PHOTOSYNTHETICA
International Journal for Photosynthesis Research

DOI 10.32615/ps.2023.027 PHOTOSYNTHETICA 61 (3): 342-353, 2023

Impact of salt stress on physiology, leaf mass, and nutrient accumulation in romaine lettuce

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Abstract

The impact of salt stress is becoming more prevalent each year, largely due to the effects of climate change. Limited availability of salt-free water is rising concern for hydroponics lettuce production. Despite evidence supporting salt stress-induced quality losses and physiological changes, studies on romaine lettuce salt-stress tolerance are limited. This study examined the mechanism underlying the sodium chloride (NaCl) tolerance (0, 50, 100, and 150 mM) of lettuce on its growth and nutrition at late-rosette and early head-formation stages. Results revealed 76% fresh mass reduction under increased NaCl at both stages. The study also found unchanged carbon assimilation with reduced stomatal conductance under increased NaCl. Salt-stressed lettuce accumulated more boron and iron but had reduced phosphorus and calcium. Phenolics and sugars increased linearly under salt stress, suggesting that lettuce responds to increased oxidative stress at both stages. A positive association between salt treatment and sodium to potassium ion ratio indicated lettuce sensitivity to salt stress at both stages.

Keywords: carbon assimilation; gas exchange; nutrition; romaine lettuce; salt tolerance; sodium chloride.

Introduction

Lettuce (*Lactuca sativa* L.) is a cool-season vegetable and widely consumed leafy salad in the United States and other regions of the world (Kang and Saltveit 2002, Romani *et al.* 2002, Nicolle *et al.* 2004). It is consumed raw in relatively large quantities, with the per capita consumption of ~5.8 kg lettuce in the United States (Statista 2021).

Highlights

- Salt stress inhibits lettuce growth, resulting in a remarkable 76% decrease in fresh mass
- Elevated phenolic and sugar contents in lettuce under salt stress show an increased antioxidant response
- The sodium-to-potassium ratio reveals lettuce's increased sensitivity to salt stress at the late-rosette and early head-formation stages

Received 28 February 2023
Accepted 26 June 2023

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Abbreviations: *ANOVA* – analysis of variance; C_i – intercellular CO_2 concentration; DM – dry mass; E – transpiration; EC – electroconductivity; F_0' – minimal fluorescence of light-adapted leaves; FM – fresh mass; F_m' – maximal fluorescence of light-adapted leaves; F_s – steady-state fluorescence; g_m – mesophyll conductance; GS – growth stage; g_s – stomatal conductance; HPLC – high-performance liquid chromatography; J_{max} – the maximum rate of electron transport; LN – leaf number; L_s – the relative stomatal limitation of photosynthesis P_N – net CO_2 assimilation rate; P_N/C_i – CO_2-response curve; P_r – photorespiration; q_P – photochemical quenching coefficient; q_L – fraction of PSII centers in the open state with plastoquinone oxidized; R_d – leaf respiration in the light; SE – standard error; T_{max} – maximum temperature; T_{min} – minimum temperature; V_{cmax} – Rubisco carboxylation's maximum rate; WUE_i – intrinsic water-use efficiency; Φ_{PSII} – the effective quantum yield of photosystem II photochemistry for light-acclimated state.

Acknowledgments: The ongoing research has received backing from the USDA-NIFA Hatch Project, with the accession number 149210. This publication is a contribution of the Mississippi Agricultural and Forestry Experiment Station. The authors extend their gratitude to Thomas Horgan for his assistance during data collection at the North Mississippi Research and Extension Center.

Conflict of interest: The authors declare that they have no conflict of interest.

In addition, lettuce is also comprised of different phytochemicals and antioxidants (Kang and Saltveit 2002, Romani *et al.* 2002, Nicolle *et al.* 2004) that have been linked to the prevention of chronic illnesses such as cancer and heart disease (Husain *et al.* 1987, Cartea *et al.* 2011). A recent study reported that more than 50% of people in the USA are deprived of recommended magnesium levels, 40% of vitamin A, and 33% of vitamin C from the vegetables and foods they consume (Bliss and Moshfegh 2012). Therefore, health-promoting bioactive compounds like vitamins, minerals, and polyphenols make lettuce a preferred raw vegetable for consumers in the USA (Pérez-López *et al.* 2013, 2018). Nevertheless, the fight to reduce hunger and destitution continues (Hasegawa *et al.* 2000).

The import of lettuce in the USA grew by 124% between 2020 and 2021 due to higher demand and lower production, causing a 3–4% increase in unit prices (Davis and Lucier 2021). Abiotic factors, including salt stress, have hindered the growth and development of several horticultural crops (Gadallah 1999, Franzoni *et al.* 2022). Soil salinization due to poor agricultural practices and increased use of fertilizers and low-quality water for irrigation has led to the shift towards hydroponic culture (Rengasamy 2010). Because of efficient use of energy, water productivity (hydroponics: 21 L kg^{-1} $year^{-1}$ and conventional: 260 L kg^{-1} $year^{-1}$) and greater yield (hydroponics: 42 kg m^{-2} $year^{-1}$ and conventional: 4 kg m^{-2} $year^{-1}$) (Barbosa *et al.* 2015), growers are shifting to hydroponic lettuce cultivation systems, which are more efficient and yield more than conventional methods. However, overuse of freshwater in hydroponics has resulted in salt accumulation, which can have a negative effect on lettuce growth and productivity (Miller 2019). Short-term salt stress can reduce growth and productivity in various vegetables (tomato, broccoli, and cauliflower), including lettuce (Zribi *et al.* 2009, Giuffrida *et al.* 2012, Machado and Serralheiro 2017, Adhikari *et al.* 2021) by decreasing water potential (Zhang and Xing 2008), causing ion toxicity (Demidchik and Maathuis 2010), nutrient imbalance (Dahiya and Singh 1976, Marschner 2012), and impaired photosynthesis (Shin *et al.* 2020).

Salt stress-induced osmotic shock and ion toxicity (Isayenkov 2012, Garrido *et al.* 2014) disrupt the response of key processes including net CO_2 assimilation rate (P_N) (Carillo *et al.* 2019), intercellular CO_2 concentration (C_i) (Penella *et al.* 2016), stomatal conductance (g_s) (Bartha *et al.* 2015), PSII efficiency (Sarabi *et al.* 2019), intrinsic water-use efficiency (WUE_i) (Kurunc 2021), and Rubisco carboxylation's maximum rate (V_{cmax}), and maximum rate of electron transport (J_{max}) (Penella *et al.* 2016). In addition, salt stress also causes increased chlorophyll fluorescence and photosystem dysfunction (Penella *et al.* 2016, Shin *et al.* 2020), limiting photosynthesis through stomatal and nonstomatal pathways and affecting plant morphology (Lemos Neto *et al.* 2021). Salt stress leads to extensive sodium (Na^+) and chloride (Cl^-) ions accumulation in the plant tissue (root and leaf), inhibiting essential nutrient availability and uptake (Isayenkov and Maathuis 2019, Loudari *et al.* 2020). This osmotic or ionic shock triggers several plant pigments and nonstructural carbohydrate accumulation to combat oxidative stress (Parida *et al.* 2002).

Studies have reported changes in lettuce growth and physiology under stress, but limited information exists on the effects of salt stress on lettuce yield, nutrition, and physiology in romaine lettuce. There is also a lack of knowledge on the impact of salt stress at the economically important growth stages (late-rosette and early head-formation) in hydroponic conditions. Overall, there is an urge to elucidate the underlying physiological, biochemical, and nutritional mechanisms of romaine lettuce to improve salt-stress tolerance. We hypothesized that physiological and growth responses might vary with salt concentrations. Here, we present the results of our study on the impact of salt stress key physiological and biochemical attributes influencing lettuce's fresh mass production and nutrition.

Materials and methods

Plant materials and growth conditions: Lettuce genotype, 'Green Forest' (GF), seeds purchased from *Johnny's Selected Seeds* (Fairfield, ME, USA) were sown in Rockwool cubes (3.81 cm × 3.81 cm × 3.81 cm; Roermond, Netherlands) and germinated in growth chambers (*Percival Scientific*, Perry, IA, USA) with 70% relative humidity, 18/22°C day/night temperatures with a 16-h photoperiod. A 5–11–26 hydroponic special fertilizer (*Peters Professional*, Summerville, SC, USA) was used as a supplemental nutrient after ten days of sowing.

Thirty-day-old seedlings were transplanted into a deep-water culture hydroponic system in the greenhouse situated at Northeast Mississippi Branch Experiment Station, Verona, MS, Mississippi State University (34°09'53.2"N, 88°43'28.5"W). Each hydroponic system tub contained 10 L of full-strength fertilizer solution a mixture of 5–11–26 hydroponic special fertilizer and 15.5:0:0 *YaraLiva CALCINIT* greenhouse/solution grade (*Yara*, Tampa, FL, USA). The fertilizer solution comprises (ppm): nitrogen (150), phosphorus (48), potassium (216), calcium (116), magnesium (60), sulfur (80), iron (3), manganese (0.5), zinc (0.15), copper (0.15), boron (0.5), and molybdenum (0.1). The fertilizer solution pH was adjusted to 5.8–5.9 using diluted sulfuric acid. The plant's root zone was evenly distributed into the system and air stone was used to oxygen circulation in the root zone (Sharma *et al.* 2018). A randomized complete block design was adopted to arrange the tubs with four replications each. Relative humidity and temperature were monitored and recorded using sensors attached to a data logger (*Campbell Scientific*, UT, USA). Daily light integrals within the greenhouse setting were recorded as mentioned in the study by Olorunwa *et al.* (2022a). Throughout the experiment, the greenhouse experienced an average daily temperature, relative humidity, and daily light integral of (mean ± standard deviation) 19.24 ± 5.25°C, 55.3 ± 12.5%, and 5.98 ± 2.4 mol m^{-2} d^{-1}, respectively. Environmental conditions details are reported in Fig. 1S and Table 1S (*supplement*).

Salt stress treatments: The salt treatment was incorporated into the hydroponic system 14 d after transplanting (6–10 leaf stage). Four different levels of salt treatments were selected (Zapata *et al.* 2003): 0 (control, 1.6–2.0 mS cm^{-1}), 50 (5–7 mS cm^{-1}), 100 (10–12 mS cm^{-1}), and 150 (12–16 mS cm^{-1}) mM NaCl. Salt concentration was gradually increased at one-day intervals until desired electroconductivity (EC) was achieved to avoid an excess osmotic shock. The hydroponic solution was replaced every two weeks, along with the adjustment of electroconductivity. A pH/EC meter (*Accumet AP85*, *Fisher Scientific*, NH, USA) was used for weekly pH/EC readings.

Morphological traits: Two economically important growth stages (GS), late-rosette (GS1), and early head-formation stages (GS2) were selected to evaluate the response of lettuce to salt stress. Replicated phenotypic data such as fresh (FM) and dry mass, and leaf number (LN) were recorded across treatments. Samples were oven-dried using an electric forced air oven to determine the dry mass (DM) for both growth stages.

Leaf gas-exchange and fluorescence traits: Young and completely expanded leaves were used to record the photosynthetic traits on GS1 and GS2. The chamber environment of the *LI-6800* portable photosynthesis system (*Li-Cor Biosciences*, NE, USA) was set to a CO_2 concentration of 490 µmol mol^{-1}, chamber temperature 22°C, corresponding to the day temperature with a 50% relative humidity for both harvest days. The *LI-6800* provided a PPFD corresponding to the greenhouse environment on both harvest days. Before the values were recorded, the measured leaves were given time to acclimatize to the chamber environment. All the gas-exchange and chlorophyll fluorescence traits were measured between 10:00–14:00 h on sunny days. The gas-exchange traits, such as photosynthetic rate (P_N), transpiration (E), stomatal conductance (g_s), and intercellular carbon dioxide (C_i) were recorded. The intrinsic water-use efficiency (WUE_i) is the ratio of P_N and g_s (Martin and Ruiz-Torres 1992). The effective quantum yield of PSII reaction center in a light-adapted state was calculated as (Φ_{PSII}) = ($F_m' - F_s$)/F_m' where F_s = chlorophyll fluorescence measured in light-acclimated state, *i.e.*, steady-state fluorescence, and F_m' = maximal fluorescence of light-adapted leaves. Similarly, F_m' was estimated using the multi-phase flash fluorometer.

The CO_2-response curve (P_N/C_i) measurements were also recorded at GS1 and GS2 using the *LI-6800* auto-programming system. The CO_2 concentration (ppm) set up in the *LI-6800* was carried out as described by Olorunwa *et al.* (2022b). The curve was analyzed using the *Excel fitting tool 10.0* (https://landflux.org/tools), as reported by Sharkey *et al.* (2007). The relationship C_i/C_a was used to compute the internal to external CO_2 ratio. To dissect the stomatal and nonstomatal limitations caused by the induction of salt stress on net photosynthesis, the relative stomatal limitation of photosynthesis (L_s) was quantified using the P_N/C_i response curves and measured as $1 - C_i/C_a$ (Ma *et al.* 2011). P_N/C_i curve was further utilized to estimate nonstomatal limitations such as the maximum rate of Rubisco carboxylation (V_{cmax}) and the maximum rate of photosynthetic electron transport (J_{max}), leaf respiration in the light (R_d), J_{max}/V_{cmax}, mesophyll conductance (g_m), photorespiration (P_r), and R_d/V_{cmax} (Bernacchi *et al.* 2001).

Mineral nutrient analysis: The leaf tissue collected at GS1 and GS2 were oven dried at 70°C for two days in an electric-forced air oven. The dried tissue was ground using an electric grinder, and 0.1 g of tissue was sent to Soil Testing Laboratory, Mississippi State University (Mississippi State, MS, USA) for the mineral nutrients analysis.

Total phenolics and flavonoids: Freeze-dried tissue of 0.1 g was placed into a 16-mm borosilicate glass test tube. Five mL of 96% ACS grade ethanol was added to the tube and placed on the shaker table for 4–7 d at room temperature. Samples were covered to protect them from light. Prior to quantification, the extractant was subjected to dilution using ACS-grade ethanol at a ratio of 1:9 (100 µL of extractant in 900 µL of ethanol). Phenolics were estimated using the Folin-Ciocalteau method as described in previous research (Singleton *et al.* 1999, Ordoñez *et al.* 2006). In brief, 100 µL of diluted sample was pipetted into a 2-mL centrifuge tube. 500 µL of 0.2 N Folin-Ciocalteau reagent was added to the diluted sample and allowed for 5 min. After adding 400 µL of sodium carbonate (75 g L^{-1}), the sample was incubated for 2 h at room temperature. All samples were evaluated in triplicate by pipetting 300 µL of extracted samples into microplate wells after centrifuging them for 10 min at 1,400 rpm at 4°C. The absorbance reading was recorded at 760 nm using the spectrophotometer (*BioTek Instruments*, *Inc*., Winooski, VT). The phenolic content was calculated using a gallic acid equivalent standard curve.

Similarly, for flavonoid content, 0.5 mL of prepared $AlCl_3$-ethanol solution (2%) was added to a 0.5 mL of diluted sample in a 2-mL centrifuge tube. The sample was then incubated at room temperature for 1 h. All samples were evaluated in triplicate by pipetting 300 µL of extracted samples into microplate wells after centrifuging them at 1,400 rpm at 4°C. The absorbance reading was recorded at 420 nm using the spectrophotometer. The flavonoid content was calculated as quercetin from a calibration curve.

Nonstructural carbohydrate extraction and analysis: Nonstructural carbohydrates (glucose, sucrose, and fructose) were detected with an *Agilent* evaporative light-scattering detector and a *Zorbax* column that has strong cation-exchange resins available in differing ionic forms using HPLC as explained by Giannoccaro *et al.* (2006), and modified by Barickman *et al.* (2016). Around 0.1 g of freeze-dried lettuce leaf tissue was ground using a mortar and pestle and collected in a 2-mL microcentrifuge tube. The internal standard (100 mM lactose) of 0.2 mL and 0.8 mL of HPLC H_2O were added. After vortexing vigorously, the sample was shaken horizontally for 15 min and centrifuged for 10 min at 14,000 rpm at 4°C. Briefly, after decanting the sample into a clean microcentrifuge tube, the sample was re-centrifuged at the same centrifuge speed. A supernatant of 500 µL was extracted, and 700 µL of acetonitrile was added to it in a new microcentrifuge tube. Briefly after mixing, the mix was left for incubation for 30 min at room temperature. The supernatant of 500 µL was separated and placed in a 1.5-mL microcentrifuge tube after centrifuging samples for 10 min at 14,000 rpm. The sample was dried in a dry bath for 40–90 min. The dried sample was dissolved in a 75:25 acetonitrile: water solution and collected in a vial using a syringe (1 mL). Finally, the sample was further analyzed with HPLC.

Statistical analysis: To determine the effect of salt treatment on morphological, physiological, and biochemical traits, one-way analysis of variance (*ANOVA*) was performed using *SAS* statistical software (*SAS 9.4*, *SAS Institute Inc.*, NC, USA). *Tukey*'s HSD at $P \leq 0.05$ was employed to separate the treatment means and their differences. Treatment was fixed and replications were random effects. Graphs were made using *Sigma Plot 14.5* (*Systat Software*, CA, USA). Besides, the illustrative figure was made using *Biorender* (https://www.biorender.com/).

Results

Lettuce nutrition: Raising the salt concentration in hydroponic solution led to a significant change in leaf Na$^+$ concentration, and a significant linear negative decline in K$^+$ concentration was observed in response to increased salt stress (Table 1). These results confirmed the validity of the experiment setup. The ratio of Na$^+$ to K$^+$ and salt treatment had a significant positive association, indicating higher sensitivity of lettuce per unit increase in salt irrigation. There was a substantial accumulation of Na$^+$ ions in the leaf under 150 mM (31-fold at GS1 and 28-fold at GS2) compared to the control (Table 1). Although the late-rosette stage (GS1) had relatively high K$^+$ compared to the early head-formation stage (GS2), raising salt concentration significantly decreased the leaf K$^+$ (Table 1). Further, phosphorus decreased to 72%, and Ca^{2+} decreased to 78% at 150 mM compared to control during GS1 (Table 1). A similar decreasing pattern was observed for Ca^{2+} (67%) under 150 mM, while phosphorus remained relatively constant across treatments during GS2. Further, iron and boron in leaves of lettuce grown under the high salt concentration were significantly higher at GS1, with no salt effect observed at GS2. During GS1, Fe increased to 11% at 150 mM NaCl than that of control. There was no significant change in the manganese with the varying salt concentration compared to the control during both growth stages. Zinc concentration was not significantly varied at GS1. In contrast, there was significantly higher zinc recorded under 100 mM compared to the control. With excess salt supply, the proportion of leaf K$^+$ decreased, indicating the significant interaction between salt stress and lettuce nutritional levels.

Fresh and dry mass: An increase in salt concentration of a nutrient solution from 0 to 150 mM NaCl significantly affected fresh and dry biomass production regardless of growth stages (Fig. 1). While there was substantial variation in fresh mass between growth stages, maximum growth was recorded under 19 d (GS2) stress compared to 11 d (GS1) across treatments. The fresh mass of 11 d salt-treated lettuce decreased by 29, 54, and 75% with 50 mM, 100 mM, and 150 mM NaCl treatment (Fig. 1*C*), respectively. A similar trend was followed in the salt-treated plants at GS2, where fresh mass decreased from 32% (50 mM) to 76% (150 mM) (Fig. 1*C*). The dry mass and leaf number were always higher under control (0 mM), and this difference increased significantly with increased salt concentration. The dry mass declined up to 55% at GS1 and 59% at GS2 compared to the control (Fig. 1*D*). Similarly, leaf numbers declined up to 22–23% (Fig. 1*E*) under salt stress compared to the control during GS1 and GS2.

Table 1. Macro- and micronutrients in the leaves of lettuce exposed to 11 d (GS1) and 19 d (GS2) after salt treatment. ** and *** indicate significant difference at $p<0.01$ and $p<0.001$, respectively. NS – nonsignificant. The data presented are mean ± SE ($n = 4$). Means followed by *a common letter* within the growth stage (GS) are not significantly different by *Tukey*'s HSD test at the 5% significance level. Na – sodium; K – potassium; P – phosphorus; Ca – calcium; B – boron; Fe – iron; Mn – manganese; Zn – zinc.

Treatment		Na [mg g^{-1}]	K [mg g^{-1}]	P [mg g^{-1}]	Ca [mg g^{-1}]	B [µg g^{-1}]	Fe [µg g^{-1}]	Mn [µg g^{-1}]	Zn [µg g^{-1}]
GS1	0 mM	1.4 ± 0.4^{d}	161.3 ± 14.3^{a}	18.2 ± 1.9^{a}	18.3 ± 1.4^{a}	71.1 ± 2.5^{b}	97.3 ± 1.9^{b}	111.3 ± 5.2^{a}	90.5 ± 10.2^{a}
	50 mM	12.1 ± 0.6^{c}	79.1 ± 9.2^{b}	11.2 ± 1.9^{b}	5.3 ± 0.9^{bc}	72.1 ± 1.5^{b}	94.2 ± 3.5^{b}	113.3 ± 4.2^{a}	76.2 ± 9.2^{a}
	100 mM	31.2 ± 1.1^{b}	75.2 ± 9.7^{b}	10.4 ± 1.6^{b}	6.2 ± 0.9^{b}	75.1 ± 2.6^{ab}	101.3 ± 3.7^{ab}	119.1 ± 8.6^{a}	77.1 ± 8.4^{a}
	150 mM	44.1 ± 2.5^{a}	42.4 ± 4.6^{c}	5.6 ± 0.9^{c}	4.5 ± 0.5^{c}	78.5 ± 1.8^{a}	107.6 ± 5.2^{a}	121.3 ± 6.2^{a}	75.4 ± 9.9^{a}
	P-value	***	***	***	***	**	**	NS	NS
GS2	0 mM	1.1 ± 0.1^{d}	65.5 ± 5.4^{a}	6.3 ± 0.6^{a}	9.1 ± 1.7^{a}	11.2 ± 0.5^{a}	96.4 ± 4.4^{a}	60.5 ± 7.3^{a}	45.1 ± 6.8^{ab}
	50 mM	17.1 ± 0.9^{c}	56.5 ± 4.1^{ab}	6.1 ± 0.8^{a}	5.4 ± 0.8^{b}	11.7 ± 0.5^{a}	96.5 ± 4.6^{a}	67.1 ± 6.5^{a}	42.4 ± 3.4^{b}
	100 mM	24.1 ± 1.3^{b}	54.1 ± 5.5^{bc}	6.2 ± 0.5^{a}	4.4 ± 0.9^{bc}	12.4 ± 0.8^{a}	98.5 ± 3.5^{a}	76.4 ± 5.1^{a}	50.9 ± 4.4^{a}
	150 mM	30.9 ± 3.4^{a}	49.4 ± 4.2^{c}	6.4 ± 0.5^{a}	3.2 ± 0.6^{c}	12.6 ± 0.9^{a}	100.1 ± 4.9^{a}	78.4 ± 6.7^{a}	39.7 ± 3.6^{b}
	P-value	***	***	NS	***	NS	NS	NS	**

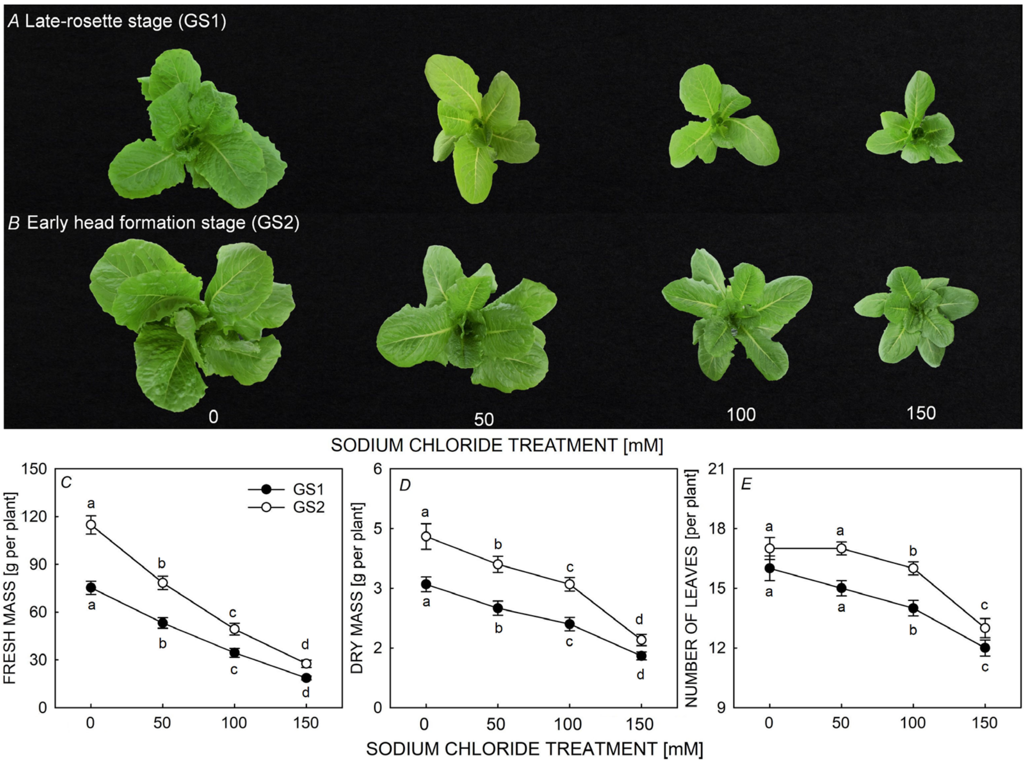

Fig. 1. Pictorial representation of salt stress (sodium chloride – NaCl) effect on leaf biomass at different growth stages [late-rosette stage, or 11 d after treatment (*A*) and early head-formation stage or 19 d after treatment (*B*)]. Graphical representation of salt stress effect on shoot fresh mass (*C*) and dry mass (*D*), and leaf number (*E*) of lettuce at different growth stages. *The vertical error bars* indicate mean ± SE ($n = 4$). Means followed by *a common letter* within growth stages (GS) are not significantly different by *Tukey*'s HSD test at the 5% significance level.

Leaf-level gas-exchange parameters: Salt treatments significantly affected all the gas parameters except for the photosynthetic rate (P_N) (Fig. 2). Stomatal conductance (g_s) and transpiration rate (E) declined with the increase in salt contents at both growth stages. g_s significantly decreased up to 51% (GS1) and 86% (GS2) compared to the control (Fig. 2*B*). A similar trend was observed for E across growth stages (Fig. 2*C*). Interestingly, WUE$_i$ significantly increased by 124% (GS1) and 142% (GS2) under 150 mM compared to the control (Fig. 2*D*). Besides, no substantial difference in the P_N/C_i curve was observed between the salt-treated and control in both growth stages (Figs. 2S, 3S; *supplement*). Salt stress caused a significant increase in stomatal limitation (L_s) compared to control in GS1 and GS2 (Fig. 2*E*). A similar increasing trend was observed for the maximum rate of Rubisco carboxylation (V_{cmax}; Fig. 2*F*) and maximum rate of photosynthetic electron transport (J_{max}; Fig. 2*G*) values during the GS1, where V_{cmax} and J_{max} increased with increased salt stress. However, at GS2, V_{cmax} decreased significantly by 63%, and J_{max} decreased by 43% with increasing salt stress compared to control. Mesophyll conductance (g_m) decreased significantly under 100 mM and 150 mM compared to 0 mM across growth stages (Fig. 2*H*).

Chlorophyll fluorescence traits: The present study demonstrated that chlorophyll fluorescence traits were significantly affected by salt stress (Fig. 3). The steady-state fluorescence (F_s) values declined significantly under 150 mM compared to GS1 (Fig. 3*A*). GS1 showed a significant increase in the effective quantum yield of PSII (Φ_{PSII}) in salt-treated lettuce compared to the control (Fig. 3*B*). Photochemical quenching of fluorescence (q_P) decreased significantly at 150 mM compared to control (Fig. 3*D*).

Leaf pigment and sugar concentration: Salt treatments significantly affected the phenolics content at GS1 (Table 2). There was a significant increase in phenolics content at 100 mM (9%) and 150 mM NaCl (11%) compared to 78% control during GS1. However, no significant changes in phenolics content were observed at GS2. Similarly, salt treatment had no significant effect on flavonoids at both growth stages. The result on nonstructural carbohydrates demonstrated that all the sugar forms (glucose, sucrose, and fructose) increased significantly with increased salt concentration compared to control during GS1 and GS2 (Table 2). Fructose content significantly increased by 33 and 56% at 100 mM and 150 mM salt concentrations compared to control at GS1 (Table 2). Glucose increased by 70, 76, and 78% at 50 mM, 100 mM, and 150 mM NaCl, respectively, compared to control during GS1. Similarly, salt-stressed (150 mM NaCl) plants had 2.5-fold higher sucrose than the control. A similar increasing trend was

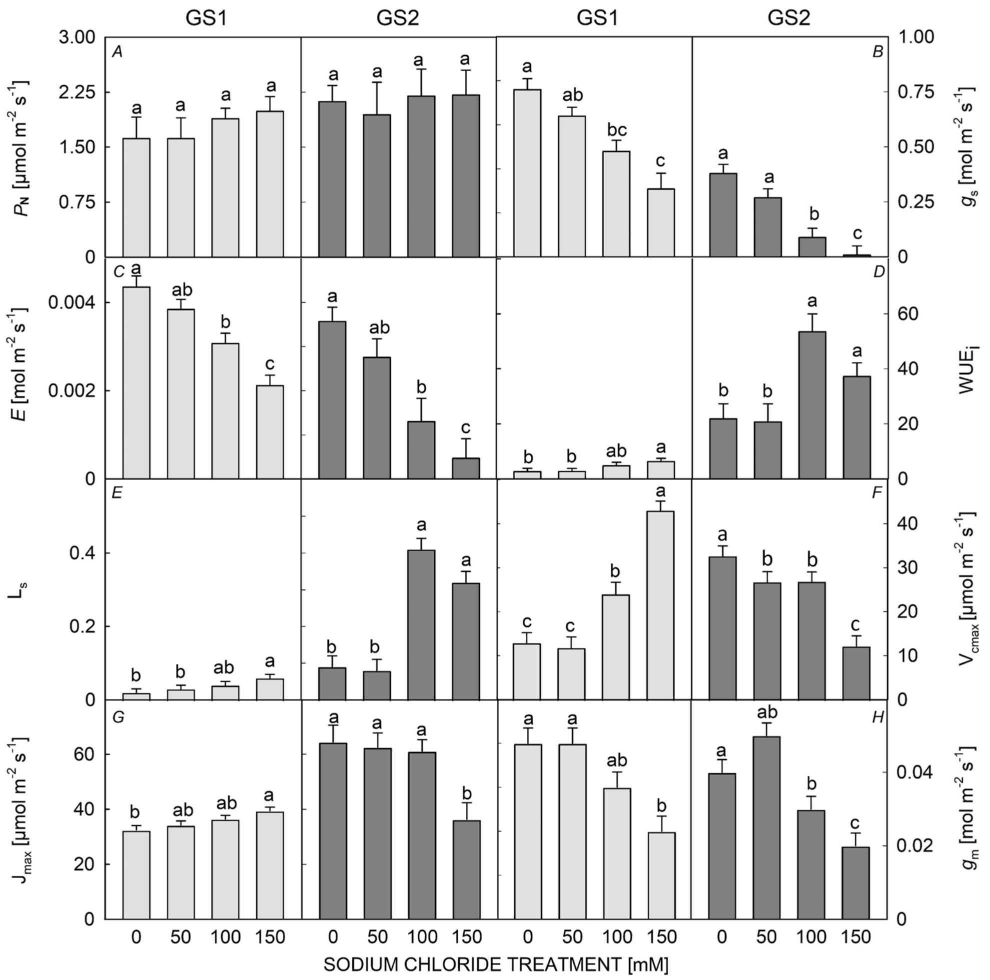

Fig. 2. Salt stress effect on photosynthesis (P_N) (*A*), stomatal conductance (g_s) (*B*), transpiration rate (E) (*C*), intrinsic water-use efficiency (WUE$_i$) (*D*), *s*tomatal limitation (L$_s$) (*E*), the maximum rate of Rubisco carboxylation (V$_{cmax}$) (*F*), maximum rate of photosynthetic electron transport (J$_{max}$) (*G*), and mesophyll conductance (g_m) (*H*) of lettuce cultivar recorded after 11 d (GS1) and 19 d (GS2) of control and different salt treatments. *The vertical error bars* indicate mean ± SE ($n = 4$). Means followed by *a common letter* are not significantly different by *Tukey*'s HSD test at the 5% significance level.

observed for all sugar forms at GS2 with increasing salt contents (Table 2).

Discussion

Salt stress is considered a major environmental stressor that threatens agriculture production (Hazell and Wood 2008, Rengasamy 2010). Salt concentrations above the threshold level around the roots affect the plant's performance in two steps: (*1*) reduces water potential and creates osmotic stress (early response), which leads to a cellular imbalance by interfering with the uptake of essential ions like K$^+$; (*2*) it leads to ion toxicity (Na$^+$) by lowering K$^+$ ion (late response) (Munns and Tester 2008, Tavakkoli *et al.* 2011). Therefore, the rate of reduction in growth is directly correlated with an increased external salt exposure. When osmotic stress and ion toxicity occur in a chain, there is a higher chance of detrimental effects on plant growth and the ultimate death of a plant (Munns and Tester 2008). Hence, this study was carried out to elucidate the impact of salt stress on the physiology, growth, and nutrition of romaine lettuce at the late-rosette and early head-formation stages (Figs. 1, 4).

An increase in salt concentrations linearly decreased lettuce biomass (Fig. 1). Lettuce was found to be sensitive to salt above 40 mM NaCl, similar to other crops (Rai *et al.* 2006, Samineni *et al.* 2011, Ondrasek *et al.* 2021) and leafy greens (Beltrão *et al.* 1997). A reduction in leaf area, not the number, appeared to cause a decrease in fresh or dry biomass (Fig. 1). Biomass decline varied with the plant growth stage, with early head-formation (GS2) being slightly more sensitive compared to late-rosette stage GS1 (Fig. 1). This has been reported in previous studies on crops such as wheat (Goudarzi and Pakniyat 2008), beans (Aydin *et al.* 2012), tomato (Zhang *et al.* 2017), melons and cucumber (Rouphael *et al.* 2012) which attributed to the low biomass production under salt stress. Increased salt contents also led to stunted leaves and a darker green color in the lettuce. The stunted growth indicates the reduction in internode elongation which could be due to a lower gibberellin content or a thick and hard cell wall (Marschner 2012). When lettuce plants were exposed to short-term salt stress (10 d), they responded by increasing the accumulation of a high amount of chlorophyll per unit area (a short-term defense mechanism), which might have led to a temporary increase in the green color of the leaves. The dark green color of leaves may also result from lower specific leaf area (Bartha *et al.* 2015, Poudel *et al.* 2023). Previous studies on lettuce and other crops, such as tomato, broccoli, cauliflower, and lettuce (Zribi *et al.* 2009, Giuffrida *et al.* 2012, Machado and Serralheiro 2017, Adhikari *et al.* 2021) reported that higher salt concentrations (100 and 150 mM) can decrease chlorophyll production or induce yellowing of leaves in the long run. At the same time, it is also worth noting that the darker green color in leaves was associated with reduced leaf area under higher NaCl concentrations compared to control, which partially explains the tradeoff between leaf area and chlorophyll content under short-term stress (Fig. 1*A*,*B*).

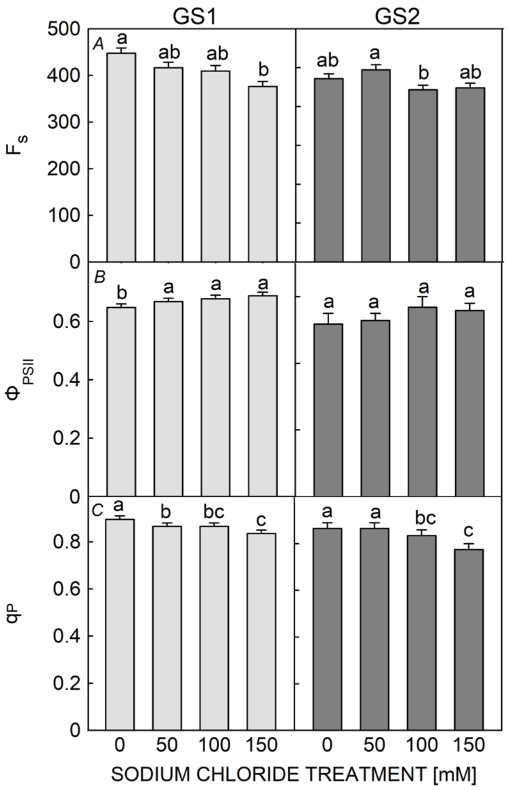

Fig. 3. Salt stress effect on steady-state fluorescence (F_s) (*A*), the effective quantum yield of PSII (Φ_{PSII}) (*B*), and photochemical quenching of fluorescence (q_P) (*C*) of lettuce cultivar after 11 d (GS1) and 19 d (GS2) of control and different salt treatments. *The vertical error bars* indicate mean ± SE (*n* = 4). Means followed by *a common letter* with growth stages (GS) are not significantly different by *Tukey*'s HSD test at the 5% level of significance.

The significant decline in lettuce biomass is linked to a high accumulation of Na^+ ions and a decrease in K^+ and Ca^{2+} ions in salt-treated lettuce (Carillo *et al.* 2020). Na^+ ions inhibit K^+ and Ca^{2+} transport and enhance Ca^{2+} efflux, leading to calcium deficiency (Azevedo Neto and Tabosa 2000, Zhao *et al.* 2007). Additionally, Na^+ also enhances Ca^{2+} efflux from plant cells while limiting Ca^{2+} ion entry through the plasma membrane which leads to Ca^{2+} deficiency in the plant (Cramer *et al.* 1989, Zhao *et al.* 2007). Higher salt concentration also reduces phosphorus movement from root to shoot and restricts the recirculation of old and young tissue (Azevedo Neto and Tabosa 2000), which corroborates with the declined phosphorus under salt stress. Boron and iron are important for plant growth and quality, with iron also involved in photosynthesis, respiration, and nitrogen fixation (Marschner 2012, Shahverdi *et al.* 2022). In addition, Fe is also involved as a constituent for several enzymatic activities involved in photosynthesis, respiration, and nitrogen fixation process (Ali *et al.* 2012, Santi *et al.* 2013). Increased boron and iron are linked to stunted stems and leaves under salt stress (Ben-Gal and Shani 2003, Yermiyahu *et al.* 2008). Thus, boron and iron toxicity are directly linked to NaCl concentrations.

Table 2. Phenolic and sugar compounds in lettuce exposed to 11 d (GS1) and 19 d (GS2) after salt treatment. ** and *** indicate significant difference at $p<0.01$ and $p<0.001$, respectively. NS – nonsignificant. The data presented are mean ± SE (*n* = 4). Means followed by *a common letter* within the growth stage (GS) are not significantly different by *Tukey*'s HSD test at the 5% significance level.

Treatment		Phenolics [mg(GAE) g^{-1}]	Flavonoids [mg(QE) g^{-1}]	Fructose [mg g^{-1}]	Glucose [mg g^{-1}]	Sucrose [mg g^{-1}]
GS1	Control	25.4 ± 0.2^{b}	16.8 ± 0.4^{a}	88.3 ± 6.2^{c}	67.9 ± 5.1^{b}	27.6 ± 5.2^{c}
	50 mM	25.3 ± 0.1^{b}	16.6 ± 0.3^{a}	91.5 ± 13.6^{c}	115.5 ± 6.5^{a}	39.8 ± 8.4^{bc}
	100 mM	27.6 ± 0.4^{a}	16.9 ± 0.5^{a}	117.1 ± 8.9^{b}	120.6 ± 9.2^{a}	50.5 ± 7.2^{b}
	150 mM	28.2 ± 0.2^{a}	16.6 ± 0.3^{a}	137.6 ± 15.4^{a}	121.0 ± 7.2^{a}	68.5 ± 7.2^{a}
	P-value	**	NS	***	**	***
GS2	Control	24.9 ± 0.3^{a}	15.9 ± 0.2^{a}	80.5 ± 7.7^{b}	68.4 ± 15.2^{b}	17.3 ± 6.6^{b}
	50 mM	25.1 ± 0.2^{a}	15.9 ± 0.1^{a}	85.1 ± 6.7^{b}	98.2 ± 9.5^{a}	24.1 ± 3.6^{b}
	100 mM	24.9 ± 0.2^{a}	15.9 ± 0.1^{a}	112.2 ± 7.5^{a}	110.1 ± 11.8^{a}	36.8 ± 5.2^{a}
	150 mM	24.8 ± 0.4^{a}	15.8 ± 0.2^{a}	124.9 ± 10.1^{a}	113.2 ± 11.6^{a}	37.1 ± 6.4^{a}
	P-value	NS	NS	***	***	***

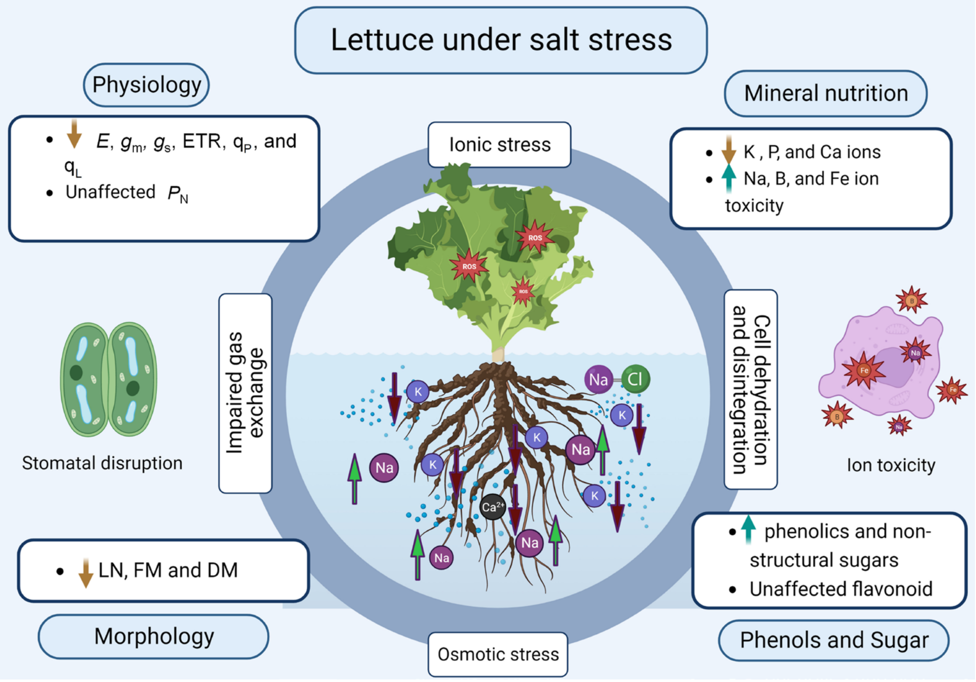

Fig. 4. The observed salt stress tolerance/resistance mechanism observed in the romaine lettuce. *E* – transpiration rate; g_m – mesophyll conductance; g_s – stomatal conductance; ETR – electron transport rate; q_P – photochemical quenching of fluorescence; q_L – fraction of PSII centers in the open state with plastoquinone oxidized; P_N – photosynthetic rate; Φ_{PSII} – the effective quantum yield of PSII; K – potassium; P – phosphorus; Ca – calcium; Na – sodium; Fe – iron; B – boron; LN – leaf number; FM – fresh mass; DM – dry mass.

Excess salt around the root zone affects leaf photosynthesis through stomatal closure and impacts other nonstomatal properties (Hamani *et al.* 2020). Na^+ or Cl^- accumulation causes imbalances in carbon metabolic and redox reactions in the thylakoid membranes and the Calvin cycle in leaves (Zhang and Xing 2008). A high Na^+ to K^+ ratio in leaf tissue significantly affects g_s in lettuce, as seen in other studies (Aroca *et al.* 2013). Despite a substantial reduction in g_s under salt stress, no effect was observed on photosynthesis and Φ_{PSII} during GS1 and GS2, potentially due to minimal damage to PSII (Ruban and Murchie 2012). Similar responses were reported in salt-tolerant species (Munns and Tester 2008, Pérez-López *et al.* 2013), due to changes in leaf morphology (small and thicker), increased chloroplast density, and modified anatomy features (Bongi and Loreto 1989, Hernandez *et al.* 1995, Delfine *et al.* 1998). It has been reported that higher WUE_i is often linked to lower stomatal density or lower stomatal conductance (g_s) in the leaves under stress such as salt or drought conditions (Chartzoulakis 2005, Yoo *et al.* 2010). In the current study, there was a linear decline in g_s and an increase in WUE_i of up to 124% in salt-stressed lettuce, which also corresponds to the results of the basil (Barbieri *et al.* 2012). In addition, the lower g_s can also create a lower potential for water loss, which increases the WUE_i. In support, studies conducted on *E. myrtifolia* and *C. citrinus* reported that plants exposed to salt stress were able to increase the WUE_i throughout the growing season despite the reduced g_s (Álvarez and Sánchez-Blanco 2014, Acosta-Motos *et al.* 2017). In addition, the findings by Munns and Tester (2008) and James *et al.* (2002) suggested that the rate of assimilation per unit leaf area often remained unchanged in the salt-treated plants even though g_s was reduced. This situation could be due to changes in leaf cell anatomy that led to smaller, thicker leaves and higher chlorophyll density per unit leaf area in salt-stressed plants (James *et al.* 2002). Additionally, the higher CO_2 supply than ambient CO_2 supply during the gas-exchange measurement (490 µmol mol^{-1}) could have resulted in unaffected photosynthetic activity or no PSII damage (Fig. 2*A*) and a P_N/C_i curve (Figs. 2S, 3S). A similar response was reported in salt-stressed lettuce under elevated CO_2 supply (Pérez-López *et al.* 2013). However, further detailed investigations are required to understand the short- and long-term effects of salt stress on resource use efficiency.

The study found that L_s increased 2–3 times in salt-stressed (150 mM NaCl) lettuce compared to the control. High salt concentrations likely cause osmotic stress and ion accumulation in the guard cells, leading to an increase in L_s (James *et al.* 2006, Maggio *et al.* 2007, Shapira *et al.* 2009). Previous research on rice and *Rhamnus* L. also stated that L_s increases with increment in NaCl and water stress (Gulías *et al.* 2002, Elbasan *et al.* 2020). Recently, g_m has been included in carbon cycle models, as its absence would result in a significant underestimation of V_{cmax} and J_{max} (Sun *et al.* 2014, Knauer *et al.* 2020). However, salt stress reduced g_m indicated limited CO_2 entry into the photosynthetic apparatus and decreased V_{cmax} and J_{max} (Cai *et al.* 2010). Despite reduced g_m, J_{max} increased in lettuce under salt stress during GS1. Reduced g_m might impaired leaf photochemical characteristics and anatomical traits, which supports the reduced size of leaves (Fig. 1) with an increased salt concentration in the study (Syvertsen *et al.* 1995, Delfine *et al.* 1999). Several studies have evaluated the salt stress effect of Na^+ and Cl^- on the photosynthetic and chlorophyll fluorescence traits in crops (Al-aghabary *et al.* 2005, Kalaji *et al.* 2011, Hamani *et al.* 2020, Shin *et al.* 2020). Photochemical quenching parameters have been used to assess PSII efficiency in stressed plants, with q_P decreasing under environmental stress like drought and salt (Smethurst and Shabala 2003, Kalaji *et al.* 2016). The current study also showed a significant decrease in q_P with increasing salt concentrations (Fig. 4). Other chlorophyll fluorescence traits were unchanged. The result from our study was on a par with the study conducted on cowpea (Larcher *et al.* 1990), cotton (Brugnoli and Björkman 1992), celery (Everard *et al.* 1994), and herbaceous crops (Lu *et al.* 2003). In response to salt stress, lettuce showed increased phenolic compounds to protect against oxidative stress, as seen under other stresses (Cartea *et al.* 2011). The study also showed that phenolic content is more important than flavonoids in conferring salt tolerance to lettuce. Despite the decrease in fresh biomass and formation of rosette leaves, there was a drastic increase in soluble sugar forms in lettuce in response to salt stress (Fig. 4). Soluble sugars showed a drastic increase in response to salt stress, which is known to enhance stress tolerance in plants by maintaining osmotic and ionic homeostasis (Ahmad *et al.* 2013) as reported in rice (Dubey and Singh 1999, Kader *et al.* 2006), lettuce (Yildirim *et al.* 2015), and sugar beet (Wang *et al.* 2017).

Conclusion: Results from the present study demonstrated that lettuce is highly sensitive to salt stress at the rosette and head-formation stages. A high Na^+-to-K^+ ratio inside the leaves affected physiology and growth and caused a decrease in fresh or dry mass. Further, a linear increase in phenolic and soluble sugar forms indicated the ability of lettuce to counter the oxidative stress under salt stress. Further field testing is needed to determine whether these traits are relevant and effective in sustaining yield (biomass) and quality (nutrition). Phenotyping diverse lettuce genotypes and identifying genetic loci associated with higher biomass at the vegetative stage would help develop salt-tolerant lettuce cultivars.

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