rise in the bound Put titer that was correlated with greater tolerance to salinity is likely one of mechanisms for different salinity response of these species.

On a molar basis, Put was the most abundant polyamine in both studied species and constitutive amounts of Spd and Spm were much lower than that of Put. The lack of Spd and Spm in conjugated and bound forms could be due to the limitation of our analyzing method. However, amounts of Spd and Spm in the range of 50 and 15 nmol g^{-1} FW respectively, were detected in the soluble fraction by the method used here. It could be concluded that, the conjugated and bound Spd and Spm, if present, would have extremely low concentration and likely could not confer considerable salinity tolerance.

In conclusion, during the osmotic phase of salinity stress both the natrophilic, salt tolerant sugar beet and the natrophobic, salt sensitive tobacco respond with increased polyamine levels. However, both species clearly differ in the involved polyamine fractions. Sugar beet has constitutively high free Spm levels and tolerance to increasing Na^+ tissue concentrations under salt stress are related to a substantial increase of the bound Put fraction in this halophyte. Contrastingly, the glycophyte tobacco responds to enhanced shoot Na^+ concentrations with a rise in the levels of free Put and Spd.

There is very limited information on the mechanisms of growth stimulation under mild salinity in natrophilic species. This work presents for the first time some evidences on the role of polyamine titers and fractions in responses of sugar beet as a natrophilic species to mild salinity. In spite of application of the same procedure for fractionation of conjugated, bound and free polyamines, we analyzed polyamines using TLC method instead of more precise HPLC method [31]. We suggest testing the hypothesis of this work and the effect of mild salinity in other natrophilic species using HPLC.

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