Does CaCl₂ Play a Role in Improving Biomass Yield and Quality of Cardoon Grown in a Floating System under Saline Conditions?

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Abstract. Supplemental calcium application has been reported to alleviate the detrimental effect of NaCl-induced salinity on crop growth. Iso-molar solutions of NaCl and NaCl plus CaCl₂ were used to study the osmotic and ionic effects of salinity on leaf dry biomass production and nutraceutical quality of cardoon (*Cynara cardunculus* L. var. *altilis* DC) grown in a floating system. A basic nutrient solution (control; T1) was enriched with 15 mM of NaCl + 10 mM of CaCl₂ (T2), 30 mM of NaCl (T3), 30 mM of NaCl + 20 mM of CaCl₂ (T4), or 60 mM of NaCl (T5). NaCl at 60 mM induced a 52% reduction of total leaf dry biomass compared with the control (T1); the iso-molar solution enriched with 20 mM of CaCl₂ (T4) increased the total leaf dry biomass production in comparison with treatment containing NaCl at 60 mM (T5). Moreover, at moderate salinity (T2 and T3), the partial replacement of NaCl with 10 mM of CaCl₂ (T2) in treatment containing 30 mM of NaCl did not help to reduce the adverse effect of NaCl on total leaf dry biomass production. Results of leaf mineral analysis demonstrated that the partial replacement of NaCl with CaCl₂ reduced the accumulation of sodium and the nutrient imbalance. Nutrient solutions enriched with CaCl₂ did not increase the accumulation of the osmoprotectant proline in leaves. Nutraceutical value of cardoon leaves was generally improved by saline treatments compared with the control. The regression analysis between phenolic compounds and antioxidant activity showed that total phenols and chlorogenic acid were the major determinants of antioxidant activity in cardoon leaf biomass. In conclusion, the partial replacement of NaCl with CaCl₂ improved the leaf dry biomass production of cardoon only at the highest salinity levels with a limited effect on nutraceutical quality of leaves.

In recent decades, the reduction of fresh water sources coupled with an increase in population and in agricultural production overall led to the use of lower quality and

saline-sodic drainage waters in agriculture. In many regions of the Mediterranean basin, the intensive greenhouse cultivation has to resort to irrigation water with high salt concentration causing salt stress problems (Colla et al., 2012).

Growth reduction and metabolic changes caused by salinity are attributable to both osmotic and ion-specific effect. The high salt concentration in the nutrient solution leads to an increase in external osmotic pressure making it harder for roots to extract water, to a direct toxicity of saline ions, and to ion imbalance (Munns and Tester, 2008).

The cultivation of salt-tolerant plants is an interesting strategy to cope with salinity problems under greenhouse conditions. Moreover, salinity stress can lead to a stimulation of

a plant's secondary metabolism improving antioxidant level (e.g., polyphenolic compounds, anthocyanins, α -tocopherol, ascorbate, and glutathione) of crops (Sreenivasulu et al., 2000). An increased antioxidant content was found in crops irrigated with saline waters under soil culture conditions, e.g., chamomile (*Matricaria recutita* L.) (Baghalian et al., 2008), as well as in soilless greenhouse cultures, e.g., Aloe spp. (*Aloe barbadensis* Miller and *Aloe arborescens* Miller) (Cardarelli et al., 2013), broccoli (*Brassica oleracea* L.) (Dominguez-Perles et al., 2011), and cucumber (*Cucumis sativus* L.) (Colla et al., 2013a).

Cultivated cardoon, recently classified as a salt-tolerant medicinal plant rich in polyphenolic compound (Ksouri et al., 2012), is a perennial herbaceous plant that has been grown from ancient times in Mediterranean areas, mainly Italy, Spain, and the south of France. Traditionally, cardoon is cultivated for the fleshy leaf petioles used to prepare typical dishes. However, cardoon is also used as cheese rennet (Verissimo et al., 1995) and in pharmacological and nutraceutical preparations (Fernández et al., 2006). *Cynara* leaf extracts have been used since ancient times for hepatobiliary system regulation and today a wide range of medicinal properties is recognized such as antioxidant effects (Kukic et al., 2008; Miccadei et al., 2008). The antioxidant capacity of cardoon extracts was found to be strongly dependent on the qualitative and quantitative phenolic profile (Pandino et al., 2011).

A previous study indicated that the application of moderate salinity stress (30 mM of NaCl) was successful in improving biomass quality of cardoon grown in a floating system (Colla et al., 2013b). However, solution enriched with 30 mM of NaCl improved the antioxidant production in cardoon leaves at the expense of the biomass yield, whereas the use of a solution enriched with 30 mM of CaCl₂ enhanced the biomass quality only in the long term without a detrimental effect on biomass yield (Borgognone et al., 2013). The addition of supplemental calcium (Ca) to irrigation water was found to mitigate the adverse effects of salinity on other crops [e.g. strawberry (Khayyat et al., 2011) or *Cichorium intybus* L. (Arshi et al., 2010)]. Starting from these considerations, we hypothesized that application of solutions enriched with both NaCl and CaCl₂ salts could reduce the detrimental effect of NaCl on leaf biomass accumulation and improve the nutraceutical value of cardoon biomass already after short-term exposure to salinity. The objectives of this study were: 1) to evaluate if CaCl₂ can mitigate the detrimental effect of NaCl salinity on leaf dry biomass yield; 2) to understand if CaCl₂ can affect the polyphenol content in cardoon leaves under NaCl salinity stress; and 3) to study the mechanisms related to CaCl₂ effects.

Material and Methods

Plant materials, growth conditions, and treatments. The experiment was conducted in

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Spring 2013 in a 300-m² polymethylmethacrylate greenhouse at the Experimental Farm of Tuscia University (lat. 42°25' N, long. 12°08' E, altitude 310 m). The daily temperature of the greenhouse was maintained between 12 and 30 °C by forced ventilation and day/night relative humidity was 55%/85%. Plants were grown under natural light conditions.

Photosynthetically active radiation above the canopy was measured using a LI-COR quantum sensor (LI-190SA, Lincoln, NE). The average photosynthetically active radiation during the growing cycle was 775 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Seeds of a cardoon cultivar Bianco Avorio (La Semiorto Sementi, Lavorate di Sarno, Italy) were sown on 7 Mar. 2013 in a floating system (Borgognone et al., 2013). The composition of the basic nutrient solution was 13 mmol·L⁻¹ NO₃-N, 1 mmol·L⁻¹ NH₄-N, 1.75 mmol·L⁻¹ sulfur, 1.5 mmol·L⁻¹ phosphorus (P), 5 mmol·L⁻¹ potassium (K), 4.5 mmol·L⁻¹ Ca, 2 mmol·L⁻¹ magnesium, 1 mmol·L⁻¹ Na, 1 mmol·L⁻¹ chloride (Cl), 20 $\mu\text{mol}\cdot\text{L}^{-1}$ iron, 9 $\mu\text{mol}\cdot\text{L}^{-1}$ manganese, 0.3 $\mu\text{mol}\cdot\text{L}^{-1}$ copper, 1.6 $\mu\text{mol}\cdot\text{L}^{-1}$ zinc, 20 $\mu\text{mol}\cdot\text{L}^{-1}$ boron, and 0.3 $\mu\text{mol}\cdot\text{L}^{-1}$ molybdenum with an electrical conductivity of 2 dS·m⁻¹. The experiment treatments were five nutrient solutions, a basic nutrient solution as a control and four saline solutions (with two different total ion concentrations) obtained by adding to the basic nutrient solution different amounts of NaCl and CaCl₂ (Table 1). Treatments started 29 d after sowing (DAS) at the two-true leaf stage. The pH of the nutrient solutions in all treatments was 6 ± 0.3. The nutrient solutions were completely renewed weekly and prepared using deionized water. The treatments were arranged in a randomized complete block design with three replicates per treatment. Each plot had an area of 0.1815 m² with 84 plants.

Biomass determination. All plants of cardoon, except for border plants, were mowed three times during the growing cycle at 48, 70, and 95 DAS. Leaves were harvested when the plant height reached 20 to 25 cm. At each

harvest, the leaf tissues were dried in a forced-air oven at 60 °C for 72 h for biomass determination. The material of each harvest was used for mineral and quality analysis.

Mineral analysis. The mineral analysis was performed separately for each replicate sample of dried tissues. The dried leaf tissues were ground in a Wiley mill to pass through a 20-mesh screen, then 0.5 g samples were analyzed for the following macronutrients: nitrogen (N), P, K, Ca, Na, and Cl. Nitrogen concentration was determined after mineralization with sulfuric acid by the Kjeldahl method (Bremner, 1965). Phosphorus, K, Ca, and Na were determined by dry-ashing at 400 °C for 24 h, dissolving the ash in HNO₃ (1:20 w/v), and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP-Iris; ThermoOptek, Milan, Italy) (Karla, 1998). Chloride ion concentration was determined by titration with AgNO₃ in the presence of K₂CrO₄ (Eaton et al., 1995).

Nitrate concentration in dry leaves of cardoon was analyzed using the salicylic acid–sulfuric acid method (Cataldo et al., 1975) by spectrophotometry (Helios Beta, Spectrophotometer; Thermo Electron Corporation, U.K.).

Evaluation of antioxidant activity. One hundred milligrams of each dried leaf sample was extracted sequentially three times in 10, 5, and 5 mL of fresh ethanol/water (80:20 v/v) containing 1% of HCl 37%, respectively (total volume 25 mL). The extraction mixture was stirred for 10 min and then solids were removed by centrifugation (503 × g, 5 min). The total content of phenolic compounds in each extract was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965) and results were expressed as gallic acid equivalents (milligrams gallic acid/gram dry weight). The Folin reagent and the salts for analysis were reagent-grade materials from Sigma Aldrich, Milan, Italy.

Total flavonoids were determined following the colorimetric aluminum chloride

method (Dutta and Maharia, 2012) and results were expressed as quercetin (reagent grade from Sigma Aldrich, Milan, Italy) equivalents (milligrams quercetin/gram dry weight).

The antioxidant activity was determined by ferric-reducing ability of plasma (FRAP) assay following the method of Pellegrini et al. (2003). Results were expressed as $\mu\text{mol FeSO}_4$ (reagent grade from Sigma Aldrich)/gram dry weight.

The method previously adopted by Colla et al. (2013b) was followed to determine caffeoylquinic acids [1,3-di-O-caffeoylquinic acid (cynarin), 3-O-caffeoylquinic acid (chlorogenic acid), and caffeic acid] and flavonoids (apigenin, luteolin, and luteolin-7-glucoside) by liquid chromatography followed by tandem mass spectroscopy with an electrospray ionization source. Pure caffeoylquinic acids and flavonoids for calibrations were purchased as certified reference materials from Sigma (St. Louis, MO). Individual stock solutions (500 $\mu\text{g}\cdot\text{mL}^{-1}$) were prepared in pure methanol with sonication and stored at -30 °C for up to 5 d. Working standard solutions were prepared daily in amber glass flasks by diluting combined aliquots of the stock solutions in the high-performance liquid chromatography mobile phase.

Proline analysis. The third and fourth leaves from the apical shoot of four plants per plot were frozen immediately in liquid N at each harvest (48, 70, and 95 DAS). The method of Bates et al. (1973) was adopted to determine the free proline content by spectrophotometry (Helios Beta, Spectrophotometer; Thermo Electron Corporation). Proline concentration was calculated on a fresh weight basis using L-proline for the standard curve.

Statistical analysis. All data were subjected to analysis of variance (ANOVA) using SPSS 14 for Windows (SPSS Inc., Chicago, IL). Duncan's multiple range test was performed at $P = 0.05$ on each of the significant variables measured. A significant level of ANOVA was reported for $P \leq 0.05$, 0.01, and 0.001. Regression analyses were conducted to identify relationships between FRAP and total phenols, total flavonoids, and target polyphenols in cardoon leaves.

Results

Leaf dry biomass. The addition of different concentrations of NaCl and CaCl₂ to the nutrient solution affected the biomass production (Table 2). At 48 DAS a significant reduction of biomass was observed in T5 treatment. At 70 DAS, the addition of NaCl with or without CaCl₂ (T2, T3, T4, and T5) caused a decrease of leaf biomass compared with T1 (control) for both iso-molar concentrations. At 95 DAS, T2 was not significantly different from T1, whereas T3, T4, and T5 induced a significant reduction of leaf biomass. Total leaf biomass was significantly reduced by 52% in T5 compared with T1, whereas T4, T3, and T2 caused a reduction of 30%, 30%, and 23%, respectively.

Table 1. Treatments tested during the trial: salt concentrations added to the basic nutrient solution, total ion concentration (Na⁺, Ca⁺⁺, and Cl⁻), and EC values of solutions.

Treatments	NaCl (mm)	CaCl ₂ (mm)	Total ion concn (mm)	EC (dS·m ⁻¹)
T1	0	0	0	2.0
T2	15	10	60	4.6
T3	30	0	60	4.6
T4	30	20	120	6.8
T5	60	0	120	6.8

EC = electrical conductivity.

Table 2. Effects of NaCl and CaCl₂ on leaf dry biomass of cardoon harvested at different days after sowing (DAS) and on total leaf dry biomass.

Treatment	Leaf dry biomass (g/plant)			
	48 DAS	70 DAS	95 DAS	Total
T1	0.76 a	1.14 a	2.67 a	4.57 a
T2	0.63 ab	0.78 b	2.12 ab	3.53 b
T3	0.61 ab	0.72 b	1.84 bc	3.17 b
T4	0.67 a	0.79 b	1.75 bc	3.21 b
T5	0.51 b	0.58 b	1.10 c	2.19 c
Significance ^z	*	**	*	**

^z*, **Significant at $P \leq 0.05$ and 0.01, respectively. Different letters within each column indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

Mineral content. Saline treatments did not affect total N and P content in leaves (data not shown). Treatments significantly affected K content in leaves (Table 3). Generally, T5 treatment caused the higher reduction of K content in comparison with the control with a decrease percentage of 35%, 47%, and 49% at 48, 70, and 95 DAS, respectively. The decrease of K content in T4, T3, and T2 treatment with respect to T1 treatment was 20%, 25%, and 17% at 48 DAS, and of 33%, 36%, and 21% at 70 DAS, respectively. At 95 DAS, T2, T3, and T4 did not decrease significantly the K content compared with T1 treatment. At 48 DAS, T3 and T5 caused a reduction of the leaf Ca content by 31% and 41%, respectively, compared with T1 treatment (Table 3). At 70 DAS, a reduction of Ca content by 34% and 46% was observed in T3 and T5 treatments, respectively, in comparison with T1 treatment. On the contrary, T2 and T4 induced an increase of Ca content by 18% and 31%, respectively, in comparison with T1. At 95 DAS, the highest Ca content was detected in T2 treatment followed by T4 > T1 > T3 > T5. At 48 and 70 DAS, all saline treatments caused an increase of leaf Na content compared with the control in the following order: T5 > T3 > T4 > T2 (Table 3). At 95 DAS, the highest Na content in leaves was observed in T5 treatment. Intermediate contents of Na were observed in the other saline treatments (T2, T3, and T4). At 48, 70, and 95 DAS, leaf nitrate content was significantly reduced in all saline treatments compared with the control (Table 3).

At 48, 70, and 95 DAS, leaf chloride content increased in response to saline treatments in comparison with the control (Table 3). At 48 DAS, the highest Cl content was recorded in T4 and T5 treatments. At 70 and 95 DAS, all saline treatments increased similarly the leaf Cl content in comparison with T1 treatment.

Proline content. Proline concentration in leaves of cardoon was significantly affected by treatments (Fig. 1). At 48 and 95 DAS, proline content of leaves was higher in T5 and T4 treatments than in T1 treatment. At 70 DAS, leaf proline content was increased in all saline treatments in comparison with the control treatment (T1).

Total phenolics, total flavonoids, antioxidant activity, and target polyphenols. A significant effect of treatments was recorded on total

phenolics (TPs), total flavonoids (TFs), and antioxidant activity (FRAP) of cardoon leaves (Table 4).

At 48 DAS, T2, T3, and T5 induced an increase of TP compared with the control treatment T1 $\times$ 38%, 42%, and 26%, respectively. At 48 DAS, TF content was highest in T3 and T5 treatments with an increase of 520% and 370%, respectively. Similarly, FRAP increased with saline treatments and the highest values were recorded with T2 and T3 treatments. At 70 and 95 DAS, all saline treatments enhanced TP, TF, and FRAP of cardoon leaves in comparison with the T1 treatment.

Caffeic acid, chlorogenic acid, and cynarin were detected in cardoon leaves with significant differences among treatments (Table 5).

At 48, 70, and 95 DAS, the highest caffeic acid content was recorded in T5 treatment. At 48, 70, and 95 DAS, chlorogenic acid content was enhanced in all saline treatments compared with the control (T1). Moreover, at 95 DAS, the highest chlorogenic acid content was recorded in T2, T4, and T5 with an increase of 336%, 391%, and 364% in comparison with T1 treatment, respectively.

At 48 and 70 DAS, leaf cynarin content was highest in all saline treatments, whereas at 95 DAS, the highest cynarin content was recorded in T5 with an increase of 425% in comparison with T1 treatment.

Flavonoids such as apigenin, luteolin, and luteolin-7-glucoside were also detected in cardoon leaves, but no significant effects of treatments were observed (data not shown).

Linear regression analysis showed an increase of FRAP as a function of total phenol content in cardoon leaves (Fig. 2). A significant positive correlation was also found between FRAP and chlorogenic acid (Fig. 2), whereas weak correlations between FRAP and total flavonoids ($R^2 = 0.55$), cynarin ($R^2 = 0.15$), and luteolin ($R^2 = 0.54$) were recorded (data not shown).

Discussion

Osmotic versus ionic effect. The use of iso-osmotic saline solutions with different ionic composition can help in discriminating the effects of specific ion toxicities during salt stress (Navarro et al., 2003).

Because salinity often leads to a significant reduction of Ca activity in solution, the

addition of supplemental Ca to irrigation water represents an interesting approach to reduce the detrimental effects of NaCl salinity on crops (Munns and Tester, 2008).

At the highest level of salinity (T4 and T5 treatments), T5 showed a more detrimental effect on biomass production than the iso-osmotic treatment with CaCl_2 (T4) indicating that cardoon response to a higher level of salinity was mainly the result of ionic effects. Calcium addition showed a beneficial effect on biomass production at the highest concentration of salts, where the ionic effect of salinity prevailed. Calcium can mitigate the detrimental ionic effect of salinity rather than the osmotic one helping to preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion-exchange behavior as well as cell wall enzyme activities (Rengel, 1992).

On the contrary, at moderate salinity (T2 and T3 treatments), there were no significant differences on leaf dry biomass production between the two equimolar solution treatments (T2 and T3) indicating that cardoon response to moderate salinity was mainly caused by the osmotic effect. Therefore, at moderate salinity, the partial replacement of Na with Ca did not help to mitigate the detrimental effect of osmotic stress on leaf dry biomass production. Silva et al. (2008) studied the effect of NaCl treatments at 30 and 60 mM in comparison with two iso-osmotic nutrient concentrations in pepper plants (*Capsicum annuum* L.) and they concluded that at the highest salt concentration, plant stress is mainly the result of ions toxicity, whereas at the lowest salinity level, the osmotic effect prevailed.

Some authors suggested that Ca can help to mitigate also the osmotic effect of salinity through the accumulation of organic solutes such as proline and glycinebetaine (Girija et al., 2002). Proline is a compatible solute involved in osmotic adjustment and acts as a non-toxic osmotic solute stabilizing the structure of macromolecules and organelles in plants subjected to salt stress (Munns and Tester, 2008; Renault and Affifi, 2009). CaCl_2 was found to mitigate the NaCl-induced stress through the enhancement of proline accumulation in many plant species [e.g. *Cicorium intybus* L. (Arshi et al., 2010), *Linum usitatissimum* L. (Nasir Khan et al., 2010), and

Table 3. Effect of saline treatments on potassium (K), calcium (Ca), sodium (Na), nitrate (NO_3), and chloride (Cl) content in leaves of cardoon at 48, 70, and 95 d after sowing (DAS).

Treatment	Mineral element ($\text{g}\cdot\text{kg}^{-1}$ dry wt)														
	K			Ca			Na			NO_3			Cl		
	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS
T1	51.8 a	50.9 a	46.6 a	12.4 a	13.1 b	12.8 c	1.8 e	1.1 e	1.1 c	16.2 a	12.3 a	15.2 a	22.1 c	22.8 b	15.1 b
T2	43.2 b	40.3 b	40.1 a	12.4 a	15.5 a	18.3 a	10.6 d	9.5 d	12.5 b	6.2 bc	3.1 b	5.7 b	55.5 b	70.2 a	72.3 a
T3	38.9 c	32.5 bc	36.4 a	8.6 b	8.7 c	8.8 d	22.8 b	20.8 b	21.2 b	7.5 b	4.6 b	6.1 b	58.2 b	69.3 a	61.1 a
T4	41.6 bc	33.9 bc	37.0 a	13.9 a	17.1 a	15.6 b	12.4 c	12.6 c	12.5 b	5.0 c	3.9 b	4.0 b	62.7 ab	68.2 a	70.1 a
T5	33.5 d	27.1 c	23.7 b	7.3 b	7.1 c	6.3 e	31.0 a	29.3 a	33.4 a	5.6 bc	3.9 b	3.7 b	68.3 a	74.5 a	75.6 a
Significance ^z	***	**	*	***	***	***	***	***	*	***	*	**	***	**	***

^z*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

Cassia angustifolia Mill. (Arshi et al., 2005)]. Differently, in this study, proline content increased with salinity independently of the salt source. This result suggested that Ca addition did not help to overcome the negative osmotic effects through proline accumulation.

Mineral analysis results supported the hypothesis that the highest biomass reduction in T5 treatment was mainly the result of the ionic effect of salinity. The highest concentration of Na ion and the nutritional imbalance associated with a reduction of K and Ca content were detected in T5 treatment. It is well known that high concentrations of Na and Cl can depress nutrient uptake and produce an extreme ratio of Na/Ca and Na/K. High concentration of Na reduced the availability and uptake of Ca as a result of the

antagonistic interaction, precipitation, and increases in ionic strength (Cramer, 1992; Rengel, 1992).

The partial replacement of NaCl with CaCl₂ in the solution improved the nutritional status of cardoon leaves. Calcium content in cardoon leaves was enhanced by equimolar solutions containing CaCl₂, and K depletion and Na accumulation were reduced in comparison with the solutions without CaCl₂.

Potassium uptake was also reduced by a high level of Na as a result of competition and loss of membrane integrity and selectivity (Grattan and Grieve, 1999). Potassium depletion was greater in cardoon plants treated with NaCl than with NaCl + CaCl₂ at both iso-molar levels. A possible explanation is that additional Ca helped to maintain K uptake,

counteracting the K/Na competition effect. The presence of a high level of Ca in the solution influences the K/Na selectivity by shifting the uptake ratio in favor of K at the expense of Na. Moreover, Ca preserves membrane integrity and then leads to the reduction of K leakage from root cells (Grattan and Grieve, 1999). K uptake was negatively affected by NaCl salinity in our previous study on cardoon (Borgognone et al., 2013) and in a number of plant species such as maize (*Zea mays* L.) (Akram, 2014), cucumber (*Cucumis sativus* L.), and melon (*Cucumis melo* L.) (Rouphael et al., 2012a). The positive role of Ca on improving K content in plant tissues has been reported in *Arabidopsis thaliana* where 10 mM of CaCl₂ completely prevented salt-induced K efflux from both roots and leaves (Shabala et al., 2006).

Antagonism between uptake of NO₃ and Cl was also observed in cardoon plants subjected to salt stress. Nitrate depletion and Cl ion accumulation could have contributed to the growth reduction induced by saline treatments. NO₃/Cl interactions are reported to be analogous to K/Na interactions and selectivity (Teakle and Tyerman, 2010). Above that, salinity can also depress NO₃ xylem transport rate and NO₃ reduction rate in leaves with a consequent reduction of NO₃ uptake, as demonstrated in a study with salt-sensitive bean (*Phaseolus vulgaris* L.) and salt-tolerant cotton (*Gossypium hirsutum* L.) (Gouia et al., 1994).

Chloride concentration was higher than Na concentration in leaves, indicating the inability of cardoon to restrict Cl uptake. In some plant species, the uptake and transport of Cl appeared to be less controlled than of Na (Alaoui-Sossé et al., 1998) as observed in cucumber (Colla et al., 2012, 2013a) and in red-osier dogwood (*Cornus sericea* L.). The critical toxicity concentration of Cl in leaf

Fig. 1. Effects of saline treatments on proline content in leaves of cardoon at 48, 70, and 95 d after sowing (DAS). Different letters within the five columns indicate differences according to Duncan's test ($P = 0.05$).

Table 4. Effects of saline treatments on total phenols, total flavonoids, and antioxidant activity (FRAP) in leaves of cardoon at 48, 70, and 95 d after sowing (DAS).

Treatment	Total phenols (mg gallic acid/g dry wt)			Total flavonoids (mg quercetin/g dry wt)			FRAP (µmol FeSO ₄ /g dry wt)		
	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS
T1	12.3 c	11.8 b	10.4 b	5.0 c	15.4 b	7.8 c	147.9 c	135.2 b	143.7 b
T2	16.9 ab	18.7 a	14.2 a	22.4 b	34.4 a	18.1 ab	208.0 ab	255.0 a	216.8 a
T3	17.5 a	18.7 a	13.6 a	31.0 a	34.8 a	16.1 ab	226.0 a	233.4 a	193.1 a
T4	14.6 bc	17.1 a	13.8 a	16.0 b	31.2 a	14.2 b	187.0 b	268.3 a	198.4 a
T5	15.5 ab	15.9 a	15.1 a	23.5 ab	27.2 a	19.6 a	194.2 b	210.7 a	232.6 a
Significance ^z	**	**	***	***	*	***	***	**	***

^z*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

Table 5. Effects of saline treatments on caffeoylquinic derivatives in leaves of cardoon at 48, 70, and 95 d after sowing (DAS).

Treatment	Caffeic acid (mg·kg ⁻¹ dry wt)			Chlorogenic acid (µg·kg ⁻¹ dry wt)			Cynarin (µg·kg ⁻¹ dry wt)		
	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS	48 DAS	70 DAS	95 DAS
T1	3.0 b	6.7 b	5.5 b	1.2 b	1.4 b	1.1 c	0.8 b	1.3 b	0.8 c
T2	8.9 b	10.4 b	6.9 b	3.7 a	4.4 a	4.8 ab	2.2 a	3.6 a	3.2 b
T3	10.6 ab	11.8 b	7.4 b	3.9 a	4.3 a	3.4 b	2.5 a	4.7 a	2.8 b
T4	8.4 b	13.5 b	8.9 b	3.5 a	6.1 a	5.4 a	2.2 a	5.0 a	3.2 b
T5	20.9 a	30.1 a	17.9 a	3.3 a	4.5 a	5.1 a	2.3 a	5.0 a	4.2 a
Significance ^z	*	**	**	***	**	***	**	**	***

^z*, **, ***Significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

Fig. 2. Relationships between total phenols and antioxidant activity (FRAP) and between chlorogenic acid and FRAP in cardoon leaves. ***Significant at $P \leq 0.001$.

tissues is from 4 to 7 and from 15 to 50 g·kg⁻¹ dry weight for Cl-sensitive and Cl-tolerant plant species, respectively (Xu et al., 2000). In this study, Cl content in leaves of cardoon plants grown under saline conditions was beyond the critical limit of 50 g·kg⁻¹ dry weight reported for salt-tolerant crops such as cardoon (Ksouri et al., 2012). However, the decrease of cardoon biomass was more related to the variation of Na content in leaves than of Cl content, which was similar among salt treatments. The use of Ca(NO₃)₂ instead of CaCl₂ could be also explored to counteract the impact of salinity matching the beneficial effect of Ca and of NO₃ vs. Cl. In strawberry under saline conditions, foliar application of Ca(NO₃)₂ was found to reduce the negative effect of NaCl on plant growth and uptake of calcium and nitrate (Kaya et al., 2002).

Nutraceutical value of leaves. Chlorogenic acid and cynarin were the most abundant caffeoylquinic derivatives in cardoon leaves, whereas among target flavonoids, the highest content was detected for luteolin. The high presence of bioactive compounds was previously observed in leaves of cardoon cv. Bianco Avorio grown in a floating system,

where, similarly, chlorogenic acid, cynarin, and luteolin were the most abundant compounds (Borgognone et al., 2013; Colla et al., 2013b; Roupheal et al., 2012b).

Nutraceutical properties of cardoon can be mainly ascribed to a synergistic effect of several active polyphenolic compounds and their antioxidant activity (Lattanzio et al., 2009). Relationships between the antioxidant compounds and antioxidant activity in cardoon leaves were studied for identifying the main polyphenolic compounds responsible of the antioxidant activity. Total phenols and chlorogenic acid showed a significant correlation with FRAP values (Fig. 2), whereas no significant correlations were detected for caffeic acid, apigenin, and luteolin-7-glucoside (data not shown). FRAP assay, based on the ability of antioxidants to reduce Fe³⁺-Fe²⁺, measures the reducing capacity of a substrate and is commonly used to measure the total antioxidant capacity of food and plants (Pellegrini et al., 2003). Falleh et al. (2008) showed a highly significant correlation between phenolics and DPPH quenching activity in cardoon.

The high level of salinity stress induced by 60 mM of NaCl (T5) did not always match

with a further increase of secondary metabolites with respect to the other saline treatments, except for caffeic acid at each harvest and cynarin at 95 DAS. At moderate salinity [25 to 50 mM of NaCl in cardoon (Hanan et al., 2008)], plant growth can be reduced more than photosynthesis; thus, the plant diverts the synthesis of carbohydrates to produce secondary metabolites. When salinity is higher, also photosynthesis can decline; therefore, growth and production of polyphenols will be decreased even further (Rezazadeh et al., 2012). A decline of polyphenols accumulation was observed with a soil salinity higher than 6.9 dS·m⁻¹ in *Cynara scolymus* L. (Rezazadeh et al., 2012) and with 15 g·L⁻¹ of NaCl in *Carthamus tinctorius* L. (Salem et al., 2014).

The addition of both NaCl and CaCl₂ (T2 and T4) did not affect polyphenol content in cardoon leaves compared with NaCl treatments (T3 and T5). This result agreed with a previous study (Borgognone et al., 2013) in which the increase of polyphenols in cardoon was similar using NaCl or CaCl₂ as salinity sources.

Conclusion

The partial replacement of NaCl with CaCl₂ mitigated the adverse effects of salinity on cardoon biomass production but only at the highest salinity level. CaCl₂ was effective in improving nutritional status of leaves increasing K and Ca content and reducing Na accumulation, especially at 48 and 70 DAS.

Nutraceutical value of cardoon biomass was increased by saline treatments, regardless of CaCl₂ addition. Results showed that the antioxidant activity of cardoon leaves was improved by salinity as a result of the increase of total phenols and chlorogenic acid contents in leaves.

Literature Cited

- Akram, M. 2014. Effect of nitrogen on solute accumulation and ion contents of maize under sodium chloride stress. *Comm. Soil Sci. Plant* 45:86–100.
- Alaoui-Sossé, B., L. Sehmer, P. Barnola, and P. Dizengremel. 1998. Effect of NaCl salinity on growth and mineral partitioning in *Quercus robur* L., a rhythmically growing species. *Trees-Struct. Funct.* 12:424–430.
- Arshi, A., M.Z. Abidin, and M. Iqbal. 2005. Ameliorative effects of CaCl₂ on growth, ionic relations, and proline content of senna under salinity stress. *J. Plant Nutr.* 28:101–125.
- Arshi, A., A. Ahmad, I.M. Aref, and M. Iqbal. 2010. Effect of calcium against salinity-induced inhibition in growth, ion accumulation and proline content in *Cichorium intybus* L. *J. Environ. Bio.* 31:939–944.
- Baghalian, K., A. Haghiry, M.R. Naghavi, and A. Mohammadi. 2008. Effect of saline irrigation water on agronomical and phytochemical characters of chamomile (*Matricaria recutita* L.). *Sci. Hort.* 116:437–441.
- Bates, L.S., R.P. Waldren, and I.N. Teare. 1973. Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–208.
- Borgognone, D., M. Cardarelli, E. Rea, L. Lucini, and G. Colla. 2013. Salinity source-induced

- changes in yield, mineral composition, phenolic acids and flavonoids in leaves of artichoke and cardoon grown in floating system. *J. Sci. Food Agr.* doi: 10.1002/jsfa.6403.
- Bremner, J.M. 1965. Total nitrogen, p. 1149–1178. In: Black, C.A., D.D. Evans, I.L. White, L.E. Ensminger, and F.E. Clark (eds.). *Methods of soil analysis*. Agronomy monographs No. 9, Part 2.
- Cardarelli, M., Y. Roupahel, E. Rea, L. Lucini, M. Pellizzoni, and G. Colla. 2013. Effects of fertilization, arbuscular mycorrhiza, and salinity on growth, yield, and bioactive compounds of two *Aloe* species. *HortScience* 48:568–575.
- Cataldo, D.A., M. Haroon, L.E. Scrandier, and V.L. Youngs. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* 6:71–80.
- Colla, G., Y. Roupahel, R. Jawad, P. Kumar, E. Rea, and M. Cardarelli. 2013a. The effectiveness of grafting to improve NaCl and CaCl₂ tolerance in cucumber. *Sci. Hort.* 164:380–391.
- Colla, G., Y. Roupahel, M. Cardarelli, E. Svecova, E. Rea, and L. Lucini. 2013b. Effects of saline stress on mineral composition, phenolics acids and flavonoids in leaves of artichoke and cardoon genotypes grown in floating system. *J. Sci. Food Agr.* 93:1119–1127.
- Colla, G., Y. Roupahel, E. Rea, and M. Cardarelli. 2012. Grafting cucumber plants enhance tolerance to sodium chloride and sulfate salinization. *Sci. Hort.* 135:177–185.
- Cramer, G.R. 1992. Kinetics of maize leaf elongation. II Responses of a Na-excluding cultivar and a Na-including cultivar to varying Na/Ca salinities. *J. Expt. Bot.* 43:857–864.
- Dominguez-Perles, R., M.C. Martinez-Ballesta, F. Riquelme, M. Carvajal, C. Garcia-Viguera, and D.A. Moreno. 2011. Novel varieties of broccoli for optimal bioactive components under saline stress. *J. Sci. Food Agr.* 91:1638–1647.
- Dutta, R.K. and R.S. Maharia. 2012. Antioxidant response of some common medicinal plants grown in copper minimizing areas. *Food Chem.* 131:259–265.
- Eaton, A.D., L.S. Clesceri, and A.E. Greenberg. 1995. Standard method for the examination of water and wastewater. 19th Ed. American Public Health Assoc., Washington, DC. p. 66–71.
- Falleh, H., R. Ksouri, K. Chaieb, N. Karraoui-Bourauoi, N. Trabelsi, M. Boulaaba, and C. Abdelly. 2008. Phenol composition of *Cynara cardunculus* L. organs, and their biological activities. *C. R. Biol.* 331:372–379.
- Fernández, J., M.D. Curt, and P.L. Aguado. 2006. Industrial applications of *Cynara cardunculus* L. for energy and other uses. *Ind. Crops Prod.* 24:222–229.
- Girija, C., B.N. Smith, and P.M. Swamy. 2002. Interactive effects of sodium chloride and calcium chloride on the accumulation of proline and glycinebetaine in peanut (*Arachis hypogaea* L.). *Environ. Expt. Bot.* 47:1–10.
- Gouia, H., M.H. Ghorbal, and B. Touraine. 1994. Effects of NaCl on flow of N and mineral ions and on NO₃⁻ reduction rate within whole plant of salt-sensitive bean and salt-tolerant cotton. *Plant Physiol.* 105:1409–1418.
- Grattan, S.R. and C.M. Grieve. 1999. Salinity-mineral nutrient relations in horticultural crops. *Sci. Hort.* 78:127–157.
- Hanen, F., R. Ksouri, W. Megdiche, N. Trabelsi, M. Boulaaba, and C. Abdelly. 2008. Effect of salinity on growth, leaf phenolic content and antioxidant scavenging activity in *Cynara cardunculus* L. p. 335–343. In: Abdelli, C., M. Ozturk, M. Ashrafand, and Y.C. Grignin (ed.). *Biosaline agriculture and high salinity tolerance*. Birkhauser Verlag, Switzerland.
- Karla, Y.P. 1998. Handbook of reference methods for plant analysis. CRC Press Inc., Boca Raton, FL. p. 165–170.
- Kaya, C., B.E. Ak, D. Higgs, and B. Murillo-Amador. 2002. Influence of foliar-applied calcium nitrate on strawberry plants grown under salt-stressed conditions. *Austral. J. Expt. Agr.* 42:631–636.
- Khayyat, M., S. Khanizadeh, E. Tafazoli, S. Rajaei, B. Kholdebarin, and Y. Emam. 2011. Effects of different calcium forms on gas exchange activities, water usage and macronutrient uptake by strawberry plants under sodium chloride stress. *J. Plant Nutr.* 34:427–435.
- Ksouri, R., W.M. Ksouri, I. Jallali, A. Debez, C. Magné, I. Hiroko, and C. Abdelly. 2012. Medicinal halophytes: Potent source of health promoting biomolecules with medical, nutraceutical and food applications. *Crit. Rev. Biotechnol.* 32:289–326.
- Kukić, J., V. Popović, S. Petrović, P. Mucaji, A. Čirić, D. Stojković, and M. Soković. 2008. Antioxidant and antimicrobial activity of *Cynara cardunculus* extracts. *Food Chem.* 107:861–868.
- Lattanzio, V., P.A. Kroon, V. Linsalata, and A. Cardinali. 2009. Globe artichoke: A functional food and source of nutraceutical ingredients. *J. Funct. Food* 1:131–144.
- Miccadei, S., D. Di Venere, A. Cardinali, F. Romano, A. Durazzo, M.S. Foddai, R. Fraioli, S. Mobarhan, and G. Maiani. 2008. Antioxidative and apoptotic properties of polyphenolic extracts from edible part of artichoke (*Cynara scolymus* L.) on cultured rat hepatocytes and on human hepatoma cells. *Nutr. Cancer* 60:276–283.
- Munns, R. and M. Tester. 2008. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59: 651–681.
- Nasir Khan, M., M.H. Siddiqui, F. Mohammad, M. Naeem, M. Masroor, and A. Khan. 2010. Calcium chloride and gibberellic acid protect linseed (*Linum usitatissimum* L.) from NaCl stress by inducing antioxidative defence system and osmoprotectant accumulation. *Acta Physiol. Plant.* 32:121–132.
- Navarro, J.M., C. Garrido, V. Martínez, and M. Carvajal. 2003. Water relation and xylem transport of nutrients in pepper plants grown under two different salt stress regimes. *Plant Growth Regulat.* 41:237–245.
- Pandino, G., S. Lombardo, G. Mauromicale, and G. Williamson. 2011. Phenolic acids and flavonoids in leaf and floral stem of cultivated and wild *Cynara cardunculus* L. genotypes. *Food Chem.* 126:417–422.
- Pellegrini, N., M. Serafini, B. Colombi, D. Del Rio, S. Salvatore, M. Bionache, and F. Brighenti. 2003. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assay. *J. Nutr.* 133:2812–2819.
- Renault, S. and M. Affifi. 2009. Improving NaCl resistance of red-osier dogwood: Role of CaCl₂ and CaSO₄. *Plant Soil* 315:123–133.
- Rengel, Z. 1992. The role of calcium in salt toxicity. *Plant Cell Environ.* 15:625–632.
- Rezazadeh, A., A. Ghasemzadeh, M. Barani, and T. Telmadarrehei. 2012. Effect of salinity on phenolic composition and antioxidant activity of artichoke (*Cynara scolymus* L.) leaves. *Res. J. Med. Plant* 6:245–252.
- Roupahel, Y., M. Cardarelli, E. Rea, and G. Colla. 2012a. Improving melon and cucumber photosynthetic activity, mineral composition, and growth performance under salinity stress by grafting onto *Cucurbita* hybrid rootstocks. *Photosynthetica* 50:180–188.
- Roupahel, Y., M. Cardarelli, L. Lucini, E. Rea, and G. Colla. 2012b. Nutrient solution concentration affects growth, mineral composition, phenolic acids, and flavonoid in leaves of artichoke and cardoon. *HortScience* 47:1424–1429.
- Salem, N., K. Msaada, W. Dhifi, F. Limam, and B. Marzouk. 2014. Effect of salinity on plant growth and biological activities of *Carthamus tinctorius* L. extracts at two flowering stages. *Acta Physiol. Plant.* 36:433–445.
- Shabala, S., V. Demidchik, L. Shabala, A. Cuint, S.J. Smith, A.J. Miller, J.M. Davies, and I.A. Newman. 2006. Extracellular Ca²⁺ ameliorates NaCl-induced K⁺ loss from Arabidopsis root and leaf cells by controlling plasma membrane K⁺-permeable channels. *Plant Physiol.* 141:1653–1665.
- Silva, C., V. Martínez, and M. Carvajal. 2008. Osmotic versus toxic effects of NaCl on pepper plants. *Biol. Plant.* 52:72–79.
- Singleton, V.L. and J.A. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *Amer. J. Enol. Viticult.* 16:144–158.
- Sreenivasulu, N., B. Grimm, U. Wobus, and W. Weschke. 2000. Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). *Physiol. Plant.* 109: 435–442.
- Teakle, N.L. and S.D. Tyerman. 2010. Mechanism of Cl-transport contributing to salt tolerance. *Plant Cell Environ.* 33:566–589.
- Veríssimo, P., C. Esteves, C. Faro, and E. Pires. 1995. The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnol. Lett.* 17:621–626.
- Xu, G., H. Magen, J. Tarchizky, and U. Kafkafi. 2000. Advances in chloride nutrition. *Adv. Agron.* 68:96–150.