Salicylic acid differently impacts ethylene and polyamine synthesis in the glycophyte *Solanum lycopersicum* and the wild-related halophyte *Solanum chilense* exposed to mild salt stress

Emna Gharbi[a,c], Juan-Pablo Martínez[b], Hela Benahmed[c], Marie-Laure Fauconnier[d], Stanley Lutts[a,*] and Muriel Quinet[a]

[a]Groupe de Recherche en Physiologie végétale (GRPV), Earth and Life Institute – Agronomy (ELI-A), Université catholique de Louvain, Louvain-la-Neuve, Belgium

[b]Instituto de Investigaciones Agropecuarias (INIA – La Cruz), La Cruz, Chile

[c]Laboratoire d'Ecologie végétale, Faculté des Sciences, Université de Tunis El Manar, Tunisia

[d]Unité de Chimie générale et organique, Faculté des Sciences agronomiques, Université de Liège, Belgium

*Corresponding author, e-mail: stanley.lutts@uclouvain.be

This study aimed to determine the effects of exogenous application of salicylic acid on the toxic effects of salt in relation to ethylene and polyamine synthesis, and to correlate these traits with the expression of genes involved in ethylene and polyamine metabolism in two tomato species differing in their sensitivity to salt stress, *Solanum lycopersicum* cv Ailsa Craig and its wild salt-resistant relative *Solanum chilense*. In *S. chilense*, treatment with 125 m*M* NaCl improved plant growth, increased production of ethylene, endogenous salicylic acid and spermine. These productions were related to a modification of expression of genes involved in ethylene and polyamine metabolism. In contrast, salinity decreased plant growth in *S. lycopersicum* without affecting endogenous ethylene, salicylic or polyamine concentrations. Exogenous application of salicylic acid at 0.01 m*M* enhanced shoot growth in both species and affected ethylene and polyamine production in *S. chilense*. Concomitant application of NaCl and salicylic acid improved osmotic adjustment, thus suggesting that salt and SA may act in synergy on osmolyte synthesis. However, the beneficial impact of exogenous application of salicylic acid was mitigated by salt stress since NaCl impaired endogenous SA accumulation in the shoot and salicylic acid did not improve plant growth in salt-treated plants. Our results thus revealed that both species respond differently to salinity and that salicylic acid, ethylene and polyamine metabolisms are involved in salt resistance in *S. chilense.*

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Abbreviations – ACC, 1-aminocyclopropane-1-carboxylic acid; ACCS, 1-aminocyclopropane-1-carboxylic acid synthase; ACCO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACN, acetonitrile; ADC, arginine decarboxylase; DW, dry weight; *EF1α*, elongating factor 1 alpha; FW, fresh weight; g_s, stomatal conductance; ODC, ornithine decarboxylase; PAs, polyamine; Put, putrescine; Ψs, osmotic potential; SA, salicylic acid; SAM, S-adenosyl-1-methionine; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; Spds, spermidine synthase; Spm, spermine; Spms, spermine synthase; WC, water content.

Introduction

Tomato (*Solanum lycopersicum*) is one of the most important fruit vegetables in the economic sphere. Under unfavorable conditions such as those occurring in arid and semi-arid conditions, irrigation is required to ensure tomato production. Intensive irrigation, however, often leads to soil salinization. Salinity is now a major critical environmental stress limiting agriculture world-wide (Flowers 2004, Munns and Tester 2008, Ruan et al. 2010). In cultivated tomato, it drastically affects plant growth and compromises yield (Bolarin et al. 1993).

Although cultivated tomato is quite sensitive to salt toxicity, several of its wild relatives are able to cope with high salinity levels and exhibit halophyte properties (Spooner et al. 2005). *Solanum chilense* is spontaneously present in a salt-affected areas of North Chile and originates from the Atacama Desert, one of the most salted and arid areas in the world (Chetelat et al. 2009). It is distributed in southern Peru to northern Chile where it is found in arid plains and deserts. This species can dwell in hyper arid areas and is distributed from sea level up to 3500 m in the Andes (Chetelat et al. 2009). This self-incompatible perennial was historically included in the polymorphic *S. peruvianum* but is now considered as a distinct species found in a geographically restricted area and characterized by narrow ecological niches (Igic et al. 2007, Nakazato et al. 2010, Tellier et al. 2011).This wild tomato species is able to grow in diverse environments and to cope with many biotic and abiotic constraints (Martínez et al. 2012, 2014). It is commonly considered as a valuable source of genes for resistance to viruses such as *Pseudomonas syringae* (Thapa et al. 2015) or tomato yellow leaf curl disease (Pérez de Castro et al. 2013). Surprisingly, physiological basis of salt-resistance in *S. chilense* has received only minor attention until now in comparison to other salt-tolerant wild-relatives, such as *S. cheesmanii, S. pimpinelifolim* and *S. pennellii* (Mittova et al. 2002, Albacete et al. 2009, Gálvez et al. 2012, Almeida et al. 2014). Comparing the behavior of the cultivated glycophyte *S. lycopersicum* with its wild-relative halophyte plant species *S. chilense* will help to unravel the strategies of plant response to salt stress and may also lead to identification of genes able to confer salt resistance to the cultivated tomato. Martínez et al. (2012, 2014) reported that *S. chilense* displays a contrasting behavior in response to prolonged exposure to moderate salinity compared with *S. lycopersicum* and that salt stress does not markedly affect plant biomass and fruit yield in this species.

Salt stress impairs water uptake and results in nutrient imbalance due to accumulation of Na^+ and

Cl^- occurring concomitantly with a decrease of K^+ (Munns and Tester 2008). Salinity induces oxidative stress, inhibits photosynthesis and hastens leaf senescence processes (Mittova et al. 2002). It also drastically modifies hormonal status and some plant growth regulators may assume positive functions in plant adaptation to salt stress (Ghanem et al. 2011). Beside the well-known abscisic acid (Yang et al. 2014) or cytokinins (Zizkova et al. 2015), salicylic acid (SA) was also found to improve plant tolerance to salt stress (Miura and Tada 2014, Jayakannan et al. 2015). Applications of exogenous SA indeed mitigated the damaging effects of salinity in Arabidopsis (Jayakannan et al. 2013) and in tomato (Szepesi et al. 2009, Poór et al. 2011, Manaa et al. 2014). Exogenous SA may induce stomatal closure (Miura and Tada 2014), improve selectivity of ion uptake and transport (Jayakannan et al. 2015), and increases antioxidative defenses in stressed tissues (Nazar et al. 2011). Salicylic acid is thought to interact in a complex way with other hormonal compounds. It has been reported to inhibit ethylene biosynthesis (Tirani et al. 2013) although a stimulation (Liang et al. 1997) or absence of effect (Poór et al. 2013) on ethylene biosynthesis has also been reported in some experimental systems.

In optimal conditions, ethylene regulates diverse aspects of plant growth and development, including germination, leaf, stem, and root growth, fruit ripening, organ abscission, leaf and flower senescence. Ethylene is also reported to act as a stress hormone, hastening senescence and ultimately contributing to programmed cell death (Koyama 2014). Salt stress has been reported to increase ethylene production from its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Bleecker and Kende 2000, Nadeem et al. 2010). According to Ghanem et al. (2008), salt stress increase of ACC in tomato leaf tissue is related to premature leaf senescence and coincides with the onset of oxidative damage and the decline in chlorophyll fluorescence prior to massive Na^+ accumulation. In contrast, ethylene was reported to improve salt tolerance in Arabidopsis mainly through the maintenance of K^+ absorption and translocation (Jiang et al. 2013), thus suggesting that ethylene may assume dual functions in plants exposed to salinity.

The complexity of ethylene involvement in salt-stressed plant response may at least partly results from its interaction with polyamine synthesis. Polyamines (PAs) are polycationic organic compounds involved in various aspects of plant development as well as in stress responses and they assume numerous protecting functions in salt-treated plants (Hu et al. 2012, Lutts et al. 2013). Polyamines and ethylene share a common precursor [S-adenosyl-L-Methionine (SAM)], and the biosynthesis of these molecules is often considered as competitive (Pandey et al. 2009, Lutts et al. 2013) although Quinet et al. (2010) showed that there is no direct antagonism between PAs and ethylene pathways in rice. Transgenic tomato plants overexpressing SAM decarboxylase accumulate high concentration of PAs and showed improved tolerance to salinity (Hazarika and Rajam 2011). Exogenous application of PAs could also enhance tolerance of tomato plants to salt stress (Hu et al. 2012, 2014). Previous data in *Medicago sativa* demonstrated that application of exogenous SA inhibits ethylene under salt stress, and concomitantly increases PAs content (Palma et al. 2013). Szepesi et al. (2009) confirmed that

tomato pre-treatment with 10^{-4} *M* SA provided protection against salinity stress in relation to higher level of free putrescine and spermine. The impact of SA on the expression of genes involved in ethylene synthesis and PAs metabolism however remains poorly studied. Although salt stress has an impact on PAs content in *S. pennellii* (Santa-Cruz et al. 1997, 1998), no data are available, to the best of our knowledge for *S. chilense*.

The present work focuses on the interaction between SA, ethylene and PAs in plant response to salinity stress in the cultivated glycophyte *S. lycopersicum* and its halophyte wild-relative *S. chilense*. Our aims were to (1) compare SA, ethylene and PAs concentrations in the two considered species, (2) investigate the impact of salt stress on their concentrations in both species, (3) compare the impact of exogenous SA application on plant behavior in the absence and presence of salt and (4) analyze the expression of genes involved in ethylene synthesis and PAs metabolism in response to salt and exogenous SA in the two species.

Material and methods

Plant material and growth conditions

Seeds of cultivated tomato *Solanum lycopersicum* cv. Ailsa Craig (TGRC accession number LA2838A) and of the wild species *Solanum chilense* (TGRC accession number LA4107) were obtained from the Tomato Genetics Resource Center (University of California, Davis, USA). After germination in peat compost, sixteen-day-old seedlings were transferred to a hydroponic culture system into growth chamber at 24/22°C under a 16/8 h day/night period. Light intensity was 245 µmol m^{-2} s^{-1}(Master TL-D reflex Super 80 58W / 840 from Philips) and relative humidity was 70 ± 5%. Seedlings were fixed on polystyrene plates floating on aerated half-strength Hoagland nutrient solution containing: 5 m*M* KNO_3, 5.5 m*M* $Ca(NO_3)_2$, 1 m*M* $NH_4H_2PO_4$, 0.5 m*M* $MgSO_4$, 25 µ*M* KCl, 10 µ*M* H_3BO_4, 1 µ*M* $MnSO_4$, 0.25 µ*M* $CuSO_4$, 1 µ*M* $ZnSO_4$, 10 µ*M* $(NH_4)_6Mo_7O$ and 1.87 g l^{-1} Fe-EDTA. Solution was renewed every week and pH was adjusted daily to 5.5–6 using 5 *M* KOH. For each treatment, seedlings were distributed among four tanks (six seedlings per tank) containing 50 l of solution in a complete randomized block design. After 1 week of acclimatization in control conditions the seedlings were randomly divided into four groups: (1) control, (2) NaCl: solution containing 125 m*M* NaCl, (3) AS: solution containing 0.01 m*M* SA, (4) NaCl + SA: solution containing 125 m*M* NaCl + 0.01 m*M* SA. Two actively growing leaves, present at the moment of treatment application (leaf number 3 and 4, numbering from the base of the plant) were tagged for subsequent growth measurements (leaf 3), and for senescence monitoring (leaf 4). After 7 days of treatment, the plants (30 days old) were harvested and divided into roots and leaves for physiological and biochemical parameter determinations.

Plant growth, water content, stomatal conductance and osmotic potential

Plant growth was determined on the basis of shoot and root dry weight (DW) per plant (estimated on 6

individual plants per treatment). Roots were quickly, blotted dry and weighed for fresh weight (FW) determination. For DW determination, roots and shoots were incubated in an oven at 70°C for 72 h. Water content (WC) was calculated as WC = (FW–DW)/FW*100. Osmotic potential (Ψs) was estimated on the extracted sap using a Wescor 5500 vapor pressure osmometer as previously detailed (Lutts et al. 1999). Leaf stomatal conductance (g_s) was measured on the fourth fully expanded leaf on 6 plants per treatment using an AP4 diffusion porometer (Delta-TDevices Ltd., Cambridge, UK). All measurements were performed between 2 p.m. and 4 p.m.

Determination of Na^+, K^+

For Na^+ and K^+ quantification, the third leaf and root tissues of three plants per treatment were oven-dried at 70°C for 3 day and 50 mg of DW were incubated in 4 ml of 35% HNO_3 at 80°C. The residue was redissolved with aqua regia (HCl 37%: HNO_3 65% 3:1) and filtered (Whatman, 11 mm). Elements were quantified by flame atomic absorption spectrophotometry (ICE 3300; Thermo Scientific; Waltham, MA).

Salicylic acid quantification

Endogenous SA was extracted according to Molinari and Loffredo (2006). The procedure was modified as follows: 200 mg of fresh plant tissue was incubated with 1.8 ml 1 m*M* HCl under mechanical shaking at 2 g. The mixture was sonicated at 5°C for 1 min and centrifuged at 15 000 *g* at 5°C for 15 min. The supernatant was collected and added to 2 ml of ethyl acetate before vortexing for 1 min and centrifugation at 15 000 *g* at 5°C for 15 min. The ethyl acetate fractions were combined and evaporated to dryness on a speedvac at 45°C. The residue was dissolved in 0.5 ml of water/acetonitrile (ACN)(1:1 v/v) before SA quantification by high performance liquid chromatography (HPLC) (5 µl of sample was injected). The system consisted of an Agilent 1260 series equipped with an automatic injector and a column (Inertsil ODS-3; 250 × 3.0 mm, 3 µm) oven both thermostated at 30°C. Salicylic acid was detected by a fluorescence detector at a 315 nm emission and a 408 nm excitation wavelengths. The mobile phase was a water/ACN gradient from 10 to 100% ACN and the flow was 1.0 ml min^{-1}. Quantification of SA was performed by external calibration using SA standards with concentrations from 0.78 to 100 µ*M*.

Ethylene quantification

The ethylene production was measured by ethylene detector ETD-300 (Sensor Sense, Nijmegen, The Netherlands) in three replicates according to Cristescu et al. (2002). Three leaves per replicate were placed in glass dishes on two layers of filter paper moistened with 5 ml of water. As a control from the obtained emission rates, the levels of ethylene were measured in a similar dish without leaves. The measurements were conducted in a growth chamber (16 h photoperiod, 200 µmol m^{-2} s^{-1} irradiance, 22°C) in a stop-and-flow mode with each cuvette being alternatively flushed with a flow of 3 l h^{-1} during 22 min.

Polyamine quantification

Free PAs were extracted and dansylated according to Quinet et al. (2014) from approximately 500 mg FW of shoots and 250 mg FW of roots (3 independent samples per condition). Samples were injected onto a Nucleodur C_{18} Pyramid column (125 × 4.6 mm internal diameter, 5 µm particle size; Macherey-Nagel) maintained at 40°C. Analyses were performed by a Shimadzu HPLC system coupled to a RF-20A fluorescence detector (Shimadzu,'s-Hertogenbosch, The Netherlands) with an excitation wavelength of 340 nm and an emission wavelength of 510 nm. The mobile phase consisted of a water/ACN gradient from 40 to 100% ACN and the flow was 1.0 ml min^{-1}.

Reverse transcription-PCR (RT-PCR)

Primers of tomato genes involved in ethylene and polyamine metabolism were designed using Primer 3 software (Rozenand and Skaletsky 2000) (Table S1). Total RNA was isolated from 150 mg of plant material, cDNA was synthetized using 1 µg of total RNA and PCR fragments were amplified (33 cycles) according to Quinet et al. (2014). Expression differences were analyzed by gel densitometry using ImageJ software and expressed as relative values compared to EF1 α expression (peak size of target gene/peak size of EF1 α). Gene expression analyses were repeated three times on two independent cultures and gave similar results.

Statistical treatment

Normality distributions and homoscedasticity were verified using Shapiro-Wilk and Levene's tests respectively and data were transformed when required. Data were analyzed using two-way analysis of variance (ANOVA). The model was defined on the basis of fixed main effects (treatment and duration of stress). When the ANOVA was significant at $P \leq 0.05$, differences between means were scored for significance according to Student-Newman-Keuls test. Data were analyzed using SAS Enterprise Guide 6.1 (SAS 9.4 system for windows). Results are presented as means ± standard errors.

Results

Shoot and root dry weight, water content and stomatal conductance

Plant weight was higher in *S. lycopersicum* than in *S. chilense* mainly regarding shoot growth (Fig. 1A, B, $P < 0.001$). In *S. chilense*, both salinity and exogenous SA application increased shoot DW, compared to control condition (Fig. 1A). Shoot DW was increased in response to SA in the absence of salt compared to controls in *S. lycopersicum* but SA had no impact in salt-treated plants (Fig. 1A). The root DW was lower in *S. chilense* plants submitted to both SA and NaCl compared to the other treatments while none of the treatments affected root growth of *S. lycopersicum* after one week of stress (Fig. 1B). The stomatal conductance (g_s) was differently affected by salt stress in the two considered species (Fig. 1C, $P < 0.0001$). In *S. chilense*, NaCl either applied alone or combined to SA increased g_s by more than 100%, while exogenous SA application alone did not affect g_s. In *S.*

lycopersicum, all treatments decreased g_s compared to controls.

Salinity had no significant impact on the leaf water content (Table 1). Salicylic acid decreased the leaf WC in *S. chilense* and such a decrease was especially conspicuous in the case of the mix treatment (NaCl + SA). Root water content was slightly decreased in response to salt stress in *S. lycopersicum.* Salinity reduced Ψs in leaves of *S. chilense* and *S. lycopersicum* but to a higher extent in the former than in the latter. A decrease in the leaf Ψs was also observed in response to SA in *S. lycopersicum* but not in *S. chilense*. In contrast, the NaCl + SA treatment strongly reduced Ψs value in both species. Salinity reduced the root Ψs but exogenous SA had no impact on this parameter. However, NaCl + SA also reduced Ψs value in *S. chilense* root.

Sodium and potassium concentrations

Salt stress induced an increase of Na^+ in leaves and roots in both species whatever the SA treatment (Fig. 2A, B). In leaves, sodium concentration was higher in *S. chilense* than in *S. lycopersicum* while an opposite trend was recorded for roots (Fig. 2B). Potassium accumulated to significantly higher levels in response to all treatments in the shoots and roots of the salt resistant species *S. chilense* compared to controls (Fig. 2C, D). In *S. lycopersicum*, leaves K^+ concentration was not significantly affected by the applied treatments (Fig. 2C). However, the shoot K^+ concentration was higher in plants treated with SA alone compared to salt stressed plants treated or not with SA (Fig. 2C). At the root level, NaCl + SA treatment significantly decreased K^+ concentration in *S. lycopersicum*. (Fig. 2D)

Endogenous SA concentration

Salicylic acid concentration was higher in the shoots than in the roots (Fig. 3A, B, $P = 0.0009$). The shoot SA concentration was higher in *S. chilense* than in *S. lycopersicum* ($P < 0.0001$, Fig. 3A). After 7 days of treatment, shoot SA concentration increased in *S. chilense* treated with NaCl, SA and combined treatment compared to controls (Fig. 3A) while root SA concentration was not affected by the applied treatments (Fig. 3B). In *S. lycopersicum*, NaCl had no impact on root and shoot endogenous SA concentration which increased in response to exogenous SA application and combined treatment only (Fig. 3A, B). At the shoot level, the highest SA concentration was observed in plants treated with SA alone in both species and addition of NaCl to SA decreased the endogenous SA shoot concentration (Fig. 3A).

Ethylene production

The salt resistant *S. chilense* exhibited a higher ethylene production than the salt sensitive *S. lycopersicum* even in control conditions. Ethylene biosynthesis strongly increased in response to salt, SA and combined treatments in the salt-resistant species *S. chilense* (Fig. 3C). In *S. chilense*, the highest ethylene concentration was observed in response to NaCl and additional exogenous SA application significantly decreased ethylene production compared to plants treated with NaCl alone. *In S. lycopersicum* none of the treatment affected ethylene production.

Polyamine concentration

The leaf PAs concentration was always higher in *S. chilense* compared to *S. lycopersicum* (Fig 6, $P < 0.0001$).

Salt stress decreased Putrescine (Put) concentration of *S. chilense* while exogenous SA increased it compared to controls whatever the plant organ but plants subjected to combined treatment had the same Put concentration than controls (Fig. 4A, B). In *S. lycopersicum*, the shoot Put concentration remained unaffected by the applied treatments (Fig. 4A) while application of SA increased the root Put concentration compared to control and salt stressed plants (Fig. 4B).

Spermidine (Spd) concentration increased in the shoots of *S. chilense* in response to SA treatment only (Fig. 4C). In *S. lycopersicum*, Spd concentration was similar whatever the treatment (Fig. 4C). In the roots, the applied treatments had an opposite effect on Spd depending on the species: Spd content increased in *S. chilense* plants treated with NaCl, SA and NaCl + SA compared to controls while in *S. lycopersicum*, salt decreased the Spd concentration (Fig.4D).

Salt stress induced a conspicuous increase in leaf spermine (Spm) concentration in the halophyte *S. chilense*. Although SA increased leaf Spm in the absence of stress, it reduced it in salt-treated plants. In contrast, the root Spm content was not markedly affected by the treatments in *S. chilense* (Fig. 4E). Similarly, both leaf and root Spm concentrations remained stable irrespective of the treatment in *S. lycopersicum* (Fig. 4E, F).

Expression of genes encoding enzymes involved in ethylene and polyamine biosynthesis

Since SAM is the common precursor of ethylene and PAs, the expression of genes coding for S-adenosyl-1-methionine (SAM) synthase, namely *SAMS1, SAMS2, SAMS3, SAMS4* and SAM decarboxylase (*SAMDC*) were compared in *S. chilense* and *S. lycopersicum.* Transcripts were not detected for *SAMS1, SAMS2* and *SAM3*. In *S. chilense*, NaCl and SA decreased the *SAMS4* expression level compared to the controls and NaCl + SA treated plants in the shoot (Fig. 5A) and all treatments decreased *SAMS4* transcript level in the root (Fig. 5B). In *S. lycopersicum*, salt did not markedly affect *SAMS4* expression but exogenous SA application resulted in an increase in its expression in the shoot, when applied alone, and a decrease in the root (Fig. 5A, B).

The *SAMDC* was expressed to a higher extent in *S. lycopersicum* than in *S. chilense* (Fig. 5C, D, $P < 0.0001$). Both NaCl and SA decreased *SAMDC* transcript level in the shoot of *S. chilense* while the root expression was not affected (Fig. 5C, D). In *S. lycopersicum*, exogenous SA application increased *SAMDC* expression in the shoot when applied alone but the simultaneous presence of NaCl suppressed the response (Fig. 7).

Ethylene biosynthesis

The expression of genes coding for aminocyclopropane-1-carboxylate (ACC) synthase (*ACCS2, ACCS3, ACCS4, ACCS5, ACCS6*) and ACC oxidase (*ACCO0, ACCO1, ACCO3, ACCO4, ACCO5,*

ACCO6, ACCO7) was investigated and their expression levels depended on the species, the treatment, the organ and the considered gene.

Overall, our RT-PCR results showed that NaCl applied alone or in combination with SA increased the total *ACCS* expression level in the leaves of both *S. chilense* (Fig. 6A, $P < 0.0001$) and *S. lycopersicum* compared to the other treatments (Fig. 6C, $P < 0.0001$) mainly due to the expression of *ACCS2* (Fig. 6A) and *ACCS3* (Fig. 6C) respectively. At the root level, there was no significant differences between treatments in *S. chilense* (Fig. 6 B, $P = 0.1220$). In *S. lycopersicum* roots, NaCl and SA decreased the total *ACCS* expression level compared to control while NaCl + SA caused an increase in the expression of two genes (*ACCS3* and *ACCS5*) (Fig. 6D).

Regarding *ACCO* expression, the total transcript levels increased in response to both NaCl and SA in *S. chilense* shoot with the highest level observed for the shoot of plants exposed to NaCl + SA (Fig. 6E, $P < 0.0001$). In *S. chilense* roots, SA applied alone or in combination with NaCl increased the total *ACCO* expression level compared to roots of control or NaCl treated plants (Fig. 6F, $P < 0.0001$), mainly in relation to *ACCO5* and *ACCO7* stimulation. In the shoot of *S. lycopersicum*, the *ACCO* transcript abundance decreased in plants treated with SA but increased in plants treated with SA + NaCl (Fig. 6G, $P < 0.0001$). At the root level, NaCl and SA applied alone decreased the total *ACCO* expression level compared to control and NaCl + SA ($P < 0.0001$) (Fig. 6H).

Polyamine metabolism

Expressions of genes coding for arginine decarboxylase (*ADC1, ADC2*), ornithine decarboxylase (*ODC*), spermine synthase (*Spms*) and spermidine synthase (*Spds*) were compared (Fig. 7).

Total *ADC* expression level decreased in response to NaCl in *S. chilense* (Fig. 7A, B, $P = 0.0009$) and increased in response to SA and NaCl + SA in the shoot (Fig. 7A, $P < 0.0001$). In *S. lycopersicum*, ADC genes were more expressed in NaCl treated plants at the shoot level (Fig. 7C, $P = 0.0329$) while their expression decreased in response to both NaCl and NaCl + SA at root level (Fig. 7D, $P < 0.0001$).

The level of *ODC* expression decreased in *S. chilense* shoot under NaCl and SA conditions but increased under combined treatment relative to controls (Fig. 7A, $P < 0.001$) while it was not affected by the applied treatments at the root level (Fig. 7B, $P < 0.05$). In *S. lycopersicum*, the *ODC* transcript abundance increased with NaCl and NaCl + SA in the shoot (Fig. 7C, $P < 0.0001$) and with all treatment in the roots (Fig. 7D, $P < 0.0001$).

Spms was more expressed in the roots than *Spds* whatever the species (Fig. 7F, H). The *Spms* expression increased in *S. chilense* shoots (Fig. 7E, $P < 0.0001$) but decreased in its roots (Fig.7F, $P < 0.0001$) in response to SA and NaCl + SA. However, the *Spds* expression was not affected by the treatment in *S. chilense* shoot (Fig. 7E, $P < 0.05$) and increased in NaCl treated plants at the root level compared to controls (Fig. 7F, $P < 0.0001$). In *S. lycopersicum* shoots, both *Spms* and *Spds* expression was reduced in NaCl and NaCl + SA treated plants compared to the other treatments (Fig. 7G, $P <$

0.0001, respectively). At the root level, *Spms* expression was highly stimulated in the SA treated plants. (Fig. 7H, $P < 0.0001$) and *Spds* was almost not expressed (Fig. 7H).

Discussion

Solanum lycopersicum and *S. chilens*e exhibited contrasting levels of salt-resistance

Improvement of salt and drought resistance is an important challenge for tomato breeding (Cuartero et al. 2006, Bai and Lindhout 2007). The present work demonstrates that *S. chilense* typically behaves as a halophyte plant species. Salt indeed slightly stimulated shoot growth (Fig. 1) and this was not related to any Na^+ exclusion since this element accumulated to higher concentration in the aerial parts of *S. chilense* comparatively to *S. lycopersicum*. Some transporters play crucial roles in Na^+ homeostasis. High Affinity K^+ Transporter (HKT) are involved in retrieving Na^+ from transpiration stream and Almeida et al. (2014) recently demonstrated that the Na^+ includer behavior of the halophyte *S. pennellii* correlated with a lower affinity of HKT1;2 for Na^+ comparatively to the glycophyte *S. lycopersicum*. Similarly, NHX proteins promote Na^+ accumulation in the vacuole: the corresponding coding genes were shown to be more expressed in the wild salt-tolerant *S. pimpinelifolium* than in the salt-sensitive cultivated species *S. lycopersicum* (Gálvez et al. 2012).

Beside regulation of ion accumulation, adaptation to the water stress component of salt stress also appears as an important determinant of salinity resistance (Flowers 2004, Munns and Tester 2008). Some studies analyzed the genetic basis of drought tolerance in *S. chilense* which occurred as the result of a positive selection (Giombini et al. 2009, Xia et al. 2010, Fischer et al. 2013). We demonstrated here that the water stress resistance of *S. chilense* was associated with a conspicuous capacity of osmotic adjustment in the leaves (Table 1). Such a property probably allowed the plant to keep the stomata open for CO_2 assimilation while the salt-sensitive *S. lycopersicum* adopted a water-saving strategy through a strong decreases in g_s values. A higher leaf dry mass per area (Gharbi, unpublished results) may also help the wild tomato species to allow greater photosynthetic rate under high irradiance and drought conditions occurring in its native area (Muir et al. 2014).

Pillay and Beyl (1990) previously suggested that *S. chilense* displays specific pattern of hormonal responses to abiotic stress but these authors did not consider ethylene. The present study demonstrated that NaCl-treated plants of *S. chilense* increased ethylene production to higher extent than *S. lycopersicum*. In most terrestrial plants, ethylene was until recently considered as a senescing hormone (Ghanem et al.2008, Koyama 2014). Recent studies, however, provided evidences that an optimal ethylene synthesis may be required for salt-resistance processes (Nazar et al. 2014). Yang et al. (2013) argued that ethylene may help to retain K^+ in the shoot rather than limiting Na^+ translocation and our data partly confirmed this view since ethylene overproduction in *S. chilense* was partly correlated with NaCl-induced increase in both shoot and root K^+ concentrations (Fig. 2). Jiang et al. (2013) also recently demonstrated that ethylene may enhance sodium/potassium homeostasis. It might thus be

hypothesized that optimal ethylene synthesis for salt resistance is high in *S. chilense* and it has already been reported to be involved in stress-induced gene expression in this species (Tapia et al. 2005). In the present case, ethylene oversynthesis triggered by NaCl in *S. chilense* appeared to be directly related to *ACCS2* gene induction which was never observed in *S. lycopersicum* under our experimental conditions.

Salicylic acid had different impact on *S. lycopersicum* and *S. chilense*

Salicylic acid is an important determinant for salt stress tolerance in plants. In tomato, it was reported to improve membrane stability (Stevens et al. 2006), to maintain redox homeostasis through glutathione transferase induction (Csizár et al. 2014), to induce abscisic acid accumulation (Szepesi et al. 2009, Horváth et al. 2015), antioxidant enzyme activities (Molina et al. 2002) and photosynthetic performances (Poór et al. 2011). It is therefore noteworthy that endogenous concentration of SA was higher in control and NaCl-treated plants of *S. chilense* than in *S. lycopersicum*. Contradictory information are available regarding SA impact on g_s. Some authors reported that exogenous SA may decrease g_s values (see Miura and Tada 2014 for review) while others reported to increase g_s in salt-treated plants (Stevens et al. 2006, Eraslan et al. 2007). In our study, a NaCl-induced increase in endogenous SA may be, at least partly, related to the above-mentioned NaCl-induced increase in g_s value in *S. chilense*.

An exogenous application of SA may appear as an attempt to confirm or to infirm its putative involvement in salt-resistance mechanisms. It is however based on the assumption that endogenous SA is not sufficient to provide protection to stressed tissues and constitutes the main limiting factor of salinity resistance. In most if not all studies dealing with exogenous application of SA, this compound is applied before stress imposition, as a priming agent allowing hardening process, and the impact on such exogenous application on the endogenous concentration is rarely quantified to check the plant's ability to absorb and accumulate it. In the present case, SA was applied either on unstressed plants, or concomitantly with NaCl. We showed that exogenous SA improved shoot growth in the absence of NaCl only and had no impact on the root system. It is noteworthy that in response to exogenous SA, SA concentration increased in the shoot but not in the roots, thus suggesting that this compound was efficiently absorbed by the root and translocated to the shoot but does not accumulate in the root system. However, the presence of NaCl somewhat impaired SA translocation to the shoot in both species and thus reduced its accumulation.

Jayakannan et al. (2013) demonstrated in Arabidopsis that SA improves salinity tolerance by preventing salt-induced K^+ loss via GORK channel. The situation appeared different in tomato since exogenous SA had no significant impact on Na^+ or K^+ concentration of salt-treated plants, although it significantly improved the K^+ nutrition of *S. chilense* in the absence of stress. In contrast to this limited impact of exogenous SA on ion nutrition, a strong positive effect of SA was recorded on the osmotic adjustment of salt-treated plants, thus suggesting that salt and SA may act in synergy on osmolyte

synthesis.

Salicylic acid has been reported as an effective inhibitor of ethylene synthesis (Tirani et al. 2013, Jayakannan et al. 2015). The present work, however, showed that in *S. chilense* SA increased ethylene synthesis in the absence of NaCl while it decreased it in NaCl-treated plants. However, when all individual plants exposed to a given treatment were considered, a highly significant positive linear correlation was recorded between SA content and ethylene synthesis for *S. chilense* (R^2=0.90 and 0.93 for NaCl and NaCl + AS treatments, respectively). In contrast, no correlation was observed for *S. lycopersicum*, thus suggesting that SA impact on ethylene synthesis may be species specific. Salicylic acid had no obvious impact on *ACCS* and *ACCO* gene expression in leaves of *S. chilense*, except regarding specific *ACCO1* induction.

Salicylic acid impact on polyamine metabolism

Polyamines are present in all organisms and are involved in the control of plant growth and development during the whole cycle, from germination to fruit maturation. Small aliphatic amines (Put, Spd, Spm) are also involved in plant response to abiotic stresses and assume positive functions in free radical scavenging, stabilization of biological membranes, regulation of ion homeostasis and delaying of stress-induced senescence processes (Lutts et al. 2013). In the present study, the shoot polyamine concentrations were always higher in the halophyte *S. chilense* than in the glycophyte *S. lycopersicum*. Salt stress drastically increased endogenous concentration of the tetramine Spm in the former species, but not in the latter. Conversely, NaCl reduced endogenous concentration of the diamine Put. According to Hu et al. (2012), Put accumulation is a negative factor for salinity resistance in tomato, while higher Spd and Spm contents directly assume protective functions. The salt-induced decrease in Put titer in *S. chilense* might be related to a decreased expression of genes coding for ODC, ADC1 and ADC2 while NaCl clearly increased the expression of those genes in *S. lycopersicum*.

Salicylic acid was reported to increase PAs concentration in plant tissues (Németh et al. 2002) and this process may have an impact on the tomato hardening process (Szepesi et al. 2011). In our study, exogenous SA increased endogenous PAs in the aerial part of *S. chilense* while it had almost no impact on *S. lycopersicum*. Comparatively to control plants, SA-treated plants of *S. chilense* increased *ADC1* and *ADC2* expression in the shoot. It may be hypothesized that the produced Put is partly used for Spd synthesis which accumulated to high extent in SA-treated *S. chilense*. Spermidine is expected to assume important roles in tomato salt-tolerance. Transgenic tomato plants overexpressing *MdSPDS1* exhibited higher protection against oxidative stress in the presence of NaCl (Neily et al. 2011) while exogenous application of Spd stabilized the photosynthetic apparatus and prevented thylakoid membrane photodamage induced by salt stress (Hu et al. 2014). This may once again afford an advantage if plants are exposed to NaCl after SA treatment. However, when exogenous SA and NaCl are applied simultaneously, Spd accumulation was not recorded in the shoot, despite *ODC,*

ADC1, ADC2 and *Spds* stimulation, thus suggesting that post-transcriptional parameters may strongly influence PAs metabolism in response to NaCl + SA treatment. In contrast, Spd accumulated in the roots of *S. chilense* in response to NaCl, SA and NaCl + SA treatment, although the mixed treatment strongly inhibited *Spds* gene expression, once again confirming the influence of post-transcriptional regulation processes.

The PAs and ethylene synthesis pathways are sharing SAM as a common precursor. Only *SAMS4* expression was detected in our study and this may be due to the fact that S-adenosyl-L-methionine synthase genes in plant species are strongly developmentally regulated (Gómez-Gómez and Carrasco 1998). In the present study, NaCl reduced both *SAMS4* and *SADMC* gene expression in *S. chilense* suggesting that the observed ethylene and Spm accumulation will not be due to an increase of the expression of these genes. It is well known that the increase of a compound is not always linked to the accumulation of transcripts coding for the enzymes involved in its synthesis and that there are several steps between gene expression and compound synthesis. It might be argued that precursors are produced in the roots and then translocated to the shoots but *SAMS4* expression was also inhibited by NaCl in the roots while *SAMDC* was not affected. The SAMDC activity is frequently considered as an important limiting factor in PAs synthesis (Lutts et al. 2013). The *SAMDC* gene was clearly more expressed in *S. lycopersicum* than in *S. chilense*, while an inverse trend was recorded for PAs accumulation. It may therefore be hypothesized that the corresponding mRNA is more efficiently translated in the halophyte species and/or that the SAMDC enzyme displays strong difference between the two species in terms of substrate affinity or kinetics properties.

Conclusion

Our results confirmed the salt stress resistance of the wild species *S. chilense* compared to *S. lycopersicum* and showed that this salt tolerance may be related to an increase of SA, ethylene, Spm and a decrease of Put production in this former while their production seemed not affected by salt stress in the latter. *S. chilense* displays thus specific pattern of SA, ethylene and PAs responses to salt stress. Oversynthesis of ethylene in response to salt stress in *S. chilense* could be due to *ACCS2* gene induction and Put reduction to the decrease of *ODC* and *ADC* gene expression. Exogenous SA application increased shoot growth in both species and induced ethylene and PAs production compared to controls in *S. chilense* without affecting their production in *S. lycopersicum*. However, when applied simultaneously to salt, its beneficial impact was less obvious and this could be partly explained by the impaired shoot SA translocation and accumulation due to salt stress. Nevertheless, SA application improved the osmotic adjustment of salt-treated plants in both species. In summary, *S. lycopersicum* and *S. chilense* exhibited contrasting levels of salt-resistance and the involvement of SA, ethylene and PAs in this resistance was species specific.

Author contributions

S.L., J.M. and H.B. conceived the study. E.G., M.Q., M.F. performed the experiments and carried out the analysis. E.G., M.Q. and S.L. designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

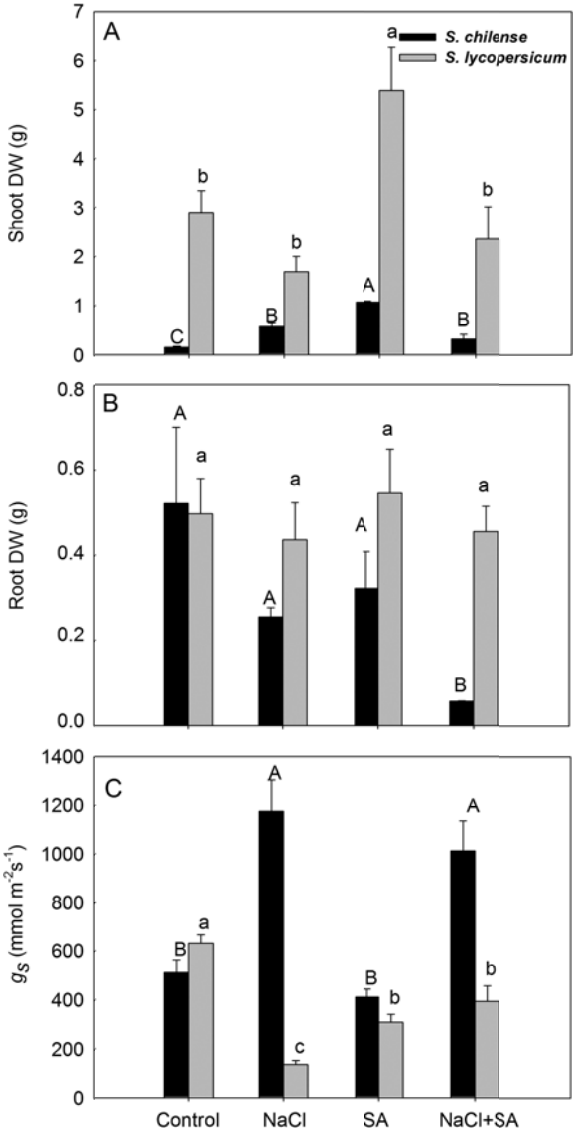

Fig. 1. Plant growth parameters and stomatal conductance (g_s) of *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA): (A) shoot and (B) root dry weight, (C) stomatal conductance. Note that vertical scales are not the same for shoots and roots. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species. Vertical bars are the SE.

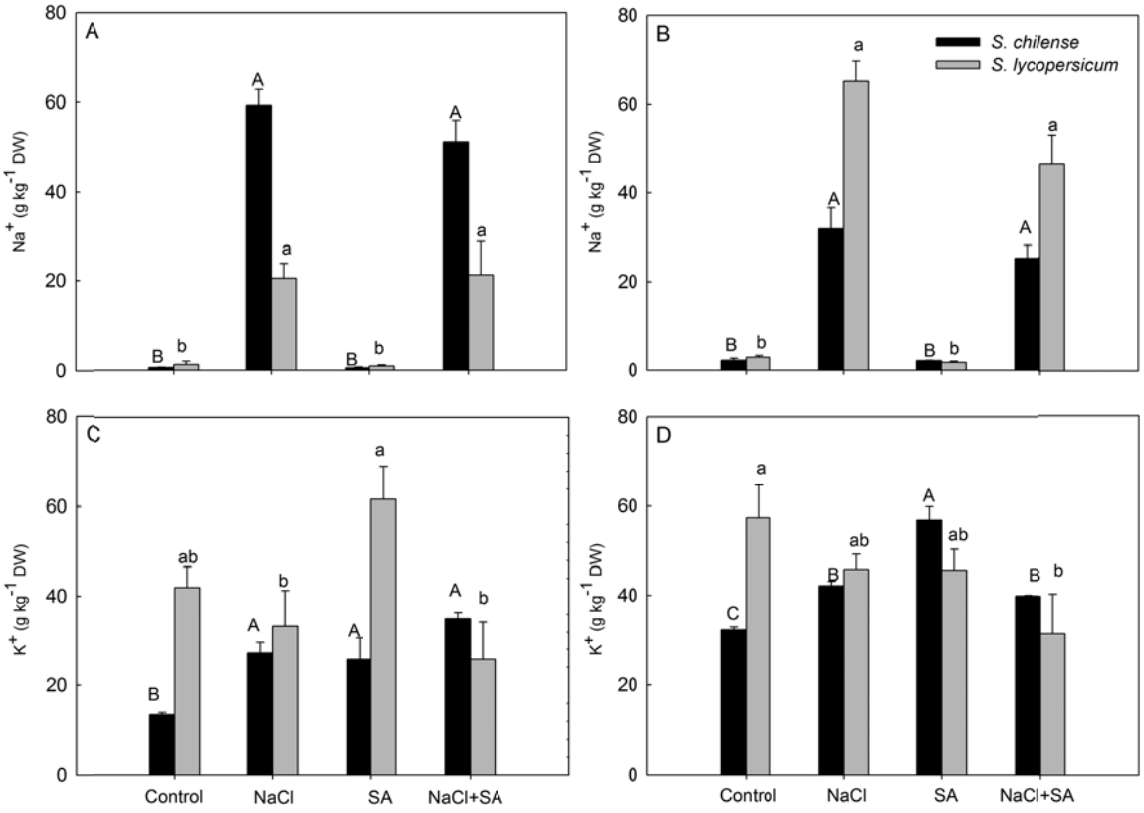

Fig. 2. Sodium (A, B) and potassium (C, D) concentration in the third leaf (A, C) and the roots (B, D) of *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA). Values sharing a common letter are not significantly different at $P < 0.05$ for a same species. Vertical bars are the SE.

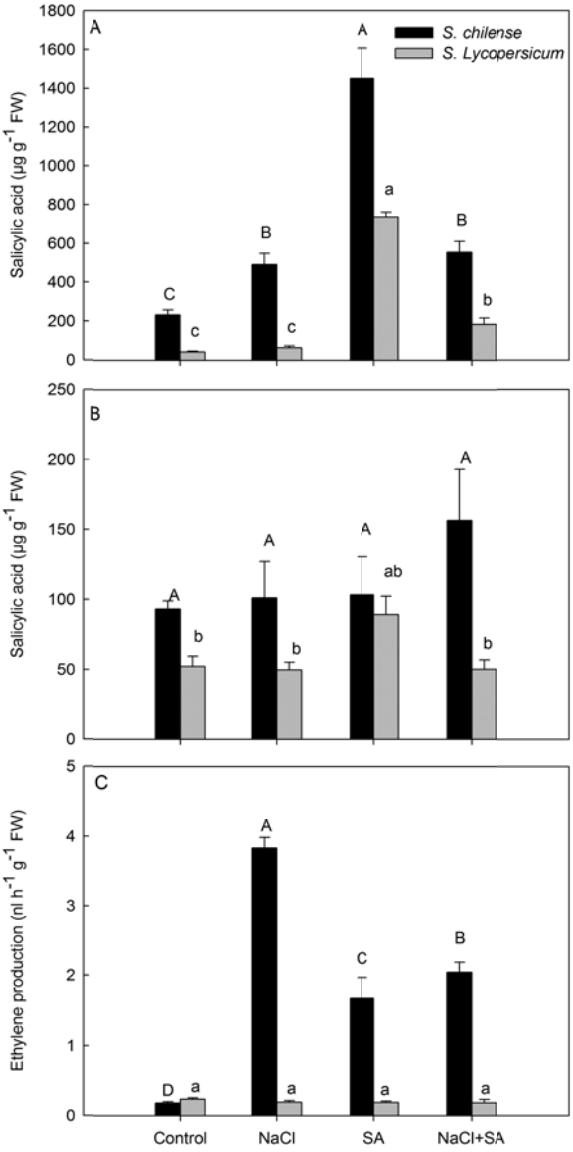

Fig. 3. Endogenous salicylic acid (SA) concentration (A, B) in the fourth leaf (A) and the roots (B) and leaf ethylene production (C) of *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA). Note that vertical scales are not the same for shoots and roots. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species. Vertical bars are the SE.

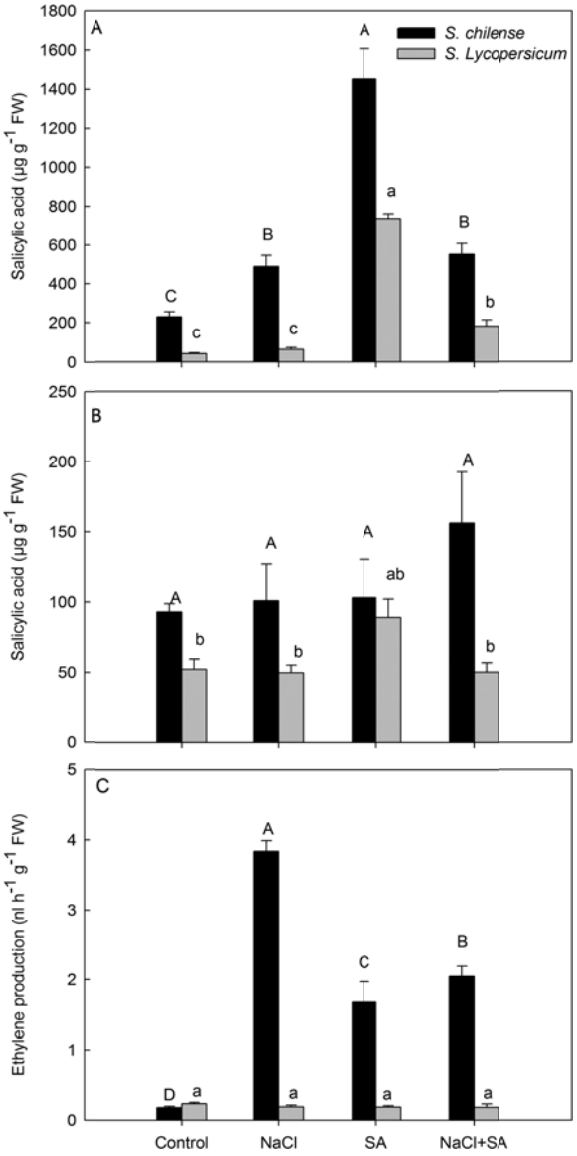

Fig. 4. Endogenous polyamine concentrations in *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA): putrescine (A, B), spermidine (C, D) and spermine (E, F) concentrations in the shoots (A, C, E) and the roots (B, D, F). Values sharing a common letter are not significantly different at $P < 0.05$ for a same species. Vertical bars are the SE.

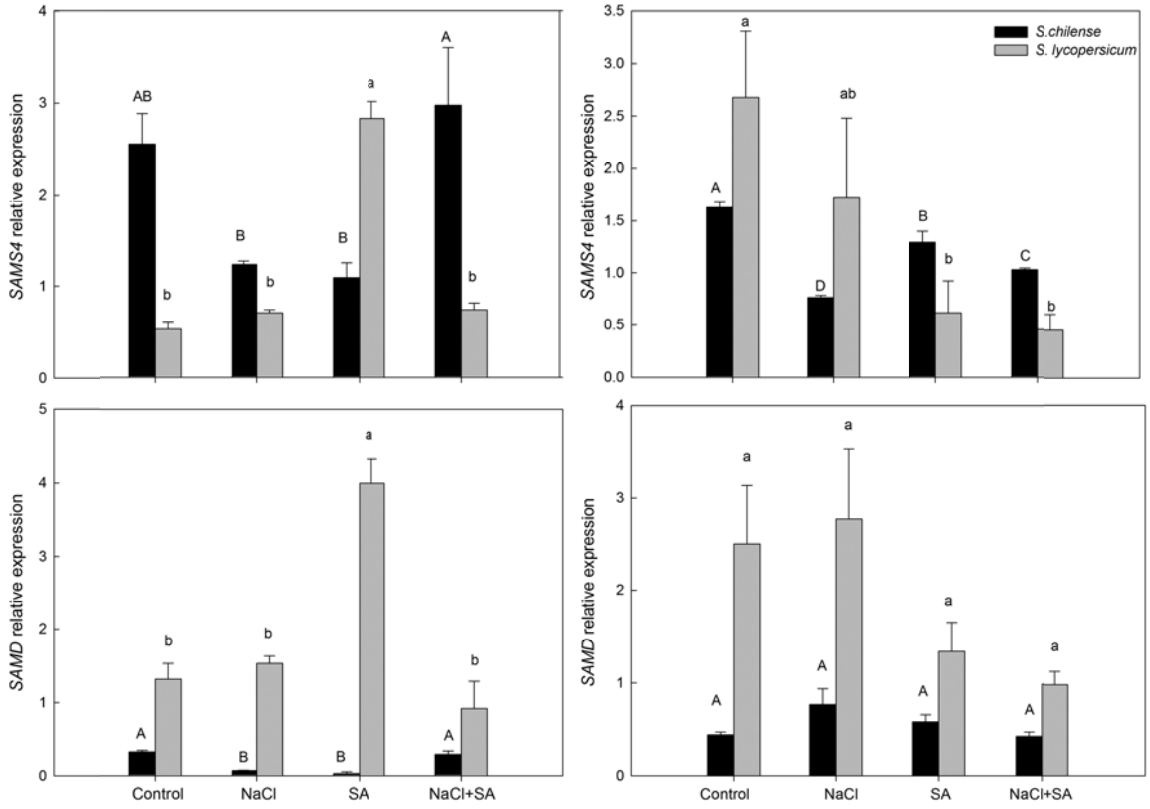

Fig. 5. Semi-quantitative RT-PCR expression analysis of genes coding for (A, B) S-adenosyl-l-methionine synthase (*SAMS4*) and (C, D) S-adenosyl-l-methionine decarboxylase (*SAMDC*) in (A, C) shoots and (B, D) roots of *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA). Actin transcripts were used as a PCR control. Relative expression level was analyzed by gel densitometry. Note that vertical scales are not the same for shoots and roots. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species. Vertical bars are the SE.

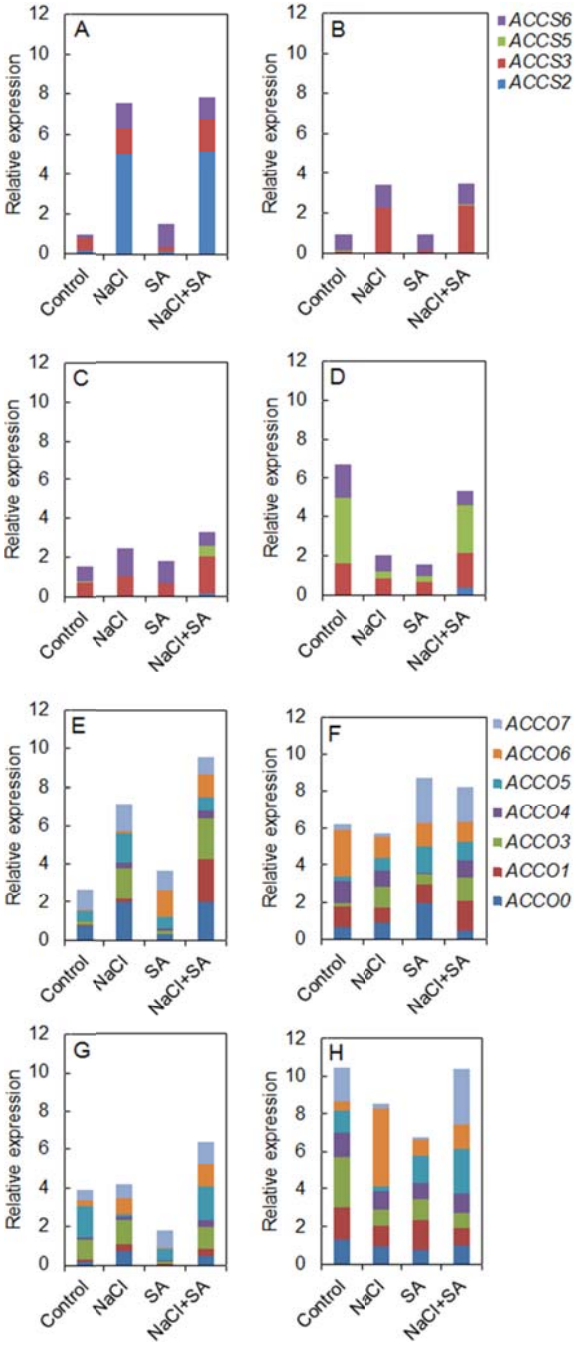

Fig. 6. Semi-quantitative RT-PCR expression analysis of genes coding for (A–D) aminocyclopropane-1-carboxylate (ACC) synthase (*ACCS2,3,5,6*) and for (E–H) aminocyclopropane-1-carboxylate (ACC) oxidase (*ACCO0-7*) in (A, E, C, G) shoots and (B, D, F, H) roots of (A, B, E, F) *Solanum chilense* and (C, D, G, H) *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA- and combined treatments (NaCl + SA). Actin transcripts were used as a PCR control. Relative expression level was analyzed by gel densitometry. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species.

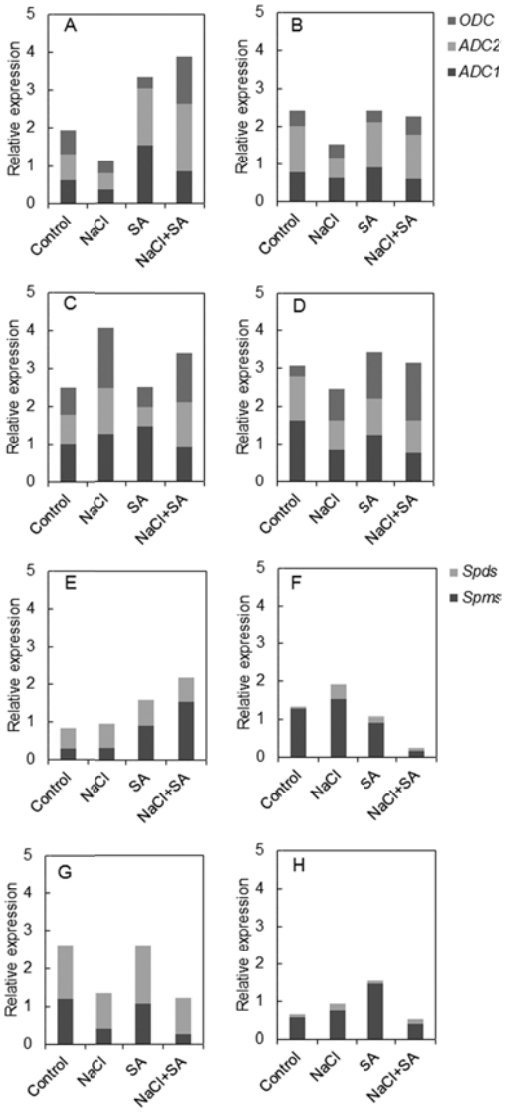

Fig. 7. Semi-quantitative RT-PCR expression analysis of genes coding for (A–D) arginine decarboxylase (*ADC 1,2*) and ornithine decarboxylase (*ODC*) and (E–H) spermine synthase (*Spms*) and spermidine synthase (*Spds*) in (A, C, E, G) shoots and (B, D, F, H) roots of (A, B, E, F) *Solanum chilense* and (C, D, G, H) *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA). Actin transcripts were used as a PCR control. Relative expression level was analysed by gel densitometry. Note that vertical scales are not the same for shoots and roots. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species.

Table 1. Water content (WC) and osmotic potential (Ψ_s) in the leaves and the roots of *Solanum chilense* and *Solanum lycopersicum* cv Ailsa Craig plants exposed during 7 days to control conditions (Control), 125 m*M* NaCl (NaCl), 0.01 m*M* salicylic acid (SA) and combined treatments (NaCl + SA). Mean ± SE. Values sharing a common letter are not significantly different at $P < 0.05$ for a same species and organ.

	Leaves		Roots	
	S. chilense	*S. lycopersicum*	*S. chilense*	*S. lycopersicum*
WC (%)				
Control	94.5 ± 2.1 A	95.0 ± 0.30 a	87.4 ± 1.5 B	95.3 ± 0.28 a
NaCl	95.9 ± 1.6 A	94.2 ± 1.2 a	94.1 ± 1.7 A	93.6 ± 0.33 b
SA	90.3 ± 0.33 A	94.1 ± 1.2 a	94.1 ± 0.16 A	95.9 ± 0.42 a
NaCl + SA	75.6 ± 0.16 B	93.6 ± 0.63 a	95.7 ± 0.13 A	94.2 ± 0.45 b
Ψ_s (MPa)				
Control	−0.69 ± 0.02 A	−0.49 ± 0.02 a	−0.45 ± 0.02 A	−0.42 ± 0.007 a
NaCl	−0.90 ± 0.03 B	−0.79 ± 0.03 c	−0.90 ± 0.01 B	−0.99 ± 0.04 b
SA	−0.72 ± 0.02 A	−0.68 ± 0.01 b	−0.43 ± 0.01 A	−0.39 ± 0.01 a
NaCl + SA	−1.78 ± 0.01 C	−1.25 ± 0.007 d	−0.95 ± 0.003 C	−0.96± 0.02 b