

## Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato

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### Abstract

The effects of salt and water stress on growth and several stress markers were investigated in cherry tomato plants. Some growth parameters (stem length and number of leaves) and chlorophyll contents were determined every third day during plant growth, and leaf material was collected after 25 and 33 days of treatment. Both stresses inhibited plant growth; chlorophyll levels, however, decreased only in response to high NaCl concentrations. Proline contents largely increased in leaves of stressed plants, reaching levels high enough to play a major role in cellular osmotic adjustment. Despite reports indicating that tomato does not synthesize glycine betaine, the stress-induced accumulation of this osmolyte was detected in cherry tomato, albeit at lower concentration than that of proline. Therefore, it appears that the plants are able to synthesise glycine betaine as a secondary osmolyte under strong stress conditions. Total sugars levels, on the contrary, decreased in stress-treated plants. Both stress treatments caused secondary oxidative stress in the plants, as indicated by a significant increase in malondialdehyde (MDA) contents. Water stress led to an increase in total phenolics and flavonoid contents and a reduction of carotenoid levels in the leaves; flavonoids also increased under high salinity conditions.

**Keywords:** proline, glycine betaine, carbohydrates, MDA, carotenoids, phenolics, flavonoids

### Introduction

Tomato is grown in a wide range of climatic conditions, from the tropics to subarctic regions, but its optimal cultivation areas are found in warm and rather dry regions, such as Mediterranean countries (Cuartero and Fernández Muñoz, 1999). In these arid or semi-arid zones, water stress and high soil salinity are common environmental factors that can significantly reduce crop yields. One of the most general responses to drought and salinity - as well as to other abiotic stress conditions, also causing cellular dehydration in plants, such as cold, elevated temperatures or exposure to heavy metals - is based on the synthesis and cytoplasmic accumulation of osmolytes, a conserved phenomenon observed in all plants, tolerant as well as sensitive to stress (Munns and Tester, 2008; Parida and Das, 2005; Parvaiz and Satyawati, 2008). Osmolytes are 'compatible solutes', very soluble, low-molecular-weight organic compounds that do not interfere with normal metabolism even when present at high concentrations. While toxic inorganic ions are sequestered in vacuoles, organic osmolytes accumulate predominantly in the cytoplasm, preventing or limiting cellular dehydration (Büßis and Heineke, 1998; Handa *et al.*,

1986; Stewart and Lee, 1974). Reduction of the osmotic potential due to accumulation of osmolytes in response to stress improves the ability of the plant cells to maintain turgor pressure at low water potentials, which is essential for biological processes such as photosynthesis or cell expansion, as well as for maintaining enzymatic activities (Tyree and Jarvis, 1982). Besides their role in osmotic adjustment, osmolytes act as osmoprotective substances, directly stabilising proteins and cell membranes under dehydration conditions. Osmolytes also protect cells from oxidative stress by inactivating 'reactive oxygen species' (ROS) (Hare *et al.*, 1998; Szabados and Saviouré, 2010).

The amino acid proline (Pro) and glycine betaine (GB), a quaternary amine, are probably the most common compatible solutes synthesised by plants as a response to abiotic stress (Ashraf and Foolad, 2007; Chen and Murata, 2008; Verbruggen and Hermans, 2008). As for other osmolytes, besides their role in osmoregulation, both compounds can act as 'low-molecular-weight chaperons', contributing to maintain the active conformation of macromolecules in stressed plants, and participate in detoxification of ROS. Moreover, Pro and GB seem to be

involved, directly or indirectly, in the regulation of gene expression as signalling molecules, and also serve as metabolites for the cellular storage of carbon and nitrogen during stress, which would be used by the cell once stress has ceased (Munns and Tester, 2008; Szabados and Savouré, 2010). Compatible solutes also include soluble carbohydrates, such as sugars (e.g. sucrose, glucose, fructose or trehalose), sugar alcohols (sorbitol, mannitol, as well as different inositol isomers and derivatives), and the raffinose family of oligosaccharides (Gavaghan *et al.*, 2011; Parida *et al.*, 2002). Although sugars have been shown to act as functional osmolytes in several species, it is not so easy to assess their specific functions in the responses to stress, which can be masked by their multiple additional roles as direct products of photosynthesis, components of the primary metabolism and regulatory molecules (Gil *et al.*, 2013).

A secondary effect of abiotic stresses, including drought and salinity, is the increased generation of 'reactive oxygen species' (ROS), including highly reactive free radicals such as superoxide, singlet oxygen, hydroxyl or perhydroxyl radicals, as well as hydrogen peroxide, molecular oxygen, ozone and other strong oxidant molecules (Apel and Hirt, 2004). ROS are continuously generated by plants as by-products of different metabolic pathways, but under stress their production increases leading to oxidative damage of cellular membranes, proteins, carbohydrates and DNA (Van Breusegem and Dat, 2006). In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., superoxide dismutase, catalase, glutathione reductase, several peroxidases) and non-enzymatic (vitamins C and E, carotenoids, flavonoids and other phenolic compounds etc.) (Apel and Hirt, 2004).

Carotenoids are pigments with several functions in plants, besides their direct role in photosynthesis, including their involvement in the mechanisms of oxidative stress tolerance (Gill and Tuteja, 2010). Phenolic compounds also fulfil multiple roles in plants, as structural components of cell walls, participating in the regulation of growth and developmental processes, as well as in the mechanisms of defence against herbivores and pathogens; in addition, they are involved in the responses of plants to practically all types of abiotic stress: UV radiation, extreme temperatures, mineral nutrient imbalance, drought, salinity, heavy metals and herbicides among others (Cheyner *et al.*, 2013; Gould and Lister, 2006). Flavonoids represent the main and most complex subgroup of polyphenols, including more than 9,000 different compounds with a wide array of biological functions (Di Ferdinando *et al.*, 2012; Pollastri and Tattini, 2011; Treutter, 2005, 2006; Winkel-Shirley, 2002). In the last decade, they have been the object of many studies, not only due to the academic interest in elucidating their multiple functions in plants, but also because their alleged beneficial effects for human health, as powerful dietary antioxidants (Wiseman, 2006).

Responses to salinity in tomato, within its salt-tolerance range under natural conditions, have been extensively studied, but only few authors have analysed its responses to higher salt concentrations (Maggio *et al.*, 2004). Subjecting the plants to 'shock treatments' at very high NaCl concentrations, well above those they encounter in the field, may trigger adaptation mechanisms that cannot be observed at low or moderate salinity levels. In addition, there are only a few

papers reporting the effects of both salinity and drought on the same plant material (Giannakoula and Ilias, 2013). These two adverse environmental conditions, which will worsen as a consequence of climate change, could seriously affect tomato production in Mediterranean countries in the coming years.

The aims of the present study were to analyse the effects on the growth of tomato plants of water and salt stress treatments, applied under different conditions, including high NaCl concentrations beyond the tolerance threshold, to allow detection of time- and salt concentration- dependent effects. These growth responses were correlated with accumulation of several biochemical stress markers, associated to general mechanisms of abiotic stress tolerance: the main osmolytes (proline, glycine betaine and total soluble sugars), responsible for cellular osmotic adjustment; malondialdehyde (MDA), as a marker of oxidative stress; and some non-enzymatic antioxidants (total carotenoids, phenolics and flavonoids), synthesised in response to secondary oxidative stress. The experiments were carried out in cherry tomato, a variety that has not been extensively studied despite its growing commercial interest.

## Materials and methods

### *Plant material*

Cherry tomato (*Solanum lycopersicum* L. var. *cerasiforme*) is clearly differentiated from other tomatoes by the smaller size of its fruits. Plants of this variety are vigorous in their growth and have a higher leaves/fruits ratio; therefore, the fruits receive more photoassimilates, in comparison to larger-size varieties, and have higher sugar and acid contents. Thus, cherry tomatoes are more flavoured and are used mostly for fresh consumption. Seeds of cherry tomato were kindly provided by Pilar Corella, from Rijk Zwaan Ibérica S.A. (Almería, Spain).

### *Plant growth*

The seeds were sown in seed trays containing a mixture of commercial peat and vermiculite (1:1) and were placed in a greenhouse with regulated temperatures ranging between 17 and 23 °C, under a long-day photoperiod (16 h light / 8 h dark). Young plants were transferred to individual pots (11 cm in diameter) on the same substrate 36 days after sowing and were watered for 26 additional days with a standard nutritive solution. Then, salt and water stress treatments started, by watering the plants with increasing NaCl concentrations (150, 300 and 450 mM NaCl in nutritive solution) and by stopping irrigation altogether, respectively; 10 plants were used per treatment. Watering was carried out twice a week by adding 1.5 L of the salt solutions to each tray, which contained 12 standard pots. Control plants were grown in parallel, maintaining the standard irrigation regime with nutritive solution. After starting the treatments, plant height was measured and the number of leaves per plant was counted every three days, to assess the effect of stress on vegetative plant growth. Chlorophyll content was also determined at the same times, using a portable 'Chlorophyll meter' SPAD-502Plus (Konica Minolta).

For each stress treatment, including non-treated controls, leaves in equivalent positions were detached from all plants, 25 and 33 days after starting the treatments, and weighed on a precision balance to calculate the average leaf fresh weight (FW) corresponding to each treatment. Part of the leaf material was stored at -75 °C until further use, and part was dried at 65 °C until constant weight, to measure the average leaf dry weight (DW) for

each treatment. Average leaf 'water content' after each treatment, expressed in percentage, was calculated as  $[(FW - DW) / FW] \times 100$ .

#### *Electric conductivity of the substrate*

Soil  $EC_{15}$  was checked at the beginning and at the end of the treatments. Soil samples were taken from three pots per treatment, air-dried and then passed through a 2-mm sieve.

A soil:water (1:5) suspension was prepared in Milli Q water and stirred for one hour at 600 u/min at 21 °C. Electric conductivity was measured with a Crison Conductivity meter 522 and expressed in dS/m.

#### *Osmolyte contents*

Pro contents were determined according to the method of Bates *et al.* (1973) with minor modifications (Vicente *et al.*, 2004). Frozen plant material (100 mg) was ground to a fine powder in a mortar, in the presence of liquid nitrogen. Extraction was carried out with 3% sulfosalicylic acid and cell debris was removed by filtration. One volume of the filtrate was mixed first with one volume of freshly prepared acid ninhydrin (25 mg/mL ninhydrin in 10.44 M acetic acid and 2.4 M phosphoric acid); then one volume of glacial acetic acid was added, the sample was mixed and incubated at 95 °C for 1 h. After stopping the reaction by cooling the samples on ice, they were extracted with two volumes of toluene. The absorbance of the organic phase was determined at 520 nm, using toluene as a blank. A calibration curve was obtained for each assay, using solutions of Pro in 3% sulfosalicylic acid of known, increasing concentrations and subjected to the same treatment as the samples. Pro contents in the plant samples were expressed as 'μmol Pro per gram of dry weight'.

The extraction and quantification of glycine betaine was performed following the method of Grieve and Grattan (1983) with the modifications proposed by Nawaz and Ashraf (2010). Frozen plant material (100 mg) was ground, using a homogenizer (Ultraturrax) in a volume of 2 mL Milli-Q water and centrifuged for 10 minutes at -4 °C and 13,000 rpm. One mL was removed from the supernatant of each extract and mixed with 400 μL HCl 2N. After stirring, 200 μL of each tube was mixed with 80 μL of potassium iodide and brought to 100 mL with Milli-Q water. The tubes were quickly placed in ice and stirred every 20 seconds for 15 min. After 90 min, 800 μL of cold Milli-Q water and 4 mL of 1,2-dichloroethane - kept in the -20 °C freezer until used - were added to each tube. The samples were vortexed for one minute and allowed to settle until the water and the organic phases separated; finally, 1 mL of the lower aqueous phase was aspirated and its absorbance at 265 nm was measured in a spectrophotometer. A standard curve was obtained for each assay, using glycine betaine solutions in Milli-Q water of increasing concentration subjected to the same treatment as the plant samples. Glycine betaine contents in the plant samples were expressed as 'μmol GB per gram of dry weight'.

Total sugars were quantified according to the method described by Dubois *et al.* (1956). Dried material (0.1 g) was crushed and diluted with 3 mL of 80% methanol, left on an agitator for 20-48 h and then mixed with concentrated sulfuric acid and 5% phenol; finally, absorbance of the samples at 490 nm was measured in a spectrophotometer. Glucose solutions were used as standard and the amount of total soluble sugars in each sample was calculated as 'mg. equivalent of glucose per gram of dry weight'.

#### *Malondialdehyde and non-enzymatic antioxidants*

Malondialdehyde (MDA), total phenolics and flavonoids contents were measured in the same extracts used for total soluble sugars determination (0.1 g of dried material in 3 mL of 80% methanol). MDA, a product of membrane lipid peroxidation, is considered as an excellent marker of oxidative stress (Del Rio *et al.*, 2005) and is routinely used to assess the degree of oxidative damage induced in plants by different types of stress (e.g. Aghaleh *et al.*, 2009; Li *et al.*, 2010). MDA content was determined by the thiobarbituric acid-reactive-substances (TBARS) assay, modified to correct for the presence of interfering compounds, as described by Hodges *et al.* (1999).

Total phenolic compounds were quantified by reaction with the Folin-Ciocalteu reagent, according to Blainski *et al.* (2013). Absorbance was measured at 765 nm and the results expressed in equivalents of gallic acid (mg eq. GA g<sup>-1</sup> DW), used as standard. Flavonoid contents were determined following the method described by Zhishen *et al.* (1999); the absorbance was measured at 510 nm, and the amount of flavonoids was expressed in equivalents of catechin (mg eq. C g<sup>-1</sup> DW), used as standard.

Total carotenoids were measured and quantified following Sims and Gamon (2002): 0.2 g fresh material was crushed and diluted in 80% ice-cold acetone/Tris buffer for 1 hour on a shaker at 4 °C, then centrifuged for 15 min at 3,000 x g. The supernatant was separated and optical density was measured at 663 nm, 646 nm and 470 nm in disposable plastic cuvettes. Total carotenoids values were then converted into μg g<sup>-1</sup> DW.

#### *Statistic analysis*

Data were analysed using the SPSS programme for Windows, v.16.00. Prior to the analysis, the Levene test was applied to check the ANOVA requirements. The significance of differences between treatments was assessed using one-way ANOVA, at 95% confidence level. The post-hoc Tukey test was used to estimate homogeneous groups when more than two samples were compared.

## **Results**

#### *Substrate electric conductivity*

The electric conductivity ( $EC_{15}$ ) of the plants' substrate was determined before and after the stress treatments (Table 1). In the control pots, not treated with NaCl, the  $EC_{15}$  value at the end of the experiment was significantly higher than the initial one; this can be easily explained by accumulation of ions present in the nutritive solution. As expected,  $EC_{15}$  increased even more after watering the plants with NaCl solutions of increasing concentration, in a concentration-dependent manner. The mean  $EC_{15}$  also increased slightly in the substrate of water stressed plants, as compared to the initial value - most likely due to concentration of ions from the nutritive solution - but the difference was not statistically significant.

#### *Stress-induced inhibition of plant growth*

Both salt and water stress negatively affected vegetative plant growth, which was estimated by the increase in plant length and in the number of leaves during the 33 days of treatment (Fig. 1). As compared to the unstressed controls, all NaCl concentrations tested totally blocked growth of tomato plants, with a clear concentration effect: the higher the salt concentration used, the shorter the lag period before growth inhibition was observed

Table 1. Substrate electric conductivity EC<sub>1:5</sub> (dS/m), measured in soil:water (1:5) extracts at the beginning (day 0) and at the end (day 33) of the salt and water stress treatments

Treatment	(day 0)	0 mM NaCl	150 mM NaCl	300 mM NaCl	450 mM NaCl	Water Stress
EC <sub>1:5</sub>	0.21 ± 0.05a	0.94 ± 0.08b	2.56 ± 0.39c	3.37 ± 0.35d	4.78 ± 0.51e	0.38 ± 0.18a

Different lower case letters indicate significant differences between treatments

Table 2. Effects of increasing salt concentration on MDA and non-enzymatic antioxidant levels in plants after 25 days (sampling 1) and 33 days (sampling 2) of treatment

Parameters	Sampling	Salt treatments (mM NaCl)			
		0	150	300	450
MDA (nmol g <sup>-1</sup> DW)	1	145.07 ± 13.00a	163.45 ± 8.60ab	169.03 ± 10.00ab	214.16 ± 2.90b
	2	149.30 ± 7.00a	188.24 ± 10.00b	206.02 ± 4.00bc	237.56 ± 20.00c
Total carotenoids (µg g <sup>-1</sup> DW)	1	648.02 ± 31.00b	613.48 ± 31.00b	546.00 ± 42.00ab	344.37 ± 19.00a
	2	659.53 ± 36.00c	473.01 ± 27.00b	352.98 ± 16.00ab	306.17 ± 18.00a
Total phenolics (mg eq. GA g <sup>-1</sup> DW)	1	9.71 ± 0.17a	11.64 ± 1.60ab	13.05 ± 0.90b	14.70 ± 0.83c
	2	12.41 ± 0.60a	17.09 ± 0.30b	15.85 ± 3.30b	17.64 ± 2.20b
Total flavonoids (mg eq. C g <sup>-1</sup> DW)	1	10.19 ± 0.30a	11.24 ± 0.80b	12.24 ± 1.10b	12.87 ± 0.50b
	2	8.74 ± 0.60a	11.03 ± 1.00b	11.64 ± 0.50b	13.74 ± 0.50c

Values shown are means followed by ± SD (n = 5). Different lower case letters in a file indicate significant differences between treatments, for each compound and sampling; Abbreviations: MDA, malondialdehyde; GA, gallic acid; C, catechin; DW, dry weight

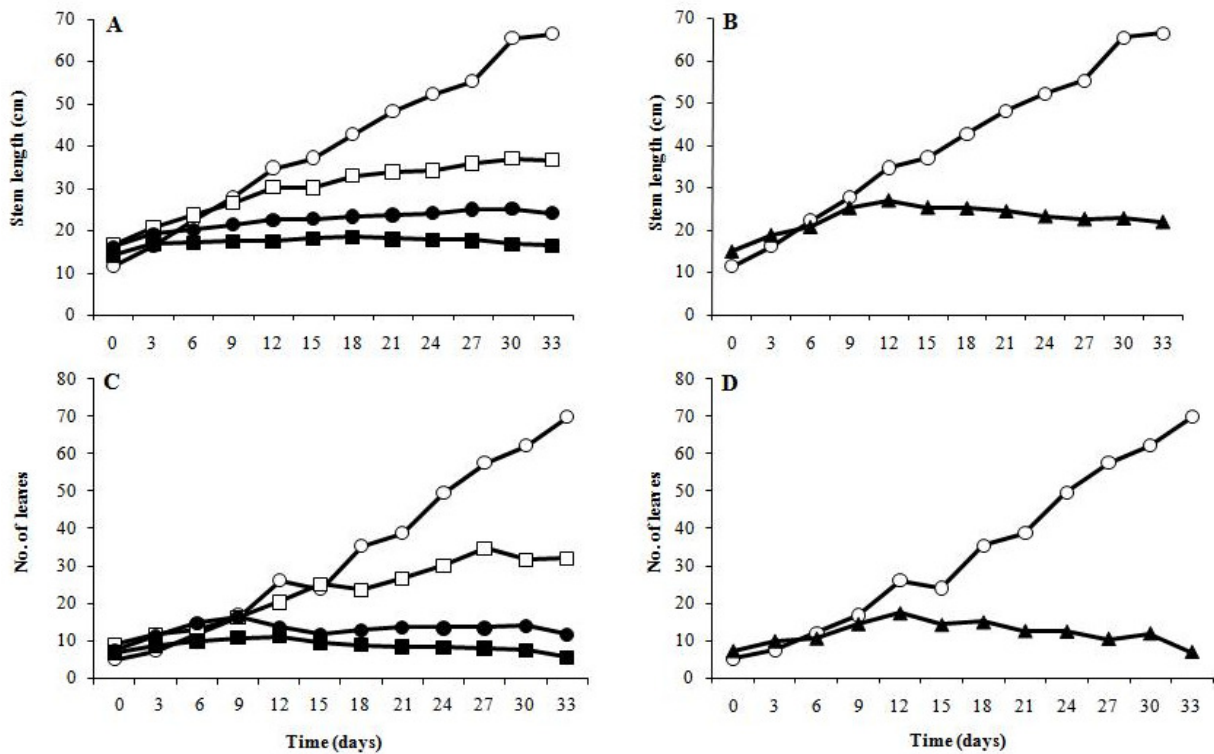


Fig. 1. Mean stem length (A, B) and mean number of leaves (C, D) changes during the salt (A, C) and water (B, D) stress treatments. Control, non-stressed plants (-○-, all panels). 50 mM (-□-), 100 mM (-●-) and 150 mM (-■-) NaCl (panels A, C). Water-stressed plants (-▲-, panels B, D)

(Figs. 1A and C). Water stress also stopped stem growth and the increase in leaf number; this effect was clearly observed 9 days after the last irrigation with nutritive solution, once the substrate had dried (Figs. 1B and D).

Apart from the growth parameters measured continuously, stress-induced inhibition of plant growth was also assessed by determining the mean fresh weight (FW) and dry weight (DW) of the tomato leaves after 25 days (sampling 1) and 33 days (sampling 2) of salt or water stress treatment (Fig. 2). A significant concentration-dependent decrease of FW was observed in salt-treated plants, reaching approximately 75%

reduction in those irrigated with 450 mM NaCl, with respect to the non-treated controls. In the presence of this high salt concentration, the plants were already strongly affected after 25 days of treatment and no significant differences were found between the two samplings; at lower salt levels - 150 or 300 mM NaCl - reduction of FW increased with the duration of the treatment (Fig. 2A). Similar qualitative results were obtained when the plants were submitted to water stress, although with stronger differences between the two samplings: after 25 days without irrigation plant FW was reduced by ca. 25%, as average, but by almost 90% after 33 days of treatment (Fig. 2B). The

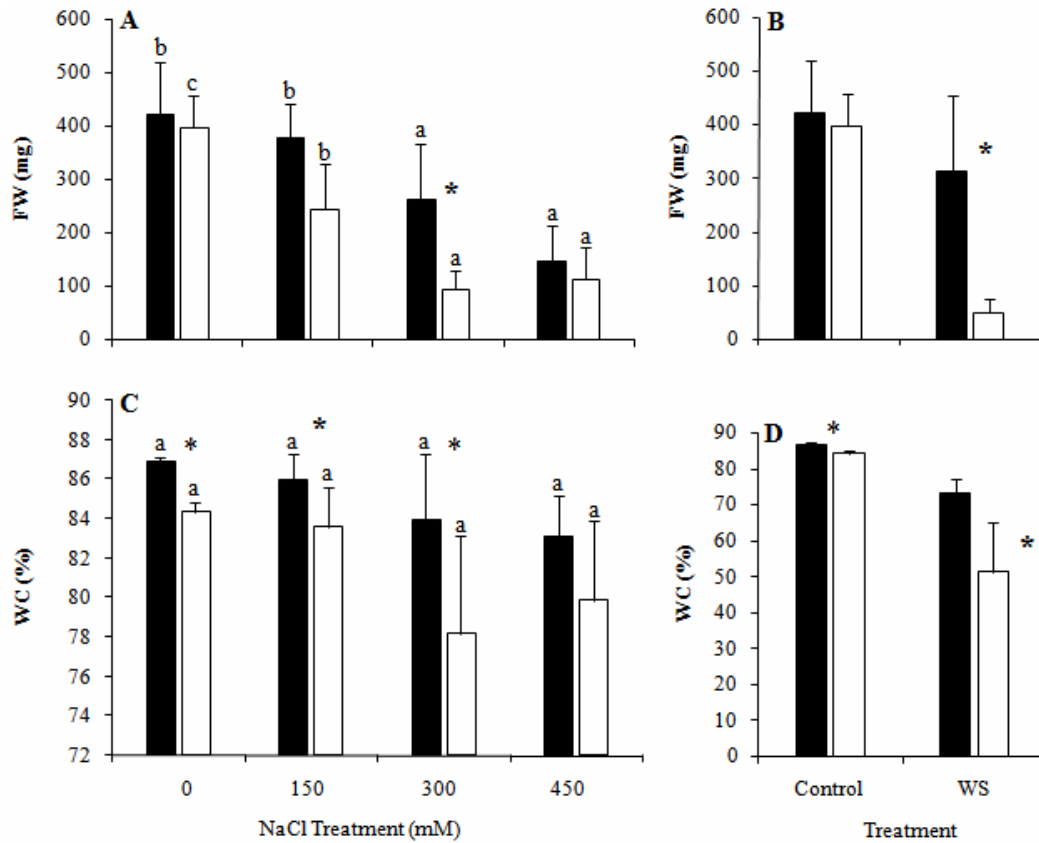


Fig. 2. Average fresh weight (A, B) and relative water content (WC) as percentage of fresh weight (C, D) of tomato leaves after 25 days (sampling 1, black bars) or 33 days (sampling 2, white bars) of salt (A, C) or water (B, D) stress treatments (means  $\pm$  SD,  $n = 3$ ). Different lower case letters indicate significant differences between treatments, for the same sampling, according to Tukey test ( $\alpha = 0.05$ ); asterisks indicate significant differences between sampling 1 and sampling 2, for each treatment

relative decrease in FW under water stress conditions - as compared with the control plants - was not only a consequence of the inhibition of growth, but also due to a significant loss of water, as shown by the calculated water content of the tomato leaves: it was only 50% of the FW for the plants maintained for 33 days without water, while for those irrigated normally with nutritive solution water content was about 85% of the total leaf weight (Fig. 2D). In the presence of salt, a general, but not statistically significant, decrease of the mean leaf water content was observed with increasing NaCl concentrations, both after 25 and 33 days of treatment, whereas significant differences were detected between the samples collected at different times for each NaCl concentration, up to 300 mM (Fig. 2C).

#### Chlorophyll content

The chlorophyll content of crop plants is positively correlated with their photosynthetic activity (Gummuluru *et al.*, 1989) and a reduction of chlorophyll level contributes to the inhibition of photosynthesis observed under abiotic stress conditions. A decrease in chlorophyll levels with respect to the controls was indeed detected in plants irrigated with high salt concentrations - 300 and 450 mM NaCl - for two weeks or longer periods (Fig. 3A). On the other hand, no significant differences with non-stressed plants were observed when a moderate - 150 mM - NaCl concentration was used (Fig. 3A) or when the tomato plants were subjected to the water stress treatment (Fig. 3B); this latter observation was consistent with

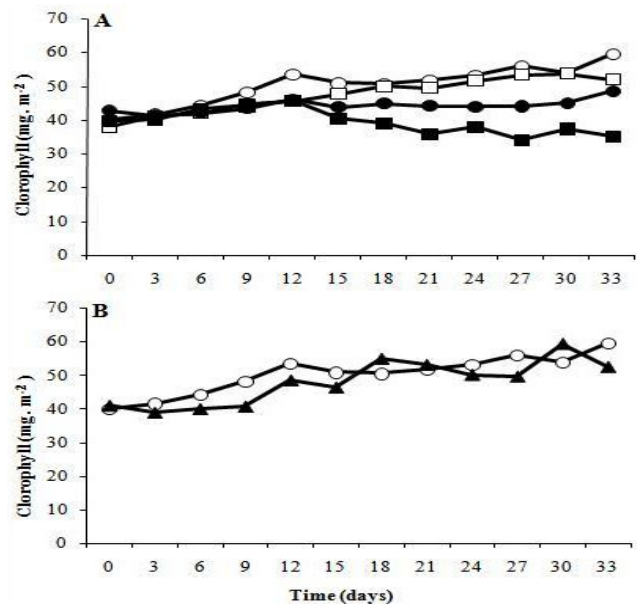


Fig. 3. Mean chlorophyll contents (mg per square meter) measured with a portable chlorophyll meter in leaves of tomato plants during the salt (A) and water (B) stress treatments ( $n = 10$ ). Control, non-stressed plants ( $\circ$ ), in both panels). 50 mM ( $\square$ ), 100 mM ( $\bullet$ ) and 150 mM ( $\blacktriangle$ ) NaCl (panel A). Water-stressed plants ( $\blacktriangle$ , panel B)



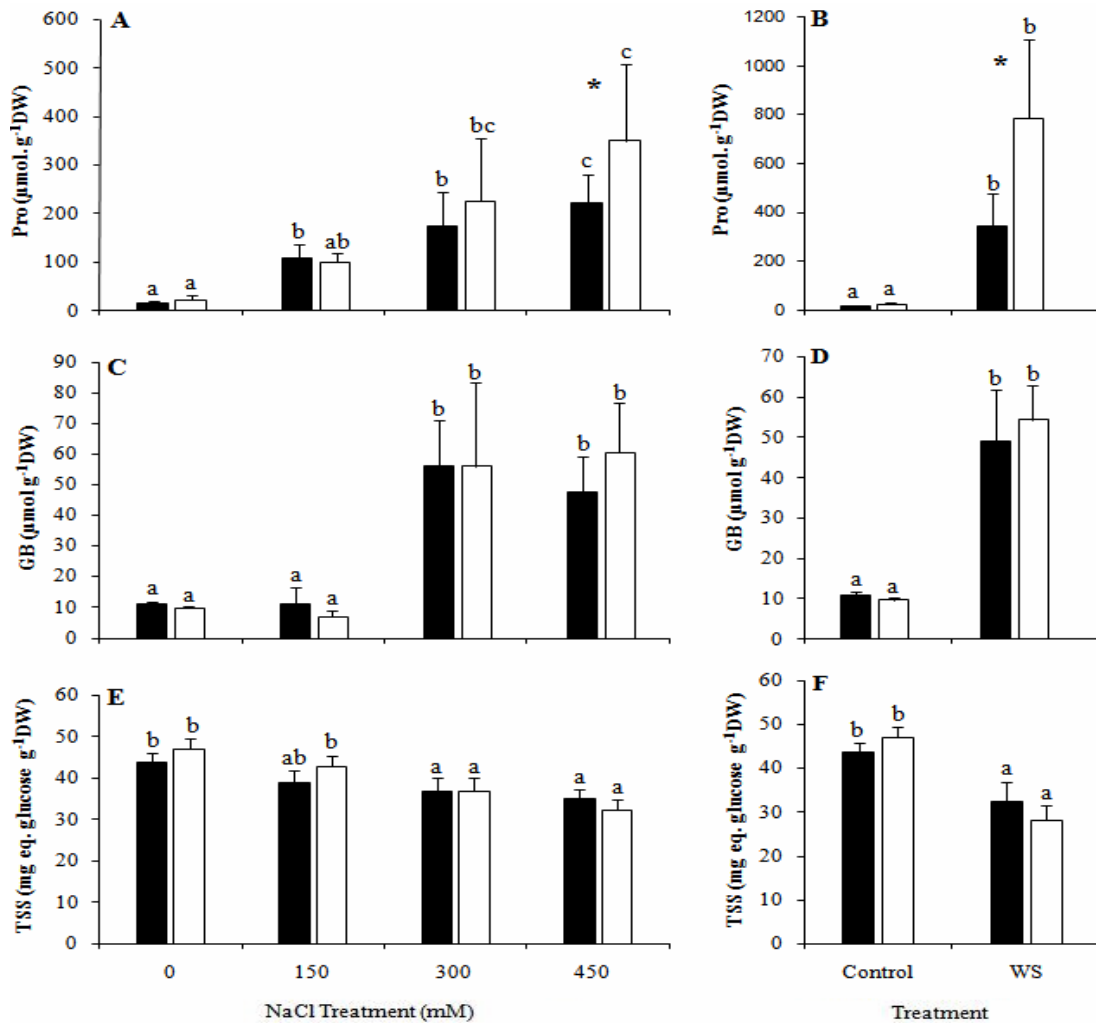


Fig. 4. Proline (Pro,  $\mu\text{mol per gram DW}$ ) (A, B), glycine betaine (GB,  $\mu\text{mol per gram DW}$ ) (C, D), and total soluble sugars (TSS, mg equivalent of glucose per gram DW) (E, F) in tomato leaves after 25 days (sampling 1, black bars) or 33 days (sampling 2, white bars) of salt (A, C, E) or water (B, D, F) stress treatments (means  $\pm$  SD,  $n = 3$ ). Different lower case letters indicate significant differences between treatments, for the same sampling, according to Tukey test ( $\alpha = 0.05$ ); asterisks indicate significant differences between sampling 1 and sampling 2, for each treatment

the visual appearance of the plants, which were clearly wilted, but had not lost their green colour (data not shown).

#### Proline content

Application of salt treatments significantly increased Pro levels in tomato leaves, in a concentration-dependent manner. Mean Pro values in the presence of 300 mM and 450 mM external NaCl also increased with longer times of treatment although the differences observed between the two samplings of plant material were significant only at 450 mM NaCl. Under the strongest salt stress conditions tested, Pro levels reached about 350  $\mu\text{mol per gram DW}$ , which represented an increase of 16-fold with respect to the non-treated controls (Fig. 4A).

Pro accumulation was more clearly observed in plants kept without irrigation, with increases of about 20-fold and 36-fold after 25 and 33 days, respectively, of water stress (Fig. 4B). Pro contents in the plants subjected to the strongest water stress treatment (33 days without irrigation), in terms of dry weight (ca. 800  $\mu\text{mol g}^{-1}$ ) were about double than those determined in plants treated for the same time with 450 mM NaCl (Figs. 4A

and B); however, considering the drastic reduction of water content in non-irrigated plants, it can be said that both treatments induced Pro accumulation to similar levels, in absolute terms.

#### Glycine betaine

Salt and water stress also induced the synthesis and accumulation of GB in tomato (Figs. 4C and D). Yet the absolute values of GB content reached in plants watered with 300 or 450 mM NaCl (50-60  $\mu\text{mol per gram DW}$ ) and its relative increase as compared to the control plants (ca. 5-fold) were lower than those of Pro. Moreover, no time-dependent increase in GB levels was detected in this experiment, as the values measured for the two samplings of plant material were not significantly different (Fig. 4C). The same results were obtained for plants subjected to 25 or 33 days of water stress (Fig. 4D).

#### Total soluble sugars

The total amount of soluble sugars slightly decreased in salt-stressed plants, but significant differences were registered only

starting with 300 mM NaCl in both samplings (Fig. 4E). Under water stress conditions the decrease was also significant and more accentuated after a longer drought treatment (Fig. 4F).

#### Malondialdehyde

MDA is a product of membrane lipid peroxidation and, as mentioned above, is considered a reliable marker of oxidative stress; thus, higher MDA contents should correspond to a higher degree of oxidative stress. As expected, MDA levels increased with all treatments, mostly after longer exposure to stress, so higher values were registered in the second sampling, after 33 days of treatment (Tables 2 and 3). The most affected plants by oxidative stress were those treated with 450 mM NaCl, in which the highest MDA contents were measured (Table 2).

Table 3. Effects of drought stress on MDA and non-enzymatic antioxidant levels in plants after 25 days (sampling 1) and 33 days (sampling 2) of treatment

Parameters	Sampling	Treatments	
		Control	Water stress
MDA (nmol g <sup>-1</sup> DW)	1	145.07 ± 13.00a	173.18 ± 5.20b
	2	149.30 ± 7.00a	198.35 ± 7.60b
Total carotenoids (µg g <sup>-1</sup> DW)	1	648.02 ± 31.00b	474.73 ± 11.00a
	2	659.53 ± 36.00b	357.15 ± 3.60a
Total phenolics (mg eq. GA g <sup>-1</sup> DW)	1	9.71 ± 0.17a	12.10 ± 1.90b
	2	12.41 ± 0.60a	19.48 ± 0.90b
Total flavonoids (mg eq. C g <sup>-1</sup> DW)	1	10.19 ± 0.30b	7.03 ± 0.20a
	2	8.74 ± 0.60b	5.10 ± 0.50a

Values shown are means followed by ± SD (n = 5). Different lower case letters in a file indicate significant differences between treatments, for each compound and sampling; Abbreviations: MDA, malondialdehyde; GA, gallic acid; C, catechin; DW, dry weight

#### Total carotenoids, phenolics and flavonoids

Carotenoid levels in leaves decreased in all treatments with respect to the control. In plants treated with 450 mM NaCl this reduction was of about 50%. When comparing the two samplings, lower values were registered in plants exposed for a longer time to stress in all treatments (Tables 2 and 3). Contrary to the pattern of variation of carotenoids, total phenolics increased in plants under stress, especially in those treated with high salt concentrations. Values were significantly higher in plants from the second sampling than in those from the first one in all stress treatments (Tables 2 and 3). A different response to the type of stress was observed in total flavonoid contents, which increased significantly in the presence of NaCl (Table 2) but decreased under drought conditions (Table 3).

#### Discussion

All our major crops and most wild species are relatively sensitive to environmental stress conditions such as drought or salinity, but tomato is moderately resistant to salt, withstanding a soil EC<sub>sat</sub> up to 6 dS m<sup>-1</sup>, so that it can be cultivated in regions exposed to a certain degree of soil salinisation (Maggio *et al.*, 2004). Yet high soil salinity inhibits seed germination and plant growth, and causes reduction in crop yields (Dalton *et al.*, 1997; Romero-Aranda *et al.*, 2002). In this study, young tomato plants have been grown in the presence of increasing concentrations of NaCl, and a concentration- and time- dependent inhibition of vegetative growth has been observed, which was better shown by the reduction of the fresh weight of the plants; this was not

unexpected, since inhibition of growth is probably the most general response of plants to stress (Munns, 2002; Munns and Tester, 2008). Despite the fact that high salinity should cause cellular dehydration, in the experimental conditions used here the water content of the plants was reduced very slightly in the presence of salt, suggesting that tomato plants activate relatively efficient mechanisms to cope with the osmotic component of salt stress, and that growth inhibition is mostly due to the ionic toxicity of the salt. Water stress also inhibited growth but, contrary to salinity, in this case a severe dehydration of the plants was observed.

Another difference between salt and water stress treatments refers to changes in chlorophyll contents. In plants treated with high NaCl concentrations, a significant reduction in chlorophyll levels, up to ca. 40% of the non-treated controls, was observed. The decrease in chlorophyll levels in plants affected by salt is due to the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Santos, 2004). Yet, reduction of chlorophyll contents is not the only reason for the inhibition of photosynthesis in the presence of salt, since NaCl also inhibits key enzymes involved in this process, such as Rubisco and PEP carboxylase (Soussi *et al.*, 1998). Although water stress should also reduce chlorophyll levels and photosynthetic activity (Alberte *et al.* 1977; Mafakheri *et al.*, 2010; Sanchez *et al.*, 1983), this was not observed in the present study, probably because the time that the plants were maintained without irrigation was not long enough to detect these effects.

Proline is generally considered as a good indicator of environmental stress in tomato (Clausen, 2005) and there are many reports describing an increase in Pro contents as a response to water or salt stress in this species (Babu *et al.*, 2012; Ghorbanli *et al.*, 2013; Giannakoula and Ilias, 2013; Inal, 2002; Handa *et al.*, 1986; Nahar and Gretzmacher, 2002; Umebese *et al.*, 2009; Yokas *et al.*, 2008), although it should be mentioned that data specific for cherry tomatoes are rather scarce (Maggio *et al.*, 2007; Rosales *et al.*, 2007). What is not so clear is the possible contribution of Pro accumulation to the (relative) resistance of tomato to salinity. When comparing tomato cultivars with differences in their degree of salt tolerance, in some cases the more tolerant cultivars were found to synthesise higher amounts of Pro under stress (Ali *et al.*, 2011), but in others there was no correlation between tolerance and Pro levels (Alian *et al.*, 2000). In the present study, a clear positive correlation between Pro accumulation and the intensity of the applied stress treatments has been established; that is, of Pro levels with the electric conductivity of the substrate - reflecting increasing NaCl concentrations in the nutritive solution - and with the time of exposure to stress. Moreover, Pro reached levels high enough to play a significant role in cellular osmotic adjustment under these stress conditions. Taken together, these data strongly support the notion that Pro is the major physiological osmolyte in cherry tomato, as it has been suggested for other tomato varieties (Nahar and Gretzmacher, 2002).

Usually, a given plant species accumulate preferentially a particular type of osmolyte in response to environmental stress; there have even been attempts to use this preference for one specific type of compatible solute as a taxonomic criterion in wild species (Gorham *et al.*, 1980; Tipirdamaz *et al.*, 2006). In agreement with this idea, and since Pro appears to be the major osmolyte in tomato, it is generally accepted that tomato does not accumulate glycine betaine in natural conditions; in fact, it has

been reported that this species lacks two enzymes required for GB biosynthesis (Goel *et al.*, 2011). Nevertheless, exogenous application of GB to tomato plants improves their resistance to drought (Rezaei *et al.*, 2012) and salinity (Chen *et al.*, 2009; Heuer, 2003; Makela *et al.*, 1998). On the other hand, there are some examples of plant species that under severe stress conditions can activate the simultaneous synthesis of several osmolytes to help alleviate the detrimental effects of stress, by contributing to osmotic adjustment and/or acting as 'osmoprotectants', as it has been recently discussed (Tipirdamaz *et al.*, 2006; Gil *et al.*, 2013). This seems to be the case also for tomato, at least for the cherry variety: contrary to the general assumption, in the present study the accumulation of GB, in response to water or salt stress treatments, has been detected in tomato plants. It is true that the maximum levels of GB measured (ca. 50  $\mu\text{mol g}^{-1}$  DW) were about 10-fold lower than those of Pro in the same plants - and also much lower than those measured in taxa that are clear GB accumulators (Boscaiu *et al.*, 2011; Gil *et al.*, 2014). Therefore, GB will have only a modest effect on osmotic balance in stressed tomato plants, but may still contribute significantly to stress resistance due to its putative functions as low-molecular-weight chaperon and/or ROS scavenger. Most published data support the absence of detectable GB in tomato; yet the present results are not the only evidence for accumulation of endogenous GB in response to stress, which has also been reported in plants submitted to chilling, where a threshold level ensures sufficient protection to low temperatures (Park *et al.*, 2006).

The increase of sugars after mild salt and water stress is well-known in tomato fruits, including the cherry variety, and moderate abiotic stress was even recommended as a strategy for improving the quality of tomatoes (Fernández-García *et al.*, 2004; Sgherri *et al.*, 2008; Ullah *et al.*, 1994). Salt stress was found to produce an increase in carbohydrates accumulation, in the form of starch, in the early stages of fruit development, but as soluble sugars in ripe fruits, thus improving the flavour of tomatoes (Yin *et al.*, 2010). There are also several reports of increased amount of sugars in tomato berries under drought (e.g. Nahar and Grezmacher, 2002). However, there are relatively few data published on stress-induced sugar accumulation in vegetative organs in this species, and they generally indicate a reduction of total carbohydrates in leaves (e.g. Amini and Ehsanpour, 2005; Li, 2009), as it has been found in the present study.

In addition to sugars, tomato fruits are rich in several compounds considered as 'health-promoting', such as carotenoids, flavonoids and other phenolics. These secondary metabolites play multiple roles in plants, including scavenging of ROS induced under different stress conditions and causing oxidative stress. A clear symptom of oxidative damage is cell membrane degradation; therefore, MDA - a product of membrane lipid peroxidation - is an excellent marker of oxidative stress (Del Rio *et al.*, 1996). In this study a significant increase of MDA levels in tomato leaves upon salt and water stress treatments of the plants was observed, in agreement with previous reports showing a salt-induced increase of MDA contents in tomato leaves (Shalata and Thal, 1998), roots (Shalata *et al.*, 2001) and fruits (Rosales *et al.*, 2006).

Mild and moderate saline and water stress produce also an increase in carotenoids levels in tomato fruits. Lycopene, the main carotenoid in tomato, is known as an important natural

antioxidant with anti-carcinogenic properties (Krauss *et al.*, 2006). In addition, lycopene is responsible for the red colour of tomatoes, aspect of great economic importance and therefore salt treatments are recommended as an alternative strategy to transgenic crops for obtaining tomatoes with higher amounts of carotenoids (Borghesi *et al.*, 2011). In the present work, carotenoid levels were measured in tomato leaves (not fruits) and a reduction was detected under stress conditions; this is in agreement with previous reports that found a negative correlation between  $\text{Na}^+$  and carotenoid contents in tomato leaves (Juan *et al.*, 2005; Tuna *et al.*, 2014).

Salt stress led to a significant increase of phenolics and flavonoids in leaves of plants submitted to the salt treatment and an enhancement of the former in water-stressed plants. There are many publications reporting an increase in the levels of phenolics and flavonoids in tomato fruits in conditions of abiotic stress, which is a topic of direct interest for human health (e.g. Krauss *et al.*, 2006; Ali and Ismail, 2014); in fact, consumption of tomatoes has been recommended to reduce the risk of cardiovascular diseases and cancer (Holiman *et al.*, 1996). Similar studies on leaf material are much scarcer; for example, Sánchez-Rodríguez *et al.* (2011) found an enhancement of some phenolics and flavonoids under moderate water stress (50% of the field capacity) in the more tolerant cultivars of cherry tomatoes, but a reduction in the more sensitive ones, in partial agreement with the data presented here.

## Conclusions

Salt and water stress treatments inhibited vegetative growth in cherry tomato, a variety that has not been extensively studied yet, despite its growing commercial interest. In non-irrigated plants, a strong dehydration was partly responsible for reduction of leaf fresh weight, an effect not detected in the presence of NaCl. Both stresses led to the accumulation in the leaves of high levels of Pro, which function as the major osmolyte in cherry tomato, responsible for osmotic adjustment under stress conditions. Glycine betaine also accumulated as a response to salt and water stress - albeit at lower levels than Pro - despite numerous publications reporting its absence in tomato plants; therefore, GB appears to act as a secondary osmolyte that could contribute to stress tolerance in cherry tomato. Both stress treatments caused secondary oxidative stress in the plants, as indicated by a significant increase in malondialdehyde (MDA) contents. The increase in antioxidant phenolic compounds levels in leaves can be considered as part of the response induced to cope with oxidative stress. Contrary to what has been reported for tomato fruits, other metabolites such as total soluble sugars or carotenoids do not increase, but rather decrease in leaves in response to the stress treatments.

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