RESEARCH

Effect of NaCl and harvest time on antioxidant compounds and morphological cell changes in Lollo Bionda and Lollo Rosso lettuces

Mónica Flores[1], Asunción Amorós[2], and Víctor Hugo Escalona[1, 3*]

[1]Universidad de Chile, Facultad de Ciencias Agronómicas, Centro de Estudios Postcosecha (CEPOC), Av. Santa Rosa 11315, 8820808, Santiago, Chile.
[2]Universidad Miguel Hernández, Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO-UMH), Ctra. Beniel, km 3.2, 03312, Orihuela, Alicante, España.
[3]Universidad de Chile, Facultad de Ciencias Agronómicas, Departamento de Producción Agrícola, Av. Santa Rosa 11315, 8820808, Santiago, Chile. *Corresponding author (vescalona@uchile.cl).

Received: 9 February 2022; Accepted: 24 May 2022; doi:10.4067/S0718-58392022000400537

ABSTRACT

It is known that lettuce (*Lactuca sativa* L. var. *crispa* L.) phenological stage and environmental changes can cause changes in the plant cell morphology and an important variation in secondary plant metabolites. Antioxidant compounds are beneficial to health and salinity can increase them. The aim of this study was to determine the effect of salt concentration (NaCl 0.05 and 0.1 mol L^{-1}) and harvest time (days 10, 20 and 30) on mass, color parameters, antioxidant compounds and proline concentration as well as the morphological tissue changes on two lettuce cultivars: Lollo Bionda 'Levistro' (green leaves) and Lollo Rosso 'Carmoli' (red leaves). NaCl treatments and time of harvest affected fresh matter (FM), DM and DM%, total phenol content, concentration of anthocyanin and antioxidant capacity of both cultivars. High NaCl concentrations decreased FM (from 16.8 to 8.7 g in 'Levistro' in 3rd harvest) and the intracellular space of the leaf tissue but increased the concentration of proline (from 19.6 to 292.2 µg 100 g^{-1} FM in 'Levistro') and antioxidant compounds (from 412.9 to 487.3 mg Trolox eq. 100 g^{-1} FM in 'Levistro'). In addition, concentrations of antioxidant compounds (from 458.2 to 506.4 mg Trolox eq. 100 g^{-1} FM in 'Levistro') and cell density were significantly higher in late than early harvested plants. Thus, successive harvesting may lead to higher antioxidant capacities of lettuces leaves. A moderate decline in FM, a higher cell density and proline concentrations may indicate the better adaptability to salinity stress of red 'Carmoli' than the green 'Levistro' lettuces.

Key words: Antioxidant compounds, cell morphology, harvest, salinity stress.

INTRODUCTION

Nowadays, the consumer is more aware of the relation between fresh product consumption and good health. Diets enriched with fruit and vegetables are associated with a decreased risk of obesity, diabetes, cancer, or cardiovascular diseases. This beneficial effect has been related to secondary plant metabolites such as antioxidant compounds (Kim et al., 2016). In the same way, consumers require not only a high-quality product but also seek out health benefits. Hence the demand for fresh products like leafy salads with a high concentration of antioxidant compounds is growing (Collado-González et al., 2022). About 35% of consumers are even willing to pay a premium for food that offers benefits beyond basic, inherently healthy nutrition (Sloan, 2019).

According to FAO data, worldwide lettuce and endive production reached 26 364 723 t in 2015, and increased to 27 660 187 t in 2020, occupying an area from 1 221 898 to 1 226 370 ha respectively (FAO, 2021). Lettuce (*Lactuca sativa* L.) is the most popular vegetable in salads due to the perception as a healthy fresh product (Llorach et al., 2008).

Its health properties are attributed to vitamins E and C, carotenoids, fiber, minerals, and polyphenol concentration (Kim et al., 2016). The latter compounds have antioxidant properties that protect human cells from the detrimental effects of reactive oxygen species (ROS) (Kim et al., 2016). Among different polyphenols compounds, flavonols and anthocyanins have been described as having greater antioxidant activity than vitamins C and E (Llorach et al., 2008).

Due to the interest in enhanced antioxidant compounds in vegetables, combined with an increased demand for different types of colored leaves in salads, lettuces stand out among others due their high variety of types, shapes, colors, and cultivars (Kim et al., 2016). Nevertheless, there is a paucity of information about antioxidant compounds in different cultivars. Moreover, a slight environmental change can generate a significant variation in antioxidant compound concentration in vegetables like lettuce (Collado-González et al., 2022).

Shabala et al. (2015) mentioned that salinity is the greatest environmental factor that restricts plant growth. During the last decade, it has caused a direct reduction in global mean yield by up to 20% (Carillo et al., 2019). The 19.5% of agricultural lands are considered saline, and every year two million hectares become saline soils; hence salinization is predicted to impact 50% of all arable land by 2050 (Butcher et al., 2016).

High levels of NaCl can be toxic for plants and reduce growth (Shabala and Munns, 2012; Lucini et al., 2015). However, in response to salinity, plants have developed various mechanisms to integrate exogenous stress with endogenous development signals to optimize the balance between growth and stress response (Shabala et al., 2015). One of the mechanisms that acts against salt stress is the accumulation of protective compounds such as antioxidants that ensure the elimination of ROS generated by this stress (Bartha et al., 2015). In addition, plants can use organic solutes like proline as osmoregulation compounds that accumulate in the cytosol and organelles to balance the osmotic pressure that Na^+ and Cl^- ions generated in the vacuole (Lucini and Bernardo, 2015). The increase in osmolytes in cells can facilitate water movement into the cell, reducing the impact of osmotic stress and cellular dehydration (Butcher et al., 2016). The accumulation of proline plays an important role in stress conditions, in addition to the osmotic adjustment, protecting and stabilizing proteins, membranes, and subcellular structures quenching ROS (Lucini and Bernardo, 2015).

Salinity decreases the water uptake and thus affects morphology and histology, e.g., producing denser leaves, which affect the overall texture of lettuces plants (Garrido et al., 2013). The changes caused by accumulations of salts in the root zone bring about osmotic stress, which decreases the water uptake. In case of the moderately salinity stress-sensitive lettuce plants (Lucini and Bernardo, 2015), decreased water uptake also reduces transpiration rates, inhibits cell division and expansion (Carillo et al., 2019), reduces overall metabolic activity, and impedes growth (Butcher et al., 2016).

Therefore, lettuce is a widely consumed vegetable throughout the world, with beneficial effects on health. Saline treatments increase beneficial compounds for health, and due to climate change, there are more and more salinized lands. As far as we know, very little has been reported about cell structural modifications in lettuce leaves due to salinity, salt tolerance and concentration of antioxidant compounds in different cultivars (Bartha et al., 2015). There is also insufficient data about lettuce responses after successive harvests grown in salinity treatments. Therefore, the aim of this study was to determine the effect of NaCl concentrations applied to the nutrient solution and harvest times on fresh and dry mass, antioxidant compounds and histological changes of lettuce leaves of 'Levistro' Lollo Bionda (green and crispy leaves) and 'Carmoli' Lollo Rosso (red and crispy leaves) grown in a hydroponic system in a greenhouse. This way it will be possible to verify if saline treatments in lettuce increase the beneficial effects for health.

MATERIALS AND METHODS

Plant material, growth conditions and experimental design

The experiment was carried out in the Postharvest Study Center (CEPOC in Spanish) of the Faculty of Agricultural Science at the University of Chile, La Pintana (32°40' S, 70°32' W; 625 m a.s.l.), Santiago, Chile. Lettuce plants (*Lactuca sativa* var. *crispa* L.) of green 'Levistro' Lollo Bionda and red 'Carmoli' Lollo Rosso cultivars (seeds obtained from Rijk Zwaan Zaadteelt und Zaadhandel B.V., De Lier, The Netherlands) were grown in floating root hydroponic culture. The growing periods were carried out in a plastic chapel greenhouse of 8 m wide, 33 m long and 5.8 m high at the zenith. The greenhouse was covered with a 200 μm thick polyethylene film on the top and sides which allowed 90% and 20% of global light transmission and diffusion, respectively (Proamco, Santiago, Chile). The greenhouse also had a manual zenith window and an automatic system for opening and closing side windows, both covered with anti-aphid mesh.

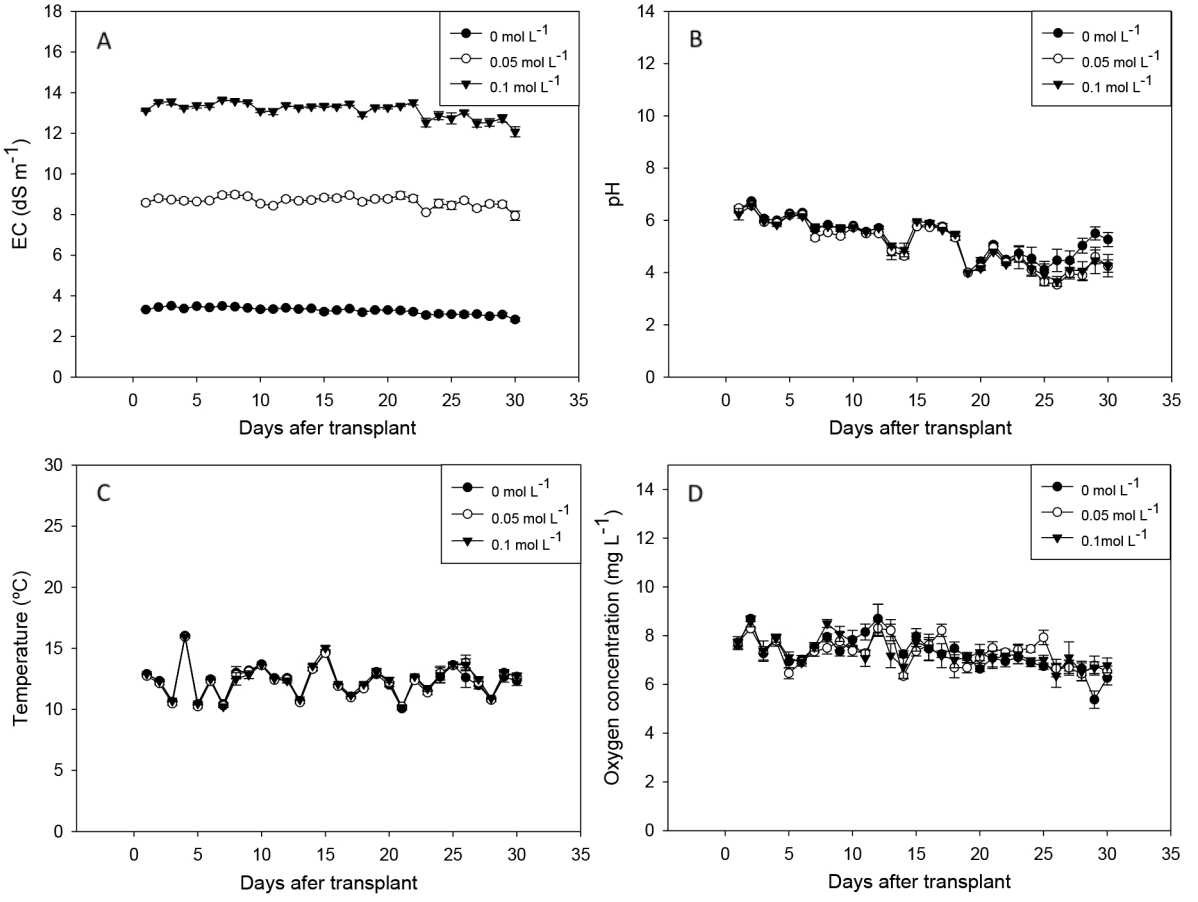
Lettuce seeds were germinated on 162 cell-tray on pre-hydrated granulated rock wool (Agrolan, El Volcán S.A., Santiago, Chile) and expanded perlite A6 (Harbolite Chile Ltda., Santiago, Chile) in a dark germination chamber (22 °C and 80% RH for 24 h). After the root emission, trays were transferred to the greenhouse and irrigated with tap water until the expanded cotyledon stage. Then, 50% of the nutrient solution used by Flores et al. (2022) for leafy vegetables was used until transplant. The nutrient solution composition was (mol L^{-1}): 1.25×10^{-3} NH_4, 11.0×10^{-3} K, 4.5×10^{-3} Ca, 1.0×10^{-3} Mg, 19.0×10^{-3} NO_3, 1.125×10^{-3} SO_4, 2.0×10^{-3} H_2PO_4, 40×10^{-6} Fe, 5×10^{-6} Mn, 4×10^{-6} Zn, 30×10^{-6} B, 0.75×10^{-6} Cu, and 0.5×10^{-6} Mo. The pH of the solution was kept between 5.5 and 5.8 to maximize nutrient absorption for the crop. Plants at the true four-leaf stage were transplanted to a closed floating root hydroponic system formed by a water reservoir of 0.5 × 1.5 × 0.12 m that contained 100% of the nutrient solution; a floating raft made by high-density expanded polystyrene of 0.5 × 1.5 × 0.025 m and 25 kg m^{-3} and a small pump (HJ-542, Super Aquatic, China) that moved and incorporated air bubbles into the nutrient solution were used.

The rafts were perforated with a 3-bobbin arrangement with a density of 50 plants m^{-2}. The plants were fastened with a low-density sponge. Two days after transplant, nutrient solution (pH 5.5 to 5.8, adjusted with nitric acid 10% solution) was added following the recommendations of Flores et al. (2022).

Three NaCl levels were evaluated: 0 mol L^{-1} as control, 0.05 and 0.1 mol L^{-1} NaCl; NaCl was incorporated as water dissolved salts, amounts were calculated to obtain the mentioned concentrations on each independent floating root hydroponic system.

The electric conductivity (dS m^{-1}), pH, temperature (°C), and dissolved oxygen (mg L^{-1}) in the nutrient solution were measured every 2 d as shown in Figure 1.

Figure 1. Means (± SE) of electric conductivity (EC) (A), pH (B), temperature (C) and oxygen concentration (D) on the nutrient solution during cultivation of lettuce plants growing with different NaCl concentrations.



Harvest times and analytical parameters of plant growth

Fully expanded leaves of the same plants were cut with sterilized (70% ethanol solution) stainless steel scissors at days 10 (5^{th} + 6^{th} leaves), 20 (7^{th} + 8^{th} leaves) and 30 (9^{th} + 10^{th} leaves), and named as first, second and third harvest, respectively.

Five plants per replicate (n = 15 per treatment) were randomly selected for fresh mass (FM) and dry mass (DM) measured using a precision analytical balance (AS 100/C/2, RADWAG, Radom, Poland). To obtain DM, samples were dried in a forced air oven (LDO-150F, Labtech, Gagok-ri, Korea) at 70 °C until constant mass; FM and DM were expressed in g. Finally, the percentage of DM was estimated by the DM:FM ratio and expressed as a percentage.

Color parameters

Five random leaves per replicate were selected to obtain color parameters (n =15 per treatment). Two points on the upper side of leaves, avoiding the central rib, were measured using a tristimulus compact colorimeter (Konica Minolta spectrophotometer CM-2500d, Ramsey, New Jersey, USA) with a D65 light source and an observer angle of 10°. Color parameters were expressed as luminosity (L), chroma (C*) and hue angle (Hue). Due to the significant color differences among cultivars, the results were analyzed separately by cultivar.

Antioxidant extraction

About 30 g fresh lettuce leaves per replicate were collected and stored at -80 °C, lyophilized and crushed with an electric mill (ML-2006x, Fagor, Mondragon, Spain) until a fine powder was obtained. For the antioxidant extraction, the same method was followed as in Flores et al. (2022). The methanol:water extract was stored at -20 °C in amber tubes until analysis of total phenolic, flavonoid, anthocyanin compounds and antioxidant activity.

Total phenolic and total flavonoid concentrations

The total phenolic concentration (TPC) was measured using the method used by Flores et al. (2022); it was calculated using a gallic acid calibration curve (Merck KGaA, Darmstadt, Germany) and expressed as gallic acid equivalent (GAE) in mg 100 g^{-1} FM.

The total flavonoid concentration (TFC) was measured following the method used by Flores et al. (2022); it was calculated using a rutin calibration curve (Merck KGaA) and expressed as rutin equivalents (Rut Eq) in mg 100 g^{-1} FM.

Total anthocyanin concentration and antioxidant capacity

The total anthocyanin concentration (TAC) was determined using a differential pH method following Du et al. (2014) recommendations; it was expressed as cyanidin-3-glucoside equivalents (Cyn3gluc eq) in mg 100 g^{-1} FM and calculated based on total volume of extract and the mass (fresh mass) of the sample extracted.

The antioxidant capacity (AC) was determined with ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The FRAP assay was performed according to the method described by Flores et al. (2022). The results were expressed as Trolox equivalent (Trolox eq) in mg 100 g^{-1} FM. The DPPH assay was performed according to the method described by Flores et al. (2022). The equivalent AC was calculated using a Trolox calibration curve starting with 400 µg mL^{-1} stock standard solution. The results were expressed as Trolox equivalent (Trolox eq) in mg 100 g^{-1} FM.

Total proline concentration

Total proline concentration (TPrC) was determined according to Kamińska et al. (2022). Proline concentration was determined by a standard curve using D-proline (Merck KGaA). The results were expressed as µg proline100 g^{-1} FM.

Microscopic cell analysis and stomatal and cell density

For the microscopic evaluation of cell morphology changes, circular samples of fresh leaves were cut with a 1 cm^2 hole punch from five plants per replicate (n = 15 per treatment). One sample set was used to observe stomatal and cell density. Another sample set (15 samples per treatment) was fixed in formalin-aceto-alcohol (FAA) solution and used to determine the intercellular space.

Stomata were counted on the abaxial epidermis at the midpoint of the leaf lamina between the midrib and margin (Xie et al., 2012). Fresh leaf tissue was cut into 5 × 5 mm with a scalpel. Each section was put in a glass slide and scraped gently to help fix the neutral red staining. The samples were observed under a bright field trinocular microscope (BA310, Motic, Hong Kong, China) with a 400X zoom lens. The stomata and cells were photographed and counted (Xie et al., 2012). The images of the tissue were taken by a digital camera (Moticam 5.0 MP, Motic, Hong Kong, China). Seven images per treatment for each harvest time and cultivar were analyzed with the ImageJ program, a free domain processing program by the National Institutes of Health (NIH, Bethesda, Maryland, USA), version v1.51j8 (Schneider et al., 2012). Stomatal density was expressed as stomata mm^{-2} and cell density as cell mm^{-2}. Also, the stomatal index (SI) was calculated using the following formula (Xie et al., 2012):

$$\text{Stomatal index (SI)} = (\text{Stomatal density}/\text{Stomatal density} + \text{Epidermal cell density}) \times 100$$

Intercellular space

This analysis was performed only on samples from the third harvest. The fresh leaf tissue of 15 plants per treatment was cut into 5 × 5 mm with a scalpel. The tissue was fixed with FAA solution (10% formaldehyde, 5% acetic acid, 50% ethanol, v/v in water). Fixation was followed by an ethanol dilution series at 50%, 70%, 90%, 95% and 100% (Sivankalyani et al., 2016).

Three 25 mm^{-2} squares per NaCl treatment for each cultivar were randomly selected and embedded in resin with the JB-4 Embedding Kit, as per the manufacturer's instructions (Sigma Aldrich, Darmstadt, Germany).

Ten cuts per sample of 10 µm thickness were taken using a manual microtome (1516, Leitz, Midland, Ontario, Canada). The samples were put on a glass slide and stained with periodic acid-Schiff (PAS) and aniline blue, according to the method proposed by Sumner (2015) with some modifications. Samples were observed under a brightfield trinocular microscope (BA310, Motic) with 100X zoom lens. Images were taken with a digital camera (Moticam 5.0 MP) and analyzed using the ImageJ program (Schneider et al., 2012). The results were expressed as the percentage of intracellular space out of the total area.

Experimental design and statistical analysis

The experiment was arranged in three blocks with a divided plot design. Three factors were considered: the main plot NaCl concentrations (0.0, 0.05 and 0.1 mol L^{-1}), cultivars ('Levistro' and 'Carmoli') and harvest time (1^{st}, 2^{nd}, 3^{rd}). Then, three independent floating root hydroponic systems for each NaCl concentration were setting up. Also, for each NaCl concentration 75 plants per cultivar (25 per replicate) were considered. Each block represented one replicate. Means were calculated and Fisher's LSD multiple comparisons test was performed to analyze the significance among factor levels. A $p < 0.05$ value was considered to determine significant differences (Di Rienzo et al., 2017).

RESULTS AND DISCUSSION

Effect on yield of green and red lettuce cultivars

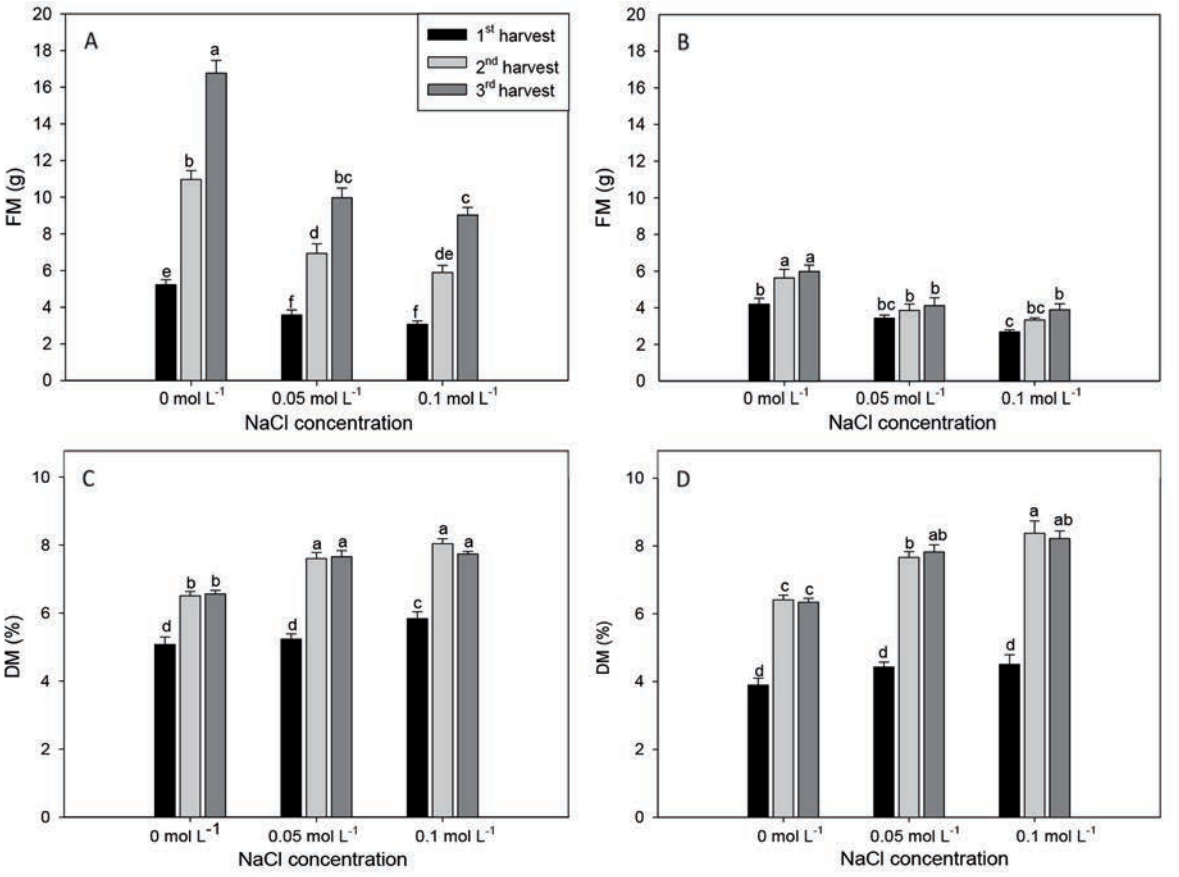
Cultivar, harvest time and NaCl concentration exerted significant triple interaction (Table 1) on the plant fresh mass. 'Levistro' presented significantly higher FM than 'Carmoli' and showed an increased by successive harvests but decreased with the NaCl presence (Figures 2A, 2B). At the first harvest, FM of plants treated with 0.05 and 0.1 mol L^{-1} NaCl significantly declined 32% and 41% respectively, compared to control, but there were not differences between both treatments. Fresh mass reduction lightly increased further the latest harvest. Treating 'Carmoli' lettuces with NaCl in the nutrient solution reduced fresh mass to a similar percentage than 'Levistro' plants. Showing at the first harvest a reduction of 33% and 46% for 0.05 and 0.1 mol L^{-1} compared to the control were observed respectively (Figure 2). However, FM of 'Carmoli' plants did not further increase from the second to the third harvest. The reduction in FM in lettuce plants because the NaCl were confirmed by Bartha et al. (2015). However, unlike Bartha et al. (2015) for 'Asparagina', 'Valdor', 'Salad B. red', 'Paris Island' and 'Parella' green lettuces, non reduction in FM between 0.05 and 0.1 mol L^{-1} NaCl were reported. Many studies have provided evidence that salinity reduces growth because of osmotic stress induction by limiting water uptake, ionic imbalance or a disturbance in ion homeostasis and toxicity caused by Na^+ and Cl^- ions at high concentrations within the plant cells (Bartha et al., 2015). The osmotic potential becoming more negative with increasing

Table 1. Fresh (FM), dry mass and dry mass content for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations for various harvest times.

Factor	Level	FM	DM	DM
		g	g	%
Cultivar (1)	'Levistro'	7.94	0.55	6.69
	'Carmoli'	4.12	0.27	6.41
Harvest time (2)	1st	3.70	0.18	4.83
	2nd	6.10	0.44	7.43
	3rd	8.29	0.60	7.39
NaCl (3)	0 mol L^{-1}	8.13	0.50	5.80
	0.05 mol L^{-1}	5.31	0.37	6.73
	0.1 mol L^{-1}	4.65	0.35	7.12
1	p value	< 0.0001	< 0.0001	< 0.0018
2	p value	< 0.0001	< 0.0001	< 0.0001
3	p value	< 0.0001	< 0.0001	< 0.0001
1 × 2	p value	< 0.0001	< 0.0001	< 0.0001
1 × 3	p value	< 0.0001	< 0.0001	0.2437
2 × 3	p value	< 0.0001	< 0.0005	< 0.0003
1 × 2 × 3	p value	< 0.0002	< 0.0046	0.4043

Values are means of 45 data per cultivar, 30 data per harvest time and NaCl concentration and means of five measurements for the interaction (1 × 2 × 3).
Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

Figure 2. Means (± SE) of fresh mass (FM) (A, B) and dry mass (DM) content (C, D) of leaves of green 'Levistro' (A, C) and red 'Carmoli' (B, D) lettuces grown on nutrient solutions with or without NaCl added for various harvest times.



Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

salt concentration, causing a reduction in leaf water content and an increase in leaf osmotic potential (Garrido et al., 2013). On the other hand, NaCl, increasing Na^+ and Cl^- ions concentration in the root zone leading to a nutritional imbalance (Lucini et al., 2015). Also, the negative effect of salt on plant growth could generate a reduction in stomatal conductance, and thus both transpiration and CO_2 gain (Garrido et al., 2013).

Cultivar, harvest time and NaCl concentration affected the percentage of DM. As it was described before for FM, an interaction among factors was detected (Table 1). Although 'Levistro' had higher DM values than 'Carmoli' and both cultivars increased DM (%) when NaCl was added. However, there were no differences between NaCl concentrations (Figures 2C, 2D). The increased DM (%) in lettuce leaves grown with an elevated salt solution was previously reported by Bartha et al. (2015). Nevertheless, unlike these authors reported for some cultivars, no differences were found for 'Levistro' and 'Carmoli' in DM (%) between 0.05 and 0.1 mol L^{-1} NaCl. The increase in DM could be due to the ion accumulation of compatible solutes like proteins, amino acids, and sugars, as a protective mechanism against salt stress. Also, the lowest DM (%) was found at the first harvest compared to the second and third harvests, showing differences among plant stages and the stress caused by the successive harvests (Sakalauskaité et al., 2012).

Effect on the color of green and red lettuce cultivars

Significant interactions were observed between cultivar and harvest time for luminosity and chroma. In the same way, significant interactions were found between cultivar and NaCl concentration for luminosity and hue angle (Table 2).

Considering color changes, 'Levistro' presented higher luminosity (L) in the control compared to NaCl treatments, which revealed increased darkening (Figure 3A). In fact, higher luminosity was found at third harvest compared to the previous ones (Figure 3A). In 'Carmoli' no differences in luminosity were found when NaCl was applied (Figure 3D).

For chroma, 'Levistro' treated with 0.1 mol L^{-1} NaCl showed lower values than the control and 0.05 mol L^{-1} NaCl (Figure 3B). 'Carmoli' also presented lower values of chroma when NaCl was applied, yielding a less intense color than control (Figure 3E). These results could be due to a reduction in chlorophyll concentration in the leaves grown under salt stress (Garrido et al., 2013).

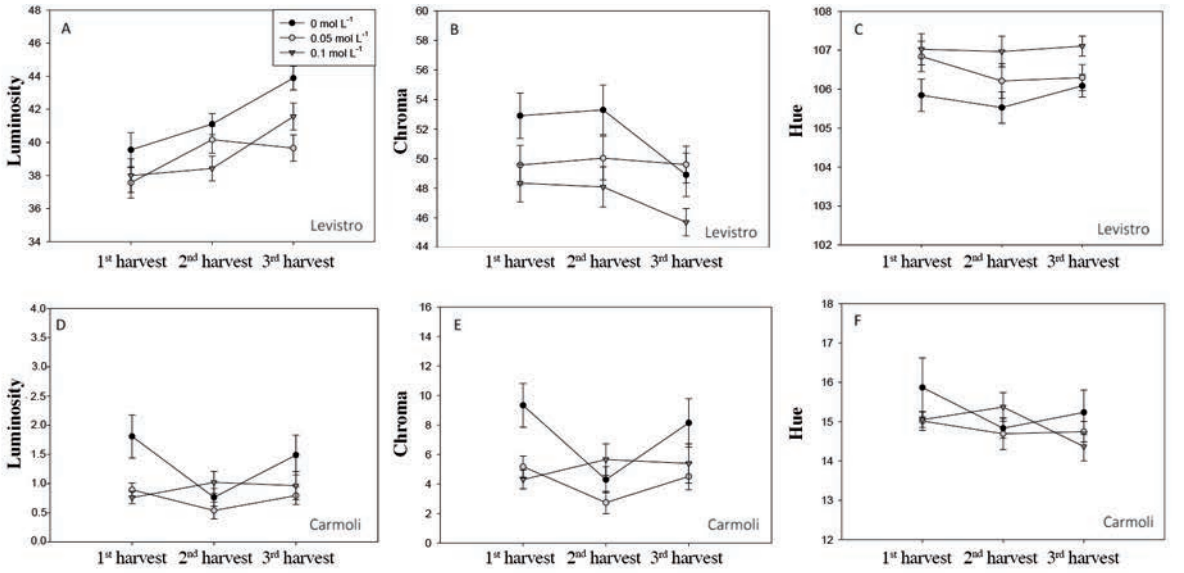
'Levistro' lettuces grown with NaCl achieved a higher hue angle compared to the control, presented values belonging to green (Figure 3C). By contrast, 'Carmoli' showed no differences in hue angle between NaCl and control, with values closer to red (Figure 3F). A direct relation between changes in color and phenolic concentrations was observed for green and red cultivars cultivated in different ambient conditions and plant stages (Pinto et al., 2014; Zapata-Vahos et al., 2020), being a simple measurement to be considered.

Table 2. Color parameters (luminosity, chroma and hue angle) for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations for various harvest times.

Factor	Level	Luminosity	Chroma	Hue
Cultivar (1)	'Levistro'	39.99	49.61	106.43
	'Carmoli'	1.00	5.51	15.02
Harvest time (2)	1st	19.76	28.27	60.94
	2nd	20.34	27.35	60.60
	3rd	21.39	27.04	60.64
NaCl (3)	0 mol L^{-1}	21.43	29.48	60.57
	0.05 mol L^{-1}	19.94	26.94	60.63
	0.1 mol L^{-1}	20.12	26.25	60.98
1	p value	< 0.0001	< 0.0001	< 0.0001
2	p value	< 0.0001	0.2086	0.2647
3	p value	< 0.0001	< 0.0001	0.1495
1 × 2	p value	< 0.0001	0.0154	0.5246
1 × 3	p value	0.0176	0.0650	< 0.0018
2 × 3	p value	0.2349	0.4268	0.4930
1 × 2 × 3	p value	0.1389	0.1662	0.6523

Values are means of 270 pieces of data per cultivar, 180 data per harvest time and NaCl concentration and means of 30 measurements for the interaction (1 × 2 × 3).
Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

Figure 3. Means (± SE) of luminosity (A, D), chroma (B, E), and hue angles (C, F) of leaves of green 'Levistro' (A-C) and red 'Carmoli' (D-F) lettuces grown on nutrient solutions with or without NaCl added for various harvest times.



Effect on phenolic compounds and antioxidant capacity of green and red lettuce cultivars

As it was expected, significant differences were found for antioxidant compounds between cultivars. 'Carmoli' had higher total phenolic concentration (TPC) between 380 to 750 mg GAE 100 g^{-1} FM than 'Levistro' with values between 230 to 340 mg GAE 100 g^{-1} FM. Which represent between 1.6 and 2.2-fold of red cultivar over green one. These results were consistent with the results found by Zapata-Vahos et al. (2020), where phenolic concentrations showed huge variations by color. According to de Souza et al. (2022), the highest TPC and AC of red lettuce might be attributed to the anthocyanin concentration. This could due to the high percentage of anthocyanins of red lettuce, mainly cyanidins and delphinidins (de Souza et al., 2022).

For both cultivars, all studied antioxidant compounds were significantly affected by the interaction of harvest time and NaCl concentration except for TPC in 'Levistro' (Table 3).

The antioxidant compound concentrations reacted to environmental growth conditions, begin an opportunity to obtain antioxidant-rich products. Liu et al. (2007) found that lettuces harvested in July had higher TPC and AC than lettuce harvested in September, showing the influence of the season. However, these responses depended on cultivar and season, showing that although there was a trend it was not a general rule. On the other hand, according to the results in this study plant stage also determined the phenolic accumulation and its response to abiotic stress (Table 3). Similarly, Mir et al. (2022) showed the effect of growth stage on the concentration and composition of phenolic fraction of tagete. In the same trend, Sakalauskaité et al. (2012) reported that the respond of basil to UV-B radiation as abiotic stress.

In 'Levistro', TPC was significantly higher at 0.05 mol L^{-1} and after the third harvest, showing a significant response to salt concentration and harvest time (Table 3). All treatments reached higher phenolic concentration at the third harvest compared to the previous ones (Table 3). 'Carmoli' also presented higher TPC at 0.05 and 0.1 mol L^{-1} compared to control. The TPC values of 'Levistro' were in the range reported by Kim et al. (2016), but greater than those mentioned by Llorach et al. (2008) and Zlotek et al. (2014) for green lettuces. On the other hand, for 'Carmoli' higher values between 380 and 750 mg GAE 100 g^{-1} FM were found compared to those reported by Llorach et al. (2008), who found values of 114 and 571 mg GAE 100 g^{-1} FM for red lettuces.

Total flavonoid concentrations (TFC) showed a similar behavior against salt treatments and harvest time than TPC, for both cultivars. 'Carmoli' had significantly higher values of 1190 and 2480 mg Rut eq 100 g^{-1} FM compared to 'Levistro' with 960 and 1500 mg Rut eq 100 g^{-1} FM. The highest flavonoid concentration was found at the third harvest particularly at high NaCl concentrations. In accordance with Garrido et al. (2013) and Lucini and Bernardo (2015), an increase in total flavonoids in saline conditions for green lettuce were found. Also, the quantity of flavonoids for 'Levistro' were near to the range mentioned by Zlotek et al. (2014) for green lettuces, but slightly lower than those reported by Kim et al. (2016) for Lollo Rosso lettuces.

Table 3. Total phenolic, flavonoid, anthocyanin contents and antioxidant capacity (AC) for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations for various harvest times.

							AC			
		Phenolics		Flavonoids		Anthocyanins	FRAP		DPPH	
Factor	Level	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
		mg GAE 100 g^{-1} FM		mg Rut eq 100 g^{-1} FM		mg Cyn3gluc eq 100 g^{-1} FM	mg Trolox eq 100 g^{-1} FM		mg Trolox eq 100 g^{-1} FM	
Harvest time (1)	1st	279.1b	408.4	1124.9	1282.2	28.6	458.2	631.3	355.9	470.1
	2nd	262.5c	596.0	1099.5	1895.1	38.5	403.7	899.0	336.0	640.8
	3rd	316.2a	685.6	1376.8	2247.2	53.3	506.4	1080.4	443.7	782.1
NaCl (2)	0 mol L^{-1}	259.2b	491.9	1075.0	1533.7	36.3	412.9	752.6	343.3	556.6
	0.05 mol L^{-1}	303.7a	598.5	1283.4	1938.0	42.3	468.1	907.7	396.2	659.3
	0.1 mol L^{-1}	294.9a	599.6	1242.8	1952.8	41.8	487.3	950.4	396.2	677.1
1 × 2	0 × 1st	260.6	384.2f	1101.2b	1191.1f	30.3e	456.1b	593.2e	349.9c	474.0d
	0 × 2nd	237.4	515.8d	962.9c	1570.4d	35.7d	361.1c	769.6d	292.8d	532.7c
	0 × 3rd	279.7	575.6c	1160.9b	1839.7c	42.7c	421.6b	895.1c	387.2b	663.0b
	0.05 × 1st	301.2	441.9e	1151.0b	1380.2e	30.9e	451.5b	672.1e	360.9c	483.3cd
	0.05 × 2nd	272.8	625.6b	1176.5b	2011.5b	39.9c	417.8b	910.6c	355.5c	673.6b
	0.05 × 3rd	337.1	728.1a	1522.7a	2422.4a	56.1b	535.1a	1140.3a	472.0a	820.8a
	0.1 × 1st	275.4	399.2f	1122.5b	1275.3ef	24.6f	467.2b	628.6e	356.8c	452.9d
	0.1 × 2nd	277.5	646.6b	1159.3b	2103.5b	39.7c	432.1b	1016.9b	359.7c	716.0b
	0.1 × 3rd	331.8	753.1a	1446.7a	2479.6a	61.2a	562.7a	1205.9a	471.9a	862.4a
1	p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
2	p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
1 × 2	p value	0.2346	< 0.0001	< 0.0016	< 0.0001	< 0.0001	< 0.0041	< 0.0001	< 0.0001	< 0.0001

Values are means of 27 pieces of data per harvest time and NaCl concentration and means of measurements for the interaction (1 × 2). Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

Total anthocyanin concentration measured in 'Carmoli' presented higher values at the third harvest in all treatments following the same trends described above for TFC and TFC (Table 3). The highest TAC was reached at the third harvest at 0.1 mol L^{-1} with 61.2 mg Cyn3gluc eq 100 g^{-1} FM, which represented an increase of 43% over the control at the same harvest. Some authors such as Llorach et al. (2008) described similar values for Lollo Rosso lettuces finding 45.6 mg anthocyanin 100 g^{-1} FM. Kim et al. (2016) also reported between 2 and 130 mg anthocyanins 100 g^{-1} FM for the same type of lettuces.

The FRAP values for 'Levistro' were from 360 to 560 mg Trolox 100 g^{-1} FM, whereas 'Carmoli' reached values between 590 and 1200 mg Trolox 100 g^{-1} FM. These values were greater than those reported by Llorach et al. (2008), who found values from 98 to 320 mg Trolox 100 g^{-1} FM for green lettuces but similar for Lollo Rosso lettuces of 810 mg Trolox 100 g^{-1} FM. Also, antioxidant capacity by FRAP showed a similar trend than TPC, TFC and TAC (Table 3). Because TPC, TFC and anthocyanins have antioxidant activity it was expected a similar behavior than AC (Zlotek, et al., 2014). 'Levistro' had lower AC than 'Carmoli' following a typical comportment for green and red lettuces (Llorach et al., 2008). Like the other parameters, the AC for 'Levistro' and 'Carmoli' showed the highest values at 0.1 mol L^{-1} at the third harvest.

The antioxidant capacity measured by DPPH showed a similar behavior to FRAP. The DPPH values for 'Levistro' were between 290 and 470 mg Trolox 100 g^{-1} FM, being higher than the values reported by Llorach et al. (2008) for green lettuce of 70 to 240 mg Trolox 100 g^{-1} FM. On the other hand, 'Carmoli' had DPPH values from 450 to 860 mg Trolox 100 g^{-1} FM, similarly to those reported previously for Lollo Rosso lettuce by Llorach et al. (2008), who found about 770 mg Trolox 100 g^{-1} FM. As it was observed, the increase in TPC, TFC and TAC under salinity and after consecutive harvests implied an increase in total antioxidant activity, stimulated by these stresses.

The patently higher amounts of TPC, TFC, TAC, and AC detected in 'Carmoli' than 'Levistro' confirmed once more the differences between cultivars in the amount of antioxidant compounds (Pérez-López et al., 2015; Sgherri et al., 2017). Interestingly, these secondary metabolites are considered an added value to basic nutritional characteristics of leafy vegetables such lettuces because of their health-promoting effects (Di Mola et al., 2017).

On the other hand, the osmotic effect generated by NaCl could lead to an increased production of ROS, resulting in oxidative stress (Shabala and Munns, 2012; Shabala et al., 2015). Under these conditions, plants would activate several physiological mechanisms to acclimate to this environment. As Neocleous et al. (2014) and Lucini et al. (2015) reported,

the metabolic response to salinity must involve the synthesis of secondary metabolites (i.e., TPC, TFC and TAC), and involves changes in the accumulation of phenylpropanoids biosynthesis enzymes as phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), flavanone-3-beta-hydroxylase (F3H) and cinnamate-4-hydroxylase (C4H). The increased in TPC, TFC, TAC and AC suggest that tested NaCl concentrations applied in the nutrient solution activated the key steps of phenolic compounds biosynthesis and accumulation.

Effect on total proline concentration of green and red lettuce cultivars

The proline concentrations showed different responses according to the cultivar. In 'Levistro', consecutive harvests and increased NaCl concentration significantly affected the proline concentration (Table 4). Proline concentration reached the highest values at 0.10 mol L^{-1}. On the other hand, proline concentration was decreased by consecutive harvests from 390 to 180 µg 100 g^{-1} FM. By contrast, 'Carmoli' only showed a rise in proline concentration when the NaCl concentration was increased, but not by the harvest time. At 0.1 mol L^{-1}, 'Carmoli' evidenced a significant increase of about 350% in proline concentration compared to control.

Proline is a low molecular weight soluble metabolite that counteracts ionic strength in vacuoles. Its accumulation under salt stress has been related with osmotic adjustment, protein and membrane protection, and quenching ROS in several species (Shabala and Munns, 2012; Lucini et al., 015; Kamińska et al., 2022). Between cultivars, a significant difference in proline concentrations was found. 'Carmoli' achieved lower values than 'Levistro' at different NaCl concentrations, indicating that green lettuces synthesized more proline as a protection mechanism against salt concentrations.

It seems that the synthesis of proline is related to phenolic compound accumulation. Stress salt concentrations could activate NADPH oxidase enzymes, which increases ROS production (Shabala et al., 2015) and according to Lucini et al. (2015) proline is involved in quenching ROS. Given that 'Carmoli' presented naturally higher values of phenolic compounds and AC than 'Levistro' (Table 4), it can efficiently protect its cells against ROS damage. So, red cultivars like 'Carmoli' do not need such a large amount of proline to react with ROS production caused by salt compared to 'Levistro' (Table 3).

Finally, the synthesis of proline occurs at the expense of plant growth because it comes with an energy cost so, it may be related to the decrease in FM by salt stress. The fact that 'Carmoli' presented only slight losses of fresh mass at higher NaCl concentrations indicates that red cultivars are more adapted to respond to this stress due to their higher concentration on TPC and AC and do not need to use energy to synthesizing proline. This reaffirms the possible relation between proline and phenolic compounds; however, further studies at the transcriptional and functional levels of proline and phenolic synthesis pathways are needed to clarify their relation.

Table 4. Proline content for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations for various harvest times.

Factor	Level	'Levistro'	'Carmoli'
		µg 100 g^{-1} FM	
Harvest time (1)	1st	168.4	28.9
	2nd	117.4	29.7
	3rd	84.2	24.6
NaCl (2)	0 mol L^{-1}	19.6	12.4b
	0.05 mol L^{-1}	58.2	14.9b
	0.1 mol L^{-1}	292.2	56.0a
1 × 2	0 × 1st	10.0f	12.1
	0 × 2nd	27.6ef	13.9
	0 × 3rd	21.3ef	11.0
	0.05 × 1st	98.2d	15.6
	0.05 × 2nd	29.5ef	13.7
	0.05 × 3rd	46.8e	15.4
	0.1 × 1st	396.9a	59.0
	0.1 × 2nd	295.2b	61.6
	0.1 × 3rd	184.5c	47.3
1	p value	< 0.0001	0.5522
2	p value	< 0.0001	< 0.0001
1 × 2	p value	< 0.0001	0.7283

Values are means of 27 data per harvest time and NaCl concentration and means of nine measurements per interaction (1 × 2).
Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

Effect on stomatal, cell density and intercellular space of green and red lettuce cultivars

Because salinity stress affects water uptake and there was a loss of water trough stoma due respiration, it is important to determinate leaf morphology in this condition. A significant effect on stomatal density for 'Levistro' and 'Carmoli' by harvest time was found (Table 5). By contrast, the stomatal index showed that neither harvest time nor NaCl concentration affected this parameter. Finally, there was a significant interaction between harvest time and NaCl concentration on cell density for 'Levistro'. On the contrary, for 'Carmoli' an independent effect was found for each factor (Table 5). Stomatal density for 'Levistro' was significantly lower than 'Carmoli' showing a mean value of 47.0 and 56.4 stomata mm^{-2}, respectively. In addition, both cultivars, at the first harvest, revealed significant lower values than second and third harvest, indicating differences according development stages of the lettuce plants (Table 5). On the other hand, there was a significantly higher cell density in 'Carmoli' than 'Levistro' with values of 708.5 and 492.9 cell mm^{-2}, respectively. Also, there was a significant increase with consecutives harvests for both cultivars and, only in the case of 'Carmoli', there were observed higher cell densities at 0.05 and 0.1 mol L^{-1} compared to control.

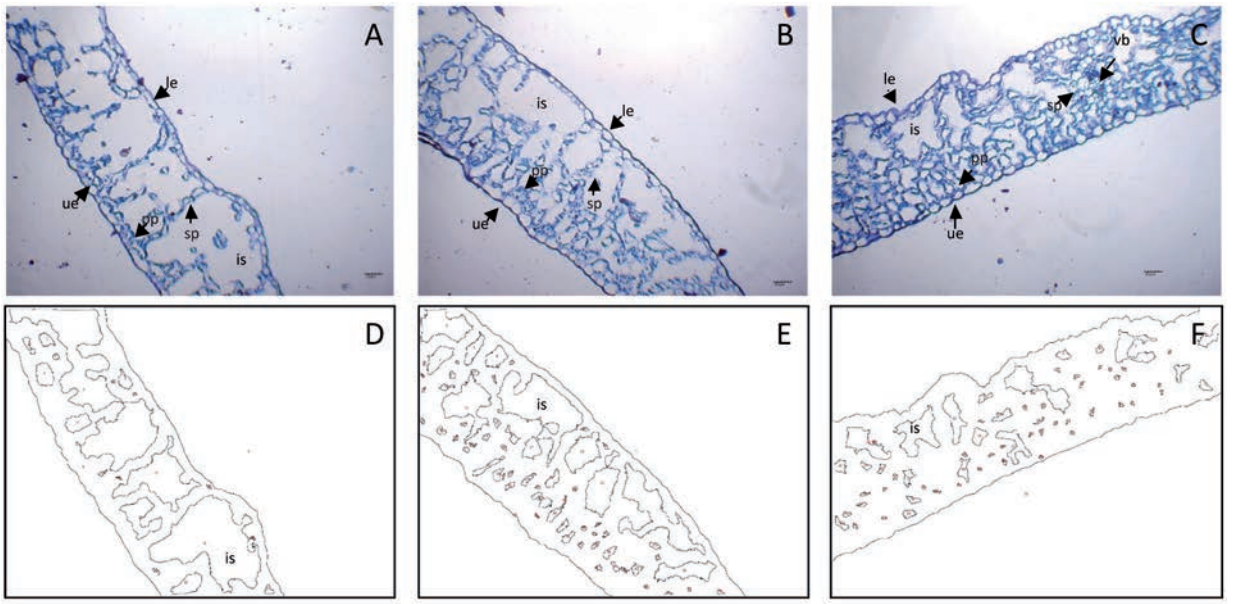
Finally, the effect of salinity on cell morphologies is presented with the microscopic view of a transversal leaf cut for 'Levistro' (Figure 4) and 'Carmoli' (Figure 5). These images display a reduction in intracellular spaces and an increase on palisade parenchyma and spongy parenchyma for both cultivars. The images were also used to calculate the intracellular percentage (Table 6). The results showed a significant interaction between cultivars and NaCl concentrations in the intracellular spaces. 'Levistro' had more intracellular spaces than 'Carmoli', and higher NaCl concentrations decreased intracellular spaces in leaves from 30.70% to 21.72%, being replaced by parenchyma tissues (Table 6). These significant reductions of intracellular spaces were confirmed by Garrido et al. (2013) for 'Capitata' green lettuce and, explain it due to high saline concentrations decrease the water potential of the leaf, which leads to less water occupying the intercellular spaces and causing little internal pressure for cell growth.

Table 5. Stomatal density, cellular density and stomatal index for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations for various harvest times.

		Stomatal density		Cellular density		Stomatal index	
Factor	Level	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
		—— Stomata mm^{-2} ——		—— Cell mm^{-2} ——			
Harvest time (1)	1st	36.5b	51.4b	395.4	579.4c	8.5	8.1
	2nd	53.1a	54.7ab	501.5	715.4b	9.6	7.1
	3rd	51.4a	63.0a	581.9	830.6a	8.2	7.2
NaCl (2)	0 mol L^{-1}	48.1	52.2	491.6	641.6b	9.0	7.7
	0.05 mol L^{-1}	47.3	60.5	470.0	746.1a	9.1	7.5
	0.1 mol L^{-1}	45.6	56.4	517.3	737.8a	8.2	7.2
1×2	0 × 1st	37.3	49.7	368.1d	499.8	9.0	9.2
	0 × 2nd	52.2	49.7	432.7cd	636.6	7.3	9.4
	0 × 3rd	54.7	57.2	385.5d	788.3	6.8	8.3
	0.05 × 1st	34.8	57.2	494.9bc	643.1	8.2	7.5
	0.05 × 2nd	57.2	54.7	480.0bc	785.8	6.5	10.8
	0.05 × 3rd	49.7	69.6	529.7b	818.2	7.9	9.1
	0.1 × 1st	37.3	47.3	611.8a	604.3	7.3	8.8
	0.1 × 2nd	49.7	59.7	497.4bc	723.7	7.5	8.7
	0.1 × 3rd	49.7	62.2	636.6a	885.3	6.7	7.2
1	p value	< 0.0001	0.0437	< 0.0001	< 0.0001	0.0761	0.1078
2	p value	0.7842	0.2122	0.0764	0.0081	0.3214	0.6445
1 × 2	p value	0.7316	0.6597	< 0.0032	0.5510	0.1739	0.2524

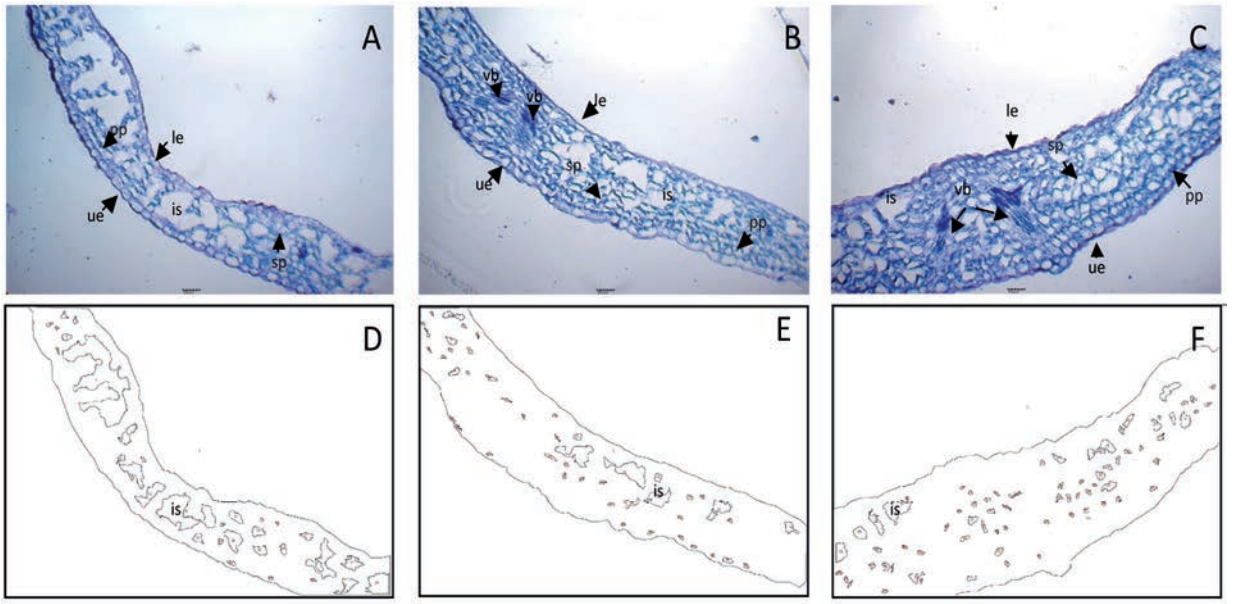
Values are means of 15 data per harvest time and NaCl concentration and means of five measurements per interaction (1 × 2).
Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

Figure 4. Transversal lettuce leaf cut images (A, B, C) captured by optical microscope with a 100X zoom of 'Levistro' grown with 0 (A), 0.05 (B) and 0.1 mol L^{-1} NaCl (C). A, B and C microscopic images processed with ImageJ software (D, E, F).



ue: Upper epidermis; le: lower epidermis; pp: palisade parenchyma; sp: spongy parenchyma, ie: intercellular spaces; vb: vascular bundle.

Figure 5. Transversal lettuce leaf cut images (A, B, C) captured by optical microscope with a 100X zoom of 'Carmoli' grown with 0 (A), 0.05 (B), and 0.1 mol L^{-1} NaCl (C). A, B and C microscopic images processed with ImageJ software (D, E, F).



ue: Upper epidermis; le: lower epidermis; pp: palisade parenchyma; sp: spongy parenchyma, ie: intercellular spaces; vb: vascular bundle.

Table 6. Intercellular space for green 'Levistro' and red 'Carmoli' lettuces, grown with different NaCl concentrations.

Factor	Level	Inter cellular space
		% Total area
Cultivar (1)	'Levistro' (L)	27.2a
	'Carmoli' (C)	13.7b
NaCl (2)	0 mol L^{-1}	23.2a
	0.05 mol L^{-1}	21.0a
	0.1 mol L^{-1}	17.2b
1×2	L × 0	30.7a
	L × 0.05	29.4a
	L × 0.1	21.7b
	C × 0	15.6c
	C × 0.05	12.9c
	C × 0.1	12.6c
1	p value	< 0.0001
2	p value	< 0.0001
1 × 2	p value	< 0.0059

Values are means of 150 images of processed data per harvest time and NaCl concentration and means of 50 image processing data per interaction (1 × 2). Different letters correspond to significant differences by Fisher's LSD ($p < 0.05$).

CONCLUSIONS

Saline concentrations increase DM and antioxidant compound concentrations of green and red lettuce cultivars but also reduce fresh mass. The increase in saline concentration and consecutive harvests stimulate the synthesis of antioxidant compounds of both cultivars, but mainly for red lettuce 'Carmoli'. The high initial antioxidant concentrations found in red lettuces protect the plants from the abiotic stress caused by salinity and cutting damage at harvest evidenced by a lower proline concentration compared to green lettuces. These results agree with the reduced intracellular space found in green lettuces grown in saline nutrient solution. This high natural antioxidant presence in red lettuces like 'Carmoli' suggests that their growth and consumption must be promoted due to environmental adaptability and healthy compound concentrations.

ACKNOWLEDGEMENTS

The authors are grateful to FIC O'Higgins 30474703-0, which financially supported this research, and to PhD scholarship (21150786, CONICYT Chile) for Ms. Mónica Flores R. The authors are also grateful to the Spanish Ministry of Science, Innovation, and Universities for the national mobility scholarship for professors and senior researchers N° PRX19/00138 granted to Dr. Asunción Amorós.

REFERENCES

Bartha, C., Fodorpataki, L., Martinez-Ballesta, M., Popescu, O., and Carvajal, M. 2015. Sodium accumulation contributes to salt stress tolerance in lettuce cultivars. Journal of Applied Botany and Food Quality 88:42-48. doi:10.5073/JABFQ.2015.088.008.

Butcher, K., Wick, A.F., DeSutter, T., Chatterjee, A., and Harmon, J. 2016. Soil salinity: a threat to global food security. Agronomy Journal 108:2189-2200. doi:10.2134/agronj2016.06.0368.

Carillo, P., Raimondi, G., Kyriacou, M.C., Pannico A., El-Nakhel, C., Cirillo, V., et al. 2019. Morpho-physiological and homeostatic adaptive responses triggered by omeprazole enhance lettuce tolerance to salt stress. Scientia Horticulturae 249:22-30. doi:10.1016/j.scienta.2019.01.038.

Collado-González, J., Piñero, M.C., Otalora, G., López-Marín, J., and del Amor, F.M. 2022. Unraveling the nutritional and bioactive constituents in baby-leaf lettuce for challenging climate conditions. Food Chemistry 384:132506. doi:10.1016/j.foodchem.2022.132506.

de Souza, A.S.N., Schmidt, H.D.O., Pagno, C., Rodrigues, E., da Silva, M.A.S., Flôres, S.H., et al. 2022. Influence of cultivar and season on carotenoids and phenolic compounds from red lettuce influence of cultivar and season on lettuce. Food Research International 155:111110. doi:10.1016/j.foodres.2022.111110.

Di Mola, I., Rouphael, Y., Colla, G., Fagnano, M., Paradiso, R., and Mori, M. 2017. Morphophysiological traits and nitrate content of greenhouse lettuce as affected by irrigation with saline water. HortScience 52:1716-1721. doi:10.21273/HORTSCI12501-17.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., and Robledo, C.W. InfoStat versión. 2017. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. Available at http://www.infostat.com.ar.

Du, W.-X., Avena-Bustillos, R., Breksa, A., and McHugh, T. 2014. UV-B light as a factor affecting total soluble phenolic contents of various whole and fresh-cut specialty crops. Postharvest Biology and Technology 93:72-82. doi:10.1016/j.postharvbio.2014.02.004.

FAO. 2021. FAOSTAT, crops. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. Available at http://www.fao.org/faostat/en/#data/QC (accessed 21 December 2021).

Flores, M., Urrestarazu, M., Amorós, A., and Escalona, V. 2022. High intensity and red enriched LED lights increased the growth of lettuce and endive. Italian Journal of Agronomy 17:1915. doi:10.408l/ija.2022.1915

Garrido, Y., Tudela, J.A., Marín, A., Mestre, T., Martínez, V., and Gil, M.I. 2013. Physiological, phytochemical, and structural changes of multi-leaf lettuce caused by salt stress. Journal of the Science of Food and Agriculture 94:1592-1599. doi:10.1002/jsfa.6462.

Kamińska, I., Lukasiewicz, A., Klimek-Chodacka, M., Długosz-Grochowska, O., Rutkowska, J., Szymonik, K., et al. 2022. Antioxidative and osmoprotecting mechanisms in carrot plants tolerant to soil salinity. Scientific Reports 12:7266. doi:10.1038/s41598-022-10835-3.

Kim, M., Moon, Y., Tou, J., Mou, B., and Waterland, N. 2016. Nutritional value, bioactive compounds, and health benefits of lettuce (*Lactuca sativa* L.) Journal of Food Composition and Analysis 49:19-34. doi:10.1016/j.jfca.2016.03.004.

Liu, X., Ardo, S., Bunning, M., Parry, J., Zhou, K., Stushnoff, C., et al. 2007. Total phenolic content and DPPH· radical scavenging activity of lettuce (*Lactuca sativa* L.) grown in Colorado. LWT-Food Science and Technology 40:552-557. doi:10.1016/j.lwt.2005.09.007.

Llorach, R., Martínez-Sánchez, A., Tomás-Barberán, F.A., Gil, M.I., and Ferreres, F. 2008. Characterization of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chemistry 108:1028-1038. doi:10.1016/j.foodchem.2007.11.032.

Lucini, L., and Bernardo, L. 2015. Comparison of proteome response to saline and zinc stress in lettuce. Frontiers in Plant Science 6:240. doi:10.3389/fpls.2015.00240.

Lucini, L., Rouphael Y., Carderelli, M., Canaguier, R., Kumar, P., and Colla, G. 2015. The effect of a plant-derived bio stimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. Scientia Horticulturae 182:124-133. doi:10.1016/j.scienta.2014.11.022.

Mir, R.A., Argal, S., Ahanger, M.A., Tomar, N.S., and Agarwal, R.M. 2022. Variation in phenolic compounds, antioxidant activity and osmotica of different cultivars of *Tagetes erecta* L. at different growth stages and effect of its leachates on germination and growth of wheat (*Triticum aestivum* L.) Journal of Plant Growth Regulation 41: 907-921. doi:10.1007/s00344-021-10348-9.

Neocleous, D., Koukounaras, A., Siomos, A.S., and Vasilakakis, M. 2014. Assessing the salinity effects on mineral composition and nutritional quality of green and red 'baby' lettuce. Journal of Food Quality 37:1-8. doi:10.1111/jfq.12066.

Pérez-López, U., Miranda-Apodaca, J., Lacuesta, M., Mena-Petite, A., and Muñoz-Rueda, A. 2015. Growth and nutritional quality improvement in two differently pigmented lettuce cultivars grown under elevated CO_2 and/or salinity. Scientia Horticulturae 195:56-66. doi:10.1016/j.scienta.2015.08.034.

Pinto, E., Almeida, A., Aguilar, A., and Ferreira, I. 2014. Changes in macro minerals, trace elements and pigments content during lettuce (*Lactuca sativa* L.) growth: Influence of soil composition. Food Chemistry 152:603-611. doi:10.1016/j.foodchem.2013.12.023.

Sakalauskaité, J., Viskelis, P., Dambrauskiene, E., Sakalauskiené, S., Samuoliené, G., Brazaityte, A., et al. 2012. The effects of different UVB radiation intensities on morphological and biochemical characteristics in *Ocimum basilicum* L. Journal of the Science of Food and Agriculture 93:1266-1271. doi:10.1002/jsfa.5879.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. 2012. NIH image to ImageJ: 25 years of image analysis. Nature Methods 9:671-675. doi:10.1038/nmeth.2089.

Sgherri, C., Pérez-López, U., Micaelli, F., Miranda-Apodaca, J., Mena-Petite, A., Muñoz-Rueda, A., et al. 2017. Elevated CO_2 and salinity are responsible for phenolics-enrichment in two differently pigmented lettuces. Plant Physiology and Biochemistry 115:269-278. doi:10.1016/j.plaphy.2017.04.006.

Shabala, S., and Munns, R. 2012. Salinity stress: Physiological constraints and adaptive mechanisms. p. 59-93. In Shabala, S. (ed.) Plant stress physiology. CABI, Wallingford, UK. doi:10.1079/9781845939953.0059.

Shabala, S., Wu, H., and Bose, J. 2015. Salt stress sensing and early signaling events in plant roots: Current knowledge and hypothesis. Plant Science 241:109-119. doi:10.1016/j.plantsci.2015.10.003.

Sivankalyani, V., Sela, N., Feygenberg, O., Zemach, H., Maurer, D., and Alkan, N. 2016. Transcriptome dynamics in mango fruit peel reveals mechanisms of chilling stress. Frontiers in Plant Science 7:1579. doi:10.3389/fpls.2016.01579.

Sloan, E. 2019. Top 10 food trends. Food Technology 73:4. https://www.ift.org/news-and-publications/food-technology-magazine/issues/2019/april/features/2019-top-10-food-trends.

Sumner, M.J. 2015. Epoxy resins for light and transmission electron microscopy. p. 83-101. In Yeung, E.C.T., Stasolla, C., Summer, M., and Huang, B. (eds.) Plant micro techniques and protocols. Springer, Cham, Switzerland. doi:10.1007/978-3-319-19944-3_5.

Xie, C., Zhang, R., Qu, Y., Miao, Z., Zhang, Y., Shen, X., et al. 2012. Overexpression of *MtCAS31* enhances drought tolerance in transgenic Arabidopsis by reducing stomatal density. New Phytologist 195:124-135. doi:10.1111/j.1469-8137.2012.04136.x.

Zapata-Vahos, I.C., Rojas-Rodas, F., David, D., Gutierrez-Monsalve, J.A., and Castro-Restrepo, D. 2020. Comparison of antioxidant contents of green and red leaf lettuce cultivated in hydroponic systems in greenhouses and conventional soil cultivation. Revista Facultad Nacional de Agronomía Medellín 73:9077-9088. doi:10.15446/rfnam.v73n1.77279.

Zlotek, U., S'wieca, M., and Jakubczyk, A. 2014. Effect of abiotic elicitation on main health-promoting compounds, antioxidant activity and commercial quality of butter lettuce (*Lactuca sativa* L.) Food Chemistry 148:253-260. doi:10.1016/j.foodchem.2013.10.031.