ORIGINAL ARTICLE

# Morpho-physiological and antioxidant response to NaCl-induced stress in in vitro shoots of pomegranate (*Punica granatum* L.)

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## Abstract

The physiological and antioxidant response to salinity was studied in pomegranate (*Punica granatum* L.) by exposing in vitro growing shoots of the Italian variety Profeta Partanna to 125 or 250 mM NaCl for 10 and 20 days. 250 mM NaCl significantly reduced shoot length, leaf area and water content of the shoots, regardless the length of the salt treatment, with respect to the control and to the 125 mM NaCl treatment. After 20 days the shoots treated with 250 mM NaCl also showed a significant reduction in relative growth rate (RGR) together with marked necroses and abscission of the oldest leaves. Salt treatments significantly decreased the contents of chlorophylls and carotenoids in both exposure times, depending on NaCl concentration. Proline, total phenolic compounds and ellagic acid did not increase or even decrease with the salt treatments. The levels of lipid peroxidation decreased, ascorbate peroxidase (APX) activity significantly increased in both treatment times and concentrations, while guaiacol peroxidase (G-POD) activity significantly increased in shoots treated with 250 mM NaCl for 20 days suggesting the rapid involvement of APX in controlling the oxidative stress in this species, even at low salt concentrations, and a delayed complementary role of G-POD.

**Keywords** Ascorbate peroxidase (APX) · Guaiacol peroxidase (G-POD) · Lipid peroxidation · Morphological parameters · Soil salinization

## Abbreviations

| | |
|---|---|
| APX | Ascorbate peroxidase |
| d.w. | Dry weight |
| f.w. | Fresh weight |
| G-POD | Guaiacol peroxidase |
| MDA | Malondialdehyde |
| MKI | McKinney index |
| RGR | Relative growth rate |
| ROS | Reactive oxygen species |
| TBARS | Thiobarbituric acid reactive substances |

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## Introduction

The worldwide extension of salt-affected soils is estimated at about 1 billion hectares and it is a consequence of both natural (primary) and human-induced (secondary) processes (FAO 2015). Soil salinization causes stress conditions to crops by altering the osmotic potential of the soil and limiting water uptake and consequently nutrient availability of the plants (Mahajan and Tuteja 2005). As a consequence, plant growth is highly reduced with severe damage to biodiversity and to yield and food production.

Salt induces alteration in cell metabolism (i.e., inhibition of photosynthetic processes and protein synthesis) and an imbalance of reactive oxygen species (ROS) which deeply affects plant growth (Mittler 2002; Miller et al. 2010; Hossain and Dietz 2016; Forni et al. 2017). Indeed, one of the main detrimental impact of salinity is alteration of redox homeostasis with over production of ROS (Mahajan and Tuteja 2005). ROS detoxification systems include enzymatic and non-enzymatic antioxidant components (Gill and Tuteja 2010). The main hydrogen peroxide-detoxification system in plant chloroplasts is the ascorbate–glutathione cycle, in which ascorbate peroxidase (APX) is the key enzyme, which

utilizes ascorbate as electron donor to reduce $H_2O_2$ to water (Caverzan et al. 2012). Guaiacol peroxidase (G-POD) is a class III plant peroxidase using guaiacol as electron donor to reduce $H_2O_2$ (Hiraga et al. 2001). Many reports evidenced the important role of these antioxidant enzymes in preserving the salt-tolerant species from the oxidative damage by ROS detoxification (Gill and Tuteja 2010; Gupta and Huang 2014).

Non-enzymatic antioxidants include carotenoids, which act as oxidative stress signals, photoprotectants and antioxidants (Nisar et al. 2015), and phenolic compounds, that can play a role in controlling oxidative damage under prolonged salt stress conditions (Rossi et al. 2016). In response to salt stress, to maintain osmotic balance, plants can also accumulate soluble organic compounds, such as proline (Hayat et al. 2012).

In vitro cultures represent a rapid and low-cost approach to study plant stress response, under controlled conditions, in particular for precocious screening of shrubs and tree species (Watanabe et al. 2000; Di Cori et al. 2013).

Pomegranate (*Punica granatum* L.) is a species, native to semitropical Asia, cultivated in the Mediterranean basin, in Southern Asia, in India and in North and South America (Syed et al. 2007; Ferrara et al. 2014). In the last years there has been an increasing market demand for pomegranate, either for fresh minimally processed arils (Sepulveda et al. 2000), or for jams, juices, wine, jellies and snacks (Gumienna et al. 2016), that is supported by the interest of consumers towards the nutritional and the antioxidant properties of the fruit (Teixeira da Silva et al. 2013). Health beneficial effects depend on the presence of different compounds, in particular flavonoids (anthocyanins, catechins, ellagitannins), synthesized in the fruit and in other plant organs (Syed et al. 2007; Adhami et al. 2009; Zarfeshany et al. 2014).

Some recent studies are available on the effects of increasing soil salinity on growth, photosynthetic activity and plant mineral constituents in pomegranate plants growing in vivo in greenhouse or in field (Khayyat et al. 2014; Hasanpour et al. 2015; Mastrogiannidou et al. 2016), and only limited in vitro (El-Agamy et al. 2010). There is an increasing interest towards ancient varieties of pomegranate and the studies on their response to climate-related changes, including also salinity and drought, are fundamental for their preservation, valorization and use in breeding programs (Hummer et al. 2012).

The aim of the research was to improve the understanding of effect of NaCl on growth, chlorophylls and proline content and to determine the role of the antioxidant mechanism to counteract the salinity effect in this species.

## Materials and methods

### Plant material and culture conditions

In vitro shoot cultures of the ancient Italian pomegranate variety Profeta Partanna were established from axillary buds collected from adult plants growing in the field germplasm collection at CREA-OFA in Rome.

Plant material was sterilized by the following protocol: branch segments with at least an axillary bud were placed in 1% benzalkonium chloride for 30 min to remove surface contamination. Then, explants were rinsed and disinfected in three different steps: by immersion in 70% ethanol for 10 min, in sodium hypochlorite solution (1% active chlorine) for 20 min and, finally, in 0.1% sodium merthiolate for 20 min. Explants were rinsed after each step with sterile water. Buds were then dissected under a stereoscope and shoot meristems were taken for in vitro culture initiation.

The shoots obtained were sub-cultured every 20 days in Magenta (Sigma) vessels containing 50 ml of a growth medium (GM) consisting in salts as previously used for hazelnut multiplication phase (Gentile et al. 2016), organic compounds (1.0 mg $L^{-1}$ nicotinic acid, 2.0 mg $L^{-1}$ glycine, 2.0 mg $L^{-1}$ thiamine–HCl and 100.0 mg $L^{-1}$ myoinositol) 30 g $L^{-1}$ sucrose and 5.7 g $L^{-1}$ agar (B&V). GM was also supplied with 0.01 mg $L^{-1}$ indole-3-butyric acid and 0.04 mg $L^{-1}$ 6-benzylaminopurine. The pH of the medium was adjusted to $5.7 \pm 0.01$ before autoclaving. All reagents were purchased from Sigma-Aldrich.

The cultures were maintained at $24 \pm 1$ °C, under 16 h photoperiod and light intensity of 37.5 µmol $m^{-2}$ $s^{-1}$.

For the salt treatments in vitro growing shoots were transferred to Magenta (Sigma) vessels containing GM supplied with 0 (control cultures), 125 and 250 mM NaCl. These treatments were selected as medium and high inducing stress according to unpublished in vitro preliminary studies on this species. Six vessels containing 15 morphologically uniform shoots were used for each treatment and salt exposition time.

Sampling for both morphological observations and physiological analyses was performed 10 and 20 days after transferring to NaCl supplied media. Samples collected for analyses were placed in liquid $N_2$ and stored at $-80$ °C until used.

### Morphological observations and evaluation of chloroses and necroses

Shoots were disposed on a surface and photographs were taken at the beginning of the experiments and after

exposure to NaCl. A software for metric measurement (ImageJ) was used to measure shoot length and leaf size. Leaf area was estimated as media of upper and lower leaves of single shoots. Three samples consisting in 10 shoots were measured.

Water content ($W_{co}$) was calculated according to Zeng et al. (2013): $W_{co}(\%) = ((f.w. - d.w.)/f.w.) \times 100$, where f.w. is fresh weight and d.w. is dry weight of 8 shoots measured after 48 h at 70 °C. Relative Growth Rate (RGR) was calculated according to the formula of Hoffmann and Poorter (2002) with slight modification: RGR = (ln f.w.$_f$ – ln f.w.$_i$) $\times 100/t$, where $t$ is the application time of the treatment (10 or 20 days) and f.w.$_f$ and f.w.$_i$ represent f. w. taken at the end and at the beginning of application of the treatment, respectively.

Evaluation of the severity of visible symptoms (chlorosis and necrosis diffusion) induced by salinity was based on a rating scale, ranking each shoot into ten classes (Table 1) using a modified McKinney index (MKI, McKinney 1923). MKI was calculated according to the following formula: MKI = $\Sigma(n_i \times i)/N$, where $n_i$ is the number of shoots assigned to the class, $i$ is the numeric value of the class, $N$ is the total number of examined shoots at each salt concentration. Data are the medium average value of 15 shoots grown in 3 Magenta vessels.

## Chlorophylls and carotenoids

For each sample 5 shoots were ground in liquid $N_2$ and 200 mg f.w. were suspended in 4 ml of 90% methanol in 15 ml darkened test tubes (Falcon-Italy). The extract was shaken and incubated at 5 °C for 1 h and then centrifuged at 1050 $g$ for 10 min. The absorbance of the supernatant was determined using a spectrophotometer (Varian Cary® 50 UV–vis Spectrophotometer) at 665.2, 652.4 and 470 nm for chlorophyll $a$, chlorophyll $b$ and carotenoids, respectively. The pigment contents were estimated by Lichtenthaler (1987) formulas and expressed as µg mg$^{-1}$ f.w.

**Table 1** McKinney index of plant injury

| Class | Visible symptoms |
| --- | --- |
| 0 | No injury |
| 1 | Partial chlorosis on leaf (up to 50%) |
| 2 | Marked leaf rolling on whole shoot |
| 3 | Up to 10% brownish leaves |
| 4 | Up to 10% brownish on stem and marked leaf chlorosis |
| 5 | Up to 20% brownish on stem and up to 50% brownish leaves |
| 6 | Up to 20% brownish on stem and up to 90% brownish leaves |
| 7 | Up to 90% brownish on whole shoot |
| 8 | Necrosis on apical leaves |
| 9 | Necrosis on whole shoot |

## Proline

Proline was determined by ninhydrin reaction method, according to Bates et al. (1973). Briefly, 5 shoots were ground in liquid $N_2$ and 200 mg f.w. was extracted in 2 ml of 3% sulfosalicylic acid. After centrifugation at 3870 $g$ for 5 min at 4 °C, one milliliter of supernatant was added to 1 ml of 1% acid ninhydrin, e.g., 1% ninhydrin in a solution of glacial acetic acid:6N phosphoric acid (3:2), and 1 ml of glacial acetic acid. Mixture was vigorously shaken and incubated in a water bath at 100 °C for 1 h, then quickly cooled in ice. Proline was extracted with 3 ml of toluene and the absorbance was spectrophotometrically detected at 520 nm. Proline amount was estimated by a calibration curve using proline as standard ($R^2 = 0.998$). Results were expressed as µg/g f.w.

## Lipid peroxidation, ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD)

The degree of lipid peroxidation was evaluated by measuring the amount of thiobarbituric acid reactive substances (TBARS), i.e., malondialdehyde (MDA), in plant tissues, according to the method described by Dhindsa and Matowe (1981), modified by Hodges et al. (1999). Briefly, 5 shoots were placed in a pre-cooled mortar, ground to fine powder in liquid $N_2$ and 200 mg f.w. were extracted in 4 ml of 10% trichloroacetic acid (TCA). The homogenates were centrifuged at 1050 $g$ for 5 min and the supernatant was collected and divided into two parts: 2 ml of 20% TCA containing 0.5% thiobarbituric acid was added to 1.67 ml of the supernatant (mixture A), while 1.67 ml of the other extract fraction was added to 2 ml of 20% TCA (mixture B). The two mixtures were heated at 95 °C for 30 min and then quickly cooled in ice. After centrifugation at 1050 $g$ for 10 min, the absorbance of the supernatant was determined at 400, 532 and 600 nm. MDA equivalents was calculated by MDA extinction coefficient ($1.57 \times 10^5$ M$^{-1}$ cm$^{-1}$) and expressed as nmol of MDA equivalents per g of f.w., according to the formulas of Hodges et al. (1999).

APX activity was determined according to the method of Nakano and Asada (1981), with slight modifications. Briefly, 8 shoots were ground to fine powder in liquid $N_2$ and 300 mg f.w. were extracted in 2 ml of cold extraction buffer (0.2 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0) added with 3 mM EDTA, 0.5 mM ascorbic acid (AA), 1% Triton X-100 and 1% polyvinylpyrrolidone (PVPP). The extracts were centrifuged at 1050 $g$ for 10 min and the supernatants were collected. Enzyme reaction solution contained 1 mM EDTA, 0.5 mM AA, 10 mM H$_2$O$_2$ and 0.1 ml of enzyme extract, in a final volume of 1.5 ml in distilled water. Changes in absorbance of the reaction solution were determined at 290 nm at 25 °C for 100 s. The enzymatic activity was measured by the coefficient of ascorbic acid (2.8 mM$^{-1}$ cm$^{-1}$). The data were

expressed as enzymatic unit ($U_{APX}$) on total protein content, determined according to Bradford (1976).

For G-POD activity determination, 8 shoots were ground to fine powder in liquid $N_2$ and 300 mg f.w. were added to 2 ml of 0.2 M phosphate buffer (pH 7.2) with 1% PVPP and then mixed and incubated at 5 °C for 1 h. After centrifugation at 1050 $g$ for 25 min, the supernatant was collected and kept in ice. The G-POD enzymatic assay solution contained 3 ml of 0.2 M phosphate buffer (pH 6.5), 4.6 mM guaiacol, 0.03 M $H_2O_2$ and 0.2 ml of enzyme extract. Changes in absorbance of the reaction solution at 470 nm for 200 s were determined at 25 °C. The enzyme activity was calculated by the extinction coefficient of oxidized guaiacol (26.6 $mM^{-1}$ $cm^{-1}$) and expressed as enzymatic unit ($U_{G-POD}$) on total protein content, determined according to Bradford (1976).

## Total phenolic compounds and ellagic acid

Phenolic compounds were extracted according to the method described by Legrand (1977) with slight modifications. Briefly, five shoots were ground in liquid $N_2$ and 200 mg f.w. added to 4 ml of 0.1 N HCl. Mixture was incubated at 5 °C for 1 h and then centrifuged at 1050 $g$ for 10 min. Supernatant was collected and the residue was re-soaked in 4 ml of 0.1 N HCl, to complete the extraction. After centrifugation, supernatants were pooled and the phenolic content was estimated according to Booker and Miller (1998). Reaction mixture contained 475 µl of 0.25 N Folin and Ciocalteu's phenol reagent and 50 µl of sample extract, added with 475 µl of 1 M $Na_2CO_3$. The absorbance of the mixture was determined at 724 nm after 1 h at 25 °C in the dark. Total phenolic content was estimated by a calibration curve using chlorogenic acid as standard ($R^2 = 0.994$) and expressed as µg of chlorogenic acid equivalents per g of f.w.

Ellagic acid content was assessed applying the method described by Özer et al. (2007), modified as follows: 8 shoots were dried at 70 °C for 48 h and 40 mg of d.w. were ground and extracted in 2 ml of 80% methanol. After centrifugation at 1050 $g$ for 3 min, supernatant was collected and the residue was re-soaked in 1 ml of 80% methanol and centrifuged as above. Supernatants were pooled and lyophilized. Sample was re-suspended in 2 ml of dimethyl sulfoxide (DMSO) and centrifuged at 1050 $g$ for 3 min. One millilitre of supernatant was added to 40 µl of 37% HCl and 40 µl of 10% $NaNO_2$. Ellagic acid amount was determined by absorbance at 512 nm, detected immediately ($t_0$) and after 40 min at 30 °C. Non-specific absorbance at $t_0$ was subtracted and the ellagic acid amount was calculated by a calibration curve using ellagic acid as standard ($R^2 = 0.992$). Three samples were evaluated for each treatment. Data were expressed as µg of ellagic acid per g of f.w.

## Statistical analysis

Results are expressed as means ± standard error (SE). A randomized block design was applied. Kruskal–Wallis and Mann–Whitney pairwise comparisons test were applied to determine the effects of the salt treatments on morphological parameters and effect of length of the salt treatment (T), of salt concentration (SC) and their interaction was evaluated applying a NP Manova test (Anderson 2001). For physiological analyses were used three samples for each treatment and each exposition time. One-way ANOVA was applied and significant differences were calculated at $P < 0.05$ with Tukey's pairwise comparisons test. Two-way ANOVA was also applied to evaluate effect of T, SC and their interaction. "Past" program was used for all the statistical analyses.

## Results

### Shoot growth and morphology

Shoot growth was determined after 10 and 20 days of treatment. Shoots treated with 125 mM NaCl for 10 and 20 days did not show significant differences in length and number of leaves/shoot compared to the control, while a significant enhancement in number of nodes was detected at the end of the experiments (Table 2). On the other hand, 250 mM NaCl significantly reduced shoot length and number of leaves/shoot after 20 days, with respect to the control and to the 125 mM NaCl treatment (Table 2; Fig. 1). Similar trend was also observed for $W_{co}$ (Fig. 2); moreover, since 10 days of treatment the leaves size was also reduced (Table 3). Concerning RGR (Fig. 3), after 10 days no significant differences were observed between salt-treated shoots and control, while biomass production was significant different between the two salt treatments. After 20 days, shoots treated with 250 mM NaCl showed a significant reduction in RGR, compared to the control and to the shoots treated with 125 mM NaCl. SC and T significantly affected all the above described morphological parameters with the exception of $W_{co}$ for which the effect of the length of the treatment was not significant.

Chlorosis and necrosis diffusion in shoots (Table 4) increased in relation with the exposure time and salt concentrations. In samples treated with 125 and 250 mM NaCl MKI value was 1.61 and 4.44 after 10 days, and 2.88 and 5.43 after 20 days, respectively; no necrosis was detected in shoots treated with 125 mM NaCl. On the other hand, shoots treated with 250 mM NaCl for 20 days showed marked necrosis and abscission of the oldest leaves, while no evident injury was detected in the apical ones (Fig. 1).

**Table 2** Shoot length, number (*n*.) of leaves/shoot and nodes/shoot in pomegranate in vitro shoots treated with 0, 125 or 250 mM NaCl for 10 or 20 days

| NaCl (mM) | Shoot length (cm) | | *n*. leaves/shoot | | *n*. nodes/shoot | |
|---|---|---|---|---|---|---|
| | 10 days | 20 days | 10 days | 20 days | 10 days | 20 days |
| 0 | $4.3 \pm 0.2a$ | $5.2 \pm 0.2a$ | $11.1 \pm 1.1a$ | $14.9 \pm 0.8a$ | $4.9 \pm 0.4a$ | $5.7 \pm 0.2b$ |
| 125 | $4.4 \pm 0.4a$ | $5.9 \pm 0.5a$ | $10.9 \pm 0.9a$ | $14.7 \pm 1.6a$ | $4.9 \pm 0.4a$ | $6.7 \pm 0.3a$ |
| 250 | $3.1 \pm 0.2b$ | $3.0 \pm 0.2b$ | $8.8 \pm 0.7a$ | $8.4 \pm 0.3b$ | $3.7 \pm 0.3b$ | $5.3 \pm 0.2b$ |
| T | * | | * | | * | |
| SC | * | | * | | * | |
| T × SC | – | | – | | – | |

Different letters, within a column, for each treatment time, indicate significant differences at $P \le 0.05$ (Kruskal–Wallis and Mann–Whitney post hoc test).

*SC* salt concentration, *T* time of application

*Significantly different at $P \le 0.05$ (NP MANOVA)

## Chlorophylls, carotenoids and proline

Salt treatments significantly decreased contents in chlorophylls and carotenoids in both exposure times, depending on NaCl concentration (Table 5; Fig. 4). However, no significant differences were observed in the chlorophyll *a*/*b* ratio between salt-treated samples and controls, except for shoots exposed to 250 mM NaCl for 10 days (Table 5). A significant reduction in proline content, compared to the control, was observed after 10 days of exposure in both salt treatments, while after 20 days no significant differences with respect to the control were observed (Fig. 5). SC, T and their interaction significantly affected chlorophylls, carotenoids and proline content.

## Lipid peroxidation and antioxidant enzymes activities

Lipid peroxidation, evaluated by measuring MDA, showed a significant reduction after salt exposure in both treatment times, while no significant differences were observed between the two salt concentrations (Fig. 6).

A significant increase of APX activity, with respect to the control shoots, after 10 and 20 days at both salt concentrations without differences between the salt treatments was also observed (Fig. 7a). Compared to the controls, G-POD activity significantly increased only in shoots exposed to 250 mM NaCl for 20 days (Fig. 7b). SC, T and their interaction significantly affected lipid peroxidation, APX and G-POD.

## Phenolic compounds and ellagic acid

Shoots exposed to 250 mM NaCl for 20 days had a significant reduction of total phenolic compounds respect to the control and to 125 mM NaCl-treated shoots (Fig. 8a).

Concerning ellagic acid (Fig. 8b), after 10 days, a significant decrease of the content was found in shoots treated with

250 mM NaCl as compared to those treated with 125 mM NaCl but not with respect to the control; after 20 days a significant reduction in the ellagic acid content was observed in 250 mM NaCl-treated shoots with respect to the control. SC and T significantly affected total phenolic compounds and ellagic acid content.

## Discussion

Accumulation of salts in the soil strongly reduces natural vegetation, biodiversity, and agricultural production, thus understanding the response of plants to salt stress is critical for a sustainable management of saline environments through selection and breeding of salt-tolerant crop varieties (Cuartero et al. 2006; Hanin et al. 2016). High salt amount in the soil hinders the plant uptake of water as well as of nutrients and, therefore, salt stress results in a loss of intracellular water, in a general water-deficit condition of the plant and in reduction of leaf size and inhibition of shoot growth (Bartels and Sunkar 2005; Mahajan and Tuteja 2005; Munns and Tester 2008; Forni et al. 2017). Various physiological and biochemical mechanisms are adopted by plants to face high salt conditions, including growth reduction, biosynthesis of osmoprotectants and antioxidant compounds, enhancement of the activity of antioxidant enzymes (Gupta and Huang 2014). Several in vivo studies have been performed to study the response in different species and in vitro cultures were also used to study salt effects, in controlled conditions, on ecophysiological parameters in woody species, such as myrtle and cherry (Di Cori et al. 2013; Erturk et al. 2007). In our study, we investigated the adaptive response of pomegranate to NaCl by evaluating the effect of application of 125 or 250 mM NaCl on in vitro growing shoots for 10 or 20 days.

The 250 mM NaCl treatment applied for 20 days significantly reduced shoot length and RGR and development of necroses and abscission of the oldest leaves were observed. Reduced leaf size was observed after 10 days of treatment
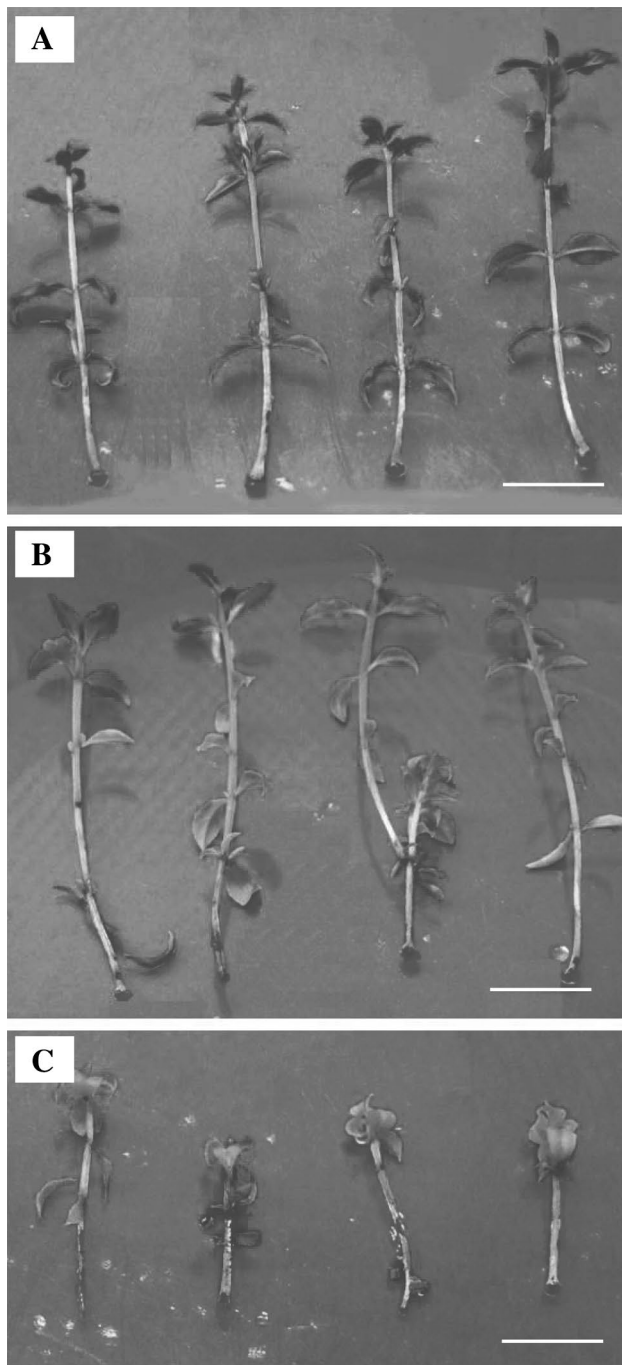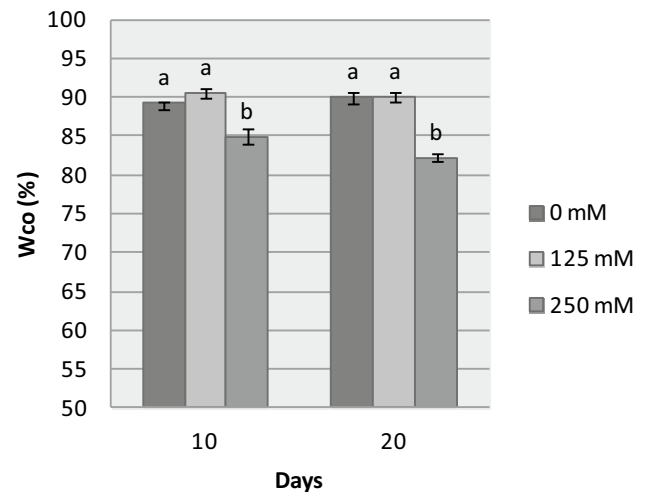
Fig. 2 Water content ($W_{co}$) of pomegranate in vitro shoots exposed to 0, 125 or 250 mM NaCl for 10 and 20 days. Different letters within the same treatment time indicate significant differences at $P \leq 0.05$ (Kruskal–Wallis and Mann–Whitney test). Salt concentration (SC) effect was significant, while time of application (T) and SC × T did not significantly affect $W_{co}$ (NP MANOVA)

**Table 3** Leaf size in pomegranate in vitro shoots treated with 0, 125 or 250 mM NaCl for 10 and 20 days

| NaCl (mM) | Leaf area (cm$^2$) | |
|---|---|---|
| | 10 days | 20 days |
| 0 | $0.1121 \pm 0.0154a$ | $0.1189 \pm 0.0258a$ |
| 125 | $0.0808 \pm 0.0119a$ | $0.0758 \pm 0.0265a$ |
| 250 | $0.0463 \pm 0.0132b$ | $0.0163 \pm 0.0025b$ |
| T | * | |
| SC | * | |
| T × SC | – | |

Different letters within a column indicate means significantly different at $P \leq 0.05$ (Kruskal–Wallis and Mann–Whitney post hoc test).

*Significantly different at $P \leq 0.05$ (NP MANOVA)



**Fig. 1** Pomegranate in vitro shoots exposed to 0 (**a**), 125 mM (**b**) or 250 mM (**c**) NaCl for 20 days. Bar = 1 cm

only at 250 mM NaCl. These responses suggest, in agreement with data on other species reviewed by Munns and Tester (2008), that "Profeta Partanna" is tolerant to salt-induced osmotic stress.

NaCl has a detrimental effect on the photosynthetic activity, in terms of pigments stability, stomatal functioning, thylakoid membranes integrity and gas exchange

(Tavakkoli et al. 2011). Reductions of pigment content, as response to salinity, was already shown in in vivo studies on pomegranate (Mastrogiannidou et al. 2016) and in in vitro studies in myrtle (Di Cori et al. 2013). Our data confirmed this behavior, in fact chlorophyll *a* and *b* decreased in the presence of salt, in manner related to NaCl concentration and length of exposure. Although these observations could seem in contrast to the suggestion of salt tolerance response coming from the above-reported morphological results, it is noteworthy that Mastrogiannidou et al. (2016) detected in pomegranate cv. Wonderful a reduced chlorophyll concentration accompanied by an increase of activity of the existing chlorophyll amount. Further studies on rate of photosynthesis under salt stress
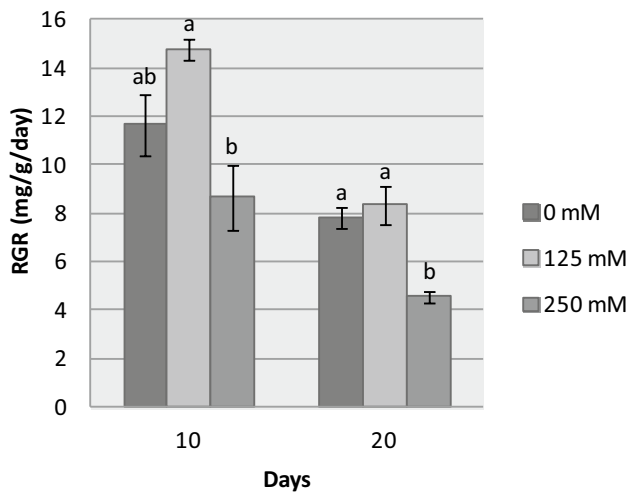
**Fig. 3** Relative growth rate (RGR) of the pomegranate in vitro shoots exposed to 0, 125 or 250 mM NaCl for 10 and 20 days. Different letters within the same treatment time indicate significant differences at $P \leq 0.05$ (Kruskal–Wallis and Mann–Whitney test). Salt concentration (SC) and time of application (T) significantly affected RGR, while SC × T effect was not significant (NP MANOVA)

**Table 4** Effects of salt on leaves determined by McKinney index of the pomegranate in vitro shoots exposed to 0, 125 or 250 mM NaCl for 10 and 20 days

| NaCl (mM) | McKinney index | |
|---|---|---|
| | 10 days | 20 days |
| 0 | 0.00 | 0.19 |
| 125 | 1.61 | 2.88 |
| 250 | 4.44 | 5.43 |

are planned to confirm this behavior also for "Profeta Partanna".

Water loss due to stress conditions can be counteracted by the accumulation of several compatible solutes, such as proline, sucrose, polyols, trehalose or glycine betaine that preserve cell turgor (Mansour 2000; Tuteja 2007; Chaves et al. 2009). Even though several studies reported the accumulation of proline in response to salt stress (Verbruggen and Hermans 2008; Huang et al. 2013; Wang et al. 2015), the significance of such accumulation in response to saline condition is still debated and varies according to the species (Verbruggen and Hermans 2008). A study performed in vivo on Iranian pomegranate cultivars reported an increased proline accumulation related to the cultivar (Khayyat et al. 2014). However, in the variety we studied, proline level did not increase in shoots exposed to salt and, considering the tolerant response to salinity in the morphological parameters above reported, we postulate that proline does not play a critical role in osmotic balancing of this genotype, as also previously observed in myrtle (Di Cori et al. 2013) and in some rice and wheat cultivars, where the level of proline was not found to be related to salt tolerance (Lutts et al. 1996; Poustini et al. 2007). The mechanism of proline accumulation are not fully understood, and in some cases other molecules can contribute to the total osmotic potential in glycophytes expose to saline environments (Cram 1976; Forni et al. 2017), thus the involvement and accumulation of other osmolytes, to be still investigated, cannot be excluded.

An increase of ROS in chloroplasts, mitochondria and peroxisomes is an essential step for plants to perceive and control salt stress; in fact, ROS act, at low concentrations, as secondary cell messengers for hormonal responses, but, at high concentrations, can induce oxidative damage to lipids,
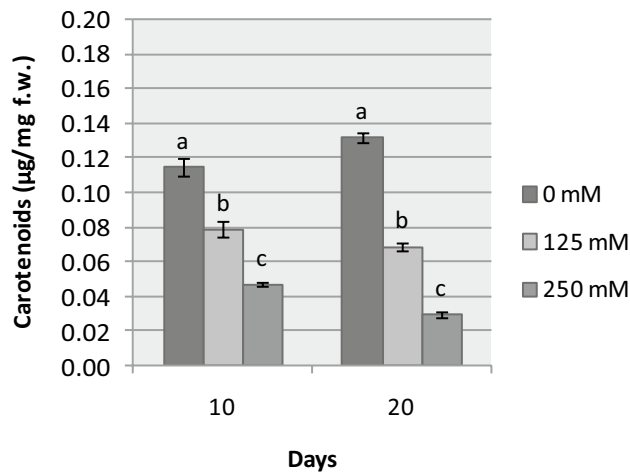
**Table 5** Chlorophylls content and Chl *a*/Chl *b* ratio of pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days

| NaCl (mM) | Chl *a* | Chl *b* | Total chlorophyll | Chl *a*/Chl *b* |
|---|---|---|---|---|
| *10 days* | | | | |
| 0 | 0.440 ± 0.021a | 1,0611 ± 0.0080a | 0.5933 ± 0.0292a | 2.8849 ± 0.0369a |
| 125 | 0.269 ± 0.018b | 0.1017 ± 0.0034b | 0.3704 ± 0.0210b | 2.6366 ± 0.1007a |
| 250 | 0.155 ± 0.001c | 0.0662 ± 0.0015c | 0.2214 ± 0.0025c | 2.3472 ± 0.0405b |
| *20 days* | | | | |
| 0 | 0.514 ± 0.017a | 0.178 ± 0.007a | 0.692 ± 0.030a | 2.893 ± 0.031a |
| 125 | 0.233 ± 0.010b | 0.078 ± 0.004b | 0.312 ± 0.014b | 2.977 ± 0.049a |
| 250 | 0.106 ± 0.002c | 0.045 ± 0.006c | 0.151 ± 0.007c | 2.741 ± 0.127a |
| T | * | * | * | * |
| SC | * | * | * | * |
| T × SC | * | * | * | * |

Different letters, within a column, for each treatment time, indicate means significantly different at $P \leq 0.05$ (One-way ANOVA and Tukey's test).

*SC* salt concentration, *T* time of application

*Significantly different at $P \leq 0.05$ (Two-way ANOVA)

**Fig. 4** Carotenoids content in pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days. Different letters within the same treatment time indicate means significantly different at $P \leq 0.05$ (one-way ANOVA and Tukey's test). Salt concentration (SC), time of application (T) and SC × T significantly affected carotenoids content (two-way ANOVA)
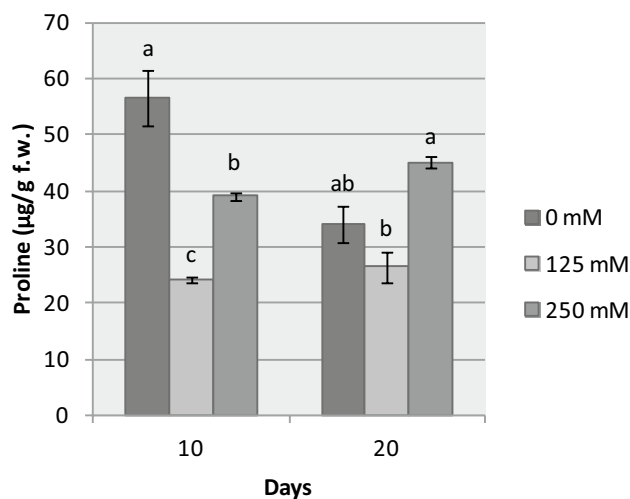


**Fig. 6** Lipid peroxidation (TBARS expressed as MDA content) in pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days. Different letters within the same treatment time indicate means significant different at $P \leq 0.05$ (One way ANOVA and Tukey's test). Salt concentration (SC), time of application (T) and SC × T significantly affected lipid peroxidation (two-way ANOVA)



**Fig. 5** Proline content in pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days. Different letters within the same treatment time indicate means significantly different at $P \leq 0.05$ (one-way ANOVA and Tukey's test). Salt concentration (SC), time of application (T) and SC × T significantly affected proline content (two-way ANOVA)

Neto et al. 2006; Niknam et al. 2011). To maintain ROS within safe levels plants use metabolites and antioxidant enzymes including superoxide dismutase, APX, G-POD, catalase and glutathione reductase (Sharma et al. 2012).

APX uses ascorbate as the electron donor in the first step of the ascorbate–glutathione cycle and is considered the most important plant peroxidase in $H_2O_2$ detoxification (Azevedo Neto et al. 2006; Caverzan et al. 2012). G-POD removes $H_2O_2$ by guaiacol as electron donor (Mehlhorn et al. 1996). Previous studies reported an important role of G-POD and APX in response to salinity in myrtle (Di Cori et al. 2013), rice (Khan and Panda 2008), wild salt-tolerant tomato species (Mittova et al. 2004) and wheatgrass (Sheikh-Mohamadi et al. 2017).

In the present study at both treatment times and concentrations, MDA level showed a significant reduction and APX activity increased; shoots, when exposed to the highest NaCl concentration for 20 days, also showed a significant increase of G-POD activity. These results suggest the rapid involvement of APX in the antioxidant response, even at low salt concentrations, as observed in other species (Di Cori et al. 2013; Mittova et al. 2004), and a possible complementary delayed role of G-POD in controlling the oxidative stress induced by high salt concentrations. The detected reduction of MDA level is consistent with previous studies which found that increased activities of ROS scavenging enzymes, like APX and POD, maintain lipid peroxidation unchanged or even reduce it in salt-tolerant cultivars exposed to NaCl, e.g., tomato, rice and olive (Shalata et al. 2001; Mittova et al. 2004; Khan and Panda 2008; Mishra et al. 2013;

proteins and nucleic acids (Mittler 2002; Apel and Hirt 2004; Mittova et al. 2004; Møller et al. 2007; Jaspers and Kangasjärvi 2010; Sharma et al. 2012; Golldack et al. 2014; You and Chan 2015; Choudhury et al. 2017). The level of MDA, a product of lipid peroxidation of polyunsaturated fatty acids of the membrane, is considered an indicator of oxidative damage and it has been utilized to characterize genotypes response to salinity in several species (Azevedo
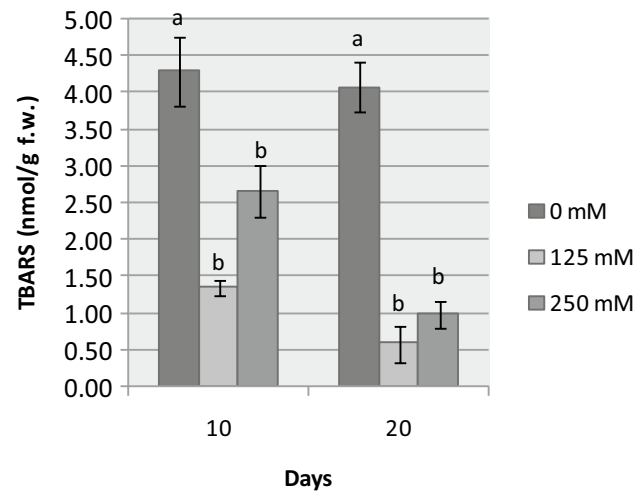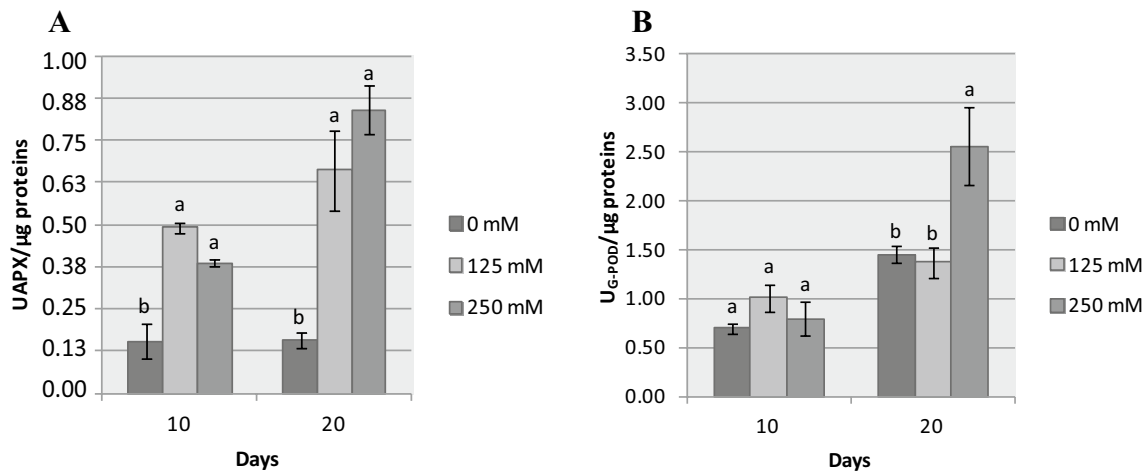
**Fig. 7** APX (**a**) and G-POD (**b**) activities of pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days. Different letters within the same treatment time indicate means signifi- cantly different at $P \leq 0.05$ (one-way ANOVA and Tukey's test). Salt concentration (SC), time of application (T) and SC × T significantly affected APX and G-POD activity (two-way ANOVA)



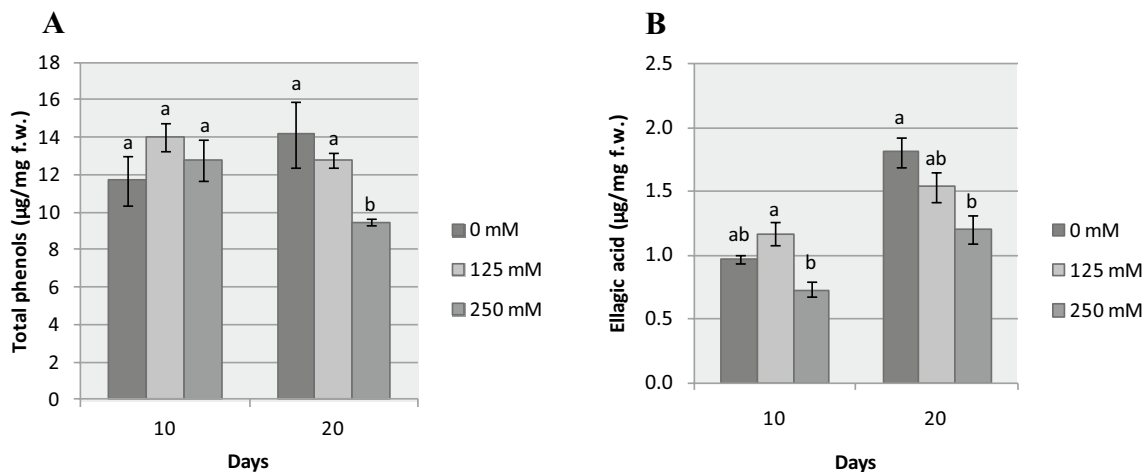**Fig. 8** Total phenolics (**a**) and ellagic acid (**b**) content in pomegranate in vitro shoots treated with NaCl (0, 125 or 250 mM) for 10 and 20 days. Different letters within the same treatment time indicate means significantly different at $P \leq 0.05$ (one-way ANOVA and Tukey's test). Salt concentration (SC) and time of application (T) significantly affected total phenolics and ellagic acid content, while SC × T effect was not significant (two-way ANOVA)

Sheikh-Mohamadi et al. 2017). Similar response was also reported in an *in vitro* study on shoots of the wild Mediterranean evergreen *Myrtus communis* (Di Cori et al. 2013). However, the involvement of other antioxidant enzymes in alleviating oxidative damage, such as catalase and superoxide dismutase, cannot be excluded (Jbir-Koubaa et al. 2015; Sofo et al. 2015 and references therein) and further studies are in progress to improve this information.

Among not enzymatic antioxidant, carotenoids are reported to play an important role in scavenging the excess of ROS induced by salt stress (Parida and Das 2005) and polyphenols can also outperform as ROS scavengers under stress conditions (Agati et al. 2012; Brunetti et al. 2013).

Carotenoids content decreased in "Profeta Partanna" salt-treated shoots and this result, in agreement with those previously obtained in myrtle (Di Cori et al. 2013) and grass pea genotypes (Piwowarczyk et al. 2016), suggests that they are not involved in the antioxidant responses to salt stress in pomegranate. We did not detect any increase of total phenolic compounds or of ellagic acid content in shoots as response to salinity; however, *M. communis, Pistacia lentiscus* (Tattini et al. 2006) and *Olea europaea* (Rossi et al. 2016) were shown to be able to utilize carbon sources for the synthesis of flavonoids involved in the antioxidant response to salt stress. Thus, the role of phenolic compounds as ROS scavengers cannot be excluded until further studies will have

been performed to characterize the role of class of polyphenols or of single compounds.

All the physiological and antioxidant parameters were affected by SC, T as previously observed (Di Cori et al. 2013).

In conclusion, we have reported the characterization of some mechanisms adopted by pomegranate to counteract the salinity using in vitro shoot cultures. A coordinated enhancement of G-POD and APX activities, coupled with a reduction of lipid peroxidation, was highlighted indicating the triggering role of these enzymes in control of the oxidative stress induced by salinity in this species. We suggest that, at least in our experimental conditions, an important response to NaCl exposure in in vitro growing shoots of "Profeta Partanna" pomegranate is the precocious activation of the antioxidant enzymatic system, and that this activation is able not only to counteract (G-POD), but also to prevent (APX) the oxidative damage on the exposed shoots, indicating "Profeta Partanna" as a variety quite tolerant to salt-induced osmotic stress. The use of the in vitro approach allowed a rapid characterization of the response to NaCl in controlled conditions without fluctuation of temperatures or watering, as previously underlined in other species (Watanabe et al. 2000; Woodward and Bennett 2005) and provided a further insight into the mechanism of salt response in pomegranate.

**Author contribution statement**  GU executed tissue culture experiments and physiological analyses, collected the data and performed data analysis. AF cooperated in execution of tissue culture experiments. PdC cooperated in execution of physiological analyses. SL and PN contributed to plant physiological analyses, to perform data analysis and to edit the manuscript. CF and EC planned the experiments, wrote and edited the manuscript.

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