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Effects of increased seawater salinity irrigation on growth and quality of the edible halophyte *Mesembryanthemum crystallinum* L. under field conditions

Giulia Atzori[a,*], Arjen C. de Vos[b,c], Marc van Rijsselberghe[b], Pamela Vignolini[d], Jelte Rozema[e], Stefano Mancuso[a], Peter M. van Bodegom[f]

[a] Department of Agrifood Production and Environmental Sciences (DISPAA) – University of Florence, Sesto Fiorentino, Italy
[b] Salt Farm Texel, Den Burg, The Netherlands
[c] Royal Institute for Sea Research (NIOZ), Den Burg, The Netherlands
[d] Phytolab (Pharmaceutical, Cosmetic, Food supplement Technology and Analysis)-DISIA- University of Florence, Sesto Fiorentino, Italy
[e] Systems Ecology, Department of Ecological Science, Faculty of Earth and Life Sciences, VU University Amsterdam, Amsterdam, The Netherlands
[f] Institute of Environmental Sciences, Leiden University, Leiden, The Netherlands

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ABSTRACT

Saline agriculture may answer to the declining availability of fresh water and to the worldwide expanding area of salinized soils by exploiting seawater and salt-affected soils for sustainable food production. Potential salt tolerant crops can be found among edible halophytes. Moreover, plants growing in saline environments are often associated with an enhanced endogenous concentrations of high-nutrient compounds. *Mesembryanthemum crystallinum* L. provides an interesting perspective in becoming a salt-tolerant and high-value crop at saline conditions, but has never been tested at representative agricultural conditions. This study aimed at assessing the effects of increasing levels of seawater salinity irrigation (electrical conductivity: 2, 4, 8, 12, 16, 20, and 35 dS m^{-1}) on growth and productive performance in a field experiment. Also, impacts of salinity on the functional value of edible leaves were evaluated by investigating the mineral elements, carotenoids, soluble sugars, and phenolic concentrations, along with antioxidant activity. Our results demonstrate that none of the salinity treatments negatively affected *M. crystallinum* biomass production. Furthermore, increased salinity extended the vegetative stage, leading to one extra month of harvest compared to non-saline conditions. Juvenile edible leaves' biomass, succulence and calcium concentrations even increased with increasing salinity. No differences were assessed in the phenolics concentration and antioxidant activity of high salinity treatments plants compared to the control. This paper demonstrates the perspective to cultivate *M. crystallinum* in saline agriculture, up to EC of 20–35 dS m^{-1}, or perhaps even higher, since we did not identify a threshold of biomass reduction. Only the Na^+ concentration in the edible leaves could constitute a health concern or allow it acting as a natural salt substitute. This excellent performance in combination with the appreciated taste and its glistening appearance, may pave the way for use of the ice plant as high-value saline crop.

1. Introduction

Because of the growing population, the human pressure on global water resources will tremendously increase. Moreover, as a result of global warming, increasing saline and dry conditions may affect arable land and sea level rise is becoming an issue in coastal areas. Many conventional crops are salt sensitive although some may be grown up to 30% seawater salinity (Koyro et al., 2011). Above this salt concentration, major yield reductions are observed, due to water stress, ion toxicity, nutritional disorders, oxidative stress, metabolic processes alterations, membrane disorganization and genotoxicity among other factors (Munns, 2002). Therefore, saline agriculture, working with salt-tolerant crops, may represent an answer to the declining availability of fresh water and to the worldwide expanding area of salinized soils. Salt tolerant species allow exploitation of the great availability of brackish and sea water, making coastal and salt affected areas productive.

* Corresponding author at: V.le delle Idee, 30, 50019, Sesto Fiorentino (Florence), Italy.
E-mail address: giulia.atzori@unifi.it (G. Atzori).

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Salt-tolerant species – halophytes – native from salt marshes and inland saline sites, can grow and reproduce on saline soils on which 99% of the other species get deprived (Flowers and Colmer 2008). Their adaptation to saline environments may be characterized as salt tolerance or as salt avoidance (Koyro et al., 2011). A wide range of morphological, physiological, and biochemical adaptations exists in halophytes, varying widely in their degree of salt tolerance (Flowers and Colmer 2008). In some cases, salinity may even result in favorable effects for yield and its quality (Flowers and Muscolo, 2015; Shannon and Grieve, 1998). Although halophytes represent just 2% of the terrestrial plant species, they are represented in half of the higher plants' families (Glenn et al., 1999). Because many of those salt tolerant plants are edible, their domestication and cultivation in a saline agriculture context could be regarded as an interesting approach to consider (Glenn et al., 1998; Rozema and Flowers, 2008; Rozema and Schat, 2013; Ventura et al., 2015).

Saline agriculture may pursue relevant goals. To begin with, an increased sustainable food production could be achieved exploiting resources which would not be used for conventional agriculture: seawater or brackish waters as complementary irrigation waters and salt-affected soils (Glenn et al., 1999). Importantly, agricultural areas lost because of salinization would be regained for staple food production (Bruning and Rozema, 2013). The numerous biochemical strategies adopted by plants to cope with salinity include the selective accumulation or exclusion of ions; the synthesis of osmotic solutes; the induction of antioxidant compounds (Parida and Das 2005), or secondary metabolites (Ramakrishna and Ravishankar, 2011). The high endogenous concentration of such compounds, known to have healthy properties for human consumption, can be precious for the nutritive characteristics of food (Di Baccio et al., 2004; Sgherri et al., 2008). Thus, halophytes grown in saline conditions could also be a source of compounds with a potential added nutritive and economic value (Flowers and Muscolo 2015), representing high-value crops.

Mesembryanthemum crystallinum L., the common ice plant, Aizoaceae, Caryophyllales, is a succulent, prostrate annual herb with high potential for becoming a salt-tolerant high-value crop. Native to southern and eastern Africa, and nowadays widespread along the coastal areas of Europe, USA, Mexico, Chile, the Caribbean and western Australia (Adams et al., 1998), this species is already being consumed as a vegetable crop in several countries such as India, California, Australia and new Zealand and in some countries of Europe (Agarie et al., 2009), i.e. in Germany (Herppich et al., 2008) and in The Netherlands. It has been known as a traditional medicine as well, in particular for its demulcent and diuretic effects (Bouftira et al., 2012), a relevant content of superoxide dismutase (SOD) and related anti-oxidants molecules, which have a role in the protection of the skin against radiation exposure (Bouftira et al., 2008), and for its antiseptic properties (Ksouri et al., 2008). The ice plant main particularity is that its entire above ground surface is covered with unicellular trichomes, ranging from 1 to 3 mm in diameter (Vivrette and Muller, 1977), called bladder cells, filled with a water solution and functioning as peripheral salinity and water reservoirs providing protection from short term high salinity or water deficit stress (Agarie et al., 2007; Luttge et al., 1978). Interestingly, its morphological adaptations, together with the capability to switch from C_3 photosynthesis to Crassulacean acid metabolism (CAM), make this species able to complete its life cycle on soil containing NaCl at a concentration comparable to seawater (Adams et al., 1998).

Because of its considerable resistance to salt and drought stress (Bloom, 1979; Vivrette and Muller, 1977), the ice plant has been studied as a model species starting from the 80 s (Bohnert et al., 1988). From then onwards, a number of laboratory experiments aiming at elucidating the physiological and molecular mechanisms behind its stress resistance have been published, and among the many studies we recall the work of (Agarie et al., 2007; Barker et al., 2004; Cosentino et al., 2010; Kore-eda et al., 2004; Oh et al., 2015; Sanada et al., 1995; Thomas et al., 1992; Thomas and Bohnert, 1993; Vernon and Bohnert, 1992; Winter and Holtum, 2007). Yet, despite the physiological understanding, scientific documentation at field conditions is very scarce. Hence, there is a strong need for large-scale field experiments to evaluate the feasibility of *Mesembryanthemum crystallinum* for halophyte crop production, together with the economic perspective for future growers (Ventura et al., 2015). Such studies – ideally executed at multiple salinity levels – are essential for verifying the crop potential of this species in saline soils and/or to estimate the production of specific osmotic solutes and secondary metabolites, known to have multiple healthy properties for humans, synthesized by the plant as protection against oxidative effects. In particular, the effect of different salinity levels on such production in the ice plant has never been investigated.

The present study had thus a twofold aim: *i*) to evaluate the crop potential of *Mesembryanthemum crystallinum*, by determining the effects of seawater irrigation on growth and productive performances in a field experiment with a wide range of salinity conditions in an agricultural setting. A full screening of morphological (i.e. specific leaf area, leaf succulence, leaf dry matter content and leaf water content) and physiological (i.e. chlorophyll concentration) characteristics was conducted to investigate eventual adaptations to salinity. *ii*) to assess the effects of different salinity levels on the accumulation of ions and the production of osmotic solutes along with secondary metabolites related to physiological adaptation and to the nutritive value of the crop, i.e. mineral elements, carotenoids, soluble sugars, phenolic compounds and the antioxidant activity.

2. Materials and methods

2.1. Research location, irrigation and soil salinity

Mesambryanthemum crystallinum was grown at increasing salinity levels in an experimental field on Texel island (53.012837°N, 4.755306°E), The Netherlands, from May to August 2015. As described in detail by Bruning et al. (2015), the experimental area consisted of a field divided into 21 plots (8×20 m each) with seven salt concentrations randomly distributed and replicated three times. Three years prior to the experiment, the soil, which mainly consists of sand (3% loam, 2% clay, and 2% organic matter), was homogenized by a large power shovel mixing the top first meter of soil for 3 days. Individual drip lines were located at 40 cm intervals, with drippers each 30 cm distance. Irrigation took place on a daily basis with a surplus amount of 10.7 mm/day to keep the soil moisture content permanently close to the field capacity. The resulting high leaching (leaching fraction is close to 90%) minimized the effect of variation in rainfall and evapotranspiration. Consequently, soil salinity levels were as constant as possible.

The irrigation water was a mixture of fresh water from a rainwater basin and natural seawater from a ditch fed from the Waddensea (electrical conductivity EC of 35 dS m^{-1}). A custom-built proportional-integral-derivative (PID) controller mixed fresh and saline waters with frequency-regulated pumps from both water sources, which allowed time-based automatic pulse irrigation with an automatic accuracy check of salinity levels in the irrigation water. Drainage pipes, located 60 cm below the surface with 5 m spacing between any two pipes, assured a rapid drainage of the daily irrigation water and aeration of the soil.

The six treatments used in the experiment, each one repeated in three plots, were characterized by the following electrical conductivities (EC) values: 4 dS m^{-1}, 8 dS m^{-1}, 12 dS m^{-1}, 16 dS m^{-1}, 20 dS m^{-1}, and 35 dS m^{-1}, plots salinity gradually reaching the target values within the half of June. An additional control treat-

ment (repeated in three plots as well) was characterized by EC of 2 dS m^{-1}. Soil salinity was monitored during the experiment by means of soil pore water samples, collected in all plots three times during the experiment. The ECe was approximated from the EC of soil pore water obtained by means of suction cups that were placed at 0–10 cm, 20–30 cm and 50–60 cm depth in all the plots, as a strong correlation was found between EC_{pw} and EC_e ($EC_e = 0.69 \times EC_{pw}$, with $r^2 = 0.82$). Such EC values did not deviate from the targeted EC of the treatments.

2.2. Plant material, sampling and growth measurements

Seeds of *Mesambryanthemum crystallinum* were sown on the 14th of April 2015 and young seedlings were transferred after one month into the experimental fields with 30 plants per plot. Three sampling events were performed during the experiment: *i*) time zero sampling (T0), on 6 untreated juvenile plants 5 weeks from germination old; *ii*) as plants' young fully expanded leaves were ready to be harvested, thus at the potential commercial maturity (T1, 16th of July 2015, on three plants per plot, nine per treatment); *iii*) at the end of the crop cycle (T2, 11th of August 2015, on three plants per plot, nine per treatment), with some plants beginning seed production/drying up.

At the T0 sampling, the total fresh and dry shoot weight was collected ($n = 6$). Dry material was then used to assess the Na^+, K^+ and Ca^{2+} concentration in juvenile plants. At T1 and T2, the total shoot fresh weight and the fresh weight of three young fully expanded leaves per plant were recorded. Leaf area (LA) of young fully expanded leaves was calculated using ImageJ software ($n = 9$). Specific leaf area (SLA), leaf succulence, leaf dry matter content (LDMC) and leaf water content (LWC) were determined on young fully expanded leaves ($n = 9$) to investigate possible morphological adaptations to salinity. Samples of young fully expanded leaves ($n = 9$) were collected, fresh weighed, frozen in liquid nitrogen and stored at −20° C for further analysis of soluble sugar, chlorophyll, carotenoids and phenolics concentration. Plant shoots were oven-dried (70° C until constant weight) and total shoot dry biomass and young fully expanded leaves dry weight were determined. Dried samples from young fully expanded leaves were then used for measuring the Na^+, K^+ and Ca^{2+} concentration and the antioxidant activity.

The specific leaf area (SLA), leaf succulence, leaf dry matter content (LDMC) and leaf water content (LWC) were determined as follows:

$$SLA = L_A / L_{DW}$$

with L_A = leaf area (cm^2) and L_{DW} = leaf dry weight (g), according to Hunt et al. (2002)

$$leafsucculence = L_{FW} / L_A$$

with L_{FW} = leaf fresh weight (g) and L_A = leaf area (cm^2), often used as an estimate of leaf succulence (Agarie et al., 2007; Jennings, 1976)

$$LDMC = L_{DW} / L_{FW}$$

with L_{DW} = leaf dry weight (g) and L_{FW} = leaf fresh weight (g) (Garnier et al., 2001)

$$LWC = (L_{FW} - L_{DW}) / L_{FW}$$

with L_{FW} = leaf fresh weight (g) and L_{DW} = leaf dry weight (g), according to the commonly used formula.

2.3. Measurement of chlorophyll and carotenoid concentrations

Total leaf chlorophyll and carotenoid concentrations were determined in young fully expanded leaves (T1 and T2, $n = 9$). Cold 100% methanol was added to the frozen grounded tissues and samples were left shaking in darkness at 4 °C for 30 min to extract pigments. After that, samples were centrifuged at 1000 rpm for 10 min. The supernatant was collected and used to read the absorbance at 665, 652 and 470 nm using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). Chlorophyll a, chlorophyll *b* and carotenoid concentrations were determined according to Wellburn (1994).

2.4. Measurement of Na^+, K^+, and Ca^{2+} concentrations

The Na^+, K^+ and Ca^{2+} concentration in young fully expanded leaves (T0: $n = 6$; T1 and T2: $n = 9$) was obtained after digesting ground dried tissues in 0.5 M HNO_3 by shaking vials in the dark at 25 °C for 48 h, as in Bazihizina et al. (2015). Diluted extracts were analyzed using a Flame Photometer Digiflame2000 (Lab Services SAS, Rome, Italy). The values of the calibration curve ranged from 0 to 0.1 mg/ml for Na^+ and K^+ ($R^2 = 0.998$) and from 0 to 0.05 mg/ml for Ca^{2+} ($R^2 = 0.999$) determination.

2.5. Measurement of soluble sugars

The extraction of soluble sugars from young fully expanded leaves (T1 and T2, $n = 9$) was performed on frozen grounded samples by incubating samples with 80% ethanol in a 80 °C water bath and centrifuged, this procedure being repeated twice to extract all soluble sugars. The supernatant was collected and used to measure total sugars with the anthrone reagent (Yemm and Willis, 1954). The concentration of total soluble sugars was determined by measuring the absorbance of samples at 620 nm in a UV–vis spectrophotometer (Bio-Rad SmartSpec™ Plus), using a standard curve for glucose. The reliability of this method was verified by determining the recovery of known amounts of glucose added to ethanol only and to extra tissue samples immediately prior to extraction. The values of the calibration curve ranged from 0 to 100 mg/ml of glucose ($R^2 = 0.997$).

2.6. Measurement of phenolics content and antioxidant activity

The total phenolic concentration (T1 and T2, $n = 3$) was measured using the Folin-Ciocalteu method, according to Dewanto et al. (2002). Deionised water (0.5 ml) and 125 μl of the Folin-Ciocalteu reagent was added to 125 μl of a suitably diluted sample extract. The mixture was allowed to stand for 6 min and then 1.25 ml of a 7% aqueous Na_2CO_3 solution were added. The final volume was adjusted to 3 ml. After 90 min, the absorption was measured at 760 nm against water as a blank using a Agilent 8453 UV–vis Spectrophotometer (Agilent Technology, Palo Alto, CA, USA). The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) by using the calibration curve of gallic acid. The calibration curve ranged from 20 to 500 mg/ml ($R^2 = 0.997$).

The antioxidant activity (T1 and T2, $n = 3$) was measured with the 1,1-diphenyl- 2-picrylhydrazyl free radical (DPPH) assay on young fully expanded leaf samples of plants treated at EC of 12, 20, 35 dS m^{-1} and control. The DPPH quenching ability of plants extracts was measured according to Hatano et al. (1988): the amount of 0.5 ml of the extract at 5 different concentrations was added to 0.5 ml of a DPPH ethanolic solution. The mixture was shaken vigorously and the absorbance was read at 517 nm in a UV–vis spectrophotometer (Bio-Rad SmartSpec™ Plus), at the very

Fig. 1. Photographs of the control and of the higher salinity treatment plants at the commercial maturity time (T1).

beginning and after 20 min from the beginning of the reaction. The scavenging activity on the DPPH radical was calculated as follows:

$$\text{DPPHscavengingeffect}(\%) = [(A_0 - A_{20})/A_0] \times 100$$

where A_0 is the absorbance at the beginning of the reaction and A_{20} is the absorbance after 20 min from A_0. The antiradical activity was expressed as EC50 (mg/mg), the antiradical dose required to cause a 50% inhibition. Lower EC50 corresponds to a higher antioxidant activity of the plant extract.

2.7. Statistical analyses

Statistical analyses were conducted using GraphPad Prism 5 for Windows. One-way analysis of variance was used to assess significant differences among treatments. In particular, the Tukey's Test was chosen to enable comparisons not only between salinity and control conditions, but also among all salinity levels. Significance level was $P \leq 0.05$ or higher, as reported in Tables and Figures captions.

3. Results

3.1. Growth

Fig. 1 reports pictures of the control and of the higher salinity treatment plants at the commercial maturity time in July: the main observable difference between the two groups is the reddish color of salt-treated plants leaves, whereas plants were comparable in size suggesting that salt did not have any negative effect on plants growth. Moreover, control plants started shrinking/going to the seed production phase in August, while 35 dS m^{-1} treated plants were still maintaining the old larger leaves. In fact, the plant cycle lasted about one more month longer at the higher salinity treatments (i.e. 20 and 35 dS m^{-1}) compared to control conditions (authors field observations).

Results of total shoot fresh weight (FW) and dry weight (DW) during the experimental period are shown in Fig. 2. No significant differences among treatments were assessed at T0, when the salinity treatments had not yet started, nor at T1, plants of all treatments reaching a comparable shoot biomass production. On the contrary, at T2, plants growing at higher salinity tended to have an increasingly higher biomass. Plants of 20 dS m^{-1} treatment showed a significant increase of both fresh and dry shoot biomass compared to control.

Fig. 3 reports the growth results of young fully expanded leaves only. As for total shoot biomass production (Fig. 2), no significant differences in FW and DW were assessed between treatments and the control at T1. In contrast, at T2 both biomass parameters significantly increased in plants growing at saline conditions compared to the control, with increasing differences with increasing salinity: while total shoot DW had significantly increased compared to control only at the 20 dS m^{-1} treatment, a significant increase of young fully expanded leaves DW was assessed at 8, 12, 20 and 35 dS m^{-1}.

As shown in Table 1, increased salinity did not overall affect the morphological parameters investigated, with the only exceptions of leaf area and succulence. LA increased significantly at T2 and only at the intermediate salinity treatments compared to the control, whereas leaf succulence increased in treated plants compared to the control at T1 in 16 dS m^{-1} treatment plants and at T2 starting from 12 dS m^{-1} treatment up to the maximum salinity level, with the exception of the 20 dS m^{-1} treatment. On the other hand, SLA, LDMC and LWC did not show any significant difference among treatments at any sampling event.

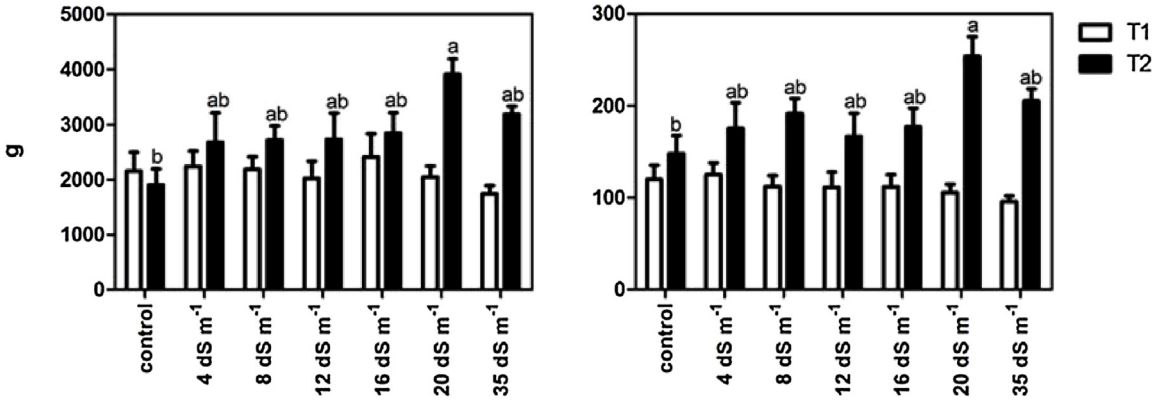

Fig. 2. FW and DW of the ice plant shoot, collected at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in grams per plant. Different letters indicate significant differences among treatments at $P < 0.01$ in FW plot and at $P < 0.05$ in DW plot (Tukey's Test).

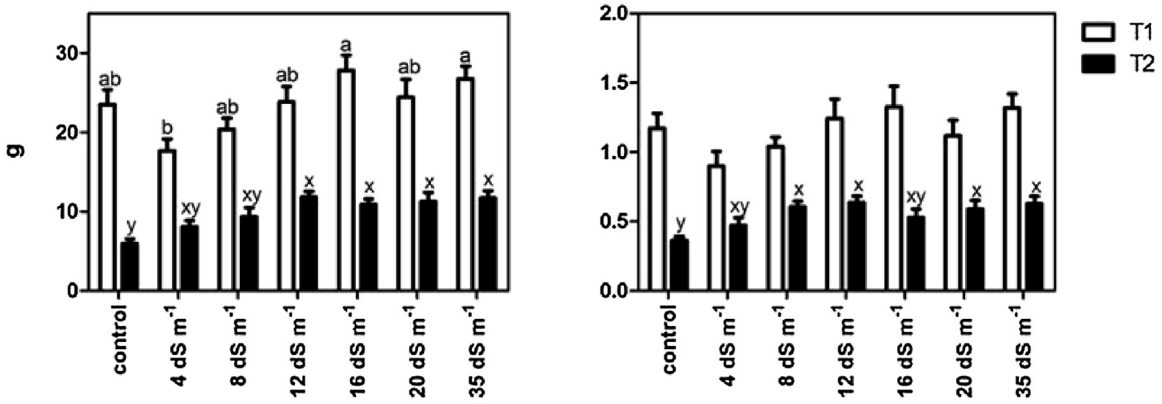

Fig. 3. FW and DW of 3 young fully expanded leaves per plant, collected at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in grams. Different letters indicate a significant difference among treatments at the same harvest event at $P < 0.01$ (Tukey's Test).

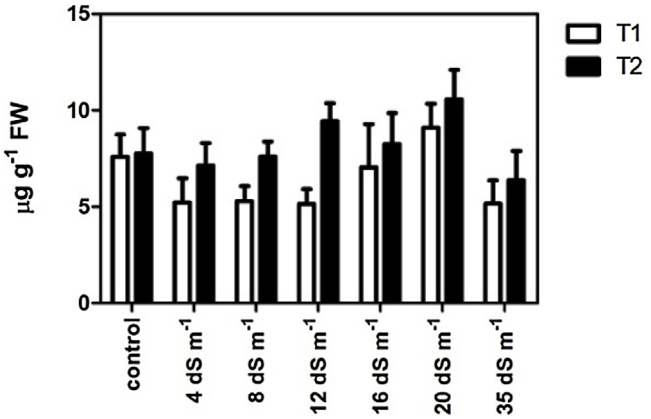

Fig. 4. Carotenoid concentration of young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in microgram per gram of fresh weight. No significant differences were assessed among treatments at $P < 0.05$ (Tukey's Test).

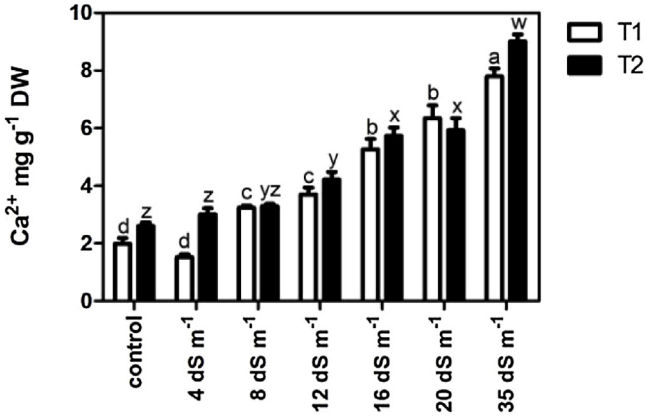

Fig. 5. Concentration of Ca^{2+} in young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in milligram per gram of dry weight. Different letters indicate a significant difference among treatments at the same harvest event at $P < 0.0001$ (Tukey's Test).

3.2. Chlorophyll and carotenoid concentrations

In Table 2, chlorophyll a, b and total chlorophyll concentrations in young fully expanded leaves are reported for T1 and T2. Salinity generally did not affect pigments concentration. Only chlorophyll a was significantly reduced in T1 35 dS m^{-1} treatment plants compared to the control. Chlorophyll decreased in time both in salt treated plants as in controls, thus not necessarily depending on salinity.

On the other hand, carotenoids concentration, reported in Fig. 4, augmented in time at increased salinity, whereas the control remained stable. Plants with the higher concentration of carotenoids at both harvests were those of the 20 dS m^{-1} treatment.

3.2.1. Concentration of Na±, K±, and $Ca^{2\pm}$

Values of Na^+, K^+, and Ca^{2+} concentration in leaves of juvenile plants collected before treatments introduction (T0) resulted to be: Na^+ 32.82 ± 2.33; K^+ 30.11 ± 4.57; Ca^{2+} 3.16 ± 0.16 (values are means ± s.e., expressed in milligram per gram of dry weight). Table 3 shows Na^+ and K^+ concentrations in young fully expanded leaves collected at T1 and T2. Sodium concentrations were significantly higher in every treatment compared to the control, at both sampling events. On the other hand, K^+ concentration significantly declined accordingly with increasing salinity compared to the control, starting from 4 dS m^{-1} treatment at T1 and from 12 dS m^{-1} treatment at T2.

Calcium concentration, reported in Fig. 5, rose in treated leaves compared to the control with increasing salinity at both sampling events.

3.2.2. Soluble sugars concentration

The soluble sugars concentration in young fully expanded leaves at T1 and T2 is reported in Table 4. At both harvests, increased salinity coincided with a decrease of soluble sugars concentrations compared to the control. At T1, the reduction was significant starting from the 12 dS m^{-1} treatment up to the higher salinity treatments. However, at T2 only at the 35 dS m^{-1} treatment a significant decrease was found. Soluble sugar concentrations proved to be more stable over time in the higher salinity treatments, while at the control and at the low to moderate salinity treatments (i.e. up to the 12 dS m^{-1} treatment), concentrations dropped from T1 to T2.

3.2.3. Phenolics content and antioxidant activity

The concentrations of phenolics in young fully expanded leaves at T1 and T2 are reported in Table 5. No significant differences were found among treatments at either sampling event.

Fig. 6 shows the volumes of extracts to be added to give a 50% reduction of the stable free radical DPPH: lower values indicate higher antioxidant activity. Our results show a significant reduction of the antioxidant activity only in 12 dS m^{-1} treatment leaves compared to the control, at both sampling events. In contrast, both

Table 1
LA, SLA, leaf succulence, LDMC and LWC of 3 young fully expanded leaves per plant, collected at T1 and T2. Values are means ± s.e. ($n = 9$). Different letters in the same column indicate a significant difference at $P < 0.05$ (Tukey's Test).

Treatment	young fully expanded leaves, T1					young fully expanded leaves, T2				
	LA (cm^2)	SLA ($cm^2\ g^{-1}$)	Leaf succulence ($g\ cm^{2-1}$)	LDMC	LWC	LA (cm^2)	SLA ($cm^2\ g^{-1}$)	Leaf succulence ($g\ cm^{2-1}$)	LDMC	LWC
control	120.67 ± 10.5	110.31 ± 12.91	0.20 ± 0.01 ^{b}	0.05 ± 0.004	0.950 ± 0.004	25.38 ± 2.46 ^{b}	70.12 ± 4.14	0.24 ± 0.02 ^{c}	0.064 ± 0.005	0.936 ± 0.005
4 dS m^{-1}	86.76 ± 6.96	101.42 ± 7.90	0.20 ± 0.01 ^{ab}	0.05 ± 0.003	0.950 ± 0.003	30.75 ± 3.42 ^{ab}	66.51 ± 4.98	0.27 ± 0.01 ^{bc}	0.059 ± 0.003	0.941 ± 0.003
8 dS m^{-1}	91.58 ± 5.91	88.68 ± 3.69	0.22 ± 0.01 ^{ab}	0.052 ± 0.003	0.948 ± 0.003	38.83 ± 3.66 ^{a}	63.86 ± 3.53	0.24 ± 0.02 ^{bc}	0.072 ± 0.009	0.928 ± 0.009
12 dS m^{-1}	102.87 ± 7.93	87.83 ± 7.70	0.23 ± 0.01 ^{ab}	0.052 ± 0.003	0.948 ± 0.003	37.92 ± 2.27 ^{a}	60.86 ± 3.56	0.31 ± 0.01 ^{ab}	0.054 ± 0.003	0.946 ± 0.003
16 dS m^{-1}	116.55 ± 9.20	93.59 ± 9.35	0.24 ± 0.01 ^{a}	0.047 ± 0.003	0.953 ± 0.003	36.48 ± 2.30 ^{ab}	72.24 ± 4.07	0.30 ± 0.01 ^{ab}	0.048 ± 0.004	0.952 ± 0.004
20 dS m^{-1}	110.66 ± 8.31	105.68 ± 11.88	0.22 ± 0.01 ^{ab}	0.047 ± 0.004	0.953 ± 0.004	37.44 ± 3.34 ^{ab}	66.52 ± 5.31	0.30 ± 0.01 ^{abc}	0.054 ± 0.005	0.946 ± 0.005
35 dS m^{-1}	113.64 ± 8.08	87.09 ± 5.23	0.24 ± 0.01 ^{ab}	0.049 ± 0.002	0.951 ± 0.002	34.81 ± 2.24 ^{ab}	56.82 ± 3.07	0.33 ± 0.01 ^{a}	0.054 ± 0.001	0.946 ± 0.001

Fig. 6. Inverse of the antioxidant activity of young fully expanded leaves at T1 and T2. The lower value indicates the higher antioxidant activity. Values are means ± s.e. ($n = 3$) and are expressed in mg of dry weight needed to reduce of the 50% 1 mg of the stable free radical DPPH. Different letters indicate a significant difference among treatments at the same harvest event at $P < 0.05$ (Tukey's Test).

20 and 35 dS m^{-1} treated leaves showed an antioxidant activity comparable to the control.

4. Discussion

4.1. Seawater irrigation did not damage the ice plant growth up to EC 35 dS m−1

Our experimental results indicated that none of the six treatments negatively affected the growth of the ice plant in terms of biomass production as compared to the control. On the contrary, the better performances of plant biomass at higher salinity levels may suggest a sort of growth stimulation by increased salinity. In fact, significant differences in total shoot biomass production were observed only at T2, with 20 dS m^{-1} salinity treatment plants showing a significant augmentation of both fresh and dry total shoot biomass compared to the control. Likewise, juvenile control leaves FW and DW were lower than salt-treated ones: FW and DW almost doubled in higher salinity treatments compared to control. This is an important result, given that in saline agriculture settings, the young leaves are the ones to be harvested.

In this sense, *Mesambryanthemum crystallinum* does not deviate from other halophytes that have their best performances in the presence of NaCl. In fact, for a number of dicotyledonous halophytes optimal growth occurs at concentrations of 50–250 mM NaCl (roughly corresponding to EC 5 and 25 dS m^{-1}) in the root medium (Flowers and Colmer 2008). Moreover, halophytes generally do not occur on non-saline sites because of their reduced growth rate and reduced competitive ability at low salinity conditions (Rozema and Schat 2013). Our growth results are in accord with Herppich et al. (2008), who did not find significant biomass differences in *Mesambryanthemum* plants grown at 150 mM NaCl, roughly comparable to EC 15 dS m^{-1}, and those grown with tap water for the first 105 days of the experiment, while a significantly higher fresh biomass in salt-treated plants compared to control was found at day 150. The good performance of the ice plant throughout the higher salinity levels indicate its potential for saline agriculture.

4.2. High salinity extended the ice plant growing season

A second important impact of saline conditions on the ice plant is that its growing season was strongly extended under seawater irrigation. Control plants started to senesce in August, to proceed to the seed production phase. In contrast, the same senescence started in high salinity treatments plants about one month after, being

Table 2
Chlorophyll a, chlorophyll *b* and total chlorophyll concentration of young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in microgram per gram of fresh weight. Different letters in the same column indicate a significant difference at $P < 0.05$ (Tukey's Test).

Treatment	Chl a (μg g^{-1} FW)		Chl b (μg g^{-1} FW)		Chl tot (μg-1 FW)	
	T1	T2	T1	T2	T1	T2
control	60.90 ± 3.84^{a}	39.91 ± 3.74	52.91 ± 1.74	28.57 ± 1.45^{ab}	113.81 ± 5.24	68.49 ± 4.63
4 dS m^{-1}	54.50 ± 3.49^{ab}	43.47 ± 4.13	53.14 ± 6.06	30.30 ± 2.57^{a}	107.64 ± 8.59	73.77 ± 6.36
8 dS m^{-1}	50.56 ± 2.79^{ab}	37.14 ± 3.24	45.17 ± 1.84	25.09 ± 3.00^{ab}	95.73 ± 4.07	62.24 ± 6.01
12 dS m^{-1}	45.96 ± 2.16^{ab}	43.90 ± 3.24	40.87 ± 3.67	23.65 ± 2.73^{ab}	86.83 ± 5.09	67.55 ± 5.39
16 dS m^{-1}	49.25 ± 6.26^{ab}	40.45 ± 3.49	37.55 ± 4.26	25.01 ± 2.75^{ab}	86.80 ± 5.79	65.46 ± 5.16
20 dS m^{-1}	59.10 ± 5.07^{a}	44.24 ± 3.97	46.41 ± 6.19	18.72 ± 3.46^{b}	105.51 ± 8.88	62.96 ± 4.63
35 dS m^{-1}	39.59 ± 3.89^{b}	32.85 ± 4.63	45.19 ± 7.00	19.31 ± 2.06^{ab}	84.77 ± 8.87	52.15 ± 6.07

Table 3
Concentration of Na^{+} *and* K^{+} in young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in milligram per gram of dry weight. Different letters in the same column indicate a significant difference at $P < 0.0001$ (Tukey's Test).

Treatment	Na^{+} (mg g^{-1} DW)		K^{+} (mg g^{-1} DW)	
	T1	T2	T1	T2
control	55.20 ± 2.24^{e}	67.13 ± 5.20^{d}	49.24 ± 2.76^{a}	34.44 ± 2.91^{a}
4 dS m^{-1}	74.11 ± 3.60^{d}	96.11 ± 7.06^{c}	35.57 ± 2.00^{b}	35.08 ± 2.96^{a}
8 dS m^{-1}	98.76 ± 3.78^{c}	117.92 ± 4.29^{bc}	32.03 ± 1.41^{b}	24.71 ± 3.1^{ab}
12 dS m^{-1}	118.39 ± 4.27^{b}	137.36 ± 4.88^{b}	26.67 ± 1.86^{bc}	17.98 ± 2.14^{b}
16 dS m^{-1}	119.50 ± 3.28^{b}	139.89 ± 4.82^{b}	26.81 ± 2.71^{bc}	17.40 ± 1.90^{b}
20 dS m^{-1}	125.20 ± 4.89^{ab}	137.66 ± 7.68^{b}	22.73 ± 1.82^{c}	19.03 ± 2.76^{b}
35 dS m^{-1}	138.38 ± 4.64^{a}	179.08 ± 3.90^{a}	23.05 ± 2.71^{c}	14.23 ± 1.05^{b}

Table 4
Concentration of soluble sugars in young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 9$) expressed in microgram per milligram of fresh weight. Different letters in the same column indicate a significant difference at $P < 0.0001$ for T1 and at $P < 0.05$ for T2 (Tukey's Test).

Treatment	soluble sugars (μg mg^{-1} FW)	
	T1	T2
control	5.83 ± 0.60^{a}	3.74 ± 1.02^{a}
4 dS m^{-1}	4.35 ± 0.64^{ab}	2.25 ± 0.61^{ab}
8 dS m^{-1}	3.93 ± 0.31^{ab}	2.09 ± 0.58^{ab}
12 dS m^{-1}	3.66 ± 0.58^{bc}	1.41 ± 0.17^{ab}
16 dS m^{-1}	3.53 ± 0.37^{bc}	2.37 ± 0.66^{ab}
20 dS m^{-1}	1.90 ± 0.17^{cd}	1.33 ± 0.09^{ab}
35 dS m^{-1}	1.11 ± 0.17^{d}	1.04 ± 0.09^{b}

Table 5
Concentration of phenolic compounds in young fully expanded leaves at T1 and T2. Values are means ± s.e. ($n = 3$) expressed in milligram of gallic acid on gram of dry weight. No letters indicate no significant differences among treatments at $P < 0.05$ (Tukey's Test).

Treatment	phenolic content (mg GAE/g DW)	
	T1	T2
control	2.39 ± 0.34	1.84 ± 0.41
4 dS m^{-1}	1.99 ± 0.33	1.24 ± 0.11
8 dS m^{-1}	1.67 ± 0.17	1.56 ± 0.23
12 dS m^{-1}	1.48 ± 0.14	0.85 ± 0.09
16 dS m^{-1}	2.24 ± 0.41	1.28 ± 0.22
20 dS m^{-1}	2.32 ± 0.23	1.35 ± 0.06
35 dS m^{-1}	2.38 ± 0.15	1.07 ± 0.18

in August still on the vegetative growth phase. The reason of the control smaller biomass compared to high salt treated plants may thus partly be found in the differences in vegetative cycle length. Control plants ended their life cycle before the salt treated ones, thus at T2 controls were already starting to shrink, unlike the salt treated plants. Likewise, Adams et al. (1998) reported that salinity slow down the plant developmental physiology. This is particularly important for our experiment purpose of testing the ice plant as potential salt tolerant crop in saline agriculture. In the particular case of the ice plant, the edible part is represented by young leaves, thus the ice plant harvest is not a destructive one, as only young leaves are picked leaving the plant undamaged. Concerning total shoot and juvenile leaves change in biomass from T1 to T2, differences are found with total shoot biomass increasing and juvenile biomass on the contrary decreasing from T1 to T2. The increase in total shoot biomass can be explained by the presence at T2 of old leaves with considerable area extension and of long shoots grown during the whole plant cycle. On the other hand, the ideal moment of harvest of juvenile leaves was identified in July because at that time leaves had the higher biomass at both saline and non saline conditions. However, as salinity proved to extend the whole vegetative stage, this also enables one extra month of harvest compared to non salinity conditions. In addition, the number of young leaves produced, thus the grams of potential harvest, were increased by the extended cycle length, as the ice plant is a fast growing species: in fact, even if smaller in T2 compared to T1, juvenile leaves were harvestable and thus marketable at August sampling too.

4.3. Growth and morphological response to increased salinity

Over time, the morphological changes were expressed more strongly with leaf succulence increasing with increasing salinity. This capability may represent an essential part of the ice plant salt tolerance. In fact, halophytes balance their growth rate with their requirement for the salt needed for osmotic adjustments (Flowers and Yeo 1986). The increase in leaf succulence, thus of the water content per unit area, is part of this balance and plays a major role in the osmotic adjustment to a low external water potential induced by salinity (Flowers and Colmer 2008). Also, it translates to an augmented carbon assimilation capacity per unit area, assuring plants growth despite a possibly relatively low SLA (de Vos et al., 2013). Indeed, in dicotyledonous halophytes the increase in leaf succulence is often connected to a SLA decrease, (Rozema et al., 2015; de Vos et al., 2013; de Vos et al., 2010; Geissler et al., 2009; Ayala and O'Leary, 1995), a morphological adaptation associated with the plants need of limiting transpiration (Flowers and Flowers 2005). Nevertheless, as reported in the results section, no significant decrease of SLA occurred in treated plants compared to the control, nor in LDMC and LWC. This is in line with Herppich et al. (2008), and confirms that none of the treatments did effectively stress the plant, but increased its physiological activity and yield. In fact, in our experiment LA of salt-treated leaves rose compared to the control (even if significantly only at the intermediate salinity treatments). It can be suggested that the ice plant leaf area was not reduced by salinity because another feature helped in regulating the leaf ion concentration: the bladder cells, filled with a water solution and functioning as peripheral salinity and water reservoirs (Agarie et al., 2007; Luttge et al., 1978). It is clear that in the agricultural field settings used here, the morphological parameters related to salt tolerance responses were induced with increasing salinity. Leaf succulence and the glistening bladder cells, in particular, provides the edible leaves with a taste, consistence and appear-

ance that make it particularly appreciated by consumers (authors personal observations).

4.4. Physiological and osmotic adaptations to salinity

The shoot color differences among treatments observable in Fig. 1 might be related to the differences in chlorophyll concentration. Indeed, both chlorophyll a and b decreased, even if not always significantly, in the higher salinity treatment 35 dS m^{-1} and in the intermediate salinity ones. Our field experimental results are in line with previous lab studies reporting a decrease in chlorophyll pigments in various halophytes species at saline conditions (Aghaleh et al., 2009; Ayala and O'Leary, 1995; Parida et al., 2002). For the ice plant, the impacts are, however, not strong, because these changes did not negatively affect the plants' photosynthetic apparatus, nor the plants' growth. Focusing on the ice plant, Barker et al. (2004) found overall no significant differences between high and low salt treated plants: this laboratory experiment testing *M. crystallinum* at 400 mM NaCl assessed that prior to any long- term adjustment in pigment composition, the studied species implements salt adaptation strategies involving a greater amount of energy in thermal dissipation over photochemistry. This strategy likely limits impacts on chlorophyll pigments.

The concentrations of Na^+, K^+ and Ca^{2+} found in control juvenile plants (T0) was in line with data reported in literature (Adams et al., 1998; Agarie et al., 2007). Results on Na^+ and Ca^{2+} concentration in salt treated plants proved to be in accord with literature as well: Agarie et al. (2007) reported a comparable sodium concentration in *M. crystallinum* leaves treated with 0 and 400 mM NaCl (roughly corresponding to EC of 40 dS m^{-1}, slightly above our higher salinity treatment). The sodium concentrations coincide with the strategy of *Mesembryanthemum* being a sodium includer, with an increasing sodium gradient from roots to shoot apices (Bohnert and Cushman 2000). Interestingly, adult leaves accumulate more Na^+ than young ones: even if juvenile leaves increase their salt content and the associated inorganic and organic ions under salinity conditions, all ions are accumulated to a lower concentration compared to adult leaves stressed for the same period (Adams et al., 1998). At maturity the ice plant accumulates and compartmentalizes sodium in its bladder cells, which sequester salt from the photosynthetic tissues that are contributing to the seed formation (Adams et al., 1998), leaving them unaffected by salinity.

Within agricultural settings, the strategy to accumulate sodium in predominantly old instead of young leaves may also be profitable because this aspect might prevent the edible leaf product from having a too high sodium content, that could otherwise have negative effects for human health. The accumulation of sodium comes at the cost of accumulating K^+ and resulted in Na^+/K^+ ratios of around six in our experiment (again coinciding with literature: Agarie et al., 2007; Ghnaya et al., 2005; Adams et al., 1998; Demmig and Winter 1986; Harvey et al., 1981). However, it has also been shown that a cytosol Na^+/K^+ ratio smaller than 1 does not seem generally essential for halophyte tolerance to high salinity conditions (Demmig and Winter, 1986). Also the increased calcium concentrations with increasing salinity (Agarie et al., 2007; Adams et al., 1998; Yang et al., 2007) coincide with the salt tolerance of the ice plant as experimental evidence correlated Ca^{2+} increase with salt adaptation (Parida and Das 2005): calcium is believed to protect membranes structure and function under salt stress (Yan et al., 1995), and its concentration increase under salinity stress can ameliorate the inhibitory effect on growth (Epstein, 1972). Moreover, the increased Ca^{2+} concentration seems to act as a second messenger that results in changes in gene expression and metabolism in salt-affected cells (Sairam and Tyagi 2004).

Besides the accumulation or exclusion of cations, the salt tolerance of halophytes is often associated with several osmotic adjustments that lead to the accumulation of a number of organic solutes, as for example soluble sugars (Parida et al., 2002; Wang et al., 2013). Nevertheless, variations in the presence of soluble carbohydrates under saline conditions are not well understood and information on physiological events involved in this process is scarce (Prado et al., 2000). In fact, evidence for free sugar responses to such stress is conflicting, both in glycophytes and in halophytes (Gorham et al., 1981). Also the ice plant in our experimental set-up did not seem to use soluble sugars as solutes to cope with salinity. Concentrations of soluble sugars decreased, at both harvests, with increasing salinity. Also Keiller et al. (1987) observed such decreases in soluble sugars. This does not seem related to a lack of energy, because photosynthesis rates were hardly affected. Instead, it has been argued by Briens and Larher (1982), that halophytes do not necessarily produce high concentrations of carbohydrates as a response to salinity, and several other solutes exist that are able to act as osmoregulatory metabolites, i.e. proline and polyols (myo-inositol, pinitol, ononitol), that also have importance for nutrition (Livesey, 2003; Wu et al., 2011). Whether the ice plant makes use of these alternative osmoregulatory metabolites remains to be investigated.

Furthermore, the absence of significant differences in phenolics concentration between salt-treated plants and the control proves once again that none of the tested salt concentrations was stressful for the ice plant. Salinity thus did not lead to an increase on phenolics nor of the antioxidant activity of the ice plants extracts. This can be explained by *M. crystallinum* capability of avoiding the C_3 mode of carbon fixation by switching to CAM. In general, this seems to be one of the main strategies utilized by halophytes to decrease ROS production while maintaining photosynthesis during stress (Bose et al., 2013). Also thanks to the efficient mechanisms of Na^+ exclusion from the cytosol, salt-tolerant species may not require a high level of antioxidants because they do not allow excessive production of ROS (Bose et al., 2013).

4.5. Nutritive quality of ice plant with increased salinity

The significant increase of Ca^{2+} concentration may represent an interesting nutritional improvement achievable in salinity conditions. Calcium is among the main mineral elements lacking in the diet of over two-thirds of the world's population (White and Broadley, 2009). To address this issue, agronomic approaches optimizing mineral fertilization to increase the concentrations of several mineral elements in agricultural products are of interest (Lynch, 2007). The ice plant is able to acquire and accumulate important mineral elements and the saline environment provided the appropriate conditions to enrich its calcium concentration. Encouragingly, a strong correlation exists between Ca^{2+} and Mg^{2+} plants accumulation capability (White and Broadley, 2009). Furthermore, species from families within the Caryophyllales tend to accumulate uncommonly high Mg^{2+} leaf concentrations (White and Broadley, 2009; Broadley et al., 2004; White 2001), as well as showing remarkable shoot Zn^{2+} concentration, which is generally higher in Caryophyllales in comparison with other plants orders (White and Broadley, 2009). Accordingly, investigations on possible patterns between salinity and other mineral elements with an important role in human diet (i.e. Cu^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}) could add important information on the ice plant nutritional enrichment opportunity in a saline environment.

Furthermore, the carotenoids – another nutritive goal – rose between T1 and T2 in all salt treated plants, while no increase was found in the control. At each harvest, the carotenoids concentrations were unaffected by saline conditions (this study, Barker et al., 2004). Also in this feature, the ice plant seems to distinguish itself positively compared to some other halophyte plants in which

the carotenoid concentration may decrease with increasing salinity (Aghaleh et al., 2009; Qiu et al., 2003; Redondo-Gomez et al., 2010).

4.6. Perspective of ice plant crop in saline agriculture

As none of the tested salt concentrations has resulted in biomass loss, it seems possible to cultivate *M. crystallinum* in saline agriculture, up to a salinity level characterized by EC of 20–35 dS m^{-1}. Perhaps even higher salinity levels are possible since we did not identify a threshold of biomass reduction, although the highest biomass production was suggested to occur around an EC of 20 dS m^{-1}. The already appreciated taste of saline agriculture vegetables in different countries (Rozema and Schat 2013), and of the ice plant in particular, helped by its glistening appearance (Agarie et al., 2009; Herppich et al., 2008), also encourage such possibility. At the higher salinity levels of 20–35 dS m^{-1} the vegetative cycle of the species proved to be extended, enabling a longer productive phase, thus higher yield. According to our results, only the Na^+ concentration in the edible leaves could constitute a concern that may constrain the characterization of the crop as being healthy. Elevated sodium intake has been associated with a number of human diseases which include hypertension, cardiovascular disease and stroke (WHO, 2012). Nevertheless, monitoring its content or allowing the ice plant to act as a natural salt substitute would prevent possible health issues.

5. Conclusions

This species' ability to achieve remarkable growth rates under saline conditions validates its crop potential in saline environments. The results of this study provide a clear evidence that the production of the ice plant with edible purposes can be obtained in saline conditions characterized by EC of 20–35 dS m^{-1} with an extended growing season and high nutritive characteristics. Also, the increased leaf succulence and glistening bladder cells at increased salinity provide the edible leaves with a taste, consistence and appearance particularly appreciated by consumers.

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