 doi:10.20944/preprints202206.0197.v1

Article

Salt Stress Affects the Photosynthetic and Chlorophyll Fluorescence Responses in Lettuce (*Lactuca sativa* L.)

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Abstract: Lettuce is a salt-sensitive crop and has a threshold electrical conductivity of 1.3–2.0 mS cm^{-1} and above that is considered detrimental. As there has been very little information on the physiological response of different critical stages of lettuce under different salt stress (SS), the current study is focused on investigating the effects of SS on the critical physiological traits influencing the carbon assimilation in different growth stages of lettuce. The experiment was conducted in deep-water culture hydroponic system in a greenhouse condition. Four levels of sodium chloride salt treatments (EC: 20, 16, 8, and 1.8 mS cm^{-1}) were applied. During both growth stages (day 11 (GS1) and day 19 (GS2) after salt treatment), the leaf transpiration rate, stomatal conductance, and intercellular CO_2 were severely decreased. However, the carbon assimilation rate remained unchanged under SS. Similarly, the water use efficiency increased under the SS. It is concluded that the increasing SS increased stomatal and non-stomatal limiting factors during GS1 suggesting the enhanced limitation in photosynthetic activity while no such trend was observed during GS2. The decreased g_m with increased SS at GS1 and GS2 suggested that SS induced the irreversible decrease of g_m, which in turn can be responsible for the transient reduction in the V_{cmax} and J_{max} during GS2. Taken together, the evidence from this research recommends that varying the SS levels can significantly affect the physiological performance of lettuce at both growth stages

Keywords: sodium chloride; photosystem II; Green Forest; carbon assimilation; salt-sensitive; C_3 plant; climate change; abiotic stress

1. Introduction

Lettuce is the most ubiquitous salad vegetable which is known to include phytochemicals such as vitamins A, C, E, calcium, iron, carotenoids, and other antioxidants [1–3]. As lettuce is consumed raw in relatively large quantities and constitutes different phytochemicals and antioxidants, it is often credited with aiding in the prevention of many chronic illnesses such as cancer and cardiovascular disease [4,5]. Globally, lettuce is one of the most important salad vegetable crops grown in the world. However, lettuce productivity is below the optimum potential yield of 30000-45000 kg ha^{-1} [6,7], particularly in Arizona and California, USA, where its average yield is less than 30000 kg ha^{-1} over the last decade. There can be a variety of biotic (insect pests, illnesses, parasitic weeds, and nematodes) and abiotic (salt, temperature extremes, water, and low fertility stress) limitations that impede crop growth and development [8,9]. Salt stress is indeed a threat and the primary abiotic stress limiting plant growth and productivity across many parts of the world, particularly in countries with irrigated agriculture, owing to increasing soil salinization as a result of poor agricultural practices and the use of low-quality water for irrigation [10].

Meanwhile, world agribusiness is confronting a lot of difficulties such as creating 70% more nourishment for an extra 2.3 billion individuals by 2050 [11]. Meanwhile there is simultaneous fighting of destitution also, hunger, consuming scarce natural resources more efficiently, and adjusting to environmental change [12]. However, the efficiency of

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yields isn't expanding in correspondence with the food interest. The lower efficiency and crop production in most cases are credited to different abiotic stresses. Reducing crop misfortunes due to different ecological stressors is a critical area of concern to adapt to the expanding food necessities [13]. Salt stress (SS) is one of the major ecological and abiotic estressors in food production. SS is one of the major factors for negative changes in the chemical and physical properties of soils, as well as crops in the hydroponic system, which is usually generated by natural and industrial sources [14]. It is of utmost concern that 7% of the total world area (930 million hectares) is affected worldwide and around 1.5 million of the productive land is affected every year by SS [15,16]. For this reason, growers are forced to irrigate crops with a relatively high concentration of water that contains mineral salts due to a scarcity of excellent quality water resources. Unfortunately, the negative impact on crop physiology and biochemical pathway could be generated by irrigation of crops with water with high EC levels containing high sodium chloride and other salts concentrations [14]. The occurrence of SS on the crop also leads to a series of changes in crop metabolic pathway and nutrient uptake including interruption in the uptake of mineral ions [17]. In detail, SS affects the plant growth and development in three steps: firstly, reduced water potential creates the osmotic stress which leads to a cellular imbalance with interference in the uptake of essential ions like potassium, calcium, and nitrates; finally, it leads to the ion toxicity (sodium (Na^+) and chloride (Cl^-)) [17]. Therefore, the intensity of growth suppression is directly correlated with the concentration of salt exposed to the crops. When the osmotic stress and ion toxicity occur in the chain, there is a higher chance of detrimental effects causing cessation of growth and ultimate death of the crop [18].

The plant also responds to salt stress through a series of physiological, and metabolic changes to overcome the detrimental effects of osmotic shock and ion toxicity [19]. Shortly after salinity is imposed on the plants, plant root and shoot metabolism changes, resulting in hyperosmotic shock and ionic imbalance causing secondary stresses such as nutritional imbalance and pathological outcomes [19,20]. For example, salt-sensitive crop like lettuce responds by suppressing water potential between the apoplast and symplast, causing a reduction in the turgor pressure with reduced photosynthetic activity [19]. This unfortunately causes growth inhibition due to cell dehydration [21,22]. There is mounting evidence that stomatal regulation of vapor loss is extremely sensitive to short-term salt stress [23,24]. Over time, salt stress reduced the net CO_2 assimilation rates (A) along with a decline in photosynthetic pigments and non-stomatal factors like $J_{max,}$ and V_{cmax} [25–27]. In addition, the photosynthetic rate drops with several stomatal and non-stomatal limitations like electron transport rate (ETR) and inhibition of Calvin Cycle enzymes, such as Rubisco, phosphoenolpyruvate carboxylase (PECP), ribulose-5-phosphate kinase, glyceraldehyde-3-phosphate dehydrogenase or fructose-1,6-bisphosphatase can occur in the long-term salt stress in the plant [28,29].

Chlorophyll fluorescence analysis, on the other hand, is a simple and common approach used in plant physiology research that can give useful information about the existing condition of photosystem II (PSII) [30]. Under saline conditions, the electron transport rate (ETR) and photochemical quenching parameters (Fv/Fm, qL, and qP) drops attributing the decrease int PSII efficiency, which is a mechanism to dispel the excess energy safely [31]. Together, salt stress can cause an imbalance between photosynthetic synthesis and electron transfer, and PSII leading to poor physiological performance of a plant. Therefore, the sensitivity of a crop like lettuce may be related to the imbalance among the PSII efficiency of plant and its chlorophyll fluorescence traits.

Although there are several well-established studies related to physiological study of lettuce under the salt stress, there has been very few information on the different critical stages of lettuce growth cultivated under the different salt stress levels. Also, there is very little information on the role of several stomatal and non-stomatal variables in overcoming the hyperosmotic stress environment created by different salt levels. Thus, the current study investigated the effects of salt stress on the critical morphological and physiological traits influencing the carbon assimilation (gas exchange, chlorophyll fluorescence,

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stomatal and non-stomatal variables) in lettuce genotype. We hypothesized that the physiological responses may varies based on the level of imposed salt environment.

2. Results

2.1. Response of leaf gas exchange parameters of lettuce during the salt stress

Salt treatments significantly affected all the gas parameters except for the CO_2 assimilation rate (A), as shown in Figure 1. leaf transpiration rate (E), stomatal conductance (gsw), intercellular CO_2 concentration (C_i) declined with the increase in NaCl level in both the growth stages. E was significantly decreased up to 51% and 86% in GS1 (11 days after salt treatment) and GS2 (19 days after salt treatment) respectively, compared to control (Figure 1B and Figure 2B). The similar trend was followed by gsw and C_i, where they declined by up to 96% and 31%, respectively during both the growth stages. Interestingly, intrinsic water use efficiency (WUE) significantly increased by 73% and 124% in 100 mM and 150 mM NaCl treated lettuce during GS1 (Figure 1E). During GS2, WUE increased up to 142% in the salt stressed lettuce as compared to control (Figure 2E).

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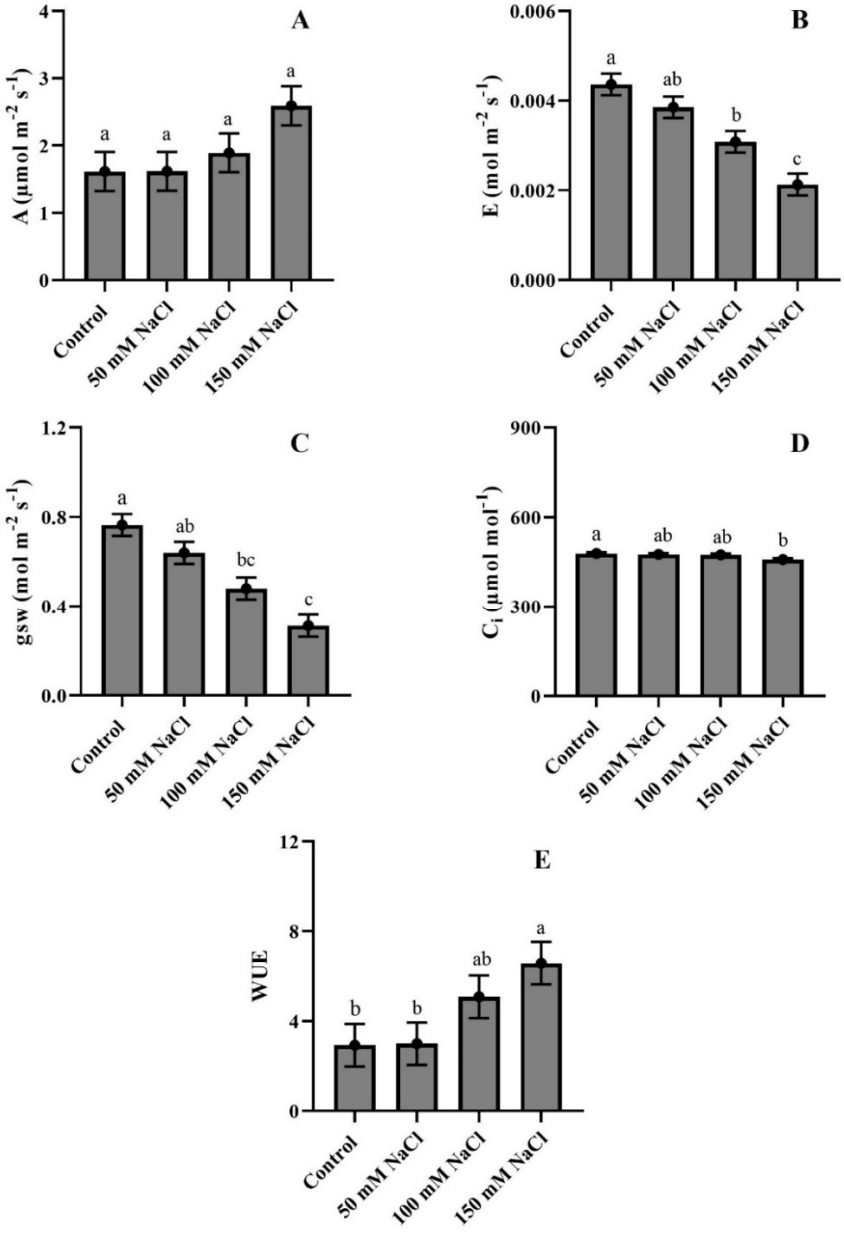

Figure 1. (A) CO_2 assimilation rate (A), (B) Leaf transpiration rate (E), (C) Stomatal conductance (gsw), (D) Intercellular CO_2 concentration (C_i), and (E) Intrinsic water use efficiency (WUE) of lettuce cultivar recorded after 11 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, A= 0.29; E = 0.0002; gsw = 0.06; C_i = 4.71; WUE = 0.95.

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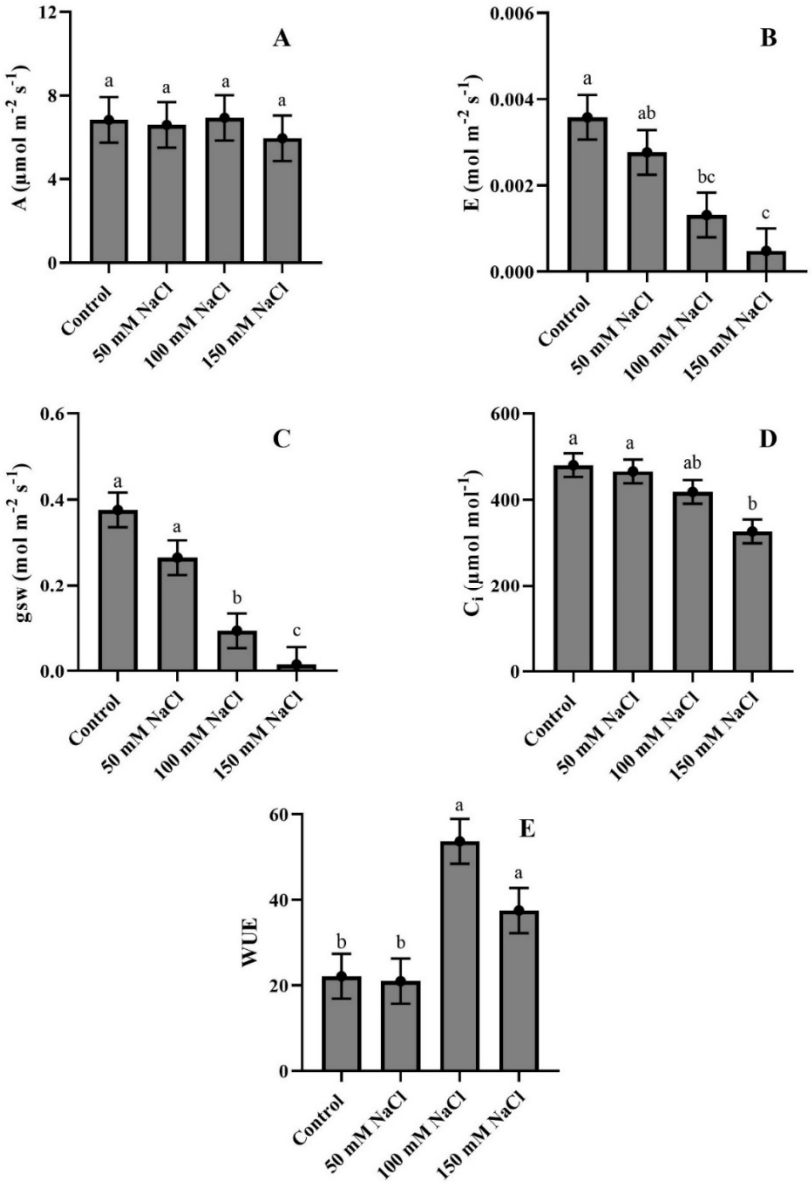

Figure 2. (A) CO_2 assimilation rate (A), (B) Leaf transpiration rate (E), (C) Stomatal conductance (gsw), (D) Intercellular CO_2 concentration (C_i), and (E) Intrinsic water use efficiency (WUE) of lettuce cultivar recorded after 19 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, A= 1.09; E = 0.0005; gsw = 0.04; C_i = 27.60; WUE = 5.26.

2.2. Salt stress and its influence on CO_2 response curve

A/C_i curve was measured at day 11 (GS1) and day 19 (GS2) after salt treatment to investigate the biochemical limitation of A's response in lettuce under SS (Figure 3 and 4). The value of A increased of lettuce subjected to SS as well as control treatments increased with increasing C_i. However, there was no substantial decline in the A/C_i curve in the salt treated plant compared to control in both the growth stages.

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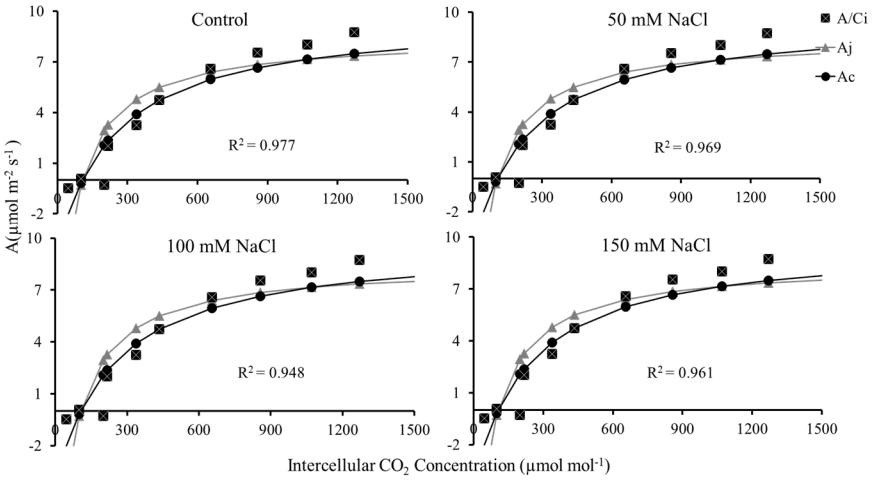

Figure 3. Response of the CO_2 assimilation rate (A) to increasing intercellular CO_2 concentration (C_i) (A/C_i Curve) in the two cucumber cultivars (Marketmore and Straight 8) after 10 days of control and waterlogging treatments. The vertical bars represent the standard error of the mean (n = 4).

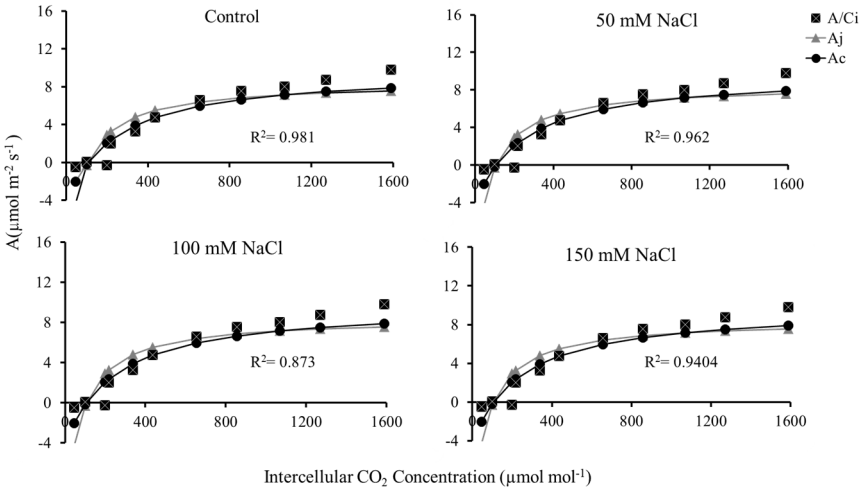

Figure 4. Response of the CO_2 assimilation rate (A) to increasing intercellular CO_2 concentration (C_i) (A/C_i Curve) in the two cucumber cultivars (Marketmore and Straight 8) after 10 days of control and waterlogging treatments. The vertical bars represent the standard error of the mean (n = 4).

2.3. Salt stress induces the stomatal and non-stomatal limitation in lettuce

The stomatal and non-stomatal limitation caused due to induction of SS in lettuce is directly related with the affected photosynthesis process in the crop. The relative stomatal limitation of photosynthesis (L_s) was quantified using the A/C_i response curves and measured as 1-C_i/C_a [38]. SS caused the significant increase in L_s with increased salt levels compared to control in both GS1 and GS2 (Figure 5A and 6A). For instance, the increase in L_s was by 192% and 197% with increased SS with 100 mM and 150 mM NaCl having the highest L_s values during GS1 and GS2, respectively as compared to control . Similar increasing trending was observed in the maximum rate of Rubisco carboxylation (V_{cmax}) (Figure 5B) and and the maximum rate of photosynthetic electron transport (J_{max}) values (Figure 5C) during the GS1 where V_{cmax} increased by 232% and J_{max} increased by 21% with increased SS compared to control . This revealed that increasing salt levels increased stomatal and some non-stomatal limiting factors during 11th day after the salt treatments suggesting the enhanced limitation in photosynthetic activity. However, during the GS2, plant was responding exactly opposite to GS1 in terms of V_{cmax} and J_{max} values where V_{cmax}

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decreased significantly by 63% (Figure 6B) and J_{max} decreased by 43% (Figure 6C) with increasing SS compared to control.

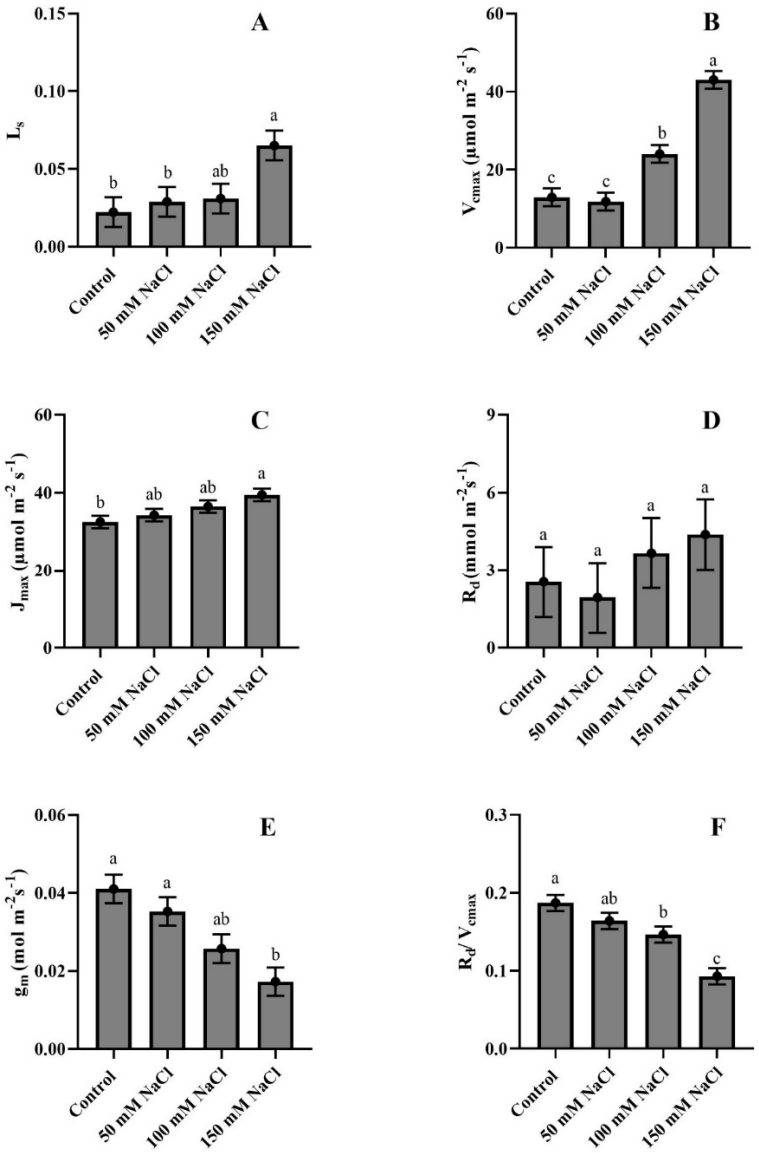

Figure 5. (A) Stomatal limitation (L_s), Non-stomatal limitations ((B) Maximum rate of Rubisco carboxylation (V_{cmax}), (C) Maximum rate of photosynthetic electron transport (J_{max}), (D) Leaf respiration in the light, also called 'day respiration' (R_d), (E) Stomatal conductance i.e., total conductance between intercelluar spaces and chloroplast (g_m) , and (F) The ratio of leaf respiration and maximum rate of Rubisco carboxylation (R_d/V_{cmax}) of lettuce cultivar recorded after 11 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, $L_s = 0.0096$; $V_{cmax} = 2.29$; $J_{max} = 1.60$; $R_d = 1.36$; $g_m = 0.0037$; $R_d/V_{cmax} = 0.011$.

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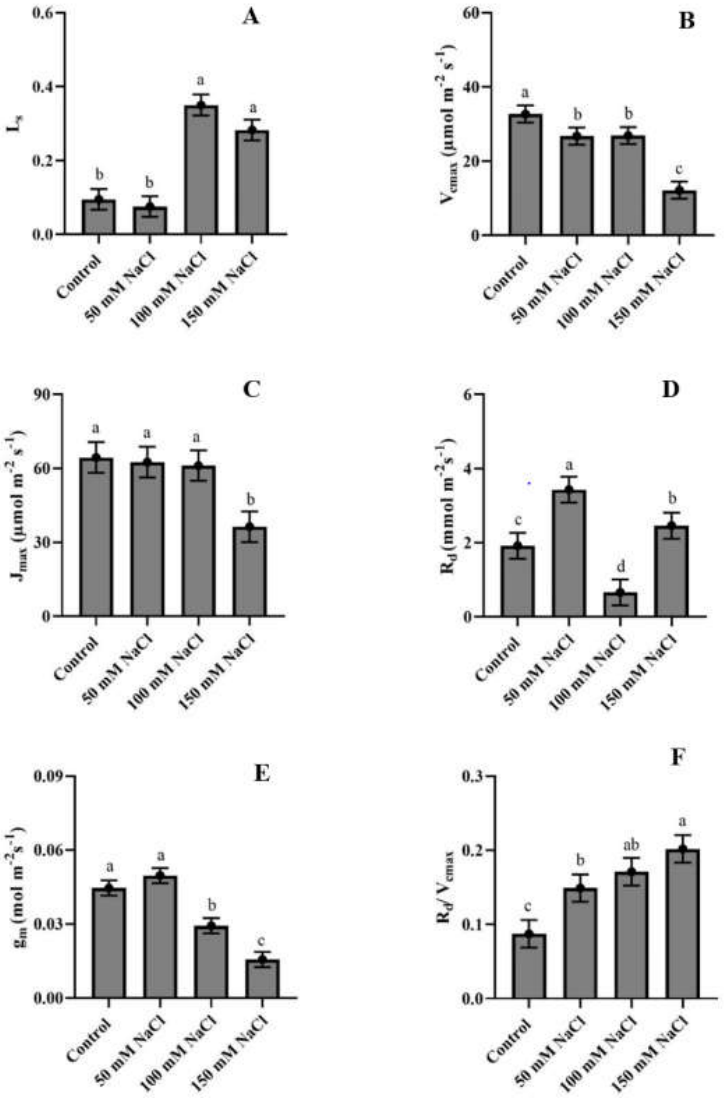

Figure 6. (A) Stomatal limitation (L_s), Non-stomatal limitations ((B) Maximum rate of Rubisco carboxylation (V_{cmax}), (C) Maximum rate of photosynthetic electron transport (J_{max}), (D) Leaf respiration in the light, also called 'day respiration' (R_d), (E) Stomatal conductance i.e., total conductance between intercellular spaces and chloroplast (g_m) , and (F) The ratio of leaf respiration and maximum rate of Rubisco carboxylation (R_d/V_{cmax}) of lettuce cultivar recorded after 19 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, $L_s = 0.028$; $V_{cmax} = 2.29$; $J_{max} = 6.21$; $R_d = 0.35$; $g_m = 0.0031$; $R_d/V_{cmax} = 0.019$.

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2.4. Salt stress influences the chlorophyll fluorescence traits in lettuce

The values of chlorophyll fluorescence traits recorded in the current study differed with the levels of salt stress imposed in the lettuce. The current study demonstrated that some of the chlorophyll fluorescence were significantly affected by SS (Figure 7-10) during both the growth stages. The steady-state fluorescence (F_s) value declined at both growth stages with increased SS compared to control (Figure 7A and 8A). During ΦPSII, qP, qL and 1-qL are critical photochemical quenching parameters for assessing PSII photochemical efficiency in stressed plants [39,40]. The values of F_s and 1-qL significantly declined with the increased SS compared to control while values of ΦPSII, ETR, qP, and qL increased after 11 days after salt treatment (GS1) (Figure 7 and 9). Similarly, during the GS2 (19 days after salt treatment), the values of F_s declined with the increased SS while the values of ETR, qP, and qL increased (Figure 8 and 10).

When comparing salt stressed lettuce with the control plants, it was observed that F_s, 1-qL, ΦPSII, ETR, qP, and qL were the most affected in different growth stages. F_s values decreased by 16% in GS1 and by 6% in GS2 when subjected to SS compared to control as shown in figure 8A and figure 9A, respectively. F'_o, F'_m, and F'_v/F'_m remained unaffected under salt treatment in both the growth stages. On the contrary, at GS1, ΦPSII, ETR, qP, and qL were significantly increased up to 7%, 7.5%, 7%, and 22%, respectively in lettuce treated with salt as compared to control. Similar trend was followed during GS2 where GS1, ΦPSII, ETR, qP, and qL were significantly increased by 9%, 17%, 44%, and 11%, respectively in lettuce treated with salt as compared to control. On the other hand, 1-qL significantly decreased by 27% and 9% during GS1 and GS2, respectively, in lettuce under SS when compared to control. Overall, the current chlorophyll fluorescence data in lettuce revealed that higher the salt concentration, higher will be the decrement on F_s and 1-qL. Similarly, higher will be the values of ΦPSII, ETR, qP, and qL with increasing SS in lettuce.

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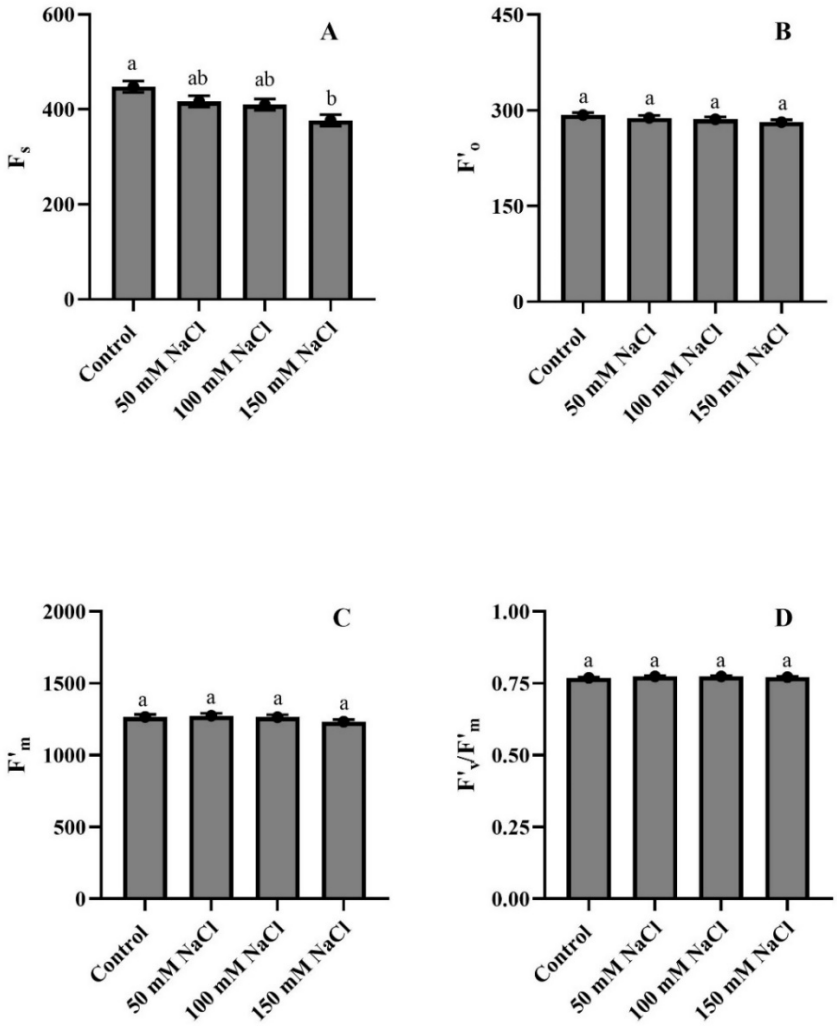

Figure 7. (A) Steady-state fluorescence (F_s), (B) Light adapted minimal fluorescence (F'_o), (C) light adapated maximal fluorescence (F'_m), and (D) Maximum quantum efficiency of PSII in the light-adapted state (Fv'/Fm') of lettuce cultivar recorded after 11 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, $F_s = 11.90$; $F'_o = 3.79$; $F'_m = 16.20$; Fv'/Fm' = 0.0024.

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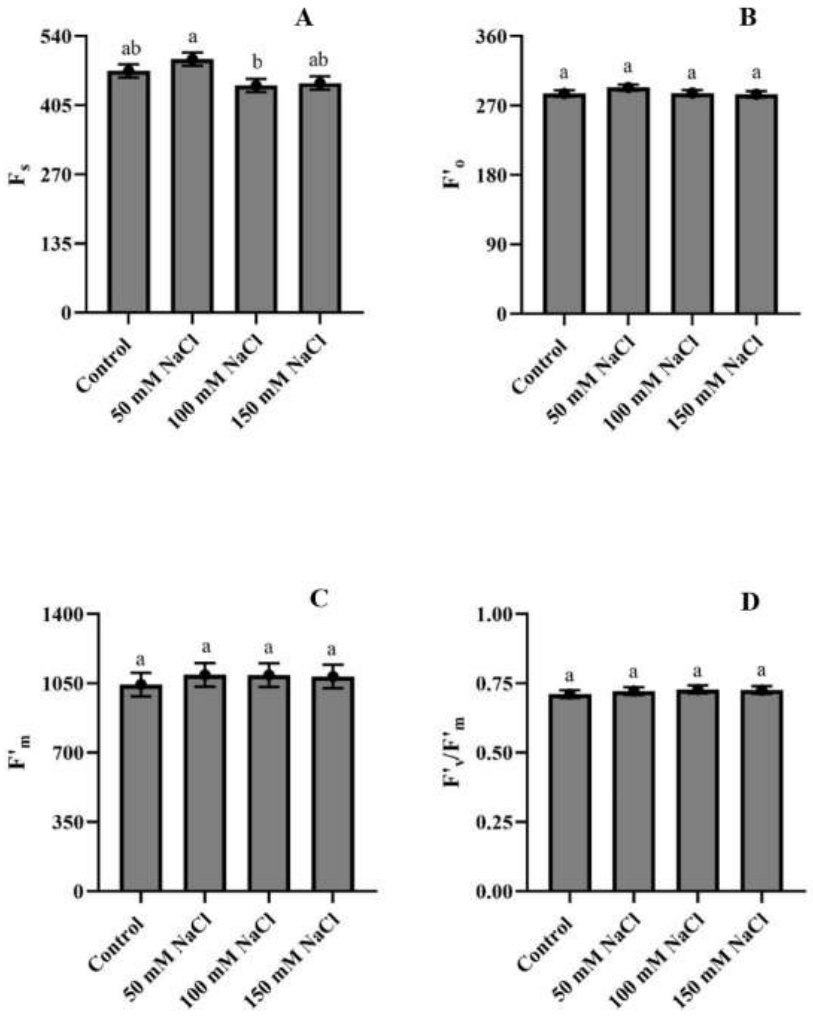

Figure 8. (A) Steady-state fluorescence (F_s), (B) Light adapted minimal fluorescence (F'_o), (C) light adapated maximal fluorescence (F'_m), and (D) Maximum quantum efficiency of PSII in the light-adapted state (Fv'/Fm') of lettuce cultivar recorded after 19 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, $F_s = 12.80$; $F'_o = 3.99$; $F'_m = 59.71$; Fv'/Fm' = 0.014.

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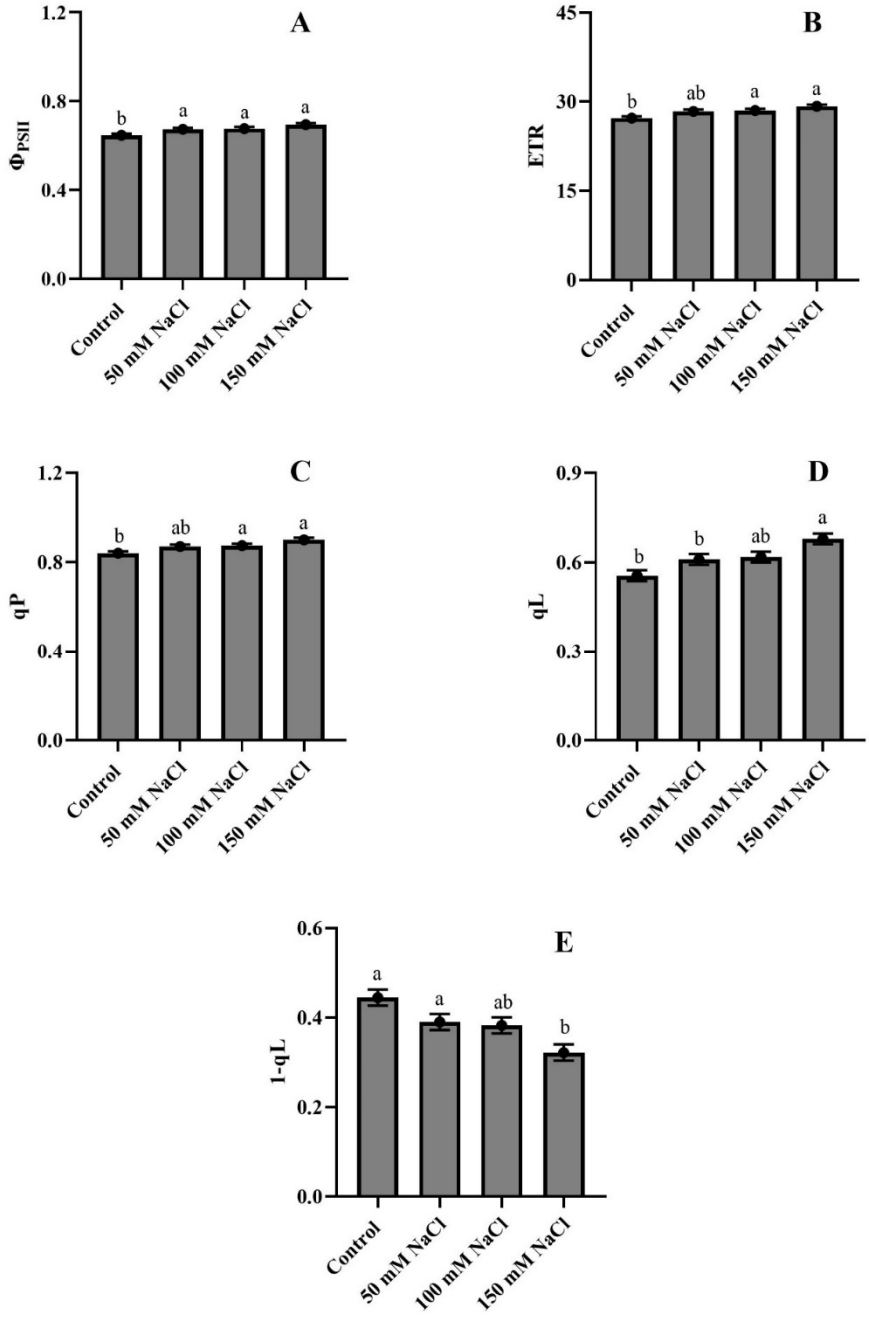

Figure 9. (A) Effective quantum yield of PSII (ΦPSII), (B) Electron transport rate (ETR), (C) Photochemical quenching of fluorescence (qP), (D) Fraction of PSII centers in the open state with plastoquinone oxidized (qL), and (E) redox state of plastoquinone pool) (1-qL) of lettuce cultivar recorded after 11 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, ΦPSII = 0.0073; ETR = 0.31; qP = 0.0089; qL = 0.018; 1-qL = 0.018.

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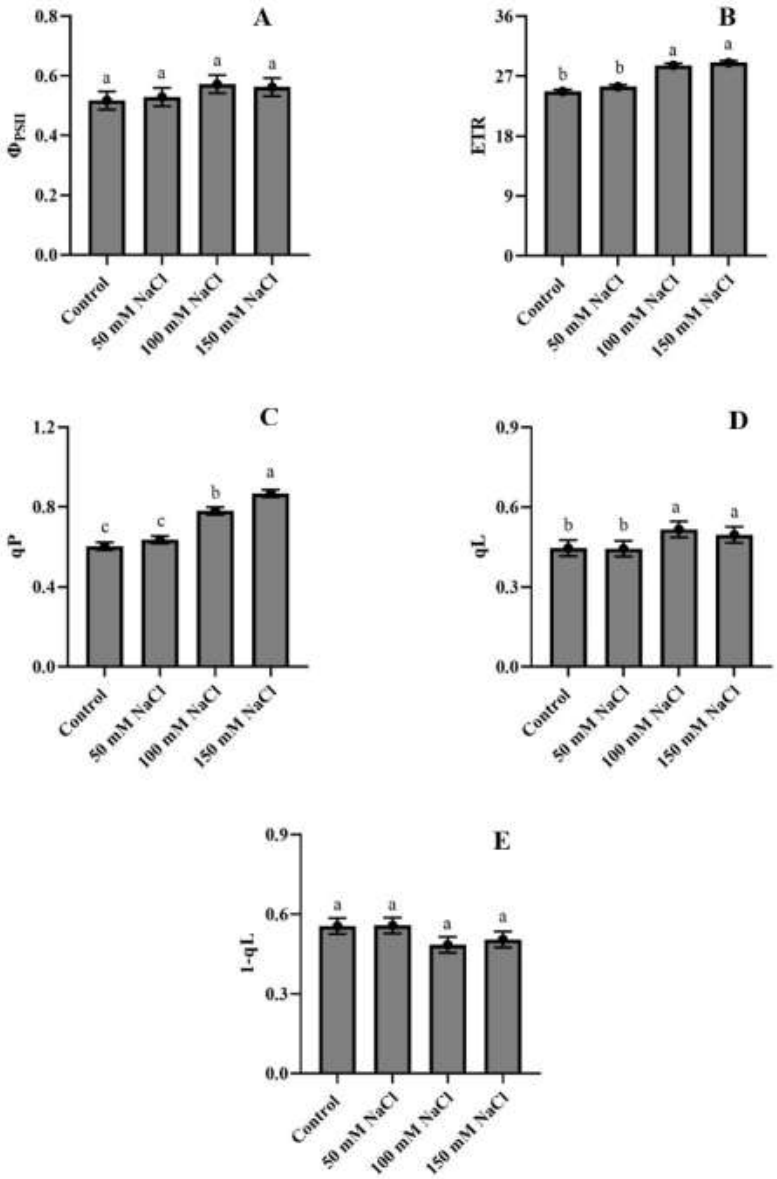

Figure 10. (A) Effective quantum yield of PSII (ΦPSII), (B) Electron transport rate (ETR), (C) Photochemical quenching of fluorescence (qP), (D) Fraction of PSII centers in the open state with plastoquinone oxidized (qL), and (E) redox state of plastoquinone pool) (1-qL) of lettuce cultivar recorded after 19 days of control and different salt treatments. Different low case and uppercase letters indicate significant differences between cultivar's means and treatments, respectively ($P < 0.05$) as determined by Tukey's HSD. The error bar on the vertical bar indicates the standard error of the mean ± 4 replications of each morphological trait. Standard error of the mean, ΦPSII = 0.031; ETR = 0.29; qP = 0.019; qL = 0.029; 1-qL = 0.029.

2.5. Correlation analysis among gas exchange and chlorophyll fluorescence parameters under SS

In Pearson's correlation analysis, the estimated morphological, gas exchange, and chlorophyll fluorescence traits were highly correlated (Figure 11 and 12). All the gas exchange parameters (E, C_i, and gsw) except for A showed the positive correlation with all the stomatal and non-stomatal limiting factors at GS1 and GS2. Interestingly, WUE was negatively correlated with J/V_{cmax}, gm, and R_d/V_{cmax} at GS1. WUE was also found strongly and negatively correlation with A. Similarly, most of the photosynthetic traits (E, C_i, and gsw) were negatively correlated with qP, qL, V_{cmax}, J_{max}. The correlation of F'_v/F'_m was moderate to weak with most of the analyzed parameters. Similarly, WUE and R_d were found moderate to weak in terms of correlation values for most of the parameters

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analyzed. Thus, correlation estimation revealed that both stomatal factors (E, C_i, WUE, gsw) as well as other non-stomatal factors are responsible for influencing the photosynthetic efficiency of lettuce under salt stress.

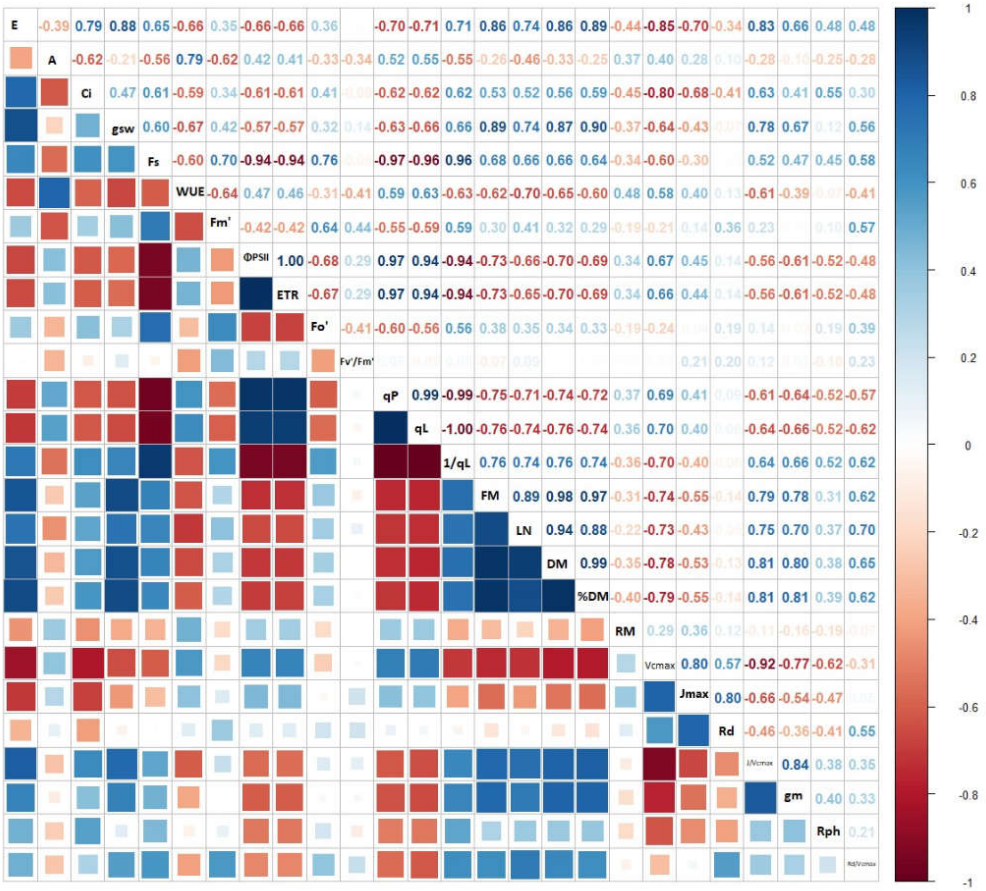

Figure 11. Pearson's correlation matrix of the changes in biomass, gas exchange, and chlorophyll fluorescence parameters of the lettuce genotype under control and salt treatments recorded at 11 days after salt treatment. Dark color represents strong correlations, and light background color represents weaker correlations. Values close to zero indicate no correlation, and values close to one indicate a strong correlation (positive – red and negative – blue) between two parameters. Larger the size of the box with dark color, lower is the significance level of correlation coefficient significance at $P \leq 0.05$.

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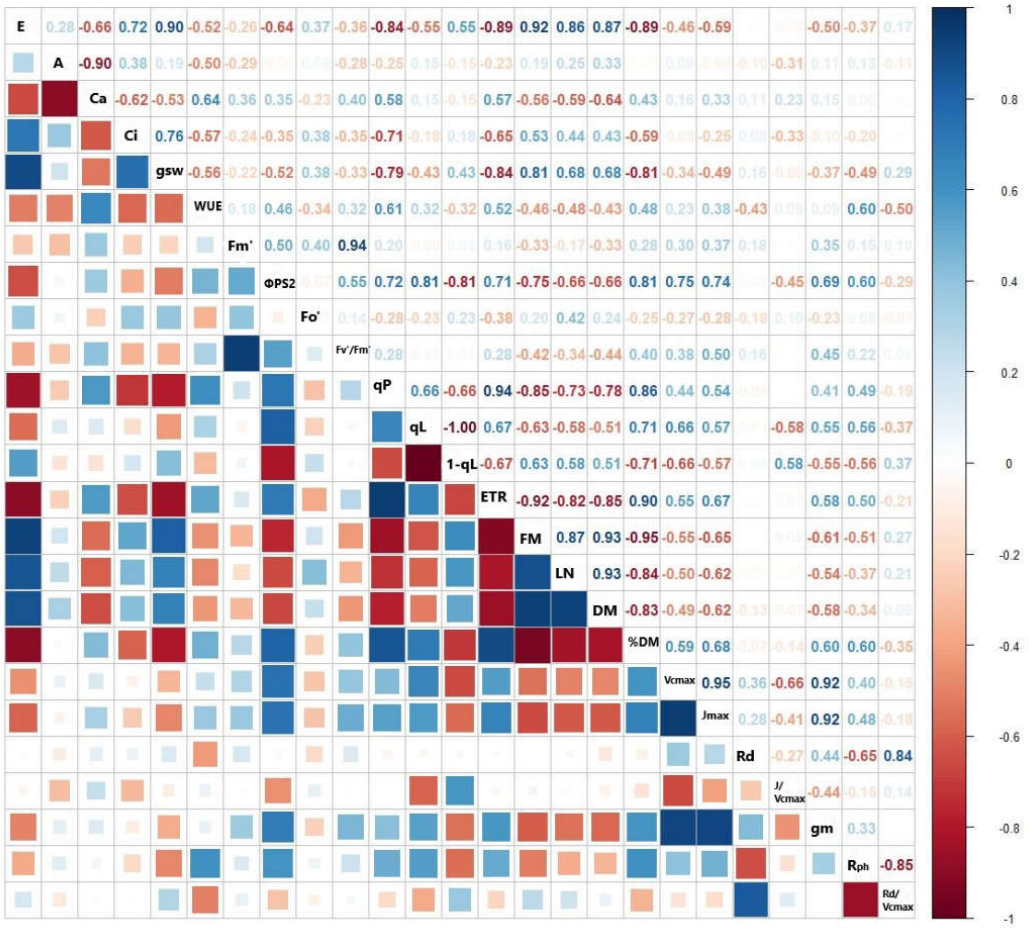

Figure 12. Pearson's correlation matrix of the changes in biomass, gas exchange, and chlorophyll fluorescence parameters of the lettuce genotype under control and salt treatments recorded at 19 days after salt treatment. Dark color represents strong correlations, and light background color represents weaker correlations. Values close to zero indicate no correlation, and values close to one indicate a strong correlation (positive – red and negative – blue) between two parameters. Larger the size of the box with dark color, lower is the significance level of correlation coefficient significance at $P \leq 0.05$.

3. Discussion

Salt stress (SS) is a primary abiotic stress limiting plant growth and productivity across many parts of the world, particularly in countries with irrigated agriculture, owing to increasing soil salinization as a result of poor agricultural practices and the use of low-quality water for irrigation [10]. Because of the disruption in plant cell turgor caused by SS, nutrient transport inside the plants is also significantly impacted [41]. SS also disrupts crop food/feed yield output and quality owing to a variety of primary (osmotic stress, decreased nutrient absorption and development) and more complicated secondary salt-induced physiological disbalances [42–44]. Salt stress reduces marketable yield and profit by increasing unmarketable production of leaves, especially in leafy vegetables, with no economic value [45]. Our previous study on screening the 38 lettuce genotypes under SS condition classified 3 as highly tolerant lettuce genotypes, 9 as moderately tolerant, 15 as less tolerant (moderately susceptible) and 10 as highly salt susceptible based on the total salt tolerance coefficient index [32]. Based on these results, it is very crucial to understand the limitation in the photosynthetic traits caused due to SS. In the present study, we evaluated a salt susceptible lettuce cultivar "Green Forest" under four different salt concentrations in two different growth stages (11 days (GS1) and 19 days (GS2) after the salt treatment). The stomatal and non-stomatal limitations, and different gas exchange and chlorophyll fluorescence traits under different SS levels were investigated.

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The intrinsic effect of SS was revealed at the stomatal capacity and stomatal conductance levels (Figure 2 and 3). SS has the great impact on the leaf photosynthesis which is accompanied by stomatal closure, lower intercellular CO_2 concentration (C_i) and several other non-stomatal properties [46]. Because of the accumulation of Na+ or Cl- in leaves, salt-sensitive plants exposed to high salt stress levels experience a reduction in leaf photosynthesis, owing to a disturbance in C metabolic pathways and redox reactions in the thylakoid membranes, as well as in the Calvin cycle [47]. In detail, the effect of salinity on plant physiology and development is divided into two stages [48]. Because Na^+ and Cl^- that enter the xylem are gathered in the vacuoles, the meristems continue to grow by feeding through the phloem and SS have no effect on plant growth in the initial phase [48]. Only the growth of leaves and roots is visible during this period. As NaCl build up in plant tissues with suppression of K^+, cells are unable to store them in vacuoles, causing the concentration in the cytoplasm to rise and the physiological function and functions of numerous enzymes to be severely reduced [48]. Also, physiological performance of plant is affected as we demonstrated in the current study. In our study, we revealed that SS rigorously affected all the gas parameters except for the CO_2 assimilation rate (*A*) and cause in decline of *E* by up to 51% and 86% in GS1 and GS2 respectively. The similar declining trend was followed by stomatal conductance (*gsw*) and C_i which was 31% to 96%. The decline in *gsw* is a common response of a plant when subjected to SS. Similarly, the interruption in the physiological performance takes place when a plant is exposed to SS. For instance, stomatal guard cells limit the stomatal conductance to reduce the transpiration rate which consequently resulted in the impairment of gas exchange parameters [49]. Also, the CO_2 assimilation rate, *A*, in general declines under the progressive salt stress which has been reported in lettuce grown under salt environment [50]. However, the value of *A* remained unaffected during the GS1 and GS2 with increased SS level in the current study which might challenge the previous finding on salt susceptible lettuce under SS. Meanwhile, in the current study, it is imperative to note that the intrinsic water use efficiency (WUE) increased with the progressing salt levels. It has been reported that higher WUE is critically determined based on the lower stomatal density or *gsw* in the leaves [51] which is often observed in plants exposed to salt or drought stress condition [41,52]. In the current study, the linear decline in gsw strongly correlated with the lower stomatal density resulting in the increased WUE up to 124% in salt stressed lettuce which also coincide with the result from the study in basil reported by [53]. Similarly, a study conducted on *E. myrtifolia* and *C. citrinus* reported that plant imposed to salt stress were able to increase the WUE throughout the growing season by maintaining *A* despite the reduced stomatal opening [31,54]. In addition, the findings by Munns and Tester [18] and James et al. [55] suggested that the rate of *A* per unit leaf area often remained unchanged in the salt-treated plants even though gsw is reduced. Thus, these findings suggest that the unaltered value of *A* in the "Green Forest" lettuce cultivar was largely caused due to increased WUE with increased SS levels rather than damage occurred in other photosynthetic apparatus like gsw. Taking together the chlorophyll fluorescence traits, these three parameters (*A*, WUE, and *gsw*) could be considered the major indicator of photosynthetic disturbance in lettuce under salt stress. The current study also revealed decreased *E*, *gsw*, and *Ci* and increased WUE which suggested an essential contribution towards the non-stomatal limitation of unaltered *A* [56]. The A/C_i curve further demonstrated that observed variability between curves doesn't co-relate with the variation in different level of SS with increasing C_i in both growth stages. This result stand with unaltered *A* in the gas exchange measurement in the current study. The possible reason could be the either photochemical or biochemical reaction that occurred and effectively fixed the internal CO_2 in salt stressed plants [57]. With this excitation, *A*/*Ci* curve remained high even for the plants treated with salt and remained comparable with control plants. Also, the fact that *A* didn't change significantly in response to salt stress could be attributed to the fact that the degree of salt stress was not severe enough to limit the net assimilation rate in lettuce plants.

In addition to unaltered *A* caused by increased WUE, there are several other stomatal (stomatal limitation (L_s)) and non-stomatal factors like maximum rate of Rubisco

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carboxylation (V_{cmax}), maximum rate of photosynthetic electron transport (J_{max}), leaf respiration in the light, also called 'day respiration' (R_d), total conductance between intercellular spaces and chloroplast (g_m). In the current study, L_s increased by two to three-fold in 150 mM NaCl salt stressed lettuce in both growth stages compared to control treatment. In support to our result, there are several studies which stated that osmotic stress and ion accumulation in the guard cells caused due to SS cause the disturbance of stomatal regulation, increasing the stomatal limitation in plants [58–60]. Also, a study on C_3 plant stated that L_s is enhanced with the NaCl stress and water stress [61,62]. Besides, during GS1, the unaltered *A* seems to correspond with the increased V_{cmax} and J_{max} which are considered to represent the carboxylation and regeneration of Rubisco and mitochondrial day respiration, respectively. Thus, our study revealed that during early stage of salt treatment (GS1), the unaltered *A* in response to SS could be due to interactive balance among V_{cmax} and J_{max}, in order contribute in ATP during photosynthesis [26,27]. On the contrary, it is interesting that V_{cmax} and J_{max} in lettuce under SS during GS2 reduced even under the unaltered *A*. In general, the decrease in *A* in plants under SS attributed to the decline in carboxylation and amount of active Rubisco resulting in decreased V_{cmax} and J_{max} [26,63] which supports the V_{cmax} and J_{max} response during the GS2. Therefore, it is apparent that the initial and total rubisco activity and photosynthetic mechanism in plants is not solely dependent upon the net assimilation rate (*A*), but also in several other non-stomatal factors V_{cmax} and J_{max} [64]. Consequently, mesophyll conductance (g_m) has lately been included in global carbon cycle models, as its absence would result in severe underestimating of V_{cmax} and J_{max}, as well as gross primary productivity [65–68]. In the current study, the downgrade of g_m with increase in the salt levels at both growth stages suggested that SS induced the irreversible decrease of g_m, which in turn can be responsible for the transient reduction in the V_{cmax} and J_{max} during GS2. Similar result was reported in a study in spinach leaves under SS [69]. Also, g_m reduction is also associated with the impairment of photochemical characteristics and anatomical changes of the leaf caused by accumulation of salt result in the 25% reduction of intercellular spaces in the mesophyll [69,70]. In turn, SS ultimately can reduce structure, and size of leaves as shown in the Figure 13. R_d, on the other hand, remained unaffected under SS during GS1 and the day respiration activity was unclear during the GS2. In support, unaffected response of R_d under SS has been reported in a study in tomato [63].

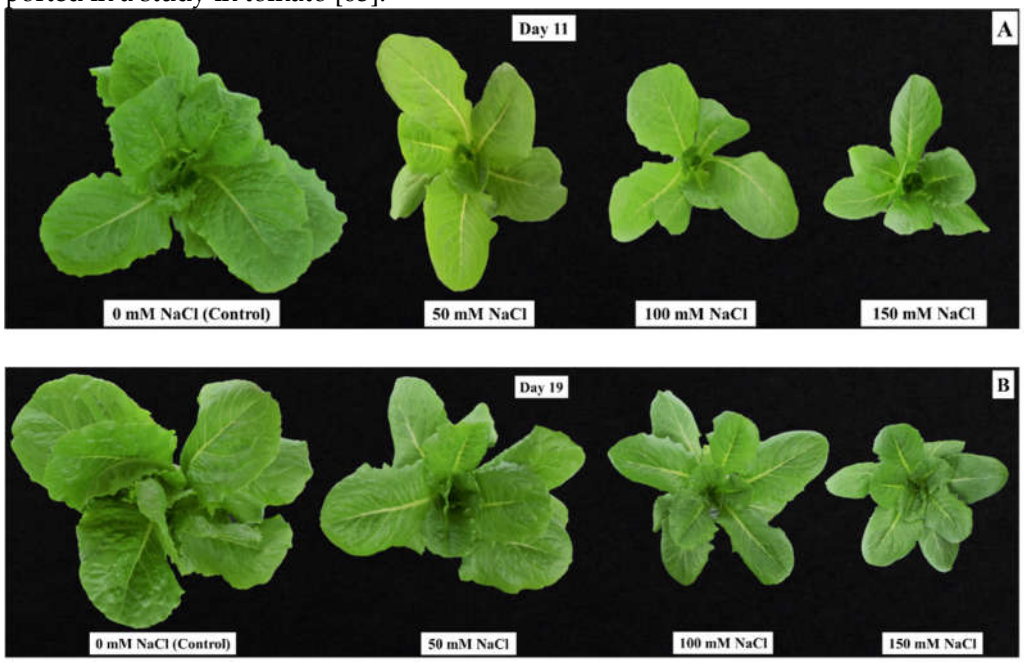

Figure 13. Visual differences in the morphological traits of lettuce cultivar treated with different salt level treatments at different growth stages ((A) 11 days after the salt treatment and (B) 19 days after the salt treatment).

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There has been several studies in evaluating the salt stress effect and how Na^+ and Cl^- can alter the entire photosynthetic system and thereby affecting the chlorophyll fluorescence traits in several crops like barley [49], cotton [46], tomato [71] and lettuce [72]. The measurements of chlorophyll fluorescence parameters were attempted to evaluate the direct effects of SS on PSII photochemistry. Result demonstrated that the light adapted minimal (F'_o) and maximal fluorescence (F'_o) and maximal quantum efficiency of PSII (F'_v/F'_m) were unaffected at GS1 and GS2 during the time course of salt treatment. Similar results have been reported in the study conducted in cowpea [73], cotton [74], celery [75] and herbaceous crops [76]. On the other hand, to investigate the possible changes in PSII phytochemistry under SS, the effective quantum yield of PSII (ΦPSII), electron transport rate (ETR), photochemical quenching of fluorescence (qP), fraction of PSII centers in the open state with plastoquinone oxidized (qL), and redox state of plastoquinone pool) (1-qL) were also investigated. The result revealed no impact of SS on ΦPSII as the value of ΦPSII under SS (0.65 and 0.55) was comparable to control 0.67 and 0.57) during GS1 and GS2 respectively. This revealed that ΦPSII remained stable even under the SS. At the same time, the current study revealed the high correlation (0.94-0.97) of ETR, qP, and qL with the progressing salt levels (Na^+ accumulation) in leaves which coincides with the study in potato [77]. These results were consistent with the result demonstrated for spinach [78] and maize [79]. In general, the ETR is inhibited with the higher level of salt as stated by Baker [80]. The present study is inconsistent with the findings by Baker [80]. The situation could be balance created by K^+ ion against Na^+ and the small change in total monovalent ions would not be expected to inhibit the ETR [78,80,81].

4. Materials and Methods

4.1. Experimental Site, growth condition, and plant materials

This experiment will be conducted in a greenhouse at North Mississippi Research and Extension Center, Verona, Mississippi State University (34°09'53.2"N 88°43'28.5"W with elevation around 100 m) from October to December 2021. One lettuce cultivar with moderate susceptibility to salt was selected for this study [32]. 'Green Forest' (GF) seeds were purchased from Johnny's (Johnny's selected seeds, Fairfield, ME, USA). The seeds will be first sown in the rockwool (3.81 cm × 3.81 cm × 3.81 cm; Roermond, The Netherlands) and germinated in the growth chamber with 18/22 °C Day/night temperature with 16 hours of photoperiod. The daily light intensity reading of photosynthetically active radiation inside the growth chamber will be measured using 50 ppm of Nitrogen was supplied after 10 days after sowing as supplemental nutrients using 5:11:26 hydroponic special fertilizer (Peters Professional, Summerville, SC, USA). The 30 days old lettuce will be then transplanted into a deep-water culture hydroponic system containing 10 liter 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 will comprise (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 pH of the fertilizer solution will be adjusted to 5.8-5.9. The root zone of the plant are evenly distributed into the system along with oxygen supplied to the roots using an air stone [33]. The root zone of the plant will be evenly distributed into the system along with oxygen supplied to the roots using an air stone (Sharma *et al.*, 2018). The plants will be arranged in a randomized complete block design with 4 replications of each treatment (n=3) where individual tub will be representing as one experimental unit. No additional artificial light intensity will be provided, and the light intensity and photoperiod will rely completely on natural photoperiod from sunlight. The photosynthetic photon flux density (PPFD) in the greenhouse will be measured using the LI-190R quantum sensor (Li-Cor, Inc., Lincoln, NE) connected to a CR1000x data logger (Campbell Scientific, Logan, UT) to measure the relative humidity and temperature.

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4.2. Salt treatments

The salt treatment will be incorporated into the hydroponic system approximately 20 days after the transplantation (6-10 leaf stage). Four different levels of salt treatment will be applied: 150 mM, 100 mM, 50 mM, and 0 mM (Control) NaCl with EC levels of 20 mS cm^{-1}, 16 mS cm^{-1}, 8 mS cm^{-1}, and 1.8 mS cm^{-1}. All NaCl treatments will be applied in split (two times) at one-day interval to avoid excess accumulation of salt, avoid osmotic shock and sudden death of plants. Weekly electroconductivity readings with a portable pH/Conductivity meter (Accumet AP85; Fisher Scientific, Hampton, NH, USA) were taken, and growth solutions will be replaced every two weeks along with the adjustment of EC with additional salt. To keep up with the lettuce plants' transpiration losses, water will be supplied to the containers to maintain a 10L level of nutritional solution.

4.3. Response Leaf gas exchange parameters assessment

Measurements on photosynthesis and fluorescence parameters was recorded on young fully expanded leaves on the day 11 and day 19 after the salt treatment. LI-6800 portable photosynthesis system was used for in-situ measurement of CO_2 assimilation rate (A), leaf transpiration rate (E), stomatal conductance (gsw), intercellular CO2 concentration (C_i), and intrinsic water use efficiency (WUE) (Li-Cor Biosciences, Lincoln, NE) at the North Mississippi Research and Extension Center (10:00 – 14:00 CST). Before the values were recorded, the measured leaves were given time to acclimatize to the measurement circumstances. The ratio of A/gsw were used to calculate intrinsic plant WUE [34].

4.4. Measurement of CO_2 response

The CO_2 response curve (A/C_i) measurements were recorded at 11 DAT and 19 DAT using the auto-programming system in LI-6800. The leaf chamber settings were fixed at 600 µmol s^{-1} flow, 70% relative humidity, 490 µmol mol^{-1} CO_2_r, 100 mmol·m^{-2}·s^{-1} PPFD, and a temperature set to mimic ambient greenhouse temperature (20 °C) to measure the steady-state response of A/C_i. CO_2 concentrations were adjusted in steps of 400, 300, 200, 50, 100, 200, 300, 400, 600, 900, 1200, and 1500 µmol mol^{-1} throughout a 50–1500 µmol mol^{-1} range. Before each A/C_i curve measurement, the lettuce leaves were acclimated to chamber conditions for at least 160 seconds (with a minimum and maximum wait time of 60 and 90 seconds, respectively). The A/C_i analysis was performed using the excel fitting tool 10.0 (https://landflux.org/tools) as reported by Sharkey et al. [35].

4.5. Other Stomatal and non-stomatal limitation estimates

The relationship C_i/C_a will be used to compute the internal to external CO_2 ratio. The stomatal limitation was calculated (L_s) was calculated using the internal to external CO_2 ratio using equation 1- C_i/C_a. The total conductance between intercellular spaces and chloroplast (g_m) was estimated using the using the estimated A/Ci response curve. A/Ci curve was further utilized to estimate non-stomatal limitation 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} , stomatal conductance i.e., (g_m), photorespiration (P_r), and R_d/ V_{cmax} according to Bernacchi et al. [36].

4.6. Chlorophyll fluorescence traits measurements

The LI-6800 provided a photosynthetic photon flux density (PPFD) of 100 µmol$m^{-2}s^{-1}$ during day 11 and 450 µmol$m^{-2}s^{-1}$ on day 19 as per to match the chamber environment with the greenhouse environment. Similarly, the chamber environment was set to CO_2 concentration of 490 µmolmol^{-1} with a 50 percent relative humidity for both harvest days. The measuring chamber temperature was maintained at 22°C, which corresponds to the daylight temperature on both harvest days. In the light, the quantum efficiency of oxidized (open) PSII reaction centers was calculated as (F'_v/F'_m) = (F'_m-F'_o)/F'_m [37],where F'_o= minimal fluorescence, which was measured at 50 s when all PSII reaction centers were opened, and F'_m = maximal fluorescence of light-adapted leaves. Steady-state fluorescence

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(F_s) was measured using the modulation light settings as recommended for light-adapted leaves. Similarly, F'_m was estimated using the multi-phase flash protocol.

4.7. Statistical Analysis

All data were subjected to analysis of variance (SAS 9.0, SAS Institute Inc., Cary, NC) to determine the treatment effects in different morphological parameters and physiological (leaf gas exchange, chlorophyll fluorescence, stomatal and non-stomatal limitation) traits. Treatment means and differences were separated using Tukey HSD test at P = 0.05. The standard errors of the mean were calculated using the pooled error term from the ANOVA table and presented in the figures as error bars. The experiment was a randomized complete block design with four salt treatments, four replications, and 3 plants in a factorial arrangement. One way analysis of variance (ANOVA) using the generalized linear mixed model (PROC GLIMMIX) was used to assess the effects of salt treatment on the replicated values of morphological, gas exchange, and chlorophyll fluorescence parameters. Treatment was considered the fixed effect while replication was treated as the random effect. A Pearson correlation analysis was utilized to study the relationship between the studied parameters. The correlation chart was plotted using R software (version: 4.1.0 (2021-05-18), RStudio, Inc., Vienna, Austria). Graphs were plotted with GraphPad Prism 9 (version. 9.1.0; GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

In this study, we explored the gas exchange and chlorophyll fluorescence parameters to reveal the key factors influencing leaf carbon fixation and the adaptive mechanism of lettuce genotype under salt stress. After 11 days (GS1) and 19 days (GS2) of salt treatment, the gas exchange parameters (*E*, *gsw*, and C_i) were severely decreased with increased salt stress level during both growth stages. However, the carbon assimilation rate (*A*) remained unchanged under salt treatment. Similarly, the WUE increased under the salt stress environment. This study confirmed that even a sensitive genotype imposed to salt stress can increase the WUE throughout the growing season by maintaining *A* despite the reduced stomatal opening (*gsw*). Moreover, the stomatal and non-stomatal limiting variables showed different response in different harvesting period under salt stress. It is concluded that the increasing salt levels increased stomatal and some non-stomatal limiting factors during GS1 suggesting the enhanced limitation in photosynthetic activity while the there was no such trend observed during GS2. The downgrade of g_m with increase in the salt levels at both growth stages suggested that SS induced the irreversible decrease of g_m, which in turn can be responsible for the transient reduction in the V_{cmax} and J_{max} during GS2. Further studies evaluating the biochemical aspects (chlorophyll, carotenoids, sugars, proline, and plant pigments like phenolics, flavonoids) of lettuce genotype subjected under different salt stress levels are required to justify the physiological results revealed in this study.

Author Contributions: B.A.: conceptualization, methodology, validation, formal analysis, investigation, writing—original draft, writing—review and editing, visualization, methodology, validation, investigation. O.J.O.: methodology, validation, formal analysis, investigation. T.C.B.: conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing review and editing, visualization, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: The project funding is based on work that is supported by the USDA-NIFA Hatch Project under accession number 149210.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Thomas Horgan and Skyler Brazel at the Vegetable Physiology Laboratory for their technical assistance for their help during data collection.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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