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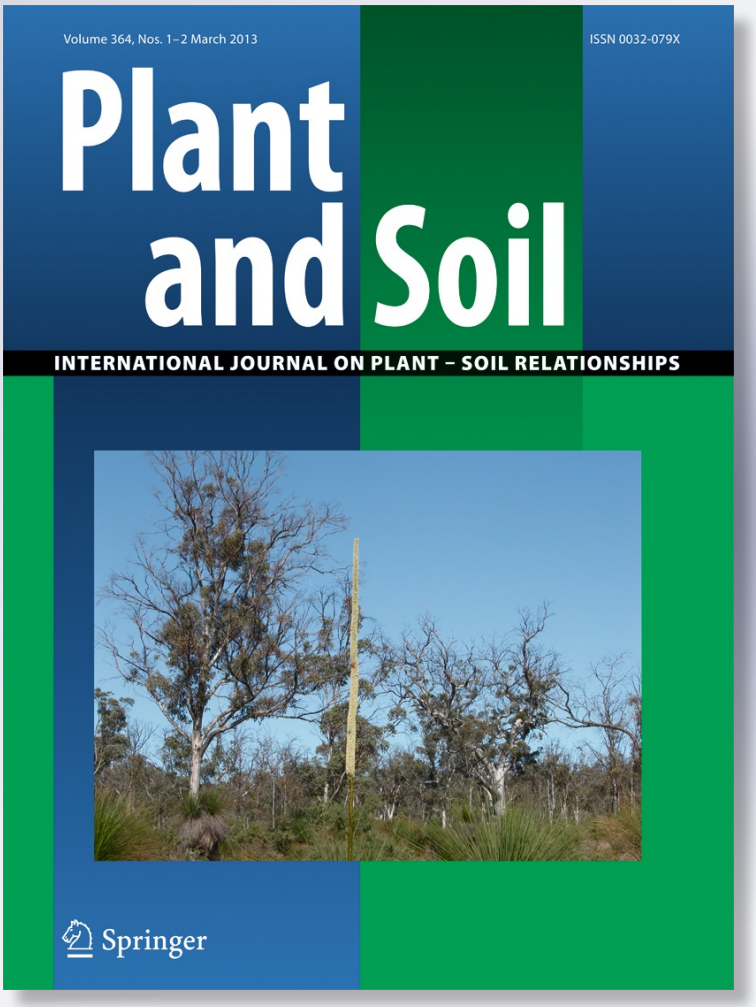

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REGULAR ARTICLE

Modulatory effects of *Mesorhizobium tianshanense* and *Glomus intraradices* on plant proline and polyamine levels during early plant response of *Lotus tenuis* to salinity

Mariela Echeverria · Analía Inés Sannazzaro · Oscar Adolfo Ruiz · Ana Bernardina Menéndez

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Abstract
Aims The study aims (1) to evaluate the effect of *Mesorhizobium tianshanense* on plant proline and polyamine levels of *Lotus tenuis* and its modulatory effect during plant response to short-term salt stress and (2) to compare these effects with those caused by mycorrhizal symbiosis.

Methods Experiments consisted of a randomized factorial design of two factors: salinity (two levels, 0 and 150 mM NaCl) and symbiosis (three levels, uninoculated, *Glomus intraradices*, and *M. tianshanense*).
Results Salinization led to increased proline levels regardless of plant organ and symbiotic status, excepting mycorrhizal *L. tenuis* roots. Salinity diminished the total polyamine level of control and rhizobial plants but not in mycorrhizal ones. Variations in the pattern response of the three individual polyamines (putrescine, spermidine, and spermine) differed in accordance with the symbiotic status of the plant, highlighting a divergence on proline and polyamine metabolisms between rhizobial and mycorrhizal symbiosis.
Conclusions Spermidine and spermine contributed the most with the salt-induced root polyamine increment observed upon salinization in roots of nodulated plants, suggesting that these polyamines might mediate an adaptive role of the plant–*M. tianshanense* symbiosis in *L. tenuis* plants growing in a saline environment.

Keywords *Mesorhizobium tianshanense* · *Lotus tenuis* · Proline · Polyamines · *Glomus intraradices*

Abbreviations

DAO	Diamine oxidase
PA	Polyamines
Put	Putrescine
Spd	Spermidine
Spm	Spermine

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Introduction

The amino acid proline is one of the compatible osmolytes that most commonly build up in the cytoplasm to prevent the osmotic imbalance resulting from the excessive accumulation of toxic ions within the vacuole (Hasegawa et al. 2000). On other hand, many authors have reported that polyamine (PA) accumulation is the immediate response to salinity in different crop plants species (Erdei et al. 1996; Chattopadhyay et al. 2002; Ghosh et al. 2011). Polyamines are aliphatic molecules of low molecular weight, absolutely required for eukaryotic cell growth. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the most common PA in plants (Cohen 1998). These compounds are thought to play an important role during the interaction between plants and root symbionts (Kytöviita and Sarjala 1997; Sannazzaro et al. 2007) and in plant responses to diverse environmental stresses (Groppa and Benavides 2008). A number of reports have shed some light about the physiological role of increased PA in salinized plants. These include maintaining cellular pH and ion balance, stabilizing membranes, and functioning as nitrogen reserve to be used after stress relief by the plant (reviewed by Mansour 2000). In addition, a number of studies have revealed a correlation between PA and proline contents in plants due to a close relationship between PA and proline metabolism (Smith 1985; Aziz et al. 1998; Santa-Cruz et al. 1999; Gaspar et al. 2000; Zhao et al. 2001; Theiss et al. 2002; Tonon et al. 2004; Sotiropoulos et al. 2007; Su and Bai 2008). Most legumes have the ability to establish mutualistic symbiotic relationships with soil N-fixing bacteria (collectively known as rhizobia) and arbuscular mycorrhizal fungi. However, symbiosis effects on plant proline and PA levels under salinity has received little attention. Diouf et al. (2005) found that, in two *Acacia* species grown under salt stress, leaf proline accumulation was lower in co-inoculated plants than in rhizobial- or mycorrhizal-inoculated ones, suggesting that co-inoculation provides the plant with improved tolerance to salt stress. Similar results were observed in *Acacia saligna* (Soliman et al. 2012). On other hand, variable effects of arbuscular mycorrhizal (AM) fungi on proline levels of plants grown under salt stress have been observed. Proline accumulation was greater in mycorrhized moong plants at 12.5 and 25 mM NaCl at 40 and 62 days after sowing (Jindal et al. 1993), whereas non-AM faba bean plants accumulated much more proline than AM plants at various salinity ranges (Rabie and Almadini 2005).

Lotus tenuis (Waldst. and Kit., syn. *Lotus glaber*; Kirkbride 2006) is a salt-tolerant glycophyte that became the best adapted legume forage in saline soils of the Flooding Pampa (the most important cattle production region in Argentina). In addition, *L. tenuis* also has the ability to establish mutualistic symbiotic relationships with diazotrophic, salt-tolerant bacteria belonging to the *Mesorhizobium* genus (Estrella et al. 2009). *L. tenuis* also associates with the arbuscular mycorrhizal fungus *Glomus intraradices* (Schenck and Smith) (Sannazzaro et al. 2004). In 2007, Sannazzaro and colleagues provided evidence supporting the idea that the regulation of plant PA levels might be a mechanism whereby the arbuscular fungus *G. intraradices* increased the tolerance of a *L. tenuis* salt-sensitive genotype to a long-term NaCl (200 mM) exposition.

In most of the studies, which have addressed the effect of biotic or abiotic factors on growth regulator balance, measurements were performed when these factors have already caused obvious changes on plant growth. However, variations in levels of growth factors might be attributable to developmental differences instead of evidence of changes induced by the studied factors themselves (Shaul-Keinan et al. 2002).

The aims of this work were: (1) to evaluate the effect of rhizobial symbiosis on plant proline and PA levels and its modulatory effect during plant response to short-term salt-stress and (2) to compare these results with effects caused by mycorrhizal symbiosis.

In plants, several physiological parameters were found to be size-dependent. For example, in *Pisum sativum* L., Spd accumulation was often associated with growing tissue activity and organogenesis, whereas higher Spd/Put ratios were related to rapid growth (Perez-Amador et al. 1995). Inversely, in a study on flexibility in growth and polyamine composition of the crucifer *Pringlea antiscorbutica* (Hennion et al. 2006), smaller plants showed the highest Spd contents and high Spd/Put ratios were more characteristic of smaller plants. Size dependency of abscisic acid and sensitivity to auxin were also observed respectively in vascular epiphytes (Zotz et al. 2001) and barley seedlings (Liptay and Davidson 1971). Besides plant regulation, other physiological parameters were found to be size-dependent, such as photosynthetic

capacity (Zotz 1997; Zotz and Ziegler 1999), stomatal regulation (Schmidt and Zotz 2001), whole-plant water relations (Schmidt and Zotz 2001), and nutrient allocation to reproduction (Zotz 2000). Therefore, to reduce eventual overlapping effects of salinity itself and those derived from differences in plant growth, we measured biochemical parameters in a phenological state where significant salt-induced morphometric changes had not become evident yet, following a similar approach to that adopted for hormonal balances by Shaul-Keinan et al. (2002).

Materials and methods

Experiments consisted of a randomized factorial design of two factors: salinity (two levels, 0 and 150 mM NaCl) and symbiosis (three levels, uninoculated, *G. intraradices*, and *Mesorhizobium tianshanense*). Five plants were grown in each Leonard jar, and there were four jars per treatment. Experiment was repeated once.

Biological material and growth conditions

Seeds of *L. tenuis* cv. INTA Pampa were scarified with sulfuric acid (95–98%), washed in distilled water, and sown in Petri plates containing water–agar (0.8%). Plates were incubated during 10 days in a growth chamber, with a 16/8 h photoperiod at 24°C/19°C (day/night) and 60/80±5% of relative humidity. Light intensity (200 μmol m^{-2} s^{-1}) was provided by daylight and Grolux fluorescent lamps (F 40 W). Seedlings were transferred to sterilized 250-ml Leonard jars (Sigma) containing a 1:1 (*v/v*) perlite/vermiculite mixture as substrate and half-strength (0.5×) Hoagland solution (Hoagland and Arnon 1950), provided by capillarity. Phosphorus and nitrogen contents in the nutrient solution were as follows: uninoculated (C) and mycorrhizal (M) plants: Hoagland 0.5× (15.5 ppm P=KH_2PO_4 0.5 mM and 210.1 ppm N ($Ca(NO_3)_2$ 5 mM+KNO_3 5 mM); nodulated (R) plants: Hoagland 0.5× (31 ppm P=KH_2PO_4 1 mM and 105.05 ppm N ($Ca(NO_3)_2$ 2.5 mM+KNO_3 2.5 mM). For saline treatments, these solutions were supplemented with 150 mM NaCl. Nutrient solutions were renewed every time it fell below 10% of the total Leonard jar volume.

G. intraradices (BAFC 3108) was multiplied in 1,000 ml pot cultures with soil/perlite mixture (1:3 *v/v*) and *Sorghum halepense* (L.) Pers. (=*Andropogon halepensis* Brot.) as host during 4 months. Fungal inoculum consisted of 1 g of root fragments with no less than 70% of their root length colonized by the fungus. For rhizobial inoculum, the *M. tianshanense* strain (BA151), isolated from a saline soil at the Flooding Pampa (Estrella et al. 2009), was grown in liquid TY medium (Beringer 1974) at 28°C. The efficiency of this strain as a *L. tenuis* growth promoter had been previously demonstrated (Estrella et al. 2009; Sannazzaro et al. 2011). Inoculum consisted of 1 ml of the bacterial suspension (DO=10^9 CFU/ml), added to the crown of each seedling. The *G. intraradices* inoculum was incorporated during seedling transference to Leonard jars, whereas *M. tianshanense* was inoculated 1 day after seedlings were transplanted. Uninoculated controls received an equal amount of autoclaved inoculum. The saline stress was applied 30 days after transplanting, when at least 50% of the root was colonized by *G. intraradices* and plants inoculated with *M. tianshanense* were nodulated.

Plants were harvested 5 days after the saline treatment was initiated. Harvested plants were divided in stems, leaf, and roots. One part of the plant material was used for dry weight measurement and the rest for biochemical determinations. For morphometric determinations, one plant from each jar was used, so that each plant was a replicate. For analytical determinations, the remaining four plants in each jar were pooled, with each pool treated as one replicate. The presence of *G. intraradices* was determined visually by clearing and staining the roots with Trypan blue in lactophenol (Phillips and Hayman 1970). Percentage of mycorrhizal root colonization was estimated according to McGonigle et al. (1990).

Proline was estimated spectrophotometrically by the ninhydrin reaction under conditions described elsewhere (Maiale et al. 2004). Briefly, proline was extracted by boiling plant material (500 mg) in 2 ml distilled water. Then, 500 μl of Na-citrate buffer (0.2 mol/l, pH 4.6) and 2 ml of 1% ninhydrin (acetic acid/water 60:40) were added to 500 μl extract. The mixture was boiled for 1 h, extracted with 2 ml toluene, and then centrifuged. Organic phase was read at 520 nm. Proline standard (Sigma, USA) was treated in the same way as the plant extracts.

Free polyamines were estimated by analyzing dansyl-derivatives by reversed-phase high-performance liquid chromatography (HPLC) as described previously

(Marcé et al. 1995). To extract free PA, pools of 300 mg of plant material were frozen in liquid N_2 and homogenized. The homogenate (300 mg) was resuspended in 1 ml of perchloric acid 5% (*v/v*), incubated on ice during 30 min and centrifuged at 15,000×*g* for 15 min. Pellet was discarded and the supernatant kept at −20°C (solution C). Aliquots (200 μl) of free PA were derivatized with dansyl chloride and determined by HPLC according to Jiménez-Bremont et al. (2007). For the dansylation reaction, 200 μl of solution C (see above) was added to 10 μl of 0.1 mM heptanodiamine (internal standard, ICN) plus 200 μl saturated Na_2CO_3 and 400 μl dansyl chloride–acetone 1% (*w/v*). After 16 h at 25°C in the dark, 100 μl of proline 100% (*w/v*) was added to stop the reaction and the dansyl-derived PA extracted with 500 μl toluene. Then, the organic phase (400 μl) was evaporated under vacuum and resuspended in 400 μl acetonitrile. PA was separated by HPLC (ISCO 2350, ISCO Inc., Lincoln, NE) with a reverse-phase column Sephasil C18 (Amersham Pharmacia) and detected with a spectrofluorometer (Variant Fluorichrom). The solvent mix was obtained with a gradient programmer ISCO 2360, flow 1.5 ml/min as follows—0–4.5 min, acetonitrile–H_2O 70:30; 4.5–9 min, acetonitrile 100; 9–15 min, acetonitrile–H_2O (70:30). Peak areas were integrated, normalized to heptanodiamine, and then interpolated into a PA standard calibration curve.

Statistical analysis

Data was subjected to two-way analysis of variance using the symbiosis, salt, symbiosis–salt interaction, and error as variation sources and means compared by Duncan's test. The Pearson's coefficient was used to attest for correlation between the increased percentages of proline and Put due to salt addition.

Results

No differences in total, shoot, and root dry weights were observed due to salt stress after 5 days of salinization (Tables 1 and 2). However, the root dry weight was influenced by a salinity×symbiosis interaction. Under the saline condition, nodulated roots were heavier than those of the corresponding control. In contrast, profound changes in proline and PA contents were observed due to the salinity×symbiosis interaction (Table 1).

Proline content

Salinization led to increased proline levels regardless of plant organ and symbiotic status, excepting roots of *G. intraradices*-inoculated plants (Table 3). Under the salt stress condition, symbiotic plants presented significantly lower proline contents than control ones. In the absence of NaCl, the symbiotic status did not largely affect the total proline level of plants. However, the root proline level of unsalinized plants was raised by the bacterial symbiosis, whereas both symbioses induced a reduction in the stem proline content.

Free PA content

Salinity and symbiosis factors interacted on PA levels (Table 1). Salinity diminished the total PA level of the plant in control and rhizobial treatments but not in the mycorrhizal one (Fig. 1). *M. tianshanense* led to a 30% higher total PA content, regardless the saline condition, whereas *G. intraradices* reduced this level in the absence of salt.

Root PA level decreased due to salt addition in non-symbiotic and mycorrhizal plants (Fig. 1). Despite this diminution, mycorrhizal salt-stressed plants showed a higher root PA level than their corresponding non-mycorrhizal control. Contrarily, root PA level of nodulated plants was increased by salinity.

Salinity diminished the stem PA content in control and nodulated plants but not in mycorrhizal ones (Fig. 1). In the leaf, salinity increased the PA level in non-inoculated and mycorrhizal plants and decreased it in nodulated ones. Under the non-saline condition, mycorrhizal colonization lowered, whereas the rhizobacteria elevated stem and leaf PA contents. On other hand, root PA contents of unsalinized plants were not affected by AM inoculation, but they were slightly raised by the bacterial symbiont (Fig. 1).

Salt effect differed according to the type of polyamine and the symbiotic status. Upon salinization, total Put decreased in control and rhizobial plants, but it remained unchanged in the AM symbiosis treatment (Fig. 2). In control plants (although not in rhizobial ones), the percentage of total Put reduction correlated with that of total proline increment, induced by salt stress (r=0.81; P<0.0001). Conversely, total Spm was incremented by salt treatment in all cases, whereas total Spd was slightly higher in mycorrhizal

Table 1 Significance of two-way ANOVA for the effect of symbiosis and salinity factors on all dependent variables measured on *L. tenuis*

	Salinity		Symbiosis		Salinity × symbiosis	
	F	*P*	*F*	*P*	*F*	*P*
Root dry weight	5.383	0.039	13.869	<0.001	4.442	0.036
Shoot dry weight	2.419	0.142	4.935	0.024	2.324	0.134
Total dry weight	2.124	0.167	5.462	0.018	2.389	0.128
Root proline	560.668	<0.001	164.133	<0.001	153.860	<0.001
Stem proline	2292.178	<0.001	706.225	<0.001	441.467	<0.001
Leaf proline	1191.140	<0.001	263.702	<0.001	342.794	<0.001
Total proline	3551.878	<0.001	867.044	<0.001	807.300	<0.001
Total polyamines	75.445	<0.001	568.444	<0.001	26.732	<0.001
Root polyamines	17.156	<0.001	307.314	<0.001	149.998	<0.001
Stem polyamines	88.066	<0.001	380.131	<0.001	30.835	<0.001
Leaf polyamines	0.0375	0.848	597.988	<0.001	73.365	<0.001
Total putrescine	258.295	<0.001	247.205	<0.001	42.366	<0.001
Total spermidine	47.693	<0.001	1026.270	<0.001	3.329	0.056
Total spermine	330.761	<0.001	385.683	<0.001	9.019	0.002
Root putrescine	172.000	<0.001	4.036	0.035	13.190	<0.001
Root spermidine	14.756	0.001	239.387	<0.001	81.555	<0.001
Root spermine	118.190	<0.001	13.116	<0.001	17.634	<0.001
Stem putrescine	126.772	<0.001	317.637	<0.001	74.433	<0.001
Stem spermidine	0.844	0.370	240.297	<0.001	8.623	0.002
Stem spermine	115.518	<0.001	140.688	<0.001	5.042	0.018
Leaf putrescine	194.094	<0.001	303.163	<0.001	117.220	<0.001
Leaf spermidine	204.445	<0.001	427.756	<0.001	28.441	<0.001
Leaf spermine	267.017	<0.001	282.867	<0.001	29.455	<0.001

Table 2 Root, shoot, and total dry weights (milligrams) of *L. tenuis* plants

Treatment	Root	Shoot	Total
C−	68.36±6.53a,b,	120.65±27.64a,b	189.55±36.37a,b
C+	41.94±8.16b	102.24±12.31a,b	144.18±20.34a,b
M−	67.69±12.06a,b	173.12±35.46a,b	241.77±51.72a,b
M+	30.99±6.64b	88.43±13.69b	120.19±26.36b
R−	85.60±7.44a	179.91±15.18a,b	265.51±22.44a,b
R+	98.64±10.55a	198.64±18.90a	297.28±29.45a

Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days

Means (±SE; n=4) with the same letter within each column era not significantly different (Duncan, P<0.05)

C uninoculated, *M* mycorrhizal, ***R*** rhizobial-inoculated plants, *plus sign* salinized, *minus sign* unsalinized plants

and control treatments, and it did not vary in nodulated plants (Fig. 2). Under the non-saline condition, AM plants diminished total Put level and presented similar Spd and Spm levels, whereas nodulated plants had higher total Put, Spd, and Spm contents compared with uninoculated ones (Fig. 2).

In the root, salinity reduced the Put content in all treatments (Fig. 3), whereas symbiosis did not affect this level under the non-saline condition. Salinity significantly increased root Spd levels in nodulated plants and root Spm levels in all treatments. In contrast, root Spd was diminished or remained invariable upon salinization in control and AM plants, respectively (Fig. 3). On the other hand, AM colonization did not affect root Spd and Spm levels of unsalinized plants, whereas inoculation with *M. tianshanense* incremented the root Spd content.

Table 3 Root, stem, leaf, and total proline contents (nanomoles per gram FW) of *L. tenuis* plants

Treatment	Root	Stem	Leaf	Total
C-	52.82±1.02d	105.56±3.78d	46.02±1.85c	204.03±4.17d,e
C+	291.52±12.21a	477.28±5.30a	392.23±11.93a	1161. 04±9.31a
M-	51.12±1.66d	61.04±1.35e	59.84±3.07c	171.99±4.76e
M+	76.36±0.87c,d	139.18±6.89c	149.55±4.97b	365. 09±6.19c
R-	92.37±3.71c	97.12±2.99d	64.95±3.16c	239.05±17.48d
R+	192.19±7.61b	249.64±7.59b	127.04±2.46b	568.88±12.07b

Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days

Means (±SE; $n=4$) with the same letter within each column are not significantly different (Duncan, $P<0.05$)

C uninoculated, *M* mycorrhizal, *R* rhizobial-inoculated plants, *plus sign:* salinized, *minus sign* unsalinized plants

In general, the patterns of PA changes due to salinity and symbiosis in stem (Fig. 4) and leaf (Fig. 5) were similar. Stem and leaf Put contents were negatively affected by salinity in control and nodulated plants. Notwithstanding this, stem and leaf Put levels in salt-treated nodulated plants were significantly higher than those observed in the corresponding uninoculated control. In AM plants, contrarily, these contents remained unaffected by salinity. Under the non-saline condition, the Put content of these organs tended to decrease and increase in mycorrhizal and nodulated plants, respectively. On other hand, salinity had no effect on the Spd level in stems (Fig. 4), but the leaf Spd level increased due to salt addition in control and AM plants (Fig. 5). In contrast, no salinity-derived change of the leaf Spd content was registered in nodulated plants. In the leaf, the pattern of Spm variations due to salinity was similar to that of Spd (Fig. 5), whereas stem Spm was raised by salt treatment, regardless of the symbiotic status (Fig. 4).

In the absence of NaCl, mycorrhizal colonization did not affect stem and leaf Spd levels (Figs. 4 and 5) but reduced the leaf Spm content (Fig. 5). In contrast, all these contents were incremented by *M. tianshanense* (Figs. 4 and 5).

Discussion

There are a number of studies supporting the view that proline accumulation in response to salt stress is a good indicator of a higher stress perception (Tal et al. 1979; Colmer et al. 1995; Vaidyanathan et al. 2003; Maiale et al. 2004; Sannazzaro et al. 2007). On this basis, our results showing variable salt-derived increases of proline accumulation among treatments (Table 3) would indicate that *L. tenuis* plants experienced different degrees of salt stress according to their symbiotic status. The fact that, under the salt stress condition, symbiotic plants presented significantly lower proline contents than control ones constitutes a hint that the last were more stressed than the former, mycorrhizal plants being the less affected ones.

Salt-derived accumulations in the proline level were formerly observed upon salinization in plants of *Lotus corniculatus*, a species phylogenetically closely related to *L. tenuis* (Márquez et al. 2005). Such similarity in the proline level response to an osmotic factor between both *Lotus* species may be linked to their common evolution in dry and saline soil environments (Allan et al. 2004).

Inoculation with *M. tianshanense* led to a 75% increase in the root proline level (Table 3), in line with findings showing that this amino acid is essential for the plant–rhizobia symbiosis and inherent to the nodulation process itself (Kohl et al. 1988; Jiménez-Zurdo et al. 1997; King et al. 2000). On the contrary, no effect by AM colonization was registered on the root proline level of unsalinized plants (Table 3), in coincidence with previous observations in *L. tenuis* genotypes (Sannazzaro et al. 2007).

Upon salinization, total PA reductions were registered in uninoculated and nodulated plants, although not in AM ones. Such reductions were primarily due to a strong depressing effect of salinity on Put contents (Figs. 2, 3, 4, and 5). In uninoculated *L. tenuis* plants, salt-induced Put diminution correlated with a proline rise, in agreement with previous observations in other uninoculated plant species subjected to saline stress

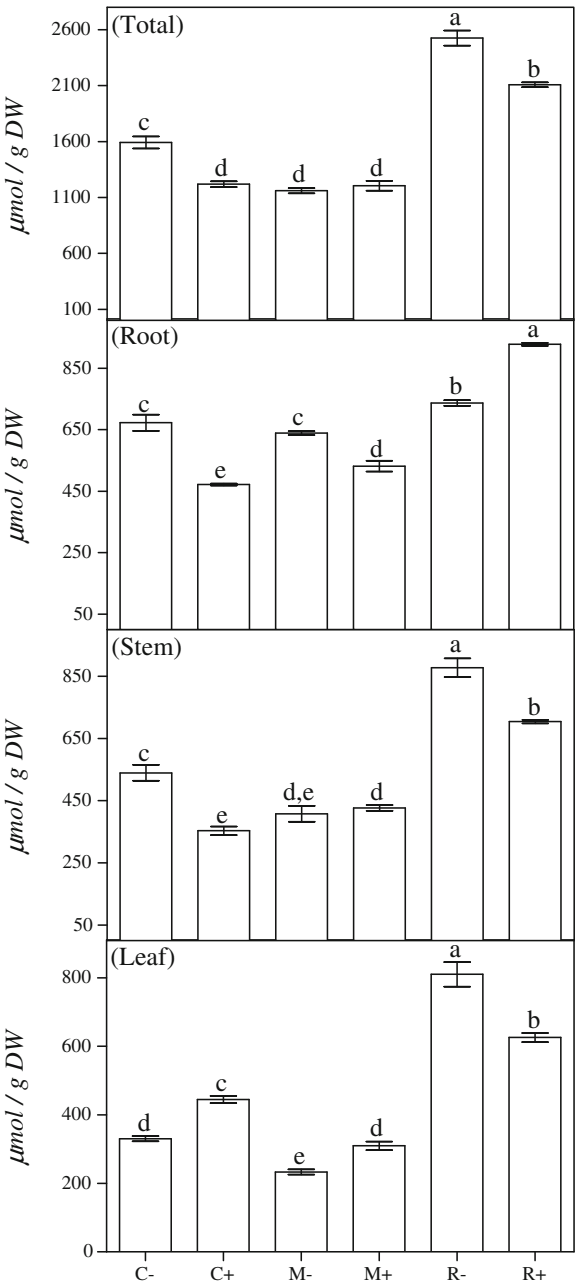

Fig. 1 Total, root, stem, and leaf free polyamine contents of *L. tenuis* plants. Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days. *C*: uninoculated, *M*: mycorrhizal, and *R*: rhizobial-inoculated plants, *plus sign*: salinized, and *minus sign:* unsalinized plants. Means (±SE; $n=4$) with the *same letter* within each column are not significantly different (Duncan, $P<0.05$)

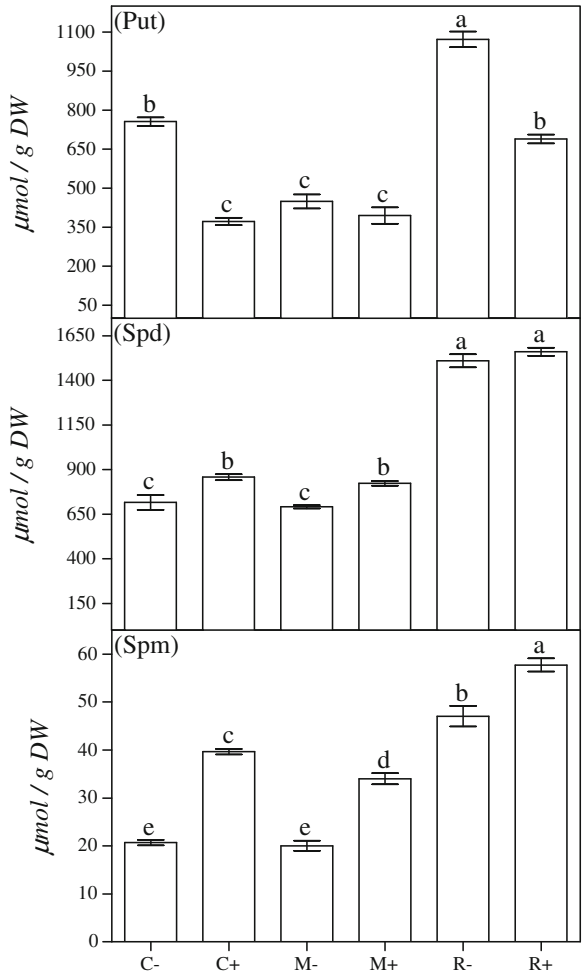

Fig. 2 Total Put, Spd, and Spm contents of *L. tenuis* plants. Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days. *C*: uninoculated, *M*: mycorrhizal, and *R*: rhizobial-inoculated plants, *plus sign:* salinized, and *minus sign:* unsalinized plants. Means (±SE; $n=4$) with the *same letter* within each column are not significantly different (Duncan, $P<0.05$)

(Aziz et al. 1998; Tonon et al. 2004; Sotiropoulos et al. 2007; Su and Bai 2008). These results find support in the fact that salinity promotes diamine oxidase (Smith 1985; Aziz et al. 1998; Su and Bai 2008), whose catalytic activity on Put may contribute to proline accumulation (Bouchereau et al. 1999; Gaspar et al. 2000). On other hand, correlations between rises in proline and declines in Put may also be registered through conversion of glutamate (a common proline and Put precursor) to proline (Santa-Cruz et al. 1999; Gaspar et al. 2000; Zhao et al. 2001; Tonon et al. 2004). This conversion was also reported to be induced under salt stress in several plant species like cashews (da Rocha et al. 2012), *Saussurea amara* (Zhang et al. 2011), and tobacco (Wang et al. 2011).

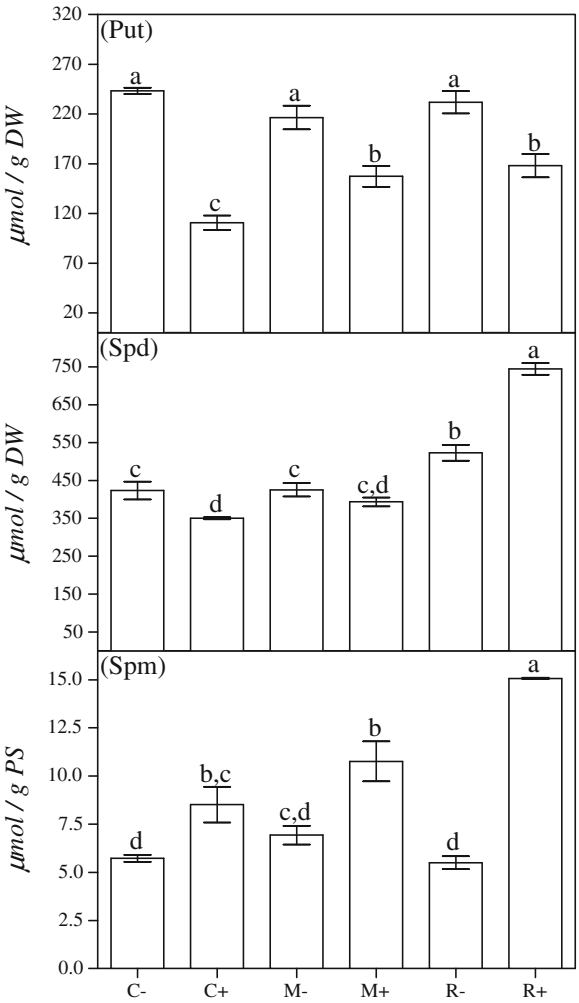

Fig. 3 Root Put, Spd, and Spm contents of *L. tenuis* plants. Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days. *C*: uninoculated, *M*: mycorrhizal, and *R*: rhizobial-inoculated plants, *plus sign:* salinized, and *minus sign:* unsalinized plants. Means (±SE; $n=4$) with the *same letter* within each column are not significantly different (Duncan, $P<0.05$)

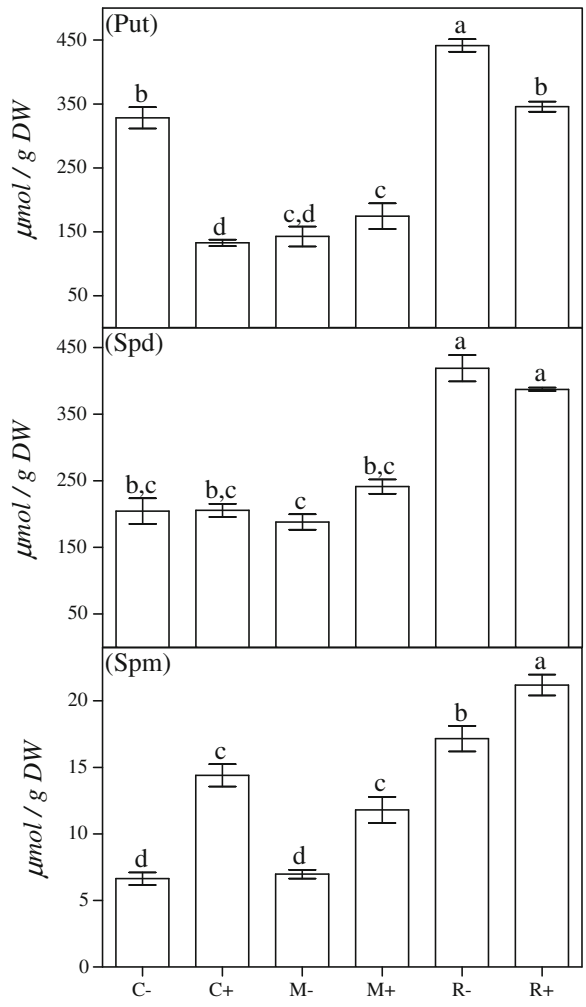

Fig. 4 Stem Put, Spd, and Spm contents of *L. tenuis* plants. Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days. *C*: uninoculated, *M*: mycorrhizal, and *R*: rhizobial-inoculated plants, *plus sign:* salinized, and *minus sign:* unsalinized plants. Means (±SE; $n=4$) with the *same letter* within each column are not significantly different (Duncan, $P<0.05$)

Interestingly, no correlation between salt-induced Put diminution and proline rise was registered in nodulated *L. tenuis* plants, what leads to suggest that the presence of *M. tianshanense* in the root may influence the interconnection of these two metabolic pathways. To date, there is very little information available about the effect of root symbionts on both proline and PA metabolism, two shared pathways, and the investment of further research efforts in this area might provide valuable information to be used for the improvement of legume tolerance to soil salinity.

Our results also showed that, in control and nodulated *L. tenuis* plants, salt-induced reduction of Put levels took place regardless the plant organ, whereas the stress affected Spm and Spm levels in an organ-dependent manner (e.g., Spd, Figs. 3 and 5). Organ-specific differences in the PA metabolism response to salinity have been formerly described in other plant species (Legocka and Kluk 2005; Stetsenko et al. 2009; Radyukina et al. 2009). Also, our results showing that shoots of rhizobial plants presented higher contents of free PA, compared with those of control and mycorrhizal ones, are in agreement with previous

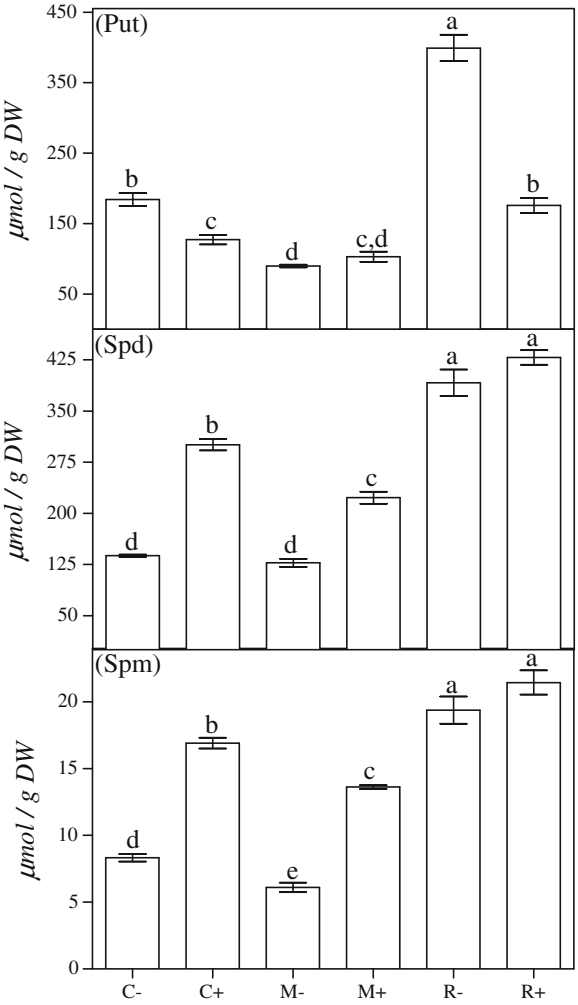

Fig. 5 Leaf Put, Spd, and Spm contents of *L. tenuis* plants. Nutrient solution containing 150 mM NaCl was applied to 40-day-old plants for 5 days. *C*: uninoculated, *M*: mycorrhizal, and *R*: rhizobial-inoculated plants, *plus sign:* salinized, and *minus sign:* unsalinized plants. Means (±SE; n=4) with the *same letter* within each column are not significantly different (Duncan, $P<0.05$)

reports in alfalfa inoculated with *Rhizobium meliloti* and *Glomus fasciculatum* (Goicoechea et al. 1998) remarking a divergence between both types of symbiosis and also, the peculiarities of their PA metabolism. In this regard, the Spd augmentation observed in roots of nodulated plants is in line with the salt-induced over-expression, in a *Sinorhizobium meliloti* strain, of the carboxynospermidine decarboxylase, enzyme playing an important role in the Spd biosynthesis (Shamseldin et al. 2006). In turn, the Spm increase in nodulated, salt-stressed *L. tenuis* plants could be explained by the rise of its precursor, Spd (Fig. 3).

Besides the mechanisms mentioned in the "Introduction" by which root PA would intervene in the salt-tolerance process by plants, a relationship between polyamine catabolism mediated by amine oxidases and cell growth has been recently proposed. On one hand, it has been reported that apoplastic reactive oxygen species (ROS) promote cellular elongation in maize coleoptile (Schopfer et al. 2001) and *A. thaliana* root (Foreman et al. 2003). On the other hand, the enzyme polyamine oxidase (PAO) oxidizes Spd and Spm on their secondary amino groups (Federico and Angelini 1991) producing hydrogen peroxide (H_2O_2) among other products. In maize, higher free PA levels were observed in segments of salt-treated plants, compared with the untreated control (Rodríguez et al. 2009). In these plants, PAO activity provided the amount of ROS in the apoplast needed to sustain the elongation of leaf blades, showing a relationship between Spd and Spm, and cell growth under salinity. A similar relationship was demonstrated in soybean hypocotyls (Campestre et al. 2011). In the present work, *L. tenuis* plants were harvested before salt-derived differences in growth became obvious. Future research should address the occurrence of possible links between combined proline and PA metabolisms, and *L. tenuis* plant growth.

Finally, it has been suggested that the salt-induced metabolic alteration of homospermidine (a Spd analog) is closely related to the salt stress tolerance of fast-growing rhizobia (Fujihara 2009). All this information, along with the fact that accumulated proline may be used as a nitrogen source or as osmoticum by plants (Stewart and Larher 1980; Trotel et al. 1996), offers the notion that these polyamines might mediate an adaptive role of the plant–*M. tianshanense* symbiosis in *L. tenuis* plants growing in a saline environment. However, the mechanisms that govern Spm and Spd (or homoSpd)-mediated salt resistance in bacteria remain unclear (Fujihara 2009).

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