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Salt in Irrigation Water Affects the Nutritional and Visual Properties of Romaine Lettuce (*Lactuca sativa* L.)

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The influence of salinity stress on the growth, appearance, and nutritional compounds, especially phenolic compounds and carotenoids, of romaine lettuce (*Lactuca sativa* L.), a low salt tolerant plant, was studied. The dry weight, height, and color of the lettuce plants were significantly changed by long-term irrigation (15 days) with higher NaCl concentration (i.e., $>$100 mM). However, no significant differences were observed in the growth and appearance among the control, all short-term treatments (2 days; 50, 100, 500, and 1000 mM), and long-term irrigation with low salt concentration. Moreover, in romaine lettuce treated with long-term irrigation with 5 mM NaCl, the total carotenoid content increased without color change, and the contents of major carotenoids in romaine lettuce, lutein and β-carotene, increased 37 and 80%, respectively. No differences were observed in lutein and β-carotene contents in short-term-treated lettuce. The phenolic content of the romaine lettuce declined with short-term salt irrigation, whereas there were no significant differences among treatments exposed to long-term irrigation. This research indicates that long-term irrigation with relatively low salt concentration, rather than short-term irrigation with high salt concentration, can increase carotenoid content in romaine lettuce without causing a tradeoff in yield or visual quality.

KEYWORDS: Salinity stress; salt; carotenoids; romaine lettuce; lutein; β-carotene; phenolic compound; secondary metabolites

INTRODUCTION

The salinity of soil and irrigation water, one of the major environmental factors decreasing crop yield, is a problem of high magnitude for food production in the world. Approximately 50% of the cropland and 20% of the irrigated land in the world are affected by high salinity, and the volume of salt-stressed land is continuously increasing (*1, 2*). Under high salinity stress, the leaf surface expansion, plant growth, and primary carbon metabolism of many plants are negatively affected due to nutritional imbalance, osmotic stress, water deficit, and oxidative stress (*3–5*).

Moreover, by being salt-affected, plants may alter their levels of secondary metabolites such as phenolic compounds and terpenoids to enhance their defense systems against stresses, especially oxidative stress (*6*). These secondary metabolites, derived mostly through phenylprophanoid and the shikimate pathways, perform multiple functions in stressed plants, such as scavenging of free radical oxidative species (ROS), lignification, herbivore protection, disease resistance, and stress signals (*7–11*). In addition to their role as defense system agents, many secondary metabolites are well-known as medicinally bioactive compounds with beneficial health-related properties, such as anticancer, antioxidant, and anti-inflammatory activities (*12–15*). For this reason, research into increasing the levels of secondary metabolites in edible plants by using gene modification techniques, elicitor treatments, and certain environment changes has increased (*16–19*).

Romaine lettuce, one of the most commonly used salad vegetables, contains various phytochemicals, including carotenoids and antioxidants (*20, 21*). The quantities and profile of these phytochemicals may be altered by wounding, high atmospheric ozone levels, and drought (*22–24*). To our knowledge, however, the effect of the period of salinity conditions on carotenoids and phenolic compounds in romaine lettuce with low salt tolerance has not been examined; therefore, in this study, the effect of short- and long-term salinity stress with various NaCl concentrations on carotenoids and phenolic compounds of romaine lettuce is investigated. The yield and appearance of the salt-stressed lettuce are also reported.

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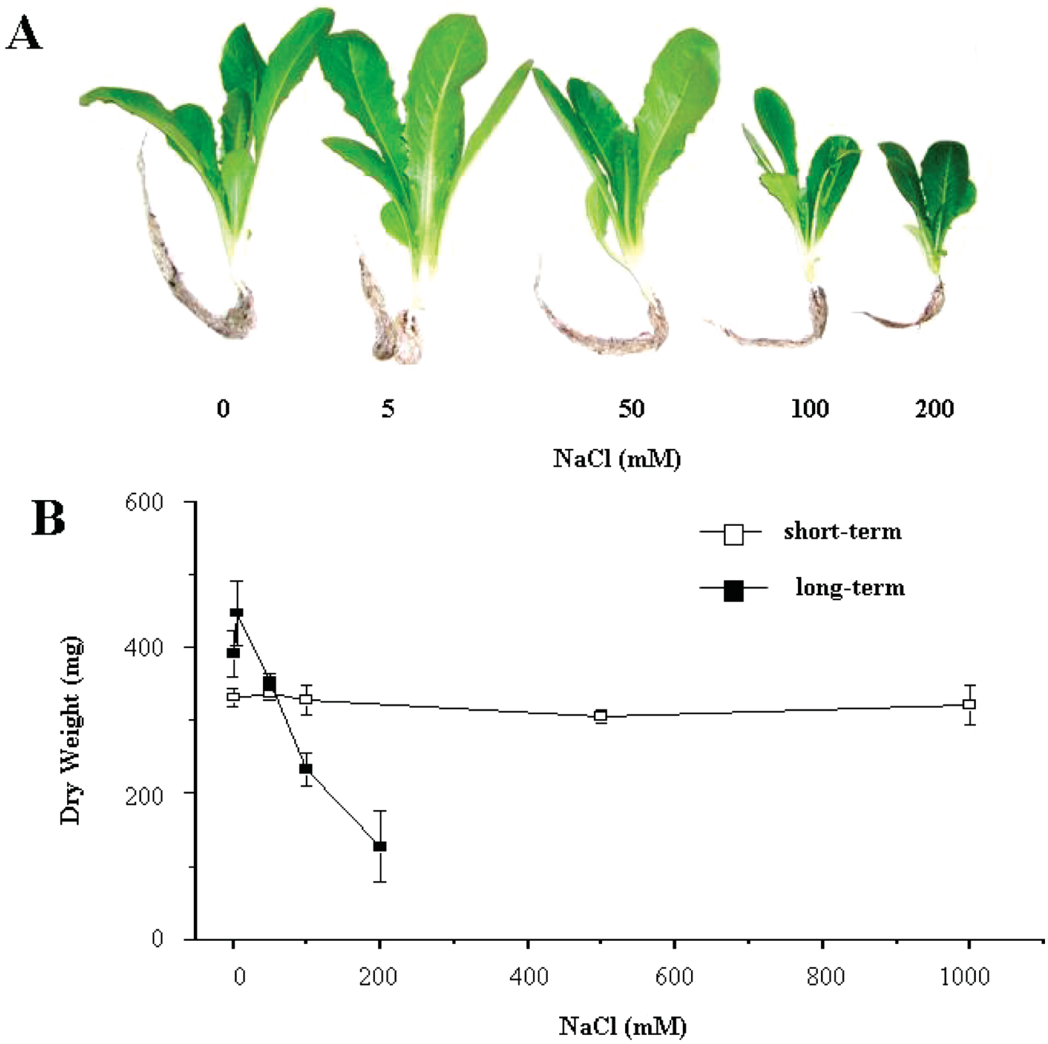

Figure 1. Effect of long-term irrigation with various NaCl solutions on appearance (**A**) and dry weight (**B**) of romaine lettuce. Lettuce was irrigated with each of 0, 5, 50, 100, and 200 mM NaCl solutions every other day for 15 days.

MATERIALS AND METHODS

Chemicals. Lutein, β-carotene, gallic acid, and Folin−Ciocalteu's reagent were purchased from Sigma Chemical Co. (St. Louis, MO), and all HPLC analytical grade solvents were sourced from Fisher Scientific (Chino, CA).

Plant Culture and Salt Treatment. Seeds of romaine lettuce (*Lactuca sativa* L. cv. Clemente) were sown in a 0.4 L plastic pot containing a commercial potting soil (Uni-Gro, Yuma, AZ). Lettuce plants were grown in a greenhouse (day/night temperature = 28/12 °C; sunlight = 290 langleys; day length = 10 h, Arizona Meteorological Network) at The University of Arizona Yuma Agricultural Research Center. Irrigation was provided once every other day, and fertilizer (Miracle-Gro, N:P:K = 24:8:16, Scott's Miracle-Gro Co., Marysville, OH) was added once every 2 weeks. For long-term irrigation with salt solution, 100 mL per pot of various NaCl solutions (0, 5, 50, 100, and 200 mM) was applied every other day for 15 days before harvest. For short-term salinity irrigation, the same volume of different NaCl solutions (0, 50, 100, 500, and 1000 mM) was irrigated 2 days before harvest.

Determination of the Growth of Romaine Lettuce Irrigated with Various NaCl Solutions. The growth of lettuce was measured in terms of dry weight, height, and number of leaves of lettuce (35 days old). The weight of all the harvested lettuce leaves and the length from the top of the root to the end of the outside leaf were measured.

Color Evaluation. The color of the lettuce leaves was determined by measuring parameters L^*, a^*, and b^* with a chromameter (Minolta CR-400, Osaka, Japan) calibrated with a white plate. Chroma (C^*), a measure of saturation, was calculated with the formula $(a^{*2} + b^{*2})^{1/2}$, and hue angle ($h°$), an indicator of the object's color in the a^*-b^* plane, was calculated as $\tan^{-1}(b^*/a^*)$ (*25*).

Extraction of Carotenoids from Romaine Lettuce. Carotenoids were extracted from the romaine lettuce following the procedure of Moros et al. (*26*), with modifications. The dried sample powder (0.2 g) was mixed with 6 mL of ethanol containing 0.1% BHT, and the sealed mixture solution was preincubated at 85 °C for 5 min. For saponification, 120 μL of 80% KOH was added to the preheated mixture solution, and the reaction solution was incubated at the same temperature for 10 min. The reacted solution was then immediately placed in ice and 3 mL of distilled water, and 3 mL of hexane was added to the solution. After centrifugation, the hexane layer containing the crude carotenoids was collected. The separation step by hexane was repeated once. The hexane layers were combined and completely dried with nitrogen gas. The residue recovered with methanol/methyl *tert*-butyl ether (MTBE) (1:1) was used as the crude carotenoids of the lettuce.

Determination of Total Carotenoids Content and Lutein Content by HPLC. The HPLC separation of carotenoids was achieved following conditions used by Moros et al. (*26*). A YMC-Carotenoid S-5 column (250 × 4.6 mm, 5 μm; Waters, Milford, MA) was connected to the LC-10AT HPLC system and equilibrated with the mixed solvent of methanol/MTBE/water (81:15:4). Twenty microliters of the crude carotenoid extract was injected and eluted with methanol/MTBE/water (81:15:4) and methanol/MTBE (9:91) at a flow rate of 1 mL/min. The absorbance of the eluant was measured at 450 nm. The total content of carotenoids was calculated by total area of all peaks separated by

Table 1. Influence of Long-Term NaCl Treatment on Color of Romaine Lettuce[a]

NaCl (mM)	*L**	*a**	*b**	*C**	*h*°
0	50.68 a	−16.50 a	22.69 a	28.07 a	−53.83 a
5	49.67 a	−16.33 a	21.27 a	26.83 a	−52.37 a
50	47.84 b	−15.53 b	18.01 b	23.79 b	−49.17 b
100	47.19 b	−14.00 c	15.73 c	21.07 c	−48.14 b
200	44.17 c	−11.33 d	10.60 d	15.54 d	−42.77 c

[a] Different letters denote significant differences at $p \leq 0.05$.

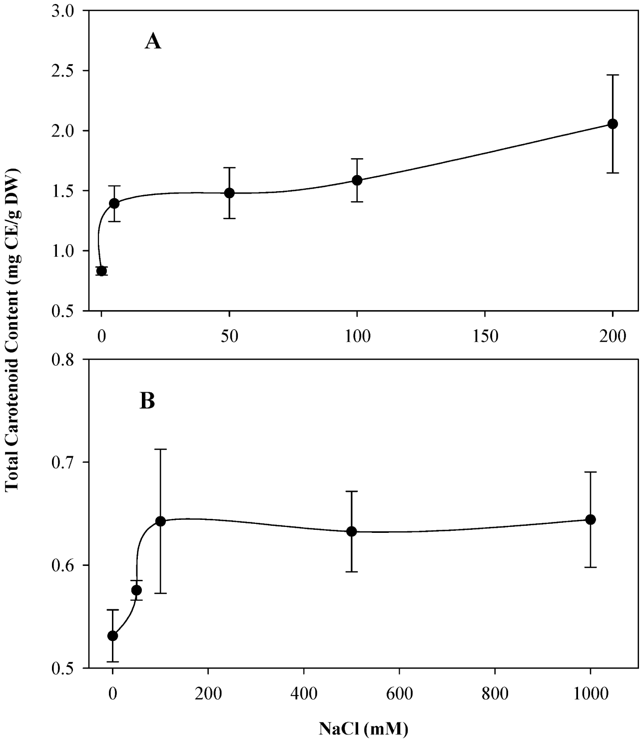

Figure 2. Total content of carotenoids in the romaine lettuce subjected to long- (**A**) or short-term (**B**) irrigation containing various NaCl solutions. Lettuce plants were subjected to irrigation with NaCl solutions (0, 5, 50, 100, and 200 mM) for 15 days. Carotenoid extracted by hexane was analyzed by YMC-Carotenoid S-5 column (250 × 4.6 mm, 5 μm) HPLC. Total content of carotenoids was calculated by total area of all peaks separated by HPLC, and the area was transformed to milligrams of β-carotene equivalent (CE) per gram of dry weight of sample.

HPLC, and the area was transformed to micrograms of β-carotene equivalent (CE) per gram of dry weight (DW) of sample. Authentic lutein and β-carotene were used to evaluate their quality and quantity in the romaine lettuce.

Determination of Total Content of Phenolic Compounds. The ground sample (0.1 g) was mixed with 5 mL of 80% methanol, and the mixture was shaken at room temperature for 12 h. After centrifugation at 2000*g* for 10 min, the supernatant was used to determine phenolic compounds. The total amount of phenolic compounds in romaine lettuce was determined using Folin−Ciocalteu's reagent according to the method of Singleton and Rossi (*27*). Fifty microliters of the methanolic extract was mixed with 450 μL of distilled water and 250 μL of 2 N Folin−Ciocalteu reagent. The mixture added to 1.25 mL of 20% Na_2CO_3 was incubated at 25 °C for 20 min and then centrifuged at 2000*g* for 10 min. The absorbance of the supernatant was measured at 735 nm, and the standard curve was prepared using gallic acid (GA). The absorbance was converted to phenolic content in terms of a milligrams of GA equivalent (GAE) per gram of DW of sample.

Experimental Design and Data Analysis. To investigate the effect of short- and long-term salinity irrigation on the physiological and biological properties of romaine lettuce, romaine lettuce was irrigated with one of five different NaCl solutions (0, 5, 50, 100, and 200 mM) for long-term irrigation (15 days) or with five different NaCl solutions (0, 50, 100, 500, and 1000 mM) for short-term irrigation (2 days). Five lettuce plants were used in each treatment for each of four replicates, and the experiments were conducted three times. The experimental data were subjected to analysis of variance (ANOVA) and analyzed (SAS 9.1, SAS Institute Inc., Cary, NC). The least significant difference was used to find the differences among all sample means.

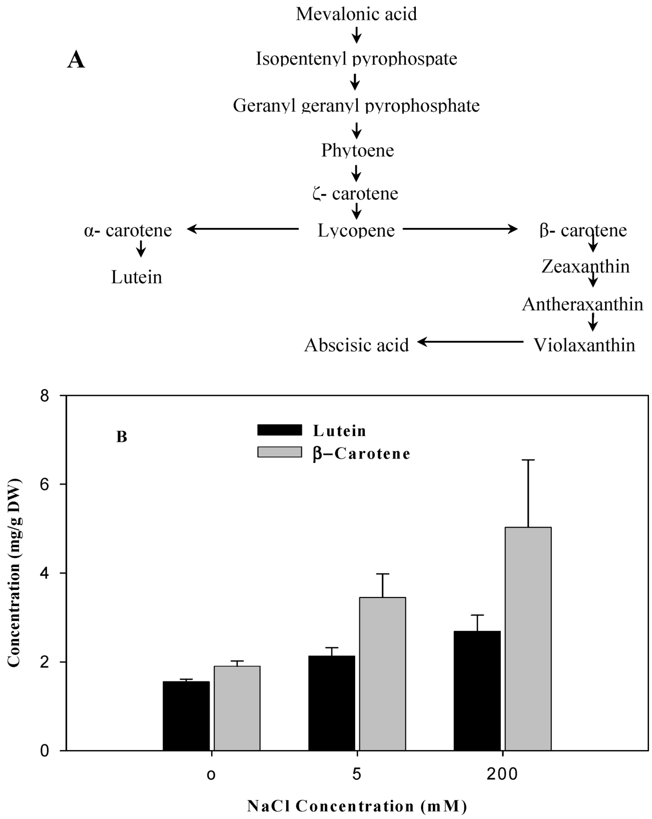

Figure 3. Carotenoid biosynthesis pathway (**A**) and lutein and β-carotene contents of the romaine lettuce irrigated with various NaCl solutions (**B**). Carotenoid extracted by hexane was analyzed by YMC-Carotenoid S-5 column (250 × 4.6 mm, 5 μm) HPLC. Authentic carotenoids were used for quantitative and qualitative determination.

RESULTS AND DISCUSSION

Effect of Salt Treatment on the Growth and Appearance of Romaine Lettuce. The growth rate and appearance of romaine lettuce were changed with the long-term irrigation water containing NaCl (**Figure 1**). We did not find any significant difference between the control and short-term treatments. In agreement with previous results that suggest salinity stress decreases the weight and the height of the leaves, stems, and roots of *Cajanus cajan* L., chickpea, guava, and pepper (*28–31*), the dry weight and the height of romaine lettuce declined when plants were subjected to a high salt concentration for 15 days. Treatments with concentrations >100 mM NaCl showed 1.7–3.1 and 1.8–2.7 times lower dry weight and height than the untreated control (**Figure 1A**). It was clearly estimated that the reduction of water potential, ion imbalance or disturbances in ion homeostasis, and toxicity by NaCl treatment led to growth reduction (*3*). Interestingly, the dry weight of the lettuce irrigated with a low NaCl concentration (5 mM) for 15 days was 14% higher than that of the control. Similar results have been reported, as the total weight of *Alhagi pseudoalhagi* increased at low salt concentration (50 mM NaCl) but decreased when the concentration was >100 mM NaCl (*32*). It is possible that

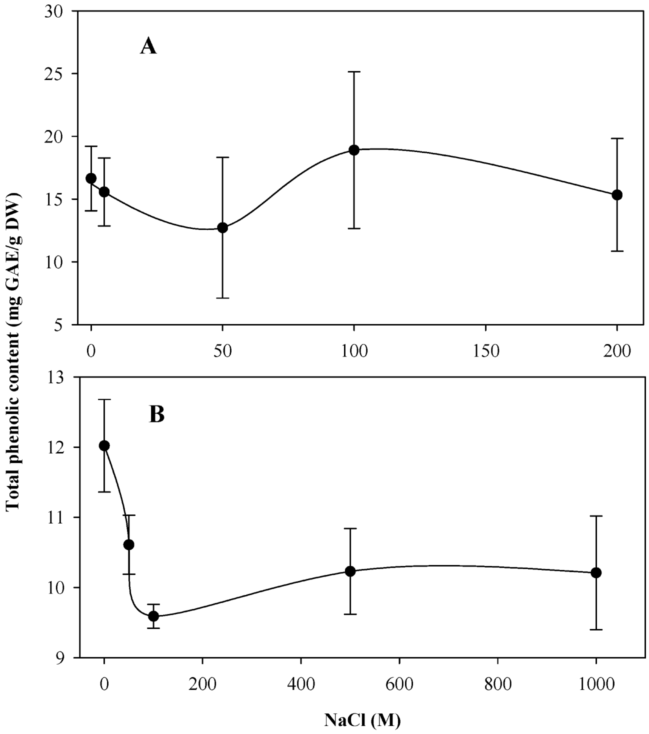

Figure 4. Total phenolic contents of romaine lettuce subjected to long- (**A**) or short-term (**B**) irrigation containing various NaCl solutions.

a low Na^+ concentration in solution might be used as a mineral source that contributes to the growth of lettuce plants; in this study, the salt concentration for the optimal growth was 5 mM NaCl. No significant difference in the number of leaves was observed (data not shown).

In addition to the effect observed on growth, the color of the lettuce changed with NaCl treatments, but significant differences in color between the control and the 5 mM NaCl treatment were not observed (**Table 1**). The lightness (L^* value), color saturation (C^*), and green color (a^* value) of the lettuce decreased with increased NaCl concentrations. In other studies, high salinity content caused significant declines in chlorophylls and carotenoids in *B. parviflora* and *Grevilea* (*33, 34*). However, in our study, the lettuce plants irrigated with NaCl solutions were greener, which was likely due to higher solute concentration, an aspect that correlated with dry mass (data not shown).

Salt Effect on Carotenoids in Romaine Lettuce. Unlike previous reports that carotenoid content in sugar cane was decreased by salt treatment (*6, 35*), the total amount of carotenoids in the romaine lettuce increased with both short- and long-term irrigation containing NaCl. The increased rate obtained with the long-term treatments was larger than that of the short-term treatments (**Figure 2**). In the long-term treatments, the carotenoid contents in the 5 mM (13.9 mg of CE/g of DW) and 200 mM NaCl (20.5 mg of CE/g of DW) treatments were 1.7- and 2.5-fold higher than that of the untreated control (8.3 mg of CE/g of DW) (**Figure 2A**). On the other hand, with short-term treatments, carotenoid content in lettuce treated with >100 mM NaCl was 27% higher than that of the control (**Figure 2B**). To investigate the effect of salinity stress on individual carotenoids in romaine lettuce, various carotenoids were separated using a HPLC-carotenoid S-5 column. Among these carotenoids, lutein and β-carotene were identified as major carotenoids in romaine lettuce. The contents of lutein—known as an eye health promoter, anticolon cancer agent, and antioxidant (*36, 37*)—and β-carotene—known as an important antioxidant (*38*)—increased with long-term treatments. The contents of lutein in lettuce treated with 5 mM (2.1 mg/g of DW) and 200 mM NaCl (2.7 mg/g of DW) were 37 and 74% higher than that of the control (1.6 mg/g of DW). Moreover, the β-carotene contents in 5 and 200 mM NaCl treatments were 1.8 and 2.7 times higher, respectively, than that of the control (1.9 mg/g of DW) (**Figure 3B**). No differences in lutein and β-carotene contents were observed with the short-term treatments (data not shown). The accumulation of secondary metabolites under stress is closely related to the induction of phytohormones (*39, 40*). Abscisic acid (ABA), which is synthesized via the mevalonic acid pathway (**Figure 3A**), has been known as one of the phytohormones; its role in regulating the development of plants under water and salinity stresses has been well established (*41*). This suggests that the accumulation of the carotenoids lutein and β-carotene, precursors to ABA, might be due to the stimulation of the ABA pathway when tolerance to water and salt stresses is required.

Effect of Salt Treatment on Phenolic Content in Romaine Lettuce. In general, phenolic compounds are elicited through the phenylpropanoid pathway by environmental stresses and/or inducers such as jasmonic acid (JA) and methyl jasmonate (MeJA), which activate the plant's defense systems (*18, 39*). The endogenous JA content of a low salt tolerant plant increased with 100 mM NaCl treatment for 72 h (*42*). However, in this study, the phenolic content of romaine lettuce, a low salt tolerant plant, declined with short-term salt treatment. The contents of phenolic compounds containing chlorogenic and caffeic acid derivatives significantly decreased by 25 and 18% with 100 and 1000 mM NaCl treatments, respectively, as compared to those of the untreated control (12 mg of GAE/g of DW) (**Figure 4**). This result disagrees with previous results showing that phenolic compounds in *B. parviflora* and in the shoots of two sugarcane clones decrease when the plants are subjected to long-term NaCl treatments (7–45 and 10–30 days, respectively) (*6, 33*). Our results, and the evidence provided by other studies, suggest that phenolic compound content in plants may be altered by salinity stress, but this is critically dependent on the salt sensitivity of plants.

The influence of salinity stress on the growth, appearance, phenolic compounds, and carotenoids in romaine lettuce was examined. The growth reduction and the change in appearance were observed by long-term irrigation with NaCl concentrations >100 mM. However, the lettuce subjected to 5 mM NaCl showed an increased growth rate without compromising appearance. Carotenoids in lettuce increased with salt treatments. The carotenoid content of 5 mM treated plants was 1.7 times higher than that of the control. Among carotenoids in romaine lettuce, lutein and β-carotene were identified as the major carotenoids, and their contents increased 37 and 80%, respectively, through long-term irrigation with 5 mM NaCl. However, no difference in concentration of lutein and β-carotene with short-term irrigated lettuce was observed. The phenolic content of romaine lettuce declined with short-term salt irrigation, whereas no significant difference was determined between the control and long-term-irrigated plants.

Despite the fact that more research into toxicity and other nutritional compounds is needed, our study indicates that long-term irrigation with 5 mM NaCl can increase the nutritional value of romaine lettuce, particularly the lutein and β-carotene contents, without a tradeoff in yield or appearance. This finding reveals that commercial production in conditions that pose mild stress to salt-sensitive plants can have an overall positive outcome.

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