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Characterizing the effects of salt stress in *Calendula officinalis* L.

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Summary

In this study the effects of salt stress on growth and several stress markers were investigated in the ornamental and medicinal plant *Calendula officinalis*. One-month-old plants were submitted to increasing salt concentrations, up to 150 mM NaCl, for a period of 30 days. Salinity affected growth in terms of relative reduction of stem length and fresh weight of the plants, but water content remained unchanged indicating a certain tolerance to low and mild NaCl concentrations. Although Na^+ and Cl^- increased in parallel to increasing salinity, the levels of K^+ and Ca^{2+} showed no significant change, while Mg^{2+} levels recorded a twofold increase upon the application of the highest salt concentration. Other measured parameters showed a more significant change, notably proline levels, which registered a nine-fold increase in the presence of 150 mM NaCl. In conclusion, although plants suffered from salt stress, as shown by the degradation of photosynthetic pigments and induction of oxidative stress (increased MDA levels), they continued their vegetative growth under low concentrations of salt. The main mechanisms of response to salt stress in this species appear to be based on the maintenance of K^+ and Ca^{2+} homeostasis and the accumulation of proline as a functional osmolyte.

Keywords: *Calendula*; ions; osmolytes; proline; salt stress

Introduction

Given the ever rising human population and the spreading of soil salinization in the context of global climate change, stress tolerance studies are becoming increasingly important. Salinity can affect growth and yield of most plants (Munns, 1993), by inducing reduced mitosis in roots (Sharp et al., 1988) and leaves (Durand et al., 1995), increase in reactive oxygen species (ROS) levels (Apel and Hirt, 2004) and disrupted ability to detoxify them (Munns and Tester, 2008), chlorophyll degradation (Kato and Shimizu, 1987), and tissue injury (leaf senescence), among other effects (Allu et al., 2014), leading to the plants' death in case of severe or prolonged exposure to salinity.

Salinity induces significant changes in plants due to ionic toxicity and osmotic stress, both of which generate oxidative stress (Grattan and Grieve, 1999; Parida and Das, 2005). Salinity modifies the nutritional balance of plants, notably on the ionic level, resulting in higher ratios of Na^+/Ca^{2+}, Na^+/K^+, Na^+/Mg^{2+}, Cl^-/NO^{3-} and Cl^-/H_2PO^{4-}, thus causing growth retardation (Grattan and Grieve, 1999).

Growing more salt-tolerant ornamentals and non-crop species with economic importance (such as medicinal plants), can open up salt affected croplands – amounting to nearly 400 million hectares worldwide (Al-Sadi et al., 2010) – for re-use, and thus make more cultivable land available for crops that could be more affected by salinity in terms of yield. For example, saline soils that cannot support standard crops might be used to grow medicinal plants as novel crops (Muhammad and Hussain, 2010). One medicinal plant – which is also used as ornamental – with unknown salt tolerance potential is *Calendula officinalis* L., commonly known as marigold (not to be confused with *Tagetes* species, commonly known as French and African marigolds). *C. officinalis* has a very long history of medicinal use, especially for its anti-inflammatory properties. *Calendula* extract-containing creams and gels are commonly used to treat skin irritation, inflammation and burns, especially after radiotherapy, and to aid wound healing (Edwards et al., 2015).

The species is a short perennial that rarely survives hot summers or hard winters. It does however tolerate a wide range of soil types, and as such is considered among the most versatile flowers for ornamental and domestic use. *C. officinalis* is native to Southern Europe, but it has been naturalized in Northern Europe, and many temperate regions of North Africa, China, and the United States.

Despite the large body of literature on salt stress, little is known about the effects of salinity on this plant. Several studies have focused on the effects of salinity on seed germination or on seedling growth in *C. officinalis* (Antonello and Espindola, 2008; Torbaghan, 2012, Gharineh et al., 2013), whereas some others discussed water relations and ionic balance (Chaparzadeh et al., 2003), or some biochemical responses (Chaparzadeh et al., 2004; Oprica et al., 2015). Taking into account the economic potential of this species, further studies regarding the effects of salt stress on *C. officinalis* are deemed necessary.

The aim of this study was to analyze the growth of *C. officinalis* at low and moderate concentrations of NaCl and to assess which are the most relevant biochemical responses to salinity in this species.

Materials and methods

Plant material and growth conditions

Seeds of *C. officinalis* were obtained from the Agricultural Research and Development Station, Secuieni, Neamt County, Romania, and were sterilized with 5% sodium hypochlorite solution before being thoroughly rinsed with milli-Q water, prior to sowing on a mixture of commercial peat and vermiculite (3:1). After germination, seedlings were transplanted into individual polyethylene pots (Ø = 11 cm) with the same substrate. During the growth period, plants were irrigated with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Both the growth (4 weeks) and the following treatment (4 weeks) periods were carried out in the greenhouse of the Institute of Plant Molecular and Cellular Biology, Polytechnic University of Valencia,

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Spain. The greenhouse maintained a controlled environment, with regulated temperatures ranging between 17 and 23 °C, under a long-day photoperiod (16 h light / 8 h dark), with light intensity of 130 μE m^{-2} s^{-1}. Four-week-old plants were treated for a period of additional four weeks, being watered twice per week with 2 L of Hoagland's nutrient solution per tray (each tray with 10 individual pots), containing NaCl to the final concentrations of 0 (for the control treatment), 50, 100, or 150 mM; therefore, 10 replicates (individual plants) were used for each treatment.

Plant growth parameters

After four weeks of treatments, the aerial part of each individual plant was harvested and the following growth parameters were determined: stem length, leaf number, fresh weight (FW), and water content percentage. Fresh material was stored at -20 °C for further studies. Fresh weight was expressed in percentage of the values corresponding to the non-stressed controls. To determine water content, part of each sample was weighed (FW), dried at 65 °C until constant weight (96 h), and then weighed again (DW); water content of each sample (%) was calculated as described in Gil et al. (2014).

Photosynthetic pigments

Total carotenoids (Caro), chlorophyll a (Chl a) and chlorophyll (Chl b) were measured following Lichtenthaler and Wellburn (1983): 0.1 g of fresh leaf material was ground in the presence of 20 ml of ice-cold 80% acetone, mixed by vortexing and centrifuged. The supernatant was collected and its absorbance was measured at 663, 646, and 470 nm. The concentrations of Chl a, Chl b, and Caro were calculated according to the equations described by Lichtenthaler and Wellburn (1983). The final values were expressed in mg g^{-1} DW.

Monovalent and divalent ions

Ion measurements were performed according to Weimberg (1987) in aqueous extracts obtained by heating the samples (0.05-0.1 g of dried, ground plant material in 25 mL of water) in a water bath, for 15 min at 99 °C, followed by filtration through a 0.45 μm filter (Gelman Laboratory, PALL Corporation). Sodium and potassium ions were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, USA), and chlorides were measured using a Merck Spectroquant Nova 60® spectrophotometer and its associated test kit (Merck, Darmstadt, Germany), while calcium and magnesium ions were measured with an atomic absorption spectrometer SpectrAA 220 (Varian, Inc., CA, USA).

Proline and total soluble sugars

Proline and total soluble sugars (some of the most common osmolytes in plants) were determined in the stressed and control plants. Proline (Pro) was extracted with 3% (w/v) sulfosalicylic acid, from 0.05 g of dry plant material, and was quantified according to the acid-ninhydrin method described by Bates et al. (1973). Pro concentration was expressed in terms of μmol g^{-1} DW. Total soluble sugars (TSS) were measured in 0.05 g dry plant material suspended in 3 ml 80% (v/v) methanol, following the phenol / sulfuric acid method, according to Dubois et al. (1956). TSS contents were expressed as 'mg equivalent of glucose' per g DW.

MDA and non-enzymatic antioxidants

Malondialdehyde (MDA) content was determined according to the method described by Hodges et al. (1999) in the same extracts as those used to measure TSS. The samples were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA (or with 20% TCA without TBA for the controls), and then incubated at 99 °C for 15 min. After stopping the reaction on ice, the absorbance of the supernatants was measured at 532 nm.

Total phenolic compounds (TPC) were quantified according to Blainski et al. (2013), by reaction with the Folin-Ciocalteu reagent. Absorbance was measured at 765 nm, and the results expressed in equivalents of gallic acid (mg·eq·GA g^{-1} DW). Total flavonoids (TF) were measured following the method described by Zhishen et al. (1999), based on the reaction of catechol groups with $AlCl_3$; the absorbance was measured at 510 nm, and the amount of flavonoids was expressed in equivalents of catechin (mg·eq·C g^{-1} DW).

Statistical analysis

Statistical analysis was performed using the program Statgraphics Centurion v. XVI (Statpoint Technologies, Warrenton, Virginia, USA). Before the analysis of variance, the Shapiro-Wilk test was used to check for validity of normality assumption and Levene test for the homogeneity of variance. If ANOVA requirements were accomplished, the significance of the differences among treatments was tested by one-way ANOVA at a 95% confidence level and post-hoc comparisons were made using the Tukey HSD test.

Results

Plant growth parameters

An inhibition of vegetative growth due to salt stress was observed in terms of stem length and fresh weight percentage (Fig. 1). Stem length decreased, as compared to the non-stressed plants, with those from the 150 mM NaCl treatment recording a reduction of 40% of the control (Fig. 1A). Concerning fresh weight, a decrease of nearly 40% was also observed in plants treated with the highest salt concentration, 150 mM NaCl (Fig. 1B). On the other hand, water content (WC%) did not change significantly in response to the NaCl treatments (Fig. 1C).

Photosynthetic pigments

Sodium chloride affected photosynthetic pigments levels in the leaves of *C. officinalis* in all treatments (Fig. 2). Chl a contents decreased by nearly 50% under the strongest salt treatment applied (Fig. 2A), while Chl b and Caro recorded reductions of about 40% in the presence of 150 mM NaCl (Fig. 2B, 2C, respectively).

Monovalent and divalent ions

Na^+ increased in parallel to increasing external salinity, from approx. 350 μmol g^{-1} DW in the leaves of control plants to 1500 μmol g^{-1} DW in the presence of 150 mM NaCl (Fig. 3A), and Cl^- from 450 μmol g^{-1} DW to 2300 μmol g^{-1} DW under the same conditions (Fig. 3B). K^+ levels did not show any statistically significant change in response to the application of NaCl (Fig. 3C). Average leaf levels of divalent cations increased with increasing NaCl concentrations in the nutrient solution; however, the observed changes in Ca^{2+} contents were not statistically significant (Fig. 3D), while a significant increase of nearly twofold over the control, non-stressed plants was measured in the case of Mg^{2+} (Fig. 3E).

Osmolytes (proline and total soluble sugars)

In order to maintain osmotic pressure under different abiotic stress conditions causing cell dehydration, plants accumulate certain sol-

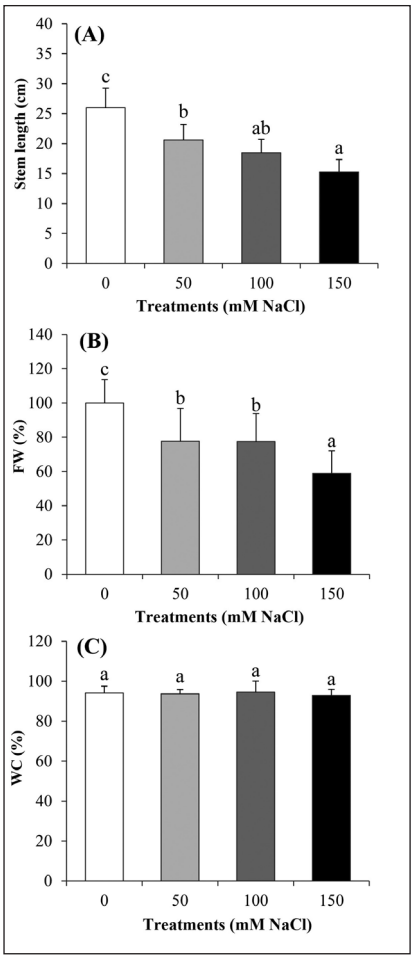

Fig. 1: Growth parameters in *C. officinalis* after four weeks of salt treatments. (A) Stem length (cm), (B) fresh weight of the aerial part (% of the control), and (C) water content percentage (WC %). Means with SD (n = 10). Different lowercase letters above the bars indicate significant differences between NaCl treatments, according to the Tukey test (α = 0.05).

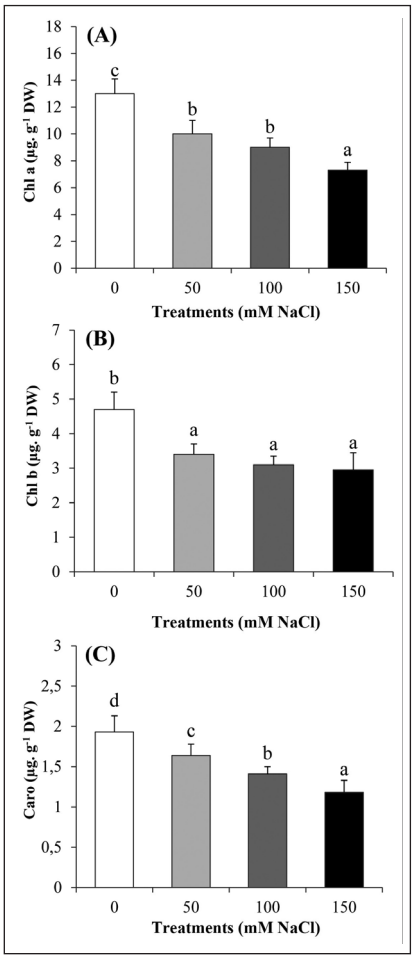

Fig. 2: Photosynthetic pigments in *C. officinalis* after four weeks of salt treatments. (A) Chlorophyll a (Chl a), (B) chlorophyll b (Chl b), and (C) total carotenoids (Caro). Means with SD (n = 10). Different lowercase letters above the bars indicate significant differences between NaCl treatments according to the Tukey test (α = 0.05).

uble inert compounds – compatible solutes or osmolytes – such as proline (Pro) and soluble sugars (TSS, e.g. glucose, fructose and sucrose, among others). The application of NaCl induced an increase in Pro levels in a concentration-dependent manner, reaching a 9-fold increment over the control in plants submitted to the 150 mM NaCl treatment (Fig. 4A). On the other hand, the changes observed in TSS levels did not correlate with the applied salt concentrations.

MDA and non-enzymatic antioxidants

Leaf contents of malondialdehyde (MDA), an oxidative stress biomarker that usually peaks under abiotic stress conditions, showed an increase in response to the salt treatments, up to twofold over the control in the presence of 150 mM NaCl (Fig. 5A). Antioxidants such as phenolic compounds and flavonoids tend to increase under stress in many species, to alleviate the secondary oxidative stress accompanying salt and other abiotic stresses. However, this does not seem to be the case in *C. officinalis*, as we did not observe such an increase in the leaf contents of total phenolic compounds (TPC, Fig. 5B) or total flavonoids (TF, Fig. 5C) upon the application of NaCl; in fact, a slight reduction in the levels of these compounds was detected in the presence of salt (Figs. 5B, 5C).

Discussion

Salt stress reduces plant growth and productivity by affecting morphological, anatomical, biochemical and physiological characteristics, processes and functions. Disturbed water and nutritional balance of plants may cause reduced crop yields in saline soils. Reduction in plant height and other growth parameters are the most distinct and obvious effects of salt stress, since inhibition of growth is probably the most general response of plants to stress (Munns, 2002; Munns and Tester, 2008). Depressed growth due to high salinity is attributed to several factors such as osmotic stress, specific ion toxicity and ion imbalance, and induced nutritional deficiency. In the present study, plant height decreased under salt stress, as found in all other studies performed on this species (Chaparzadeh et al., 2003; Bayat et al., 2012; Mirlotfi et al., 2015; Nofal et al., 2015). Although salinity affects growth in *Calendula*, low concentrations of 50 and 100 mM NaCl did not reduce drastically the stem length and fresh weight of the aerial part. Plants from the 50 and 100 mM treatments had only a 20% lower FW in comparison to the control, which is similar to the reduction of only 10 and 27% at 50 and 100 mM NaCl, respectively, reported by Chaparzadeh (2003); this degree of salt-induced growth inhibition is much lower than that reported under similar conditions for stress sensitive cultivars of different crops (see, for example, Bartha et al., 2015; Al Hassan et al., 2016a, among

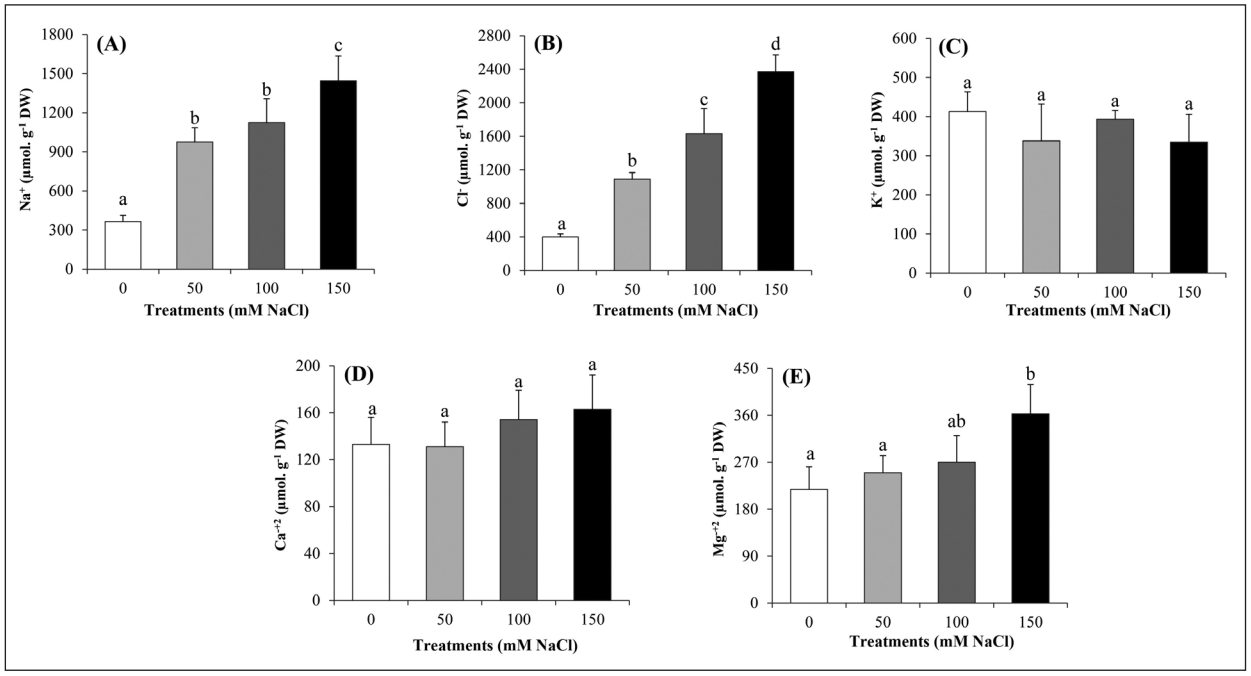

Fig. 3: Ion concentrations in *C. officinalis* after four weeks of treatments. (A) Sodium (Na^+), (B) chloride (Cl^-), (C) potassium (K^+), (D) calcium (Ca^{2+}), and (E) magnesium (Mg^{2+}). Means with SD (n = 10). Different lowercase letters above the bars indicate significant differences between NaCl treatments according to the Tukey test ($\alpha = 0.05$).

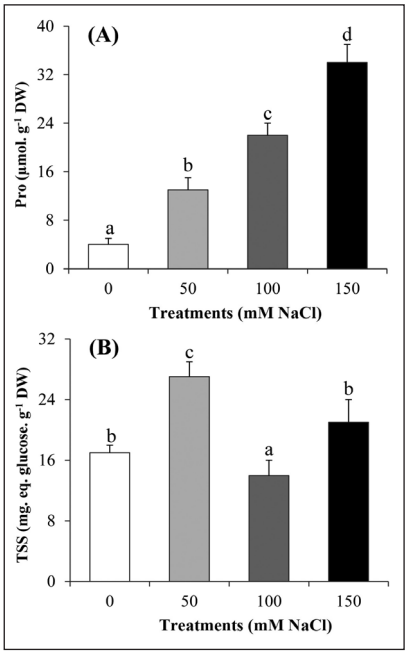

Fig. 4: Osmolytes levels in *C. officinalis* after four weeks of treatments. (A) Proline (Pro) and (B) total soluble sugars (TSS). Means with SD (n = 10). Different lowercase letters above the bars indicate significant differences between NaCl treatments according to the Tukey test ($\alpha = 0.05$).

many other references). Moreover, salt treatments did not produce a significant change in the water content of the plants leaves, indicating as well a relatively high resistance to dehydration, which will certainly contribute to some degree of salt tolerance in this species. Although clearly a glycophyte, *C. officinalis* is able to maintain vegetative growth at low concentrations of salt. Therefore, it may be considered for cultivation on soils that are not optimal for more salt sensitive crops.

Photosynthetic pigment concentrations in the leaves of salt stressed *C. officinalis* recorded a significant decrease in comparison with those measured in unstressed controls, which is likely due to chlorophyll degradation induced by toxic levels of NaCl. These results are consistent with those reported by Bayat et al. (2012), who indicated that chlorophyll content significantly decreased in the leaves of *C. officinalis* with increasing NaCl concentration. Reduction of chlorophyll levels in salt-treated plants is due to the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Santos, 2004). Yet, this 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). There are, however, some reports suggesting that chlorophyll and carotenoid contents in *C. officinalis* are not affected by salinity (Mirlotfi et al., 2015) or can even increase in the presence of salt (Oprica et al., 2015). This apparent contradiction may be due to the use of different experimental growth conditions in these reports. In any case, a reduction of photosynthetic pigment levels appear to be a general response to salt stress (Parihar et al., 2015), as we have shown in previous work with other species (Al Hassan et al., 2015, 2016a, b; Kumar et al., 2017).

An interesting pattern of variation was found in the ion content of foliar tissue. As the applied salt concentration increased, Na^+ and Cl^- levels also increased, in a concentration-dependent manner, reaching more than four-fold and five-fold, respectively, over the corresponding controls in the presence of 150 mM NaCl. Sodium accumulation in plants is usually accompanied by the inhibition of many enzymatic activities and physiological processes**,** and by a reduction in the concentrations of potassium, as both ions compete for the same membrane transporters (Rodríguez-Navarro, 2000). Moreover, K^+ deficiency has negative effects on photosynthesis, osmoregulation, protein biosynthesis and turgor driven movements (Gierth and Mäser, 2007). Intracellular K^+ and Na^+ homeostasis is important for

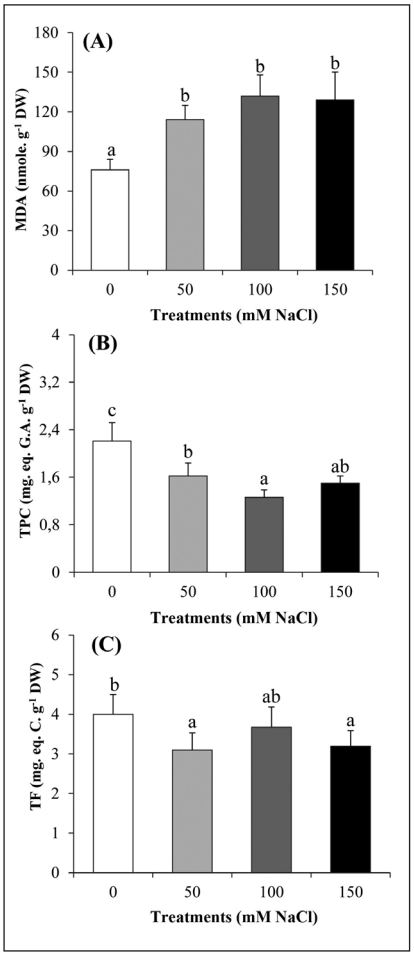

Fig. 5: Oxidative stress marker MDA and chemical antioxidants in *C. officinalis* after 4 weeks of treatments. (A) Malondialdehyde (MDA), (B) total phenolic compounds (TPC), and (C) total flavonoids (TF). Means with SD (n = 10). Different lowercase letters above the bars indicate significant differences between NaCl treatments according to the Tukey test ($\alpha = 0.05$).

the activities of many cytosolic enzymes, and for maintaining membrane potential and an appropriate osmoticum for cell volume regulation. Therefore mechanisms which allow maintaining relatively low Na^+/K^+ ratios are essential for salt tolerance of plants (NIU et al., 1995; RODRÍGUEZ-NAVARRO, 2000). Contrary to this general behavior, this study showed that the accumulation of Na^+ in the leaves of *C. officinalis* did not lead to a significant reduction of K^+, indicating that a possible defense mechanism against salt stress is the activation of K^+ transport from roots to the aerial parts of plants, avoiding excessively high Na^+/K^+ ratios in the leaves, as we have also reported for some other species (SCHIOP et al., 2015; AL HASSAN et al., 2016b).

Salt tolerance is also depending on the plant's capacity to accumulate Na^+ and Cl^- in the vacuole, to avoid reaching toxic concentrations in the cytoplasm, a mechanism that is especially efficient in some succulent, highly tolerant dicotyledonous halophytes. This 'ion compartmentalisation hypothesis' (FLOWERS et al. 1977; WYN JONES et al., 1977; GLENN et al., 1999) is generally accepted, although it is supported by few experimental data, given the technical difficulties to determine ion concentrations in the vacuole and the cytoplasm separately. Since we also measured ion contents in the whole leaf material, without any subcellular discrimination, we cannot estimate the possible contribution of this mechanisms to the salt tolerance of *C. officinalis*.

Regarding changes in divalent cations, their average leaf concentrations increased under salt treatments, but this variation was significant only for Mg^{2+} (not for Ca^{2+}). The divalent cation Ca^{2+}, an essential mineral nutrient, has an important and well-known role in the mechanisms of plant responses to salinity, reducing the harmful effects of Na^+ (BRESSAN et al., 1998; HEPLER, 2005; GUL and KHAN, 2006). The possible role of Mg^{2+} in those mechanisms is not so well established, but it has been shown that inhibition by salt of some enzymes that use Mg^{2+} as cofactor is due to its displacement from the active centre by Na^+ (ALBERT et al., 2000). Therefore, increased concentrations of intracellular Mg^{2+} (without reaching toxic levels) may partially counteract the inhibitory effect of Na^+ and contribute to salt stress tolerance in plants (BOSCAIU et al., 2011; GRIGORE et al., 2012).

Osmolyte accumulation in the cytosol is a general response to abiotic stress in all plants, both halophytes and glycophytes. Besides their main function in osmotic adjustment, osmolytes play many other important roles in the mechanisms of stress defense, as low-molecular weight chaperones, reactive oxygen species (ROS) scavengers or signalling molecules (ASHRAF and FOOLAD, 2007; SZABADOS and SAVOURÉ, 2010; GIL et al., 2013). Proline is one of the most studied compatible solutes of plants, and is considered to play the primary role in protecting plants against osmotic stress in many different species (ASHRAF and FOOLAD, 2007; VERBRUGGEN and HERMANS, 2008; AL HASSAN et al., 2016b). In this study, we found a clear positive correlation between Pro accumulation and the intensity of the applied stress treatments, suggesting its participation in the mechanisms of salt resistance in *C. officinalis*, as osmoprotectant and contributing to cellular osmotic adjustment under stress conditions.

The role of soluble sugars and polyalcohols in salt tolerance is also well documented (GIL et al., 2011, 2013). Total soluble sugars accumulate depending on the intensity of salt stress, time exposure and organs of plants where it has been measured (NEMATI et al., 2011). In our experiments we did not find a uniform pattern regarding the accumulation of total sugars in response to the exposure to salt, as changes in leaf sugar contents did not correlate with increasing external NaCl concentrations. Therefore, with the present data it is difficult to speculate about their possible role in salt stress tolerance in *C. officinalis*. It cannot be ruled out that, in addition to Pro, some specific sugar may act as a functional osmolyte in *C. officinalis*, accumulating specifically in the leaves of salt-treated plants, this being masked by changes, not related to salt stress responses, in the concentrations of other sugars. Fractionation of the extracts, for example by HPLC, identification and quantification of individual sugars would be required to check this hypothesis.

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., 2005). In this study a significant increase of MDA levels in *C. officinalis* upon salt stress treatments of the plants was observed, indicating that plants suffered from secondary oxidative stress associated with salinity.

C. officinalis is rich in several compounds considered as 'health-promoting', such as carotenoids, flavonoids and other phenolics (RAAL and KIRSIPUU, 2011). These secondary metabolites play multiple roles in plants, including scavenging of ROS induced under different stress conditions and causing oxidative stress. In our study, leaf levels of carotenoids, total phenolics and flavonoids, all showed a significant, concentration-dependent decrease in *C. officinalis* salt-treated plants; this suggests that these compounds do not play any major role in the mechanisms of tolerance to salinity in this species.

In conclusion, although *C. officinalis* is affected by salt stress at the vegetative growth stage, it shows nonetheless a certain tolerance to low and moderate concentrations of NaCl (50 - 100 mM). The responses to increasing salt concentrations contributing to this tole-

rance appear to be related to the maintenance of K^+ and Ca^{2+} leaf levels and a slight increase in Mg^{2+}, to counterbalance the negative effects of Na^+, and to the synthesis and accumulation of proline as a physiological osmolyte. Therefore, *C. officinalis* might be considered as a possible candidate for cultivation on soils regarded unfit for more sensitive crops, as it would tolerate low quality, slightly saline water for irrigation. However, if grown as a medicinal plant, it should be taken into account that the contents of some interesting secondary metabolites, such as antioxidant carotenoids and phenolics, will decrease in *C. officinalis* plants irrigated with saline water.

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