

## Antioxidative Defense Mechanism in *Callistemon citrinus* (Curtis) Skeels and *Viburnum tinus* L. 'Lucidum' in Response to Seawater Aerosol and Surfactants

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### ABSTRACT

In the last decades, resistance to salt stress has been studied intensively in plants. Many ornamental plants have widespread presence in green areas of coastal regions. In such regions, plants are subject to seawater aerosol and surfactants, both of which are frequent in the coastal areas of Mediterranean environment. The objective of this study was to investigate the antioxidant enzyme activities of two ornamental plants, namely, *Callistemon* and *Viburnum*, under the effects of these stressful conditions. To analyze the performance of these plants stressed by 8 weeks treatments with seawater aerosol and surfactants, we measured the antioxidative defense mechanism, considered as enzymatic response, Proline (Pro) levels, Chlorophyll (Chl) and MalonDiAldehyde (MDA) contents. To better understand the response mechanisms, two different growing periods were studied: from January to March and from May to July. The higher temperatures of the second period negatively affected the response of the plants. Salt stress considerably reduced the chlorophyll content in both species, especially in the second period. In particular, the sea aerosol treatments caused 29% and about 45% reduction in *Callistemon* and *Viburnum* plants, respectively. The amount of Pro in *Viburnum* was very small (154.35 nmol g<sup>-1</sup>) compared to *Callistemon* (1466.94 nmol g<sup>-1</sup>). An opposite trend was noticed for MDA. ROS-scavenging enzymes, such as SuperOxide Dismutase (SOD), catalase (CAT), and Glutathione Peroxidase (GPX) in plants exposed to treatment with sea aerosol plus anionic surfactant were significantly higher. Between the two species, *Viburnum* showed more efficient action mechanisms to overcome aerosol stress.

**Keywords:** Ion content, Lipid peroxidation, Marine aerosol, Oxidative stress, Proline.

### INTRODUCTION

Plants growth along coastal areas are affected by seawater aerosol (Ferrante *et al.*, 2011), which induces the foliar absorption of ions produced by wind blowing over seawater. Plant species typical of the coastal areas have adapted to survive the direct contact of the salt on the leaves, although the exposure to sea aerosol may reduce plant growth and the ornamental value of plant species used in green areas (Cassaniti *et al.*, 2012). The effects of salt stress strongly

depend on the intensity and on the length of time (Niinemets, 2010). The presence of surfactant, however, can enhance the foliar absorption of sea salt through stomatal and cuticular penetration (Raddi *et al.*, 2009). The widespread use and the high consumption of surfactants might contribute to the load of anthropogenic surfactants in the environment (Scott and Jones, 2000).

The stress can trigger the accumulation of Reactive Oxygen Species (ROS) and plants have evolved a variety of mechanisms to counteract the effects of ROS in cellular

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compartments (Cassaniti *et al.*, 2013). Measurement of activities of antioxidant enzymes can thus be used to indicate oxidative stress in plants (Ruley *et al.*, 2004).

Catalase (CAT), guaiacol peroxidase, Ascorbate Peroxidase (APX), SuperOxide Dismutase (SOD), Glutathione Peroxidase (GPX), and Peroxidase (POD) are all involved in many physiological processes in plants, concerning responses to biotic and abiotic stresses (Toscano *et al.*, 2016; Acosta-Motos *et al.*, 2017). In particular, they are involved in the scavenging of ROS, which are partially reduced forms of atmospheric oxygen, highly reactive, and capable of causing oxidative damage to the cell.

The superoxide anion, generated by the univalent reduction of molecular oxygen, is scavenged in plants by superoxide dismutases enzyme, which dismutates it to hydrogen peroxide and molecular oxygen (Alscher *et al.*, 2003). Moreover, superoxide anion is an essential component of the ascorbate-glutathione cycle for the detoxification of toxic ROS (Chew *et al.*, 2003).

*Callistemon citrinus* (Curtis) Skeels belongs to the Myrtaceae family and is one of the most important Australian ornamental species, which has interesting characteristics (rapid growth, abundant flowering, and great variety of forms and volumes) (Lao and Jiménez, 2002). Most *Callistemon* species have been adapted and used in Mediterranean conditions, where they show some degree of tolerance to environmental stresses such as drought, root restriction, and high salt conditions (Álvarez and Sánchez-Blanco, 2013; Mugnai *et al.*, 2009). For these reasons, *C. citrinus* has enjoyed considerable success as a flowering shrub for ornamental landscaping in the Mediterranean area. Since the general growth performance of *C. citrinus* under stress conditions is well-known, it was considered as a suitable resistant plant against the unknown behavior of *Viburnum tinus* L. 'Lucidum' under the same stress.

Also, *Viburnum tinus* 'Lucidum' L. is an ornamental flowering shrub of interest in Italy due to the high demand for them on national and European markets (Cirillo *et al.*, 2016). Tolerance to water deficit stress and high salt stress (Lippi *et al.*, 2006) has made this species popular in urban plantings, landscapes, and xeriscapes. Different authors classified *Viburnum* as saline-sensitive (Bañón *et al.*, 2012) or moderately tolerant to salinity (Cassaniti *et al.*, 2009). This different salt stress classification is probably linked to salt concentration, modality of imposed stress (by leaf or irrigation water), and intraspecific variability (the response is quite different among the different cultivars).

Indeed, the responses of these two ornamental shrubs to salinity were only investigated by morphological and physiological responses, whereas no data exist on the enzymatic response.

The highly reactive ROS, in the absence of any protective mechanism, can seriously disrupt normal metabolism by damaging photosynthetic pigments (Sharma *et al.*, 2005). Photosynthesis is one of the most severely affected processes during salinity stress, which is mediated by decreased chlorophyll content (Sudhir and Murthy, 2004).

In addition, the physiological changes in plants under stress conditions are indicated also by some indices, such as free Proline (Pro) and MalonDiAldehyde (MDA) contents, playing as plant osmotic regulator and lipid membrane oxidation indicator, respectively, in response to abiotic stresses (Deng *et al.*, 2012).

The aim of our study was to investigate the mechanism used by two different ornamental plants, namely, *Callistemon* and *Viburnum*, to resist sea aerosol with and without anionic surfactants. For this purpose, levels of leaf enzymatic antioxidants (CAT, GPX and SOD) and stress indicators (Chl, Pro and MDA contents) were determined.

## MATERIALS AND METHODS

### Location, Plant Material, and Growth Conditions

The experiment was conducted in 2016, adopting two growing periods, i.e. from January to March (GPI) and from May to July (GPII), in a greenhouse located in Catania, Italy. For each growing period, rooted cuttings of *Callistemon* [*Callistemon citrinus* (Curtis) Skeels] and *Viburnum* (*Viburnum tinus* L. 'Lucidum') grown in 7×7 cm pots were transplanted into 3 L plastic pots (16 cm) filled with peat and perlite (1:1 v/v) amended with 2 g L<sup>-1</sup> of Osmocote Plus (14:13:13 N, P, K plus microelements). Plants were sprayed with different aqueous solutions: (S1) Solution simulating the composition of sea aerosol (Elshatshat, 2010); (S2) Solution containing an anionic surfactant (sodium dodecylbenzenesulfonate 82.52%, 50 mg L<sup>-1</sup>) (Sánchez-Blanco *et al.*, 2003); (S3) Solution with sea aerosol and anionic surfactant. Another group of plants was used as control and was treated only with deionized water (C). The treatments were defined by a factorial combination of four treatments.

All the pots of the different treatments were covered with an aluminum film to prevent the different solutions from reaching the substrate.

During the experiments, plants were cultivated using standard methods and watered according to the microclimatic conditions and the substrate moisture status. The water was supplied to the plants to maintain soil moisture at container capacity (Álvarez *et al.*, 2011).

The mean air temperature, relative humidity, and global radiation under different shading conditions were recorded on a data logger CR1000 (Campbell Scientific Ltd., Loughborough, UK) during the experimental periods. The mean temperature and the relative humidity recorded during GPI and GPII were 13.4 and 21.4°C, and 86.1 and 76.0%, respectively.

## Samples Preparation

At the end of each growth period, fully developed young leaves (5<sup>th</sup> and 6<sup>th</sup> leaves below the shoot apex) were removed from each plant of each line. Three replicate samples for each species were collected. 150-200 g of Fresh Weight (FW) of the leaves were ground using a cryogenic mortar in liquid nitrogen. Powder was weighed and stored at -80°C until the time of analysis. The enzyme extraction was determined according to Bian and Jiang (2009).

## Chlorophyll Content

At the each growth period the chlorophyll content was determined using a SPAD-502 chlorophyll meter (Minolta Camera Co., Osaka, Japan). As proposed by Wang *et al.* (2005), a calibration curve was plotted considering the relationship between the SPAD values and the chlorophyll content as extracted according to Moran and Porath (1980). The following equation was used to obtain the chlorophyll content:

$$\text{Callistemon chlorophyll } (\mu\text{g cm}^{-2}) = 0.021 (\text{SPAD index})^2 - 0.1632 (\text{SPAD index}) - 6.3754 \quad (R^2 = 0.6002^{***}) \quad (n = 50);$$

$$\text{Viburnum chlorophyll } (\mu\text{g cm}^{-2}) = 1.2925 (\text{SPAD index}) - 34.158 \quad (R^2 = 0.7014^{***}) \quad (n = 50).$$

## Mineral Analysis

Almost 100 g of fresh leaves were used for mineral analyses and the dried tissues were ground in a Wiley Mill to pass through a 20-mesh screen (Sieve size= 0.841 mm). Then, Na<sup>+</sup> (0.5 g) and Cl<sup>-</sup> (2.0 g) for each replicate were weighed to obtain mineral concentrations by ion chromatograph Dionex IC 25 fitted with a 40 EG Eluent Generator, and an IonPac AS11-HC separation column for the anion, and an Ion Pac CS-12A column for the cation (Dionex Corp., Sunnyvale, CA, USA). Ion



concentrations were expressed in  $\text{g kg}^{-1}$  DW (Giuffrida *et al.*, 2013).

### Determination of Proline and MDA Content

The amount of free proline in fresh plant material was determined using the method of Bates *et al.* (1973) using *L*-proline as standard, as reported by Toscano *et al.* (2016).

Fresh material (1 g) was homogenized in 5 mL of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at  $14,000\times g$  for 15 minutes. A 2-mL aliquot of the supernatant was mixed with an equal volume of acetic acid and acid ninhydrin and incubated for 1 h at  $100^{\circ}\text{C}$ . The reaction was terminated in an ice bath and extracted with 4 mL of toluene. The extract was vortexed for 20 seconds. The absorbance was determined spectrophotometrically at 525 nm using toluene for a blank, *L*-proline as the standard.

MDA content was measured according to Heath and Packer (1986) as reported by Li *et al.* (2010). Samples of approximately 0.5-g were homogenized in 1.5 mL of 5% trichloroacetic acid (weight/volume). The homogenate was centrifuged at  $5,000\times g$  for 10 minutes, and the supernatant was diluted to 10 mL. A 2-mL aliquot of the diluted extract was mixed with the same volume of 0.67% 2-thiobarbituric acid. The mixture was incubated in boiling water ( $95\text{-}100^{\circ}\text{C}$ ) for 30 minutes, then, centrifuged at  $5,000\times g$  for 10 minutes. The MDA content in the aqueous phase was calculated based on the following formula:  $C (\mu\text{mol L}^{-1}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$ .

### Enzyme Activities Analysis

The SOD (SOD; EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of Nitro Blue Tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The CAT

(CAT; EC 1.11.1.6) was analyzed according to Aebi (1984) and Aguilera *et al.* (2002). The GPX (GPX; EC 1.11.1.7) activity was measured using the method described by Ruley *et al.* (2004). All enzyme activities were related to protein content that was determined using Bradford's method (1976).

### Statistical Analysis

A complete randomized design with three replicates was adopted; each experimental unit consisted of 10 plants. Data were subjected to a two-way Analysis Of Variance (ANOVA), using CoStat release 6.311 (CoHort Software, Monterey, CA, USA), to determine the effects of Treatments (T) and Growth Period (GP); means were compared using Student-Newman-Keuls (SNK) test ( $P \leq 0.05$ ). The interactions, when significant, are presented separately in the figures.

## RESULTS AND DISCUSSION

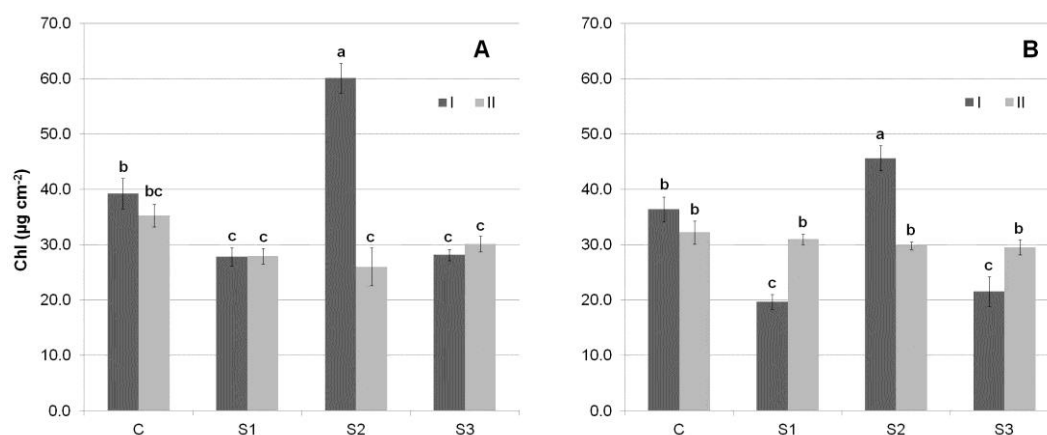
### Chlorophyll Content

In both species, the chlorophyll content changed only in the first growth period (Figure 1). This parameter increased in S2 while decreased in the treatments with salt addition (S1, S3). The chlorophyll content in *Callistemon* plants treated with sea aerosol (S1) showed a significant reduction of 29%, while a slight but always significant reduction (9%) was observed in S3 against the untreated plants (Table 1). In *Viburnum*, both treatments with sea aerosol (S1 and S3) reduced the chlorophyll content by about 45% (Table 1). Salt stress considerably reduced the contents of Chl in both species, and these results corroborate the findings of Talaat and Shawky (2012). Our results are in agreement with Farieri *et al.* (2016) where plants of *Viburnum* subjected to aerosol marine showed a significant reduction of this pigment. Also, in plants of *Arbutus unedo*, the chlorophyll content declined with

**Table 1.** Mean effects of four different spray treatments and two growing periods on Na<sup>+</sup>, Cl<sup>-</sup> concentration, and Chl, Pro, MDA content on leaves of *Callistemon* and *Viburnum*.<sup>a</sup>

Treatments	Growi ng period	Chl ( $\mu\text{g cm}^{-2}$ )	Na <sup>+</sup> ( $\text{g kg}^{-1}$ DW)	Cl <sup>-</sup> ( $\text{g kg}^{-1}$ DW)	Pro ( $\text{nmol g}^{-1}$ FW)	MDA ( $\text{nmol g}^{-1}$ FW)
<i>Callistemon</i>						
C		37.3 <sup>b</sup>	2.79 <sup>b</sup>	6.41 <sup>c</sup>	262.77 <sup>c</sup>	0.91 <sup>ab</sup>
S1		27.9 <sup>c</sup>	21.46 <sup>a</sup>	36.54 <sup>a</sup>	1414.91 <sup>a</sup>	0.77 <sup>b</sup>
S2		43.1 <sup>a</sup>	1.44 <sup>b</sup>	3.46 <sup>c</sup>	394.05 <sup>b</sup>	1.09 <sup>a</sup>
S3		29.2 <sup>c</sup>	25.11 <sup>a</sup>	33.19 <sup>b</sup>	1466.94 <sup>a</sup>	0.88 <sup>a</sup>
	I	38.8 <sup>a</sup>	10.23 <sup>b</sup>	8.80 <sup>b</sup>	1038.05 <sup>a</sup>	1.10 <sup>a</sup>
	II	29.6 <sup>b</sup>	15.16 <sup>a</sup>	31.0 <sup>a</sup>	731.29 <sup>b</sup>	0.73 <sup>b</sup>
<i>Significance</i>						
Treatments (T)		***	***	***	***	*
Growing Period (GP)		***	***	***	***	***
T×GP		***	***	***	***	***
<i>Viburnum</i>						
C		34.31 <sup>a</sup>	1.14 <sup>c</sup>	3.18 <sup>b</sup>	27.18 <sup>b</sup>	1.08 <sup>d</sup>
S1		25.35 <sup>c</sup>	18.37 <sup>a</sup>	20.92 <sup>a</sup>	39.77 <sup>b</sup>	1.34 <sup>c</sup>
S2		37.75 <sup>a</sup>	1.30 <sup>c</sup>	4.10 <sup>b</sup>	33.73 <sup>b</sup>	1.60 <sup>b</sup>
S3		29.85 <sup>b</sup>	15.32 <sup>b</sup>	21.87 <sup>a</sup>	154.35 <sup>a</sup>	2.04 <sup>a</sup>
	I	30.82	9.87	8.62 <sup>b</sup>	89.06 <sup>a</sup>	1.27 <sup>b</sup>
	II	32.81	8.20	16.41 <sup>a</sup>	38.45 <sup>b</sup>	1.76 <sup>a</sup>
<i>Significance</i>						
Treatments (T)		***	***	***	***	***
Growing Period (GP)		NS	NS	***	***	***
T×GP		***	NS	***	***	***

<sup>a</sup> Values are means for main effects of Treatments (T) and Growing Period (GP). Different letters indicate statistical differences for  $P \leq 0.05$ . Level of significance; NS: Non-Significant, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .



**Figure 1.** Interaction effects of treatments and growth period on chlorophyll content in *Callistemon* (A) and *Viburnum* (B). Values with the same letter are not significantly different by SNK  $P \leq 0.05$  test. Values are means  $\pm$  SE (n= 3).



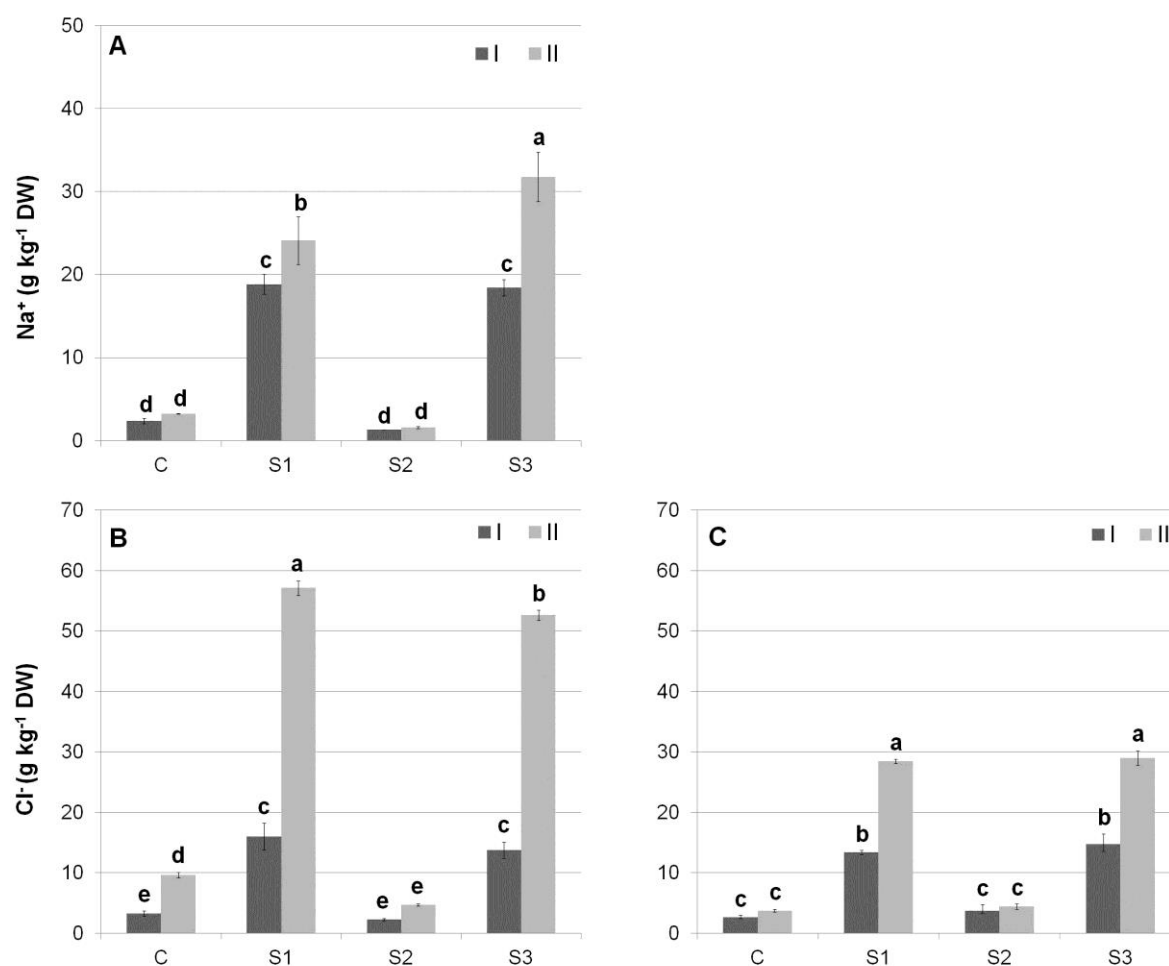
the increase of salt stress (Navarro *et al.*, 2008). The salt-induced reduction in the chlorophyll content can affect the photosynthetic process. As a mechanism to protect the photosynthesis process, tolerant species respond by maintaining or increasing their chlorophyll content (Acosta-Motos *et al.*, 2017).

### Mineral Analysis

Compared to the control, Callistemon treated with aerosol plus surfactant (S3) showed the highest leaf Na<sup>+</sup> concentrations, particularly in GPII, slightly lower but

statistically different was the content in S1, while between the mentioned samples during the GPI many differences were observed. As observed by Zollinger *et al.* (2005), environmental conditions could influence the response to salt stress: the higher temperature and irradiance meant that plants became more stressed. The amount of this compound in plants subjected only to the treatment with surfactant was similar to the untreated control for both growing periods analyzed (Figure 2).

A different trend was evident in Viburnum, where the highest leaf Na<sup>+</sup> concentrations were in S1, followed by S3. Moreover, many statistical differences



**Figure 2.** Interaction effects of treatments and growth period on Na<sup>+</sup> concentration in leaves of Callistemon (A), and Cl<sup>-</sup> concentration in leaves of Callistemon (B) and Viburnum (C). Values with the same letter are not significantly different by SNK  $P \leq 0.05$  test. Values are means  $\pm$  SE (n= 3).

linked with growing period or interaction among growing period and aerosol or surfactant treatments were seen (Table 1).

In the same way, *Callistemon* and *Viburnum* treated with aerosol gave back leaf  $\text{Na}^+$  concentrations higher than the control by nearly ten and fifteen times, respectively, while the amount of sodium dodecylbenzenesulfonate supplied by itself did not show any increase. In fact, in both species, the control and S2 showed similar  $\text{Na}^+$  concentration.

Leaf  $\text{Cl}^-$  concentration in *Callistemon* was higher in S1 followed by S3, values deeply tied to the second growing period, whereas S2 data were similar in both growing periods and also in comparison with the control in GPI. In *Callistemon*, both leaf ions considered showed highly significant differences considering treatments, growing periods, and the interaction between both variables (Figure 2 A). In *Viburnum*, considering leaf  $\text{Cl}^-$  concentrations, the tendency of S1 and S3 was the same in both growing periods, showing the highest values in GPII, while the control and S2 followed the same course and both having many statistical difference (Figure 2 C).

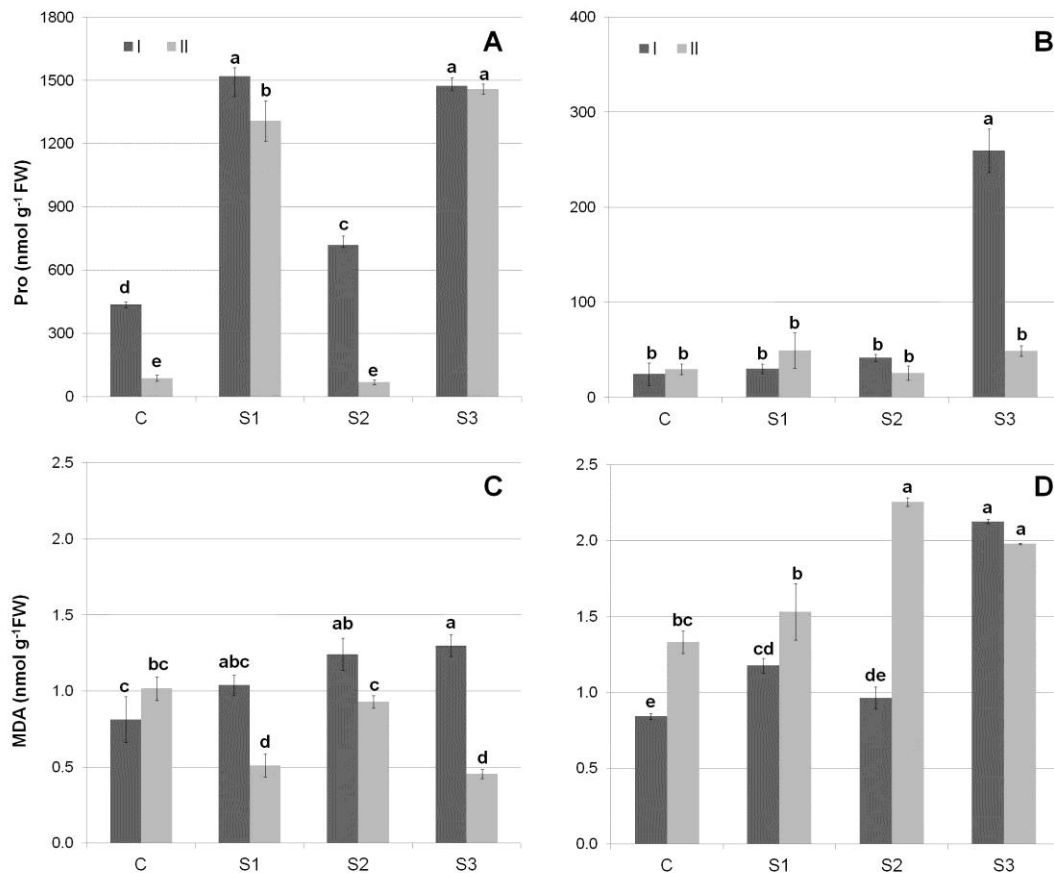
Our results confirm findings of Sanchez-Blanco *et al.* (2003) where higher  $\text{Na}^+$  and  $\text{Cl}^-$  concentration were found in treatments including sea aerosol and sea aerosol plus anionic surfactants. In their study, levels of  $\text{Cl}^-$  in the pine needles were related to the salt content in the solution, whether the solution contained surfactants or not (Bussotti *et al.*, 1995). Moreover, it has been shown that salt tolerance is associated with the plant's capacity to limit foliar absorption of  $\text{Cl}^-$  (Francois, 1982). Toxic ion accumulation can be an energetically cheap and positive way for plants to obtain osmotic regulation, but only if they are properly compartmentalized. Otherwise, the accumulation of phytotoxic ions in leaves results in a nutrient imbalance (Acosta-Motos *et al.*, 2017).

### Proline and MDA Content

Plants activate various metabolic and defense systems to survive. Proline is an osmoprotectant that confers stress tolerance. Considering the GPI, *Callistemon* has a Pro content lower in the control, slightly high in samples treated with surfactant, while the value increased fivefold under aerosol treatment (S1) and in S3, in response to the double stress caused by the sum of the treatments (Table 1, Figure 3). It is remarkable how the amount of Pro in S3 is similar in both growing periods, while in the other treatments (C, S1 and S2), the Pro content in GPI is always higher than GPII, indicating the greater sensitivity of plants subjected to both winter and treatments stresses. The amount of Pro in *Viburnum* treated with sea aerosol and anionic surfactant is very small ( $154.35 \text{ nmol g}^{-1} \text{ FW}$ ) if compared to the same treatment on *Callistemon* ( $1,466.94 \text{ nmol g}^{-1} \text{ FW}$ ). It is interesting to observe how the trend among the treatments is similar to that observed for *Callistemon*, but it is ten times less. This probably means that *Viburnum* activated a different metabolic and defense system. Also in this case, the content is higher in the first growing period and interaction between treatments and growing period are significant as reported in Figure 3 A and B, but only for S3 in GPI.

Salinity stress when combined with the higher temperature during GPII is amplified because higher transpiration results in enhanced uptake of salt (Cassaniti *et al.*, 2013).

As confirmed by Ashraf and Harris (2004), Pro content in many salt tolerant plants has been found to be higher than that in salt sensitive ones. Interestingly, Pro content did not change very much under any level of salinity in *Viburnum*. This is in contrast to many previous reports where Pro levels were increased under salinity stress to decrease the cellular water potential and improve water uptake, and possibly



**Figure 3.** Interaction effects of treatments and growth period on Pro (above) and MDA (below) content in leaves of *Callistemon* (A, C) and *Viburnum* (B, D). Values with the same letter are not significantly different by SNK  $P \leq 0.05$  test. Values are means  $\pm$  SE (n = 3).

scavenge ROS molecules (Soshinkova *et al.*, 2013).

An opposite trend can be noticed for MDA. As the final product of lipid peroxidation, MDA is one of the key indicators of damage to the cell membrane system. In *Callistemon* (Figure 3 C), the increasing rate of MDA is evident during GPI, where S3 and S2 reach the higher content (1.2 and 1.3 nmol g<sup>-1</sup> FW, respectively). In GPII, the MDA content was higher in the control and in S2 (1.0 and 0.9 nmol g<sup>-1</sup> FW, respectively) and lower in S1 and S3 (0.5 nmol g<sup>-1</sup> FW in both treatments).

In *Viburnum*, the MDA content was higher in both growing periods in S3 (2.1 and 2.0 nmol g<sup>-1</sup> FW) (Figure 3-D). Although MDA content in *Callistemon* and

*Viburnum* is not much different among the treatments, the values stayed in the range of 0.8 to 1.3 nmol g<sup>-1</sup> FW; the increasing trend is evident in all samples of *Viburnum* in GPII. As generally reported in literature, changes in leaf MDA content were opposite to those of leaf Pro content (Deng *et al.*, 2012). As is common in abiotically stressed plants, Pro serves as an osmotic regulator and protectant for a number of cellular structures during exposure to stresses (Ueda *et al.*, 2008), while MDA is one of the ultimate products of lipid peroxidation damage by free radicals. Under drought stress, MDA content increases independent of drought intensity, development stage, and plant organ (Ge *et al.*, 2006). The changes in lipid peroxidation index (MDA) is coherent



with the findings of Ashraf and Harris (2004), according to whom the salt sensitive lines increased more than in the salt tolerant lines under salt stress. Salt stress induced an oxidative stress in plants that correlates with increases in some oxidative stress parameters, such as electrolyte leakage, lipid peroxidation, and protein oxidation (Hernández *et al.*, 1993, 2001; Acosta-Motos *et al.*, 2015). These results were correlated with the morphological changes. Indeed, at the end of each growing period, in both species, aerosol marine, alone or in combination with surfactant, resulted in reductions of total dry biomass. In Callistemon, total dry matter decreased by ~35% in S1 and S3 treatments. Viburnum showed similar results: total and shoot dry biomass in S1 and S3 decreased by ~27% (data not shown). As confirmed by Cassaniti *et al.* (2012), salt stress involves an increase in the percentage of dry matter and in the root/shoot ratio, and a reduction in the leaf area.

### Enzyme Activities Analysis

Antioxidant enzymes and their gene expressions may be differentially or cooperatively involved in the defense mechanisms in the leaves of Callistemon and Viburnum exposed to different kinds of abiotic stress like marine aerosol and surfactants (Farieri *et al.*, 2016).

Different works have reported that salt stress induces an accumulation of superoxide radicals ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) in different cell compartments (Hernández *et al.*, 1993; 1995; 2001). Also, different authors have reported that the tolerance of plants to salt stress is associated with the induction of antioxidant defenses (Hernández *et al.*, 2000; Mittova *et al.*, 2002). The activities of some antioxidant enzymes, such as SOD, CAT and GPX, showed varying responses with induction at different treatments and growing period (Table 2). Plants are equipped with a defense system to repair the

damage created by ROS. Antioxidant enzymes play important roles in this process; their behavior is different depending on the kind of stress suffered by the plants.

Among treatments means, the SOD activity showed higher values during GPI ( $260\text{ U mg}^{-1}$  protein and  $200\text{ U mg}^{-1}$  protein, respectively, in GPI and GPII). In Callistemon, only the treatment with aerosol plus surfactant gave a value of SOD activity around  $400\text{ U mg}^{-1}$  protein. The results showed that in both growth periods there were similar values (Figure 4 A).

In GPI, the mean of SOD activity in plants of Viburnum was higher than in Callistemon plants. In Viburnum, however, in the first growing period, there were significantly higher values for all treatments compared to the control (884, 989 and  $1101\text{ U mg}^{-1}$  protein respectively for S1, S2 and S3). SOD activity during GPII was not different in all treatments (Figure 4 B).

SOD plays an important role in cells protection against toxicity and mutagenicity caused by superoxide radicals. Any oxidative stress occurring to vegetation causes a response from their biological systems, releasing free radicals that force plants to develop antioxidative strategies in which SOD and CAT are the most efficient antioxidant enzymes (Masia, 1998). SOD activity is the key enzyme in the active oxygen scavenger system, because it catalyzes the dismutation of superoxide free radicals into  $H_2O_2$  and  $O_2$  (Toscano *et al.*, 2016). GPX and CAT further convert  $H_2O_2$  into  $H_2O$  and  $O_2$ , and the damage caused by ROS is removed from plants (Wu *et al.*, 2012). Viburnum confirmed its role as a salt tolerant species, showing an increase in SOD activity, as was found by Mittova *et al.* (2002) who reported higher salt tolerance in wild tomato (*Lycopersicon pennellii*) compared with cultivated tomato (*L. esculentum*), where salt tolerance was correlated with increased activities of these enzymes.

Similar to the SOD, the mean of CAT activity showed higher values during GPI

**Table 2.** Mean effects of four different spray treatments and two growing periods on activity of SOD, CAT and GPX enzymes in leaves of *Callistemon* and *Viburnum*.<sup>a</sup>

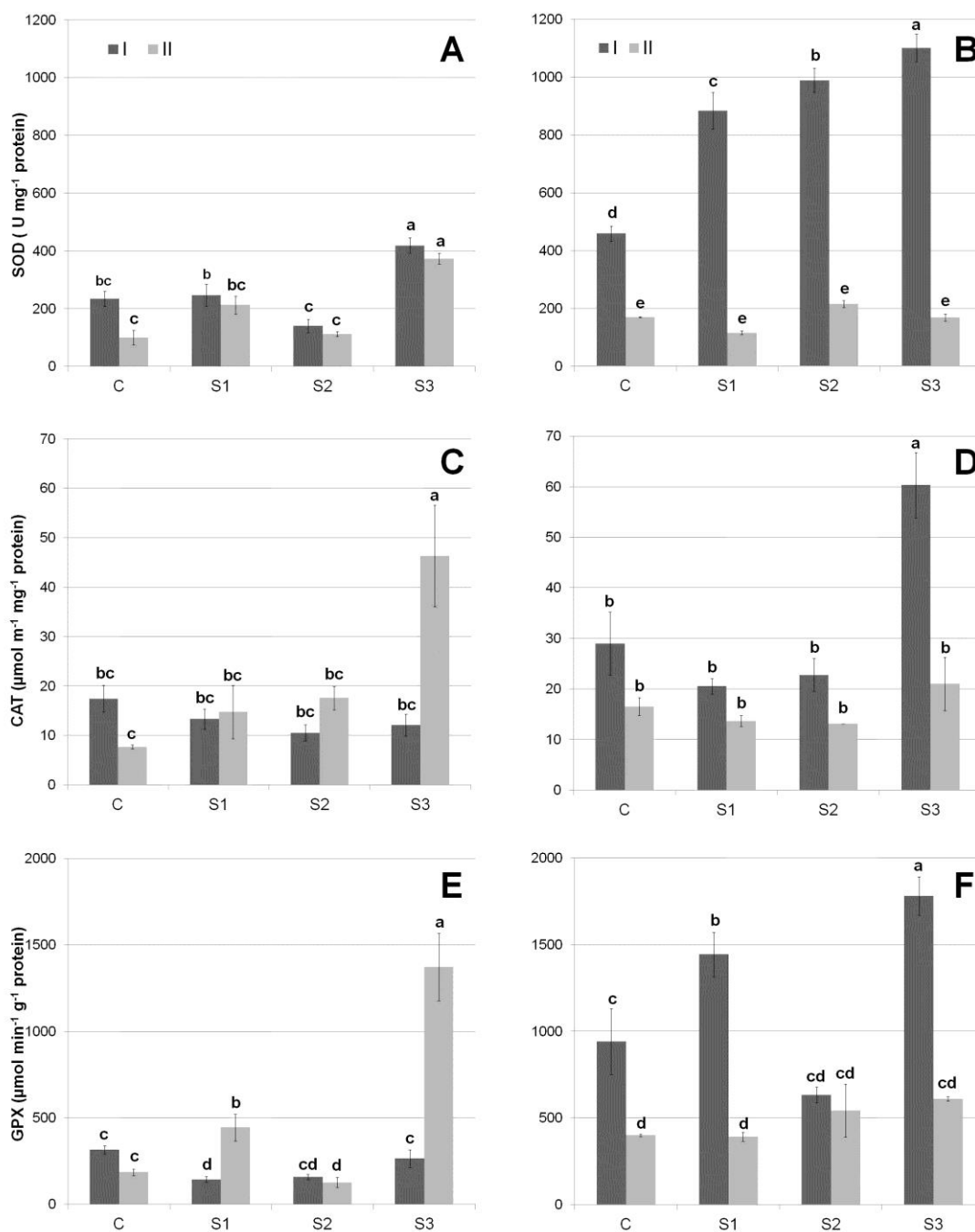
Treatments	Growing period	SOD (U mg protein <sup>-1</sup> )	CAT ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein)	GPX ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein)
<i>Callistemon</i>				
C		167.8 <sup>c</sup>	12.6	250.6 <sup>b</sup>
S1		229.9 <sup>b</sup>	14.0	294.6 <sup>b</sup>
S2		126.5 <sup>c</sup>	14.0	143.1 <sup>c</sup>
S3		396.3 <sup>a</sup>	29.2	818.2 <sup>a</sup>
	I	260.3 <sup>a</sup>	13.4 <sup>b</sup>	220.9 <sup>b</sup>
	II	200.0 <sup>b</sup>	21.6 <sup>a</sup>	532.4 <sup>a</sup>
<i>Significance</i>				
Treatments (T)		***	NS	***
Growing Period (GP)		***	**	***
T×GP		***	*	***
<i>Viburnum</i>				
C		71.39	0.45 <sup>ab</sup>	1.25 <sup>a</sup>
S1		64.26	0.34 <sup>bc</sup>	1.40 <sup>a</sup>
S2		75.98	0.28 <sup>c</sup>	0.97 <sup>b</sup>
S3		71.93	0.51 <sup>a</sup>	1.50 <sup>a</sup>
	I	97.33 <sup>a</sup>	0.36	1.29
	II	44.45 <sup>b</sup>	0.43	1.27
<i>Significance</i>				
Treatments (T)		NS	**	**
Growing Period (GP)		***	NS	NS
T×GP		**	**	**

<sup>a</sup> Values are means for main effects of Treatments (T) and Growing Period (GP). Different letters indicate statistical differences for  $P \leq 0.05$ . Level of significance; NS: Non-Significant, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .

(21.6  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein and 13.4  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein, respectively, in GPI and GPII). In *Callistemon* plant, the CAT was more active during the GPII in S3 (48  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) while there was no statistical difference between the control and other treatments during GPI (Figure 4 C), indicating that, during GPI, from January to March, the lowest temperature kept the CAT values more stable. In *Viburnum* plants, the highest values were in S3 at GPI (60  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein). During GPII, no significant difference was found between the treatments (Figure 4 D). The range of results for CAT enzyme was not too much different between the two species analyzed and only a slight decrease could be observed in *Callistemon*.

The activity of CAT increased with the increase of the stress in both species, and this increase in stressed plants might be an adaptation to eliminate  $\text{H}_2\text{O}_2$  (Ben Ahmed *et al.* 2009).

Peroxidases, like GPX, also scavenge  $\text{H}_2\text{O}_2$  indirectly by combining it with antioxidant compounds as guaiacol (Ruley *et al.*, 2004). The mean of GPX activity showed the highest values during GPII (532.4  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein in GPII compared to 220.9  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein in GPI). The GPX enzyme activity in *Callistemon* showed small values during the GPI (221  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein), but a significant increase was observed during the GPII in S3 (1372  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) followed by S1 (444  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) (Figure 4-E). An opposite trend was observed in *Viburnum*: in GPII, no



**Figure 4.** Interaction effects of treatments and growth period on SOD (above), CAT (middle) and GPX (below) enzymes in leaves of Callistemon (A, C, E) and Viburnum (B, D, F). Values with the same letter are not significantly different by SNK  $P \leq 0.05$  test. Values are means  $\pm$  SE (n = 3).



significant difference was found between the treatments, while in GPI, an increase of this activity was observed for S1 and S3 (respectively, +35 and +47% compared to the control) (Figure 4-F).

As previously stated by Ashraf and Harris (2004), salt stress caused considerable increase in the activities of GPX in the salt tolerant cultivars, whereas the activities of these enzymes remained unchanged or decreased in the salt sensitive cultivars.

The ability to maintain high SOD, CAT, and APX activity under stress conditions is essential for the balance between the formation and removal of H<sub>2</sub>O<sub>2</sub> within the intracellular environment (Joseph and Jini 2011). This ability was observed in our study in leaves of Viburnum, especially during GPII. Considering that, in general, there was high activity of the antioxidant defense system under the stress conditions evaluated. In coping with the deleterious effects of salinity, including lower accumulation of phytotoxic ions as well as greater increases and/or constitutive levels of SOD, CAT and GPX, Viburnum showed more efficient mechanisms than Callistemon plants.

## REFERENCES

1. Acosta-Motos, J.R., Ortuño, M.F., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M.J. and Hernandez, J.A. 2017. Plant Responses to Salt Stress: Adaptive Mechanisms. *Agronomy*, **7(1)**: 18.
2. Aebi, H., 1984. Catalase *In Vitro*. *Meth. Enzymol.*, **105**: 121-130.
3. Aguilera, J., Bischof, K., Karsten, U., Hanelt, D. and Wiencke, C. 2002. Seasonal Variation in Ecophysiological Patterns in Macroalgae from an Arctic Fjord. II. Pigment Accumulation and Biochemical Defense Systems against High Light Stress. *Mar. Biol.*, **140**: 1087-1095.
4. Alscher, R. G., Erturk, N. and Heath, L.S. 2003. Role of Superoxide Dismutases (SODs) in Controlling Oxidative Stress in Plants. *J. Exp. Bot.*, **53**: 1131-1341.
5. Álvarez, S., Navarro, A., Nicolás, E. and Sánchez-Blanco, M. J. 2011. Transpiration, Photosynthetic Responses, Tissue Water Relations and Dry Mass Partitioning in *Callistemon* plants during Drought Conditions. *Sci. Hortic.*, **129**: 306-312.
6. Álvarez, S. and Sánchez-Blanco, M. J. 2013. Changes in Growth Rate, Root Morphology and Water Use Efficiency of Potted *Callistemon citrinus* Plants in Response to Different Levels of Water Deficit. *Sci. Hortic.*, **156**: 54-62.
7. Ashraf, M. and Harris, P. J. C. 2004. Potential Biochemical Indicators of Salinity Tolerance in Plants. *Plant Sci.*, **166**: 3-16.
8. Bañón, S., Miralles, J., Ochoa, J. and Sánchez-Blanco, M.J. 2012. The Effect of Salinity and High Boron on Growth, Photosynthetic Activity and Mineral Contents of Two Ornamental Shrubs. *Hortic. Sci.*, **4**: 188-194.
9. Bates, L., Waldren, P. P. and Teare, J. D. 1973. Rapid Determination of the Free Proline of Water Stress Studies. *Plant Soil*, **39**: 205-207.
10. Ben Ahmed, C.H., Ben Rouina, B., Sensoy, S., Boukhris, M. and Ben Abdallah, F. 2009. Changes in Gas Exchange, Proline Accumulation and Antioxidative Enzyme Activities in Three Olive Cultivars under Contrasting Water Availability Regimes. *Environ. Exp. Bot.*, **67**: 345-352.
11. Bian, S. and Jiang, Y. 2009. Reactive Oxygen Species, Antioxidant Enzyme Activities and Gene Expression Patterns in Leaves and Roots of Kentucky Bluegrass in Response to Drought Stress and Recovery. *Sci. Hortic.*, **120**: 264-270.
12. Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.*, **72(1-2)**: 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
13. Bussotti, F., Grossoni, P. and Pantani, F. 1995. The Role of Marine Salt and Surfactants in the Decline of Tyrrhenian Coastal Vegetation in Italy. *Ann. Sci. For.*, **52**: 251-261.
14. Cassaniti, C., Li Rosi, A., Romano, D., 2009. Salt Tolerance of Ornamental Shrubs Mainly Used in the Mediterranean Landscape. *Acta Hortic.*, **807**: 675-680. doi: 10.17660/ActaHortic.2009.807.101
15. Cassaniti, C., Romano D. and Flowers T. J. 2012. The Response of Ornamental Plants to

- Saline Irrigation Water. In: "Irrigation: Water Management, Pollution and Alternative Strategies", (Ed.): García-Garizábal, I. and Abrahao, R. ISBN 979-953-307-012-3, InTech, Rijeka, Croatia, PP. 131-158.
16. Cassaniti, C., Romano, D., Hop, M. E. C. M. and Flowers, T. J. 2013. Growing Floricultural Crops with Brackish Water. *Environ. Exp. Bot.*, **92**: 165-175.
  17. Cirillo, C., Roupheal, Y., Caputo, R., Raimondi, G., Sifola, M. I. and De Pascale, S. 2016. Effects of High Salinity and the Exogenous Application of an Osmolyte on Growth, Photosynthesis, and Mineral Composition in Two Ornamental Shrubs. *J. Hortic. Sci. Biotech.*, **91(1)**: 14-22.
  18. Chew, O., Whelan, J. and Miller, A. H. 2003. Molecular Definition of the Ascorbate–Glutathione Cycle in *Arabidopsis* Mitochondria Reveals Dual Targeting of Antioxidant Defences in Plants. *J. Biol. Chem.*, **278**: 46869–46877.
  19. Deng, Y., Jiang, J., Chen, S., Huang, C., Fang, W. and Chen, F. 2012. Drought Tolerance of Intergeneric Hybrids between *Chrysanthemum morifolium* and *Ajania przewalskii*. *Sci. Hortic.*, **148**: 17-22.
  20. Elshatshat, S. A. 2010. The Effect of Simulated Seawater on Water Permeability of Isolated Leaf Cuticular Layers. *Int. J. Agric. Biol.*, **12**:150-152.
  21. Farieri, E., Toscano, S., Ferrante, A. and Romano, D. 2016. Identification of Ornamental Shrubs Tolerant to Saline Aerosol for Coastal Urban and Peri-Urban Greening. *Urban For. Urban Gree.*, **18**: 9-18.
  22. Ferrante, A., Trivellini, A., Malorgio, F., Carmassi, G., Vernieri, P. and Serra, G. 2011. Effect of Seawater Aerosol on Leaves of Six Plant Species Potentially Useful for Ornamental Purposes in Coastal Areas. *Sci. Hortic.*, **128**: 332-341.
  23. Francois, L. E. 1982. Salt Tolerance of Eight Ornamental Tree Species. *J. Am. Soc. Hortic. Sci.*, **107**: 66-68.
  24. Ge, T. D., Sui, F. G., Bai, L. P., Lu, Y. Y. and Zhou, G. S. 2006. Effects of Water Stress on the Protective Enzyme Activities and Lipid Peroxidation in Roots and Leaves of Summer Maize. *Agr. Sci. China*, **5**: 291-298.
  25. Giannopolitis, C. N. and Ries, S. K. 1977. Superoxide Occurrence in Higher Plants. *Plant Physiol.*, **59**: 309-314.
  26. Giuffrida, F., Cassaniti, C. and Leonardi, C. 2013. The Influence of Rootstock on Growth and Ion Concentrations in Pepper (*Capsicum annum* L.) under Saline Conditions. *J. Hortic. Sci. Biotec.*, **88**: 110-116.
  27. Heath, R. L. and Packer, L. 1986. Photoperoxidation in Isolated Chloroplasts. I. Kinetics and Stoichiometry of Fatty Acid Peroxidation. *Arch. Biochem. Biophys.*, **125**: 189-198.
  28. Hernandez, J. A., Olmos, E., Corpas, F. J., Sevilla, F., Del Rio, L. A., 1995. Salt-induced Oxidative Stress in Chloroplasts of Pea Plants. *Plant Sci.*, **105(2)**: 151-167.
  29. Hernández, J. A., Corpas, F. J., Gómez, M., del Rio, L. A. and Sevilla, F. 1993. Salt-Induced Oxidative Stress Mediated by Activated Oxygen Species in Pea Leaf Mitochondria. *Physiol. Plant.*, **89(1)**: 103-110.
  30. Hernández, J. A., Ferrer, M. A., Jiménez, A., Barceló, A. R. and Sevilla, F. 2001. Antioxidant Systems and O<sub>2</sub>–/H<sub>2</sub>O<sub>2</sub> Production in the Apoplast of Pea Leaves. Its Relation with Salt-Induced Necrotic Lesions in Minor Veins. *Plant Physiol.*, **127(3)**: 817-831.
  31. Joseph, B. and Jini, D. 2011. Development of Salt Stress-Tolerant Plants by Gene Manipulation of Antioxidant Enzymes. *Asian J. Agric. Res.*, **5**: 17-27.
  32. Lao, M. T. and Jiménez, S. 2002. Estudio Comparativo del Comportamiento Productivo de *Callistemon citrinus*. In: "I Jornadas Ibéricas de plantas Ornamentales". Servicio de Publicaciones y Divulgación, Consejería de Agricultura y Pesca, Junta de Andalucía, PP **113-120**.
  33. Li, G., Wan, S., Zhou, J., Yang, Z. and Qin, P. 2010. Leaf Chlorophyll Fluorescence, Hyperspectral Reflectance, Pigments Content, Malondialdehyde and Proline Accumulation Responses of Castor Bean (*Ricinus communis* L.) Seedlings to Salt stress Levels. *Ind. Crop. Prod.*, **31**: 13-19.
  34. Lippi, G., Serra, G., Vernieri, P. and Tognoni, F. 2006. Response of Potted *Callistemon* Species to High Salinity. *Acta Hortic.*, **609**: 247–250.
  35. Masia, A. 1998. Superoxide Dismutase and Catalase Activities in Apple Fruit during Ripening and Post-Harvest and with Special



- Reference to Ethylene. *Physiol. Plantarum*, **104**: 668-672.
36. Mittova, V., Guy, M., Tal, M. and Volokita, M. 2002. Response of the Cultivated Tomato and Its Wild Salt-Tolerant Relative *Lycopersicon pennellii* to Salt-Dependent Oxidative Stress: Increased Activities of Antioxidant Zymes in Root Plastids. *Free Radic. Res.*, **36**: 195-202.
  37. Moran, R. and Porath, D. 1980. Chlorophyll Determination in Intact Tissues Using n,n-Dimethylformamide. *Plant Physiol.*, **65**: 478-479.
  38. Mugnai, S., Ferrante, A., Petrognani, L., Serra, G. and Vernieri, P. 2009. Stress-Induced Variation in Leaf Gas Exchange and Chlorophyll a Fluorescence in *Callistemon* Plants. *Res. J. Biol. Sci.*, **4**: 913-921.
  39. Navarro, A., Bañón, S., Conejero, W. and Sánchez-Blanco, M. J. 2008. Ornamental Characters, Ion Accumulation and Water Status in *Arbutus unedo* Seedlings Irrigated with Saline Water and Subsequent Relief and Transplanting. *Environ. Exp. Bot.*, **62**: 364-370.
  40. Niinemets, Ü. 2010. Responses of Forest Trees to Single and Multiple Environmental Stresses from Seedlings to Mature Plants: Past Stress History, Stress Interactions, Tolerance and Acclimation. *Forest Ecol. Manag.*, **260**: 1623-1639.
  41. Raddi, S., Cherubini, P., Lauteri, M. and Magnani, F. 2009. The Impact of Sea Erosion on Coastal *Pinus pinea* Stands: A Diachronic Analysis Combining Tree-Rings and Ecological Markers. *Forest Ecol. Manag.*, **257**: 773-781.
  42. Ruley, A. T., Sharma N. C. and Sahi S. V. 2004. Antioxidant Defense in a Lead Accumulating Plant, *Sesbania drummondii*. *Plant Physiol. Bioch.*, **42**: 899-906.
  43. Sánchez-Blanco, M. J., Rodríguez, P., Morales, M. A. and Torrecillas, A. 2003. Contrasting Physiological Responses of Dwarf Sea-Lavender and Marguerite to Simulated Sea Aerosol Deposition. *J. Environ. Qual.*, **32**: 2238-2244.
  44. Scott, M. J. and Jones, M. N. 2000. The Biodegradation of Surfactants in the Environment. *BBA – Biomembr.*, **1508**: 235-251.
  45. Sharma, N., Gupta, N. K., Gupta, S. and Hasegawa, H., 2005. Effect of NaCl Salinity on Photosynthetic Rate, Transpiration Rate, and Oxidative Stress Tolerance in Contrasting Wheat Genotypes. *Photosynthetica*, **43**: 609-613.
  46. Soshinkova T. N., Radyukina N. L., Korolkova D. V. and Nosov A. V. 2013. Proline and Functioning of the Antioxidant System in *Thellungiella salsuginea* Plants and Cultured Cells Subjected to Oxidative Stress. *Russian J. Plant Physiol.*, **60**: 41-54.
  47. Sudhir, P. and Murthy, S. D. S. 2004. Effects of Salt Stress on Basic Processes of Photosynthesis. *Photosynthetica*, **42**: 481-486.
  48. Talaat, N. B. and Shawky, B. T. 2012. 24-Epibrassinolide Ameliorates the Saline Stress and Improves the Productivity of Wheat (*Triticum aestivum* L.). *Environ. Exp. Bot.*, **82**: 80-88.
  49. Toscano, S., Farieri, E., Ferrante, A. and Romano, D. 2016. Physiological and Biochemical Responses in Two Ornamental Shrubs to Drought Stress. *Front. Plant Sci.*, **7**: 645.
  50. Ueda, A., Shi, W., Shimada, T., Miyake, H. and Takabe, T. 2008. Altered Expression of Barley Proline Transporter Causes Different Growth Responses in Arabidopsis. *Planta*, **227**: 277-286.
  51. Wang, Q., Chen, J., Stamps, R. H. and Li, Y. 2005. Correlation of Visual Quality Grading and SPAD Reading of Green-Leaved Foliage Plants. *J. Plant Nutr.*, **28**: 1215-1225.
  52. Wu, G. Q., Zhang, L. N. and Wang, Y. Y. 2012. Response of Growth and Antioxidant Enzymes to Osmotic Stress in Two Different Wheat (*Triticum aestivum* L.) Cultivars Seedlings. *Plant Soil Environ.*, **58**: 534-539.
  53. Zollinger, N., Cerny-Koenig, T., Kjelgren, R., Koenig, R. and Kopp, K. 2005. Salinity Tolerance of Eight Ornamental Herbaceous Perennials. *HortScience*, **40**: 1034-1035.

سازوکارهای دفاعی آنتی اکسیدانی در واکنش به آتروسل دریایی و سورفاکتانت  
Viburnum tinus L. 'Lucidum' و Callistemon citrinus (Curtis) Skeels در

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چکیده

در دهه های اخیر، مقاومت به تنش شوری در گیاهان شدیداً مورد مطالعه بوده است. بسیاری از گیاهان زینتی در فضای سبز مناطق ساحلی حضور دارند و در چنین مناطقی گیاهان در معرض آتروسل دریایی و سورفاکتانت ها قرار میگیرند که هر دو آنها در سواحل محیط های مدیترانه ای فراوانند. هدف این پژوهش بررسی فعالیت آنتی اکسیدانی آنزیم های دو گیاه زینتی به نام های Callistemon و Viburnum تحت تاثیر چنین شرایط تنش آلودی بود. برای تجزیه تحلیل عملکرد این گیاهان، ما سازو کار دفاعی آنتی اکسیداتیو را به صورت واکنش آنزیمی، سطح پرولین (Pro) و کلروفیل (Chl) و مالون دی آلدئید (MDA) در گیاهانی که به مدت ۸ هفته با آتروسل دریایی و سورفاکتانت تیمار شده بودند اندازه گیری کردیم. برای درک بهتر سازوکار دفاعی، دو دوره رشد متفاوت مطالعه شد: از ژانویه تا مارچ و از ماه می تا ژوئیه. درجه حرارت بیشتر در دوره دوم تاثیر منفی روی واکنش و عملکرد گیاهان داشت. همچنین، تنش شوری منجر به کاهش قابل ملاحظه مقدار کلروفیل در هر دو گونه گیاهی شد، به ویژه در دوره دوم. به طور مشخص، تیمار آتروسل دریایی در گیاهان Callistemon و Viburnum باعث به ترتیب ۲۹٪ و ۴۵٪ کاهش شد. مقدار Pro در Viburnum (154.35 nmol g<sup>-1</sup>) در مقایسه با Callistemon (1466.94 nmol g<sup>-1</sup>) خیلی کم بود. در مورد MDA عکس این روند مشاهده شد. آنزیم های تخلیه کننده ROS مانند سوپراکسید دیسموتاز (SOD)، کاتالاز (CAT) و گلوکاتایون پراکسیداز (GPX) در گیاهانی که در معرض تیمار آتروسل دریایی و سورفاکتانت آبیونی قرار گرفته بودند به طور معناداری بیشتر بود. بین این دو گیاه، Viburnum در برابر تنش ناشی از آتروسل دریایی سازوکار کارآمد تری نشان داد.