

Effect of exogenously applied spermidine on growth and physiology of citrus rootstock Troyer citrange under saline conditions

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Abstract: The aim of this study was to assess the effects of spermidine (Spd) on ameliorating adverse effects of salinity in Troyer citrange seedlings. For this purpose, 6-month-old, uniform-sized seedlings of Troyer citrange were transplanted to plastic containers containing Hoagland nutrient solution. Addition of 75 mM NaCl into the nutrient solution reduced the plant growth, leaf chlorophyll content, chlorophyll fluorescence yield (Fv/Fm), net photosynthetic rate, respiration rate, and total Spd, N, and $\text{Ca}^{2+} + \text{Mg}^{2+}$ contents of the leaves. It increased the leaf proline, total putrescine (Put), total spermine (Spm), Na^+ , and Cl^- contents of the leaves. Addition of Spd (0.1 or 0.5 mM) to the saline nutrient solution and its weekly sprays (1 or 5 mM) on NaCl-stressed plants improved leaf number, chlorophyll content, Fv/Fm, net photosynthetic rate, and N content; increased total Spd and Spm contents; and reduced Na^+ contents of the plants. Leaf $\text{Ca}^{2+} + \text{Mg}^{2+}$ contents were slightly improved only when Spd (0.5 mM) was added to the saline nutrient solution. Leaf P and K^+ contents were not significantly affected by the salinity or Spd treatments. Among the polyamines, Put content was least affected, while there was a sharp increase in Spm content due to the salinity and Spd treatments. These results suggest involvement of Spm in the salinity tolerance of citrus rootstock Troyer citrange.

Key words: Citrus, mineral composition, photosynthesis, proline content, salinity stress, spermidine

Introduction

The polyamines are low molecular weight, nonprotein, straight chain, aliphatic hydrocarbon compounds with amino and imino groups. Their involvement has been postulated in a wide range of biological processes, including cell division, growth and development, senescence, and response of plants to abiotic stresses (Suleiman et al. 2002; Navakoudis et al. 2007). At physiological pH, these polycations have been shown to influence protein synthesis, DNA-protein interaction, and membrane integrity

(Chattopadhyay et al. 2002). Recent studies suggest that changes in polyamine biosynthesis and levels may be an integral part of the response of plants to stress (Bouchereau et al. 1999; Suleiman et al. 2002; Yang et al. 2007). Accumulation of polyamines, such as Put, Spd, and Spm, has been reported under various stress conditions, e.g. K^+ deficiency, increase in certain atmospheric pollutants, and acidic, ionic, osmotic, water, temperature, and cold stresses (Shen et al. 2000). However, the effects of saline conditions on polyamine metabolism are not always clear-cut, and

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the mechanism involved is generally less understood (Bouchereau et al. 1999). Differences in polyamine responses under salt-stress conditions have been reported among and within species. Salt-tolerant barley (Liu et al. 2006) and rice cultivars (Krishnamurthy and Bhagwat 1989) drastically accumulated high levels of Spd and Spm, with a relative decrease in Put content. Total Spd content increased in leaf tissues of sunflower plants subjected to salt stress, while the levels of other polyamines decreased or no significant changes occurred (Mutlu and Bozcuk 2005). This indicates the potential role of polyamines in the salinity tolerance of crop plants. However, it is still unclear which of the polyamines is important for protection against salinity (Kasinathan and Wingler 2004). There is a strong correlation between increased cellular proline and the capacity to survive under high environmental salinity (Stewart and Larher 1980). Exogenously applied proline at lower concentrations stimulates the accumulation of proline, while exogenously supplied Spd has no effect on proline accumulation (Aziz et al. 1998). The physiological significance of these responses is a matter of controversy, because direct evidence for the part played by proline or by polyamines under stress conditions remains tenuous (Munns 1993). Nevertheless, identification of intracellular solutes and the importance of the changes induced in their levels under stress conditions could be relevant as metabolic traits of interest regarding the characterization of stress tolerant cultivars of crop plants (Aziz et al. 1999).

Although polyamines play a pivotal role in the tolerance of fruit trees to environmental stresses, direct evidence for their involvement in salinity tolerance is still lacking. Furthermore, there is a lack of knowledge on the molecular mechanism underlying the role of polyamines in fruit trees (Wen et al. 2008). Citrus is mostly grown in the semiarid and arid areas of the world, where salinity is a serious problem. Once planted, it has to remain in the same soil for several years and is continuously exposed to salinity stress. Thus, development of techniques for avoidance or reduction of injury from salinity stress and an understanding of the physiological and biochemical basis of salinity tolerance in citrus rootstocks are of prime importance. As Spd is believed to ameliorate the toxic effects of salinity in plants, it

was hypothesized that Spd treatment could alleviate the adverse effects of salt stress in the salt-sensitive citrus rootstock Troyer citrange. The primary objective of the present study was to assess the effects of Spd on growth, photosynthetic efficiency, and leaf proline content, and ultimately ameliorate the adverse effects of salinity in the seedlings of Troyer citrange. The second objective was to determine whether exogenously applied Spd through root medium or in spray form was more effective in regulating the photosynthetic capacity and ion uptake in the seedlings.

Materials and methods

Plant material and growth conditions

Seeds of Troyer citrange (*Poncirus trifoliata* × *Citrus sinensis*) were obtained from the Horticultural Research Station at Sahiwal, Pakistan, and sown in earthen pots containing commercial nursery soil (pH = 6.84, EC = 0.44 dS m⁻¹, OM = 4.80%, N = 0.24%, available P = 40 µg g⁻¹, and K = 520 µg g⁻¹) in a greenhouse at Mie University, Tsu-shi, Mie-ken, Japan. Watering and fertilizer applications were performed as required. When seedlings were 3 months old, uniform-sized seedlings were selected and shifted to a hydroponic system in plastic containers; 12 seedlings were transplanted to each container, holding 37.5 L of Hoagland nutrient solution (Hoagland and Arnon 1950). The solution was circulated through a water pump and aerated using an air pump. The pH of the nutrient solution was maintained at 6.0 ± 0.1 and the solution was renewed after every 2 weeks. After 3 months, when the seedlings were 6 months old, the following treatments were applied to the seedlings.

T₁ = Hoagland solution (control)

T₂ = Hoagland solution containing 75 mM NaCl

T₃ = Hoagland solution containing 75 mM NaCl and 0.1 mM Spd

T₄ = Hoagland solution containing 75 mM NaCl and 0.5 mM Spd

T₅ = Hoagland solution containing 75 mM NaCl + weekly spray of 1 mM Spd

T₆ = Hoagland solution containing 75 mM NaCl + weekly spray of 5 mM Spd

NaCl and Spd concentrations were determined in a preliminary study. After every week, the test solution was drained and containers were filled with fresh test solutions. The plants in T₅ and T₆ were sprayed weekly for a period of 6 weeks; after that, the experiment was terminated. However, half of the seedlings in each treatment were harvested after 3 weeks. The plants were kept throughout the experiment under a 14 h photoperiod at a photon flux density of 290 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Growth measurements

Growth of the plants, in terms of shoot height and number of leaves, was recorded at the beginning and after 3 and 6 weeks of applying the treatments, and increases were estimated. Toxicity symptoms were recorded at the end of the experiment.

Photosynthesis and chlorophyll fluorescence

Relative chlorophyll contents of the leaves were measured nondestructively using a chlorophyll meter (SPAD 502; Minolta, Japan). Chlorophyll fluorescence yields (Fv/Fm) were recorded in attached leaves in the greenhouse using a photosynthesis yield analyzer (Mini Pam; H. Walz, Germany) after 30 min of dark adaptation. Both measurements were recorded after 3 and 6 weeks of applying the treatments. Rates of photosynthesis and respiration were measured at the end of the experiment using the Rank oxygen electrode system.

Proline analysis

After 6 weeks of applying treatments, leaves from each treatment were harvested separately, washed in deionized water, preserved in liquid nitrogen, and lyophilized in a freeze dryer. The lyophilized leaf material (30 mg) was extracted with 3 mL of deionized water at 80 °C for 15 min. After cooling, the samples were shaken for approximately 1 h at room temperature and then allowed to stand overnight at 4 °C. The extracts were filtered through Whatman No. 2 filter paper and analyzed for free proline content using the acid ninhydrin method described earlier (Anjum 2008).

Polyamine analysis

Polyamines were extracted by the method described earlier (Anjum 2008). Aliquots of the samples containing free plus conjugated and bound polyamines were hydrolyzed in 6 M HCl at 110 °C for

18 h to convert the conjugated and bound forms of the polyamines into the free form. 1,6-Hexanediamine (5 μL of 0.5 mM) was used as the internal standard, and after dansylation, polyamines in the supernatant and hydrolyzates were quantified using high-pressure liquid chromatography (HPLC) as described by Burtin et al. (1989).

Leaf mineral analysis

After 6 weeks of applying treatments, plants were harvested and their leaves were separated. These were washed in deionized water, preserved in liquid nitrogen, and stored in a freezer. The leaf samples were lyophilized and ground in a sample mill. Leaf N was determined using the Kjeldahl method (Jones 1991). For estimation of P, K⁺, Na⁺, Ca²⁺, and Mg²⁺, leaf samples were wet-digested in HNO₃ + HClO₄ (2:1) following the method of Ryan et al. (2001). P was determined on a Spectronic 20 (Bausch and Lomb). Total K⁺ and Na⁺ were estimated by flame photometry (Jenway, PFP-7). Ca²⁺ + Mg²⁺ was determined by the versenate solution titration method. For Cl⁻ estimation, 50 mg of ground leaf material was taken and heated in deionized distilled water at 80 °C for 3 h. The chloride content of the extract was determined by the method described by Richards (1954).

Statistical analysis

All of the presented data are mean values of at least 3 replications. The data were analyzed statistically by using the analysis of variance (ANOVA) technique. Due to variable response with time, data collected after 3 and 6 weeks were analyzed separately. The treatments were compared by using the LSD test at P \leq 0.05. For the Figures, SEs were also calculated and are indicated as vertical bars.

Results

Plant growth

The maximum increase in shoot height was recorded in the control plants (T₁). Salinity treatment (T₂) resulted in a sharp decrease in plant height. Spd treatments to the seedlings (T₃-T₆) failed to revert the decrease in shoot growth caused by the salinity treatment. The maximum number of leaves was produced in the control plants. This was followed by T₄ and T₃, where 0.5 and 0.1 mM Spd were added to

the saline nutrient solution, respectively. Weekly sprays of 1 and 5 mM Spd (T_5 and T_6) were only effective for increasing leaf number during the first 3 weeks. These results indicate that Spd slightly improved leaf number but failed to alleviate the harmful effect of salinity on the seedling growth (Figures 1a and 1b). The growth rate was high during the first 3 weeks in all of the treatments. No toxicity symptoms were observed in the nonstressed control plants. Addition of 75 mM NaCl to the nutrient solution resulted in necrosis of leaf margins and also leaf drop. Addition of Spd to the nutrient solution or weekly spray did not reduce these symptoms. However, when plants were sprayed weekly with 5 mM Spd, in addition to leaf necrosis and leaf drop, tip burning was also observed in 50% of the plants.

Photosynthesis and chlorophyll fluorescence

Both the chlorophyll content of the leaves and the photochemical efficiency of photosystem II (PSII), as determined by the ratio of the dark-adaptive variable (Fv) to the maximum fluorescence yield (Fm), were affected by the treatments applied. The maximum chlorophyll content and Fv/Fm values were recorded in the control plants (T_1). These both decreased with

salinity treatment (T_2). However, there was an improvement in the values of both parameters when 0.1 or 0.5 mM Spd was added to the saline nutrient solution (T_3 and T_4) or when the plants growing in saline nutrient solution were sprayed weekly with 1 mM Spd (T_5) (Figures 2a and 2b).

The leaves of the control plants had the maximum photosynthetic rate, which decreased sharply due to the addition of 75 mM NaCl to the nutrient solution (T_2). The rate of photosynthesis was improved with the addition and increase of Spd concentration in the saline nutrient solution. However, improvement in the photosynthetic rate by weekly sprays of Spd was less pronounced. Increased Spd level in the saline nutrient solution (0.5 mM) effectively improved the photosynthetic rate (Figure 3a), indicating some role of polyamines in salinity tolerance. The maximum respiration rate was recorded in the leaves of the control plants and the minimum in the salt-stressed plants. Respiration rates of the plants grown in saline solution with a higher concentration of Spd (0.5 mM) and those sprayed weekly with 1 or 5 mM Spd were better than those of salt-stressed plants (Figure 3a).

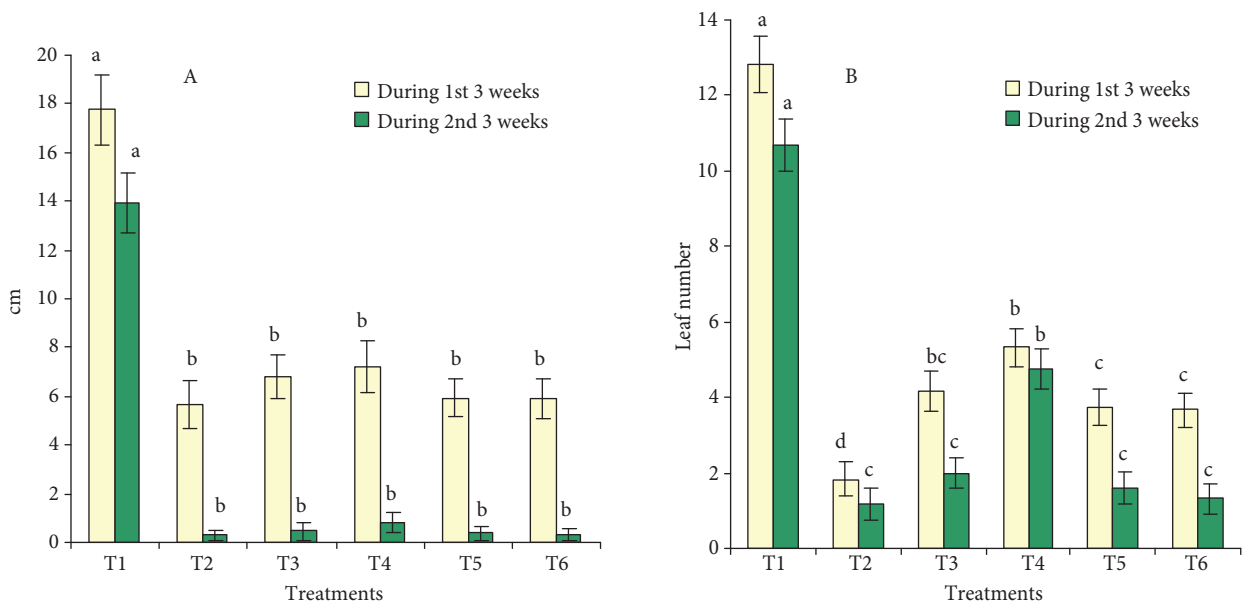


Figure 1. Increase in a) shoot height and b) leaf number of Troyer citrange seedlings during 6 weeks under different treatments. Vertical bars indicate SE (n = 3).

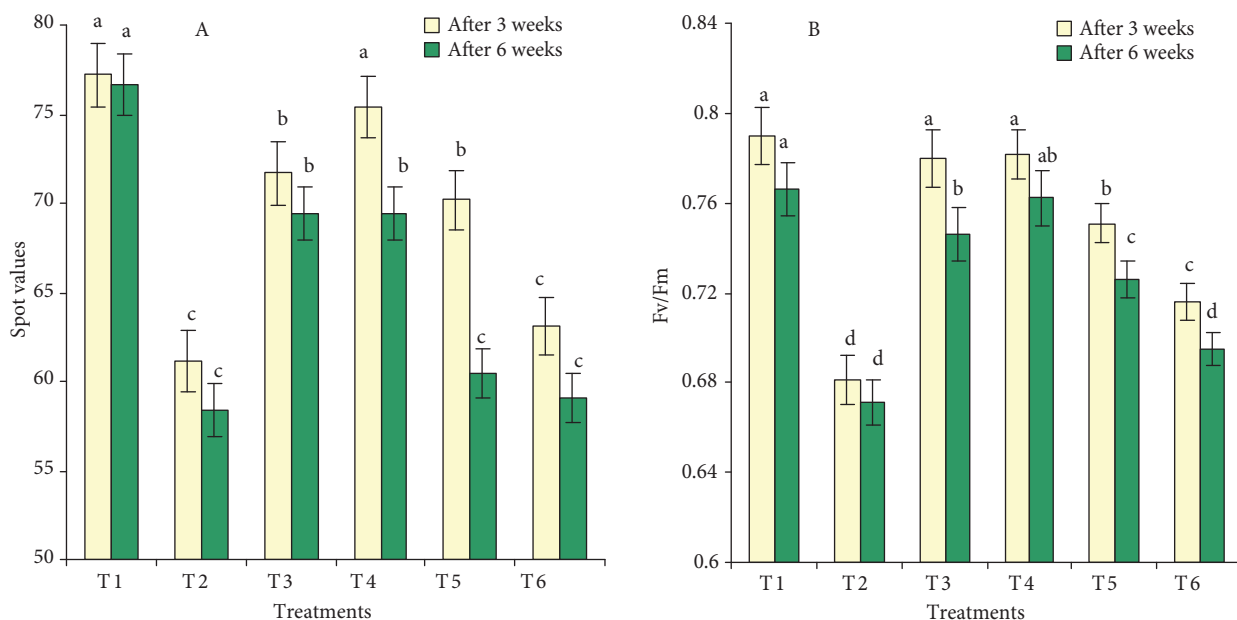


Figure 2. Changes in a) chlorophyll content and b) chlorophyll fluorescence yield of leaves of Troyer citrange seedlings after 3 and 6 weeks of applying treatments. Vertical bars indicate SE (n = 3).

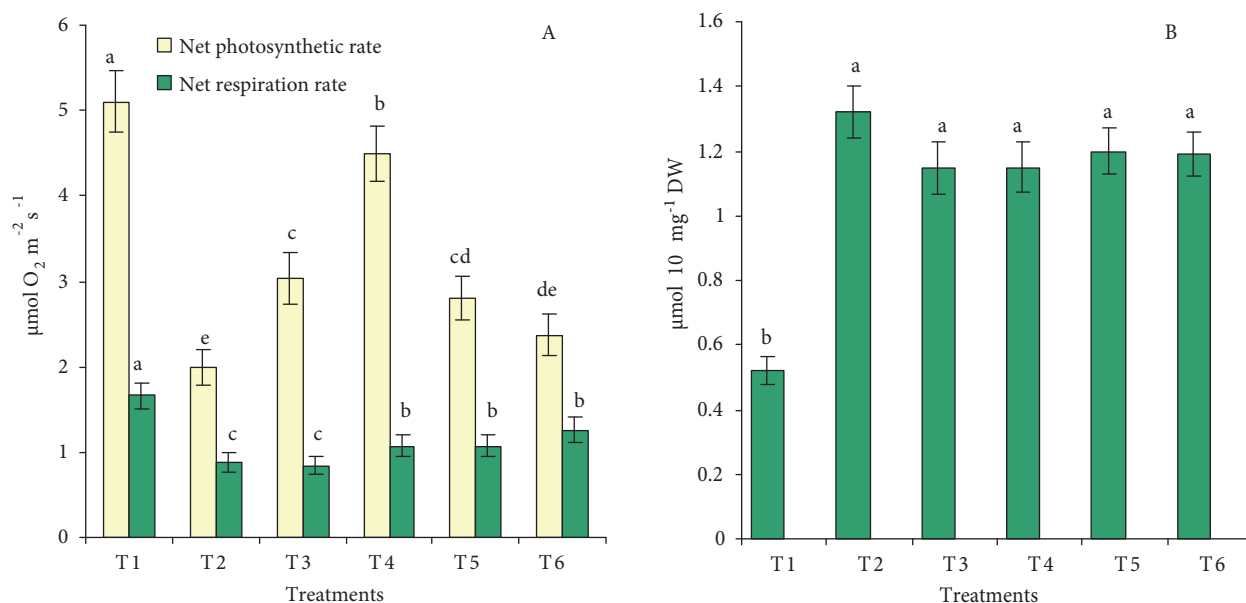


Figure 3. Changes in a) net photosynthetic and respiration rates and b) leaf proline content of leaves of Troyer citrange seedlings after 6 weeks of applying treatments. Vertical bars indicate SE (n = 3).

Proline contents

The minimum proline contents were recorded in the leaves of control plants (T_1) and the maximum in leaves of those plants grown in saline nutrient solution

(T_2). All other treatments had almost the same concentrations of proline in the leaves, which were statistically similar to salt-stressed plants. This indicates that the addition of Spd (0.1 or 0.5 mM) to

the saline nutrient solution or the weekly spray of Spd (1 or 5 mM) on plants grown in saline solution were at par and could not significantly alter the proline level (Figure 3b).

Polyamine contents

The concentrations of various forms (free, conjugated, and bound) of different polyamines, i.e. Put, Spd, and Spm, in the leaves of Troyer citrange were quite variable. Free Put titer was highest in the plants grown in saline nutrient solution (T_2) and significantly differed from all other treatments. This was followed by those grown in saline nutrient solution supplemented with Spd (T_6 , T_3 , T_5 , and T_4). All of these 4 Spd treatments were statistically at par. The minimum titer of free Put was recorded in untreated plants (T_1), followed by those grown in the saline nutrient solution containing 0.5 mM Spd (T_4), and these 2 treatments behaved statistically alike. This indicates that exogenously applied Spd effectively lowered the free Put contents of leaves; however, when added to the nutrient solution at a higher concentration (0.5 mM), it was the most effective. The untreated plants accumulated more Put in the conjugated form. The concentration of bound Put was significantly higher when plants were grown in a saline nutrient solution supplemented with a lower concentration (0.1 mM) of Spd (T_3), followed by those grown in saline nutrient solution only (T_2). Total Put content was significantly higher in the leaves of the plants grown in saline nutrient solution, followed by the leaves of those plants grown in a saline nutrient solution supplemented with a lower concentration of Spd (0.1 mM). However, it was at a minimum in the leaves of the plants grown in saline solution and sprayed weekly with 1 mM Spd (T_5), which differed significantly from the rest of the treatments. This indicates that weekly spraying of 1 mM Spd counteracted the adverse effect of salinity (Table 1). However, Put concentration alone cannot be accounted as criteria for salinity tolerance.

The leaves of the plants grown in saline nutrient solution and supplemented with 0.5 mM Spd (T_4) had a higher concentration of free Spd. This was followed by the treatment in which plants were grown in saline solution with 0.1 mM Spd (T_3) and those sprayed weekly with 5 mM Spd (T_6), possibly due to the exogenously applied Spd. These 3 treatments were

Table 1. Polyamine contents (n moles per gram of dry weight) in leaves of Troyer citrange seedlings after 6 weeks of applying treatments.

Treatments	Put	Spd	Spm
Free polyamines			
T_1 (control)	59.304 c	292.231 b	348.716 b
T_2	89.739 a	274.054 b	528.582 a
T_3	76.716 b	395.943 a	574.717 a
T_4	67.005 bc	428.402 a	605.722 a
T_5	71.697 b	278.321 b	551.090 a
T_6	76.942 b	383.594 a	544.359 a
Conjugated polyamines			
T_1 (control)	59.775 a	61.158 a	7.015 c
T_2	49.265 ab	16.806 d	20.630 bc
T_3	41.519 bc	49.485 ab	50.050 b
T_4	39.305 bc	63.855 a	99.324 a
T_5	24.276 d	41.107 bc	21.023 bc
T_6	29.533 cd	25.460 cd	43.518 b
Bound polyamines			
T_1 (control)	24.490 bc	31.051 a	17.913 b
T_2	27.882 ab	17.948 cd	19.575 b
T_3	36.482 a	13.834 d	18.029 b
T_4	20.221 bc	22.763 bc	36.406 a
T_5	8.626 d	26.481 ab	14.763 b
T_6	16.531 cd	32.595 a	18.647 b
Total polyamines			
T_1 (control)	143.569 bc	384.440 cd	373.644 c
T_2	166.886 a	308.808 e	568.787 b
T_3	154.717 ab	459.262 ab	642.796 ab
T_4	126.531 cd	515.020 a	741.452 a
T_5	104.599 e	345.909 de	586.876 b
T_6	123.006 d	441.649 bc	606.524 b

Data represent mean of 3 replicates. Values within a group followed by different letters are significantly different at $P \leq 0.05$.

statistically at par with each other but significantly differed from other treatments. Spd added to the nutrient solution also resulted in more Spd in the conjugated form, while, when sprayed weekly, it accumulated more in the bound form. However, total Spd contents of leaves were significantly higher when plants were grown in a saline solution with 0.5 and 0.1 mM Spd (T_4 and T_3), possibly due to the uptake of Spd by the plants from the nutrient solution. The lowest total Spd content was recorded in salt-stressed plants (Table 1).

The minimum free Spm was recorded in the leaves of the control plants (T_1), which significantly differed from all other treatments. All other treatments were statistically at par. The Spm in conjugated and bound forms was the highest in those plants grown in a saline solution with 0.5 mM Spd (T_4). Resultantly, the maximum amount of total Spm was found in T_4 , followed by T_3 , indicating the impact of exogenously applied Spd. The lowest Spm content was recorded in the leaves of the control plants, which were statistically different from all other treatments (Table 1).

Leaf mineral analysis

Leaf N and $Ca^{2+} + Mg^{2+}$ contents were decreased when NaCl (75 mM) was added to the nutrient solution (T_2) and significantly improved by the exogenous application of Spd (0.5 mM) through nutrient solution (T_4). However, other Spd treatments (T_3 , T_5 , and T_6) stood statistically at par with T_2 . Although there were changes in the P and K^+ contents of the leaves, the differences were statistically nonsignificant. The Na^+ and Cl levels of the leaves sharply increased with salinity treatment. Spd application through nutrient solution (T_3 and T_4) resulted in the significant reduction of the Na^+ levels of the leaves. Exogenous application of Spd through weekly sprays was not so effective at altering leaf Na^+ levels. Cl contents of the leaves were not affected significantly by the Spd treatments (Table 2).

Discussion

Accumulation of Spd in leaf tissues of sunflower (Mutlu and Bozcuk 2005), rice (Krishnamurthy and Bhagwat 1989), and barley (Liu et al. 2006) has been reported under salt stress. Transgenic plants overexpressing spermidine synthase (SPDS) demonstrated reduced susceptibility to salt stress (Kasukabe et al. 2004; Wen et al. 2008) and had higher Spd contents in response to salinity (He et al. 2008). Exogenously applied Spd prevented chlorophyll loss and inhibition of photochemical reactions of photosynthesis induced by salinity stress (Chattopadhyay et al. 2002), and reduced the adverse effects of salinity by significantly reducing accumulation of Na^+ and loss of K^+ (Roy et al. 2005). Changes in polyamine biosynthesis and levels are an integral part of the response of plants to stress. During the metabolic pathway(s), the polyamine produced is stored in the relevant plant cell compartments without any detrimental effect. Spd accumulated under stress conditions is degraded when stress conditions are released. It also behaves as a true compatible solute. When exogenously applied at physiologically relevant concentrations to nonstressed plants, it is expected to not induce deleterious effects on plant growth and development. Furthermore, it is supposed that Spd does not favor pathogenic attacks when made available on the plant surface. Therefore, Spd was used in the present study to observe its effects on salt-stressed plants.

Table 2. Mineral composition of leaves (on dry weight basis) of Troyer citrange seedlings after 6 weeks of applying treatments.

Treatments	N (%)	P (%)	K (%)	Ca + Mg (%)	Na (%)	Cl (%)
T_1 (control)	1.96 a	0.52	1.90	0.90 a	0.50 c	0.65 b
T_2	1.12 c	0.46	1.70	0.60 c	2.00 a	1.91 a
T_3	1.26 bc	0.49	1.75	0.70 bc	1.40 b	1.61 a
T_4	1.45 b	0.49	1.80	0.85 ab	1.30 b	1.52 a
T_5	1.14 c	0.44	1.70	0.60 c	1.75 ab	1.67 a
T_6	1.26 bc	0.44	1.70	0.70 bc	1.75 ab	1.64 a

Data represent mean of 3 replicates. For each parameter, values followed by different letters are significantly different at $P \leq 0.05$.

Salinity significantly affects growth and photosynthesis in crop plants. Most crop plants are salt-sensitive and display a variety of visual symptoms when exposed to salt stress during vegetative growth, i.e. leaf chlorosis, necrosis, and abscission; reduced growth rate; and plant death. However, growth suppression due to NaCl stress is one of the most obvious results in citrus and other plants in general (Anjum et al. 2001; Verslues et al. 2006; Anjum 2008). In the present study, growth in terms of shoot height and leaf number was reduced when 75 mM NaCl was added to the nutrient solution. The addition of Spd to the nutrient solution or its weekly spray did not prove so effective at reversing the growth inhibition caused by NaCl stress, although it improved the leaf number. Similarly, chlorophyll content and chlorophyll fluorescence yields (Fv/Fm) were reduced by the salinity treatment, and plants also showed toxicity symptoms. Plant growth, chlorophyll content (Sultana et al. 1999; Al-Yassin 2005), and photosynthetic rate (Bethke and Drew 1992) decrease in response to salt stress, and plants show different toxicity symptoms, including chlorosis and necrosis (Bethke and Drew 1992; Sultana et al. 1999). The progressive decrease in Fv/Fm was also recorded in green alga with an increase in salinity level (Demetriou et al. 2007). Spd and Spm, when applied to salt-stressed plants, prevented chlorophyll loss and inhibition of photochemical reactions of photosynthesis (Chattopadhyay et al. 2002). In the present study, exogenously applied Spd also improved chlorophyll content and Fv/Fm in salt-stressed plants. However, plants sprayed weekly at the higher concentration of Spd (5 mM) had lower chlorophyll content and Fv/Fm as compared to other Spd treatments. This shows that this concentration of Spd was even toxic to some extent, as also indicated by the toxicity symptoms.

Net photosynthetic rate sharply decreased with the addition of 75 mM NaCl in the nutrient solution. Reduction in photosynthesis has already been recorded in *Populus euphratica* (Ma et al. 1997), rice (Sultana et al. 1999), and Cleopatra mandarin (Anjum 2010) seedlings under salt treatments. PSII is highly susceptible to salinity stress. In *Ulva lactuca*, increasing salinity significantly reduced the photosynthetic O₂ evolution and markedly declined fluorescence yield, which was associated with a decreased activity of the PSII complex (Xia et al.

2004). Plants under salt stress exhibit a decrease in their photosynthetic efficiency; however, it is not known how this actually occurs (Ma et al. 1997). According to Sultana et al. (1999), reduction in photosynthesis in plants under salt stress depends not only on the decreased availability of CO₂ due to stomatal closure, but also on the cumulative effect of stomatal conductance, transpiration rate, relative leaf water content, water and osmotic potential, photosynthetic pigments, soluble carbohydrates, and proteins. Increased salinity provokes changes in the photosynthetic apparatus by affecting both its structure and function. The addition of polyamine to NaCl-treated cultures can compensate for these changes by reorganizing the photosynthetic apparatus, therefore conferring protection to the photosynthetic apparatus against NaCl stress (Demetriou et al. 2007). In the present study, exogenous supply of Spd to the plants resulted in an increase in the net photosynthetic rate, but this was still lower than those of the nonstressed control plants. Addition of 0.5 mM Spd to the nutrient solution was more effective as compared to other Spd treatments for inhibiting the decrease in photosynthetic rates. Rate of respiration also improved when 0.5 mM Spd was added to the saline nutrient solution or sprayed weekly at both of the concentrations used.

Proline is considered to act as a compatible osmoticum and is perhaps involved in salt and drought tolerance. In the present study, after 6 weeks of treatment application, the data showed a more than 2-fold increase in the proline content of the leaves. Similar results have been reported in a previous study (Anjum 2008). Exogenous application of Spd to salt-stressed plants reduced the proline pool, but this decrease was statistically nonsignificant. The results of the present study are in accordance with Sultana et al. (1999), who observed a significantly increased accumulation of proline by salinity in rice leaves, and Aziz et al. (1998), who found that tomato leaf explants subjected to salt stress accumulated very high amounts of proline and that exogenously applied Spd was not effective in altering the proline levels.

Free Put and Spm were accumulated in response to salt stress, resulting in increased total Put and Spm and reduced Spd contents. The higher Put content could be due to the enhanced interconversion of Spd

into Put through acetylation of Spd. Increase in Spm content could be attributed to increased activity of Spm synthase, the enzyme which catalyzes the production of Spm from Spd, while a decrease in Spd could be due to the reduced activity of enzymes responsible for its production (Roussos and Pontikis 2006) or its conversion to Spm. The results of the present study demonstrate a possible role of Spm in the salinity tolerance of Troyer citrange. Accumulation of Spm in rice (Maiale et al. 2004) and sunflower leaf tissue (Mutlu and Bozcuk 2005), and in jojoba explants in vitro (Roussos and Pontikis 2006), have already been reported during salt stress. Spm content also increased in leaves of both of the citrus rootstocks, i.e. Cleopatra mandarin and Troyer citrange, in response to salinity stress (Anjum 2008). The highest concentrations of conjugated Put were in the leaves of control plants, followed by those under salinity treatment, and they decreased with application of Spd. This indicated that the exogenously applied Spd might ameliorate harmful effects of salinity by reducing conjugated Put. In plant response to stress, polyamines may have distinct functions, and a conversion between free, conjugated, and bound forms may occur (Ndayiragije and Lutts 2006). Bound polyamines act as membrane stabilizers under high salt conditions (Roussos and Pontikis 2006). Conjugated polyamines are associated with plant resistance to stress (Liu et al. 2004) and could be a good source for free polyamines under the conditions when required in active forms. The increase in salt tolerance by free and conjugated forms of Spm or Spd could be ascribed to their ability to act as free radical scavengers (Bouchereau et al. 1999), activate antioxidant systems, and stabilize membranes due to their polycationic properties (He et al. 2008). The differences in the role of polyamines may exist between species, among cultivars of same species, and even among organs of same plant (Çavuşoğlu et al. 2007). Spm regulates the amount of chlorophyll; low amounts of exogenous Spm induce chlorophyll biosynthesis, while in higher amounts, it inhibits (Beigbeder and Kotzabasis 1994). Increased Spd and Spm play an important role in preserving the thylakoid membrane integrity (Besford et al. 1993), whereas Put causes depolarization of membranes (Tiburcio et al. 1990). Therefore, an increase in Spd and/or Spm levels might be one of the crucial factors involved in stress tolerance, including salinity.

High salt concentrations in the soil or nutrient solution cause ion-specific stresses resulting from changes in K^+ to Na^+ ratios, leading to increased Na^+ and Cl^- concentrations that are detrimental to plants. In the present study, addition of NaCl to the nutrient solution resulted in increased Na^+ and Cl^- and reduced N and $Ca^{2+} + Mg^{2+}$ contents of the leaves. The major saline ions (Na^+ and Cl^-) in nutrient solutions or soil can affect mineral uptake through competitive interaction or by affecting the ion selectivity of membranes, resulting in nutritional imbalance. Some of these changes in ion contents due to salt stress were markedly counteracted when Spd was added to the saline nutrient solution. For example, Na^+ contents of the leaves were significantly affected. However, Cl^- concentrations were not significantly changed, possibly because Troyer citrange has the capacity to restrict Na transport but is a poor Cl^- excluder. Polyamines may have a protective role due to their polycationic nature by altering ion concentrations through membrane stabilization (Chattopadhyay et al. 2002). The results suggest that Spd might inhibit uptake and translocation of Na^+ ions from roots to leaves. Exogenous application of Spd during NaCl stress reduces the accumulation of Na^+ and the loss of K^+ (Roy et al. 2005). In the present study, exogenously applied Spd decreased Na^+ and increased (to some extent) $Ca^{2+} + Mg^{2+}$ uptake. However, weekly sprays of Spd on the salt-stressed plants were not so effective, which suggests that the plants absorb more Spd through the roots. The leaf P and K^+ concentrations were not significantly affected by the salinity or Spd treatments. In citrus, NaCl-induced change in leaf K^+ content varies with rootstock. Generally, citrus leaves show no major change in K^+ content during salt stress (Syvertsen and Yelenosky 1988). There is no evidence that Na^+ transported to the shoot replaces K^+ in leaf cells (Storey and Walker 1999). However, in the present study, it is evident that as the Na^+ contents of the leaves increased, $Ca^{2+} + Mg^{2+}$ uptake was reduced and vice versa. Increased $Ca^{2+} + Mg^{2+}$ uptake may be beneficial during salt stress due to the maintenance of cellular membrane integrity and reduction of Na^+ uptake. Furthermore, reduction of Na^+ and Cl^- uptake and higher uptake of K^+ , Ca^{2+} , and Mg^{2+} is an important mechanism in salt tolerance. An increase in Na^+ and Cl^- and a decrease in Ca^{2+} contents in bell pepper (Bethke and Drew 1992), and a decrease in K^+

and Mg^{2+} content in rice plants, have been recorded due to salt stress, and exogenous polyamines helped to maintain the balance (Chattopadhyay et al. 2002). High levels of Spd and Spm bound to the plasma membrane and H^+ -ATPase stabilize the plasma membrane and maintain a low level of endogenous Na^+ (Roy et al. 2005). Spd treatments to salt-stressed plants had beneficial effects on ion homeostasis, as they lowered leaf Na^+ content and increased uptake of $Ca^{2+} + Mg^{2+}$, indicating that polyamine can confer salt tolerance by affecting ion uptake. Root salt exclusion is a strategy widely adapted by plants surviving in saline environments. Thus, reduced uptake of a toxic ion (Na^+) and higher uptake of beneficial ions ($Ca^{2+} + Mg^{2+}$) could be a mechanism of salt tolerance under Spd treatments.

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Conclusion

The adverse effects of salinity can be ameliorated, to some extent, by exogenous application of Spd. In Troyer citrange seedlings, Spd is converted into Spm, which is involved in the salinity tolerance mechanism of this rootstock.

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