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Physiological and Nutraceutical Quality of Green and Red Pigmented Lettuce in Response to NaCl Concentration in Two Successive Harvests

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Abstract: Nutritional eustress such as salinity or nutrient stress applied in soilless systems, is a convenient pre-harvest factor efficient in modulating the phytochemical components of horticultural crops, by triggering defensive mechanisms and accumulating plant secondary metabolites in plants tissues. Nevertheless, genetic material (cultivars with different pigmentation) dictates lettuce metabolites and physiological response to extrinsic eustress, with red leaf cultivars being highly nutrient packed notwithstanding the stress. Product quality can be meliorated equally by applying several cuts, a practice proven to increase bioactive compounds accumulation. In this study, we analyzed the effects of four salinity levels (1, 10, 20 and 30 mM NaCl) on green and red pigmented Salad Bowl lettuce (*Lactuca sativa* L. var. *acephala*) in two successive harvests cultivated in a floating raft system. The morphological parameters, mineral composition, leaf gas exchanges, bioactive compounds, and antioxidant activity of both cultivars were assessed. The green cultivar exhibited superior crop productivity but was more prone to salinity effect than the red cultivar. Irrespective of cultivar and cut order, the net photosynthesis decreased with increasing salinity in the nutrient solution. The second cut incurred higher dry biomass, greater accumulation of most minerals and higher photosynthetic activity. In red lettuce, 20 mM NaCl proved adequate eustress to increase phytonutrients and beneficial minerals (K, Ca, and Mg) with minimal loss of yield. Mild salinity and sequential harvest have proven effective pre-harvest tools in positively modulating the quality of lettuce. Eustress interaction with genotype was demonstrated as a promising field for future breeding programs targeting select genotypes for agronomic application of eustress to improve the nutraceutical value of vegetable crops.

Keywords: antioxidant activity; floating raft system; eustress; electrical conductivity; *Lactuca sativa* L.; leaf gas exchange; functional quality

1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the main leafy green vegetables cultivated worldwide, whose consumption has steadily increased due to its perception as a health-promoting fresh food [1–3]. In particular, baby lettuce leaves with their petiole, at an optimum size of 8–12 cm long, have become

very popular in recent years as minimally processed or fresh-cut salad vegetables ready to eat [4,5]. They represent an innovative produce corresponding to the lifestyle of modern consumers [2]. Indicatively, the sector of minimally processed agricultural products in Italy has grown from the early 1980s to 2019 by about 400%, with approximately 19.4 million regular consumers in 2017 [6]. In addition, Italy is the European leader of leafy green production with about 15,000 ha under greenhouse conditions located mainly in Campania, Veneto, and Lombardia regions [6].

Lettuce has a low calorific value and supplies a plethora of antioxidant compounds, such as vitamins C (ascorbic acid), E and folate (vitamin B_9), chlorophylls, carotenoids and polyphenols, in addition to micro- and macro-nutrients and fiber [3,7–12]. There is growing evidence that these secondary bioactive metabolites, in particular polyphenols, can act as antioxidants counteracting the post-harvest decay of lettuce tissues and the development off-odors, and also as nutraceuticals with a preventive role against oxidative stress-related diseases, such as cardiovascular diseases, atherosclerosis, neurodegenerative disorders and cancer [3,13–16]. This evidence has attracted the interest of researchers and producers toward ways of enhancing the content of these compounds [3,13–16], including genetic modification and novel agronomic techniques [1]. However, EU policymakers have introduced very restrictive regulations concerning the use of genetically modified organisms, which consequently eliminates this tool from the array of breeders and producers [17]. Therefore, agronomic techniques such as cropping system, nutrient solution management and elicitation factors (e.g., salinity, high CO_2, light intensity, and quality) are the favored methods employed to improve crop performance, to enhance the nutritional and nutraceutical qualities of products and extend their post-harvest life [14,18–21].

Recent studies on the application of mild stresses (i.e., salt or nutrient stress) to crop plants provide evidence to the existence of stress-related plasticity responsible for increased synthesis and accumulation of protective secondary metabolites that counteract stress [22–25]. These secondary metabolites, mostly deriving from phenylpropanoid and shikimate pathways, are able to scavenge reactive oxygen species (ROS), promote lignification and herbivore protection, and act as disease and stress signals [15]. For a positive stress (eustress) to increase the content of beneficial metabolites without significant detriment to plant growth and especially yield, a compromised level of stress must be found [19,26]. Salinity at an optimal mild dose (up to 6.0 dS m^{-1}, equivalent to about 60 mM NaCl) has been found to improve the content of ascorbic acid, α-tocopherol and the antioxidant activity in *Cichorium spinosum* without significant yield reduction [27]. Neocleous et al. [28] also found that 10 mM NaCl strongly increased ascorbic acid content in two baby lettuce cultivars (green "Paris Island" and red "Sanguine"). Leafy vegetable *Amaranthus tricolor* showed tremendous augmentation of carotenoids, ascorbic acid, total phenols, total flavonoids, phenolic acids, flavonoid compounds, and antioxidant activity at 100 mM NaCl stress with minimal yield reduction [29–31]. Furthermore, Colla et al. [32] found that by adding 30 mM of sodium chloride (NaCl) to the nutrient solution of cultured artichoke and cardoon, the leaf content of total phenolics, chlorogenic acid, cynarine and luteolin and the antioxidant activity increased. Long-term irrigation with 5 mM NaCl salt concentration was found to increase lutein (+37%) and β-carotene (+80%) in romaine lettuce without altering the visual quality or decreasing yield [15]. More recently, Rouphael et al. [18] showed, in a greenhouse study on green and red pigmented perilla, that the application of 10 mM NaCl enhanced the content of polyphenols while decreasing that of nitrate (an antinutrient); moreover, key aroma compounds increased, particularly in green perilla, following the application of 20 mM NaCl to the nutrient solution. The above evidence indeed highlights the motto of Paracelsus "dose makes the poison" [18].

Since the application of NaCl under soil conditions poses a high risk of plant overstress, soilless cultivation, particularly the floating raft system, may be an efficient tool to apply controlled eustress for enhancing plant secondary metabolism and sensory/functional product quality, provided the proper management of nutrient solution composition and concentration [4]. In addition, soilless cultivation presents numerous advantages over soil-based cultivation regardless of eustress application. In particular, (i) multiple cultivation cycles can be facilitated throughout the year; (ii) soil related pathogenic and abiotic stress conditions are alleviated; (iii) high density planting can be applied

without weed control pressure; (iv) labor requirements are reduced; (v) products free of soil-particles and other debris can be readily harvested [24,33–36].

Another agronomic factor widely adopted for leafy greens that may modulate their secondary metabolic profile and nutraceutical quality could be sequential harvesting. The cut number in sequential harvesting may lead to physiological changes that affect plant metabolism. In a previous work on basil Nicoletto and co-workers [37] demonstrated that several harvests/cuts (3 or 4) of the apical part of the plant during the growth cycle may strongly enhance the phenolic content. Therefore, the application of sequential harvesting merits appraisal as a potential tool to modulate the nutraceutical quality of lettuce.

To our knowledge, the application of salinity as eustress in combination with successive harvests to modulate physiological and biochemical plant parameters has never been tested. Accordingly, in our study we analyzed the effects of four salinity levels (1, 10, 20 and 30 mM NaCl) on green and red pigmented lettuce cultivars in two successive harvests with respect to morphological parameters, mineral composition, leaf gas exchange, bioactive compounds, and in vitro antioxidant activity.

2. Materials and Methods

2.1. Growth Conditions, Lettuce Cultivars, Experimental Design and Salt Application

The experimental work was carried out during the spring-summer 2015 growing cycle in a glasshouse at the experimental station 'Torre Lama Dipartimento di Agraria' (Bellizzi, Salerno 40°37′00″ N 14°57′00″ E). Two lettuce (*Lactuca sativa* L. var. *acephala*) varieties with different leaf pigmentations: 'Green Salad Bowl' and 'Red Salad Bowl' (SAIS seed company, Cesena, Italy) were used as tested crop. The two lettuce varieties were sown on 22 March in polystyrene trays (190 holes) with a planting density of 1025 plants per square meter. Eleven days after sowing (DAS) lettuce plants were moved to a floating raft system. The system consisted of polystyrene plug trays, floating in wood tanks with a constant volume (150 L) of fresh nutrient solution. The dissolved oxygen concentration in the nutrient solution was always higher than 6 mg L^{-1}. The experimental design was full factorial, with three factors: "cultivar" (CV) with two levels (i.e., green and red lettuce), "salinity" (S) four levels, namely 1, 10, 20, and 30 mM NaCl and "harvest" (cut 1 and cut 2). The concentration of the macro and microelement in the non-saline nutrient solution was: 12.0 mM nitrate, 1.0 mM ammonium, 1.75 mM sulfur, 1.5 mM phosphorus, 5.0 mM potassium, 4.0 mM calcium, 1.5 mM magnesium, 1.0 mM sodium, 1.0 mM chloride, 20 μM iron, 9 μM manganese, 0.3 μM copper, 1.6 μM zinc, 20 μM boron, and 0.3 μM molybdenum, with an electrical conductivity (EC) of 1.9 dS m^{-1}. The concentration of the macronutrients and micronutrients in the saline solutions were the same of the non-saline (NS) plus an additional 10, 20 and 30 mM NaCl. The EC of the 10, 20 and 30 mM solutions was 2.8, 4.0 and 5.1 dS m^{-1}, respectively. The pH of the nutrient solution for all treatments was 6.0 ± 0.2. The 16 combinatorial treatments were arranged in a randomized complete-block design with three replicates for each cultivar, making a total of 48 experimental units (i.e., plots).

2.2. Sample Preparation

Determinations of total phenolic, antioxidant activity assays (lipophilic and hydrophilic fractions), mineral profile (nitrate, total nitrogen, phosphorus, potassium, calcium, magnesium, sodium, and chloride) were evaluated using lyophilized samples. Whereas, total ascorbic acid was done on liquid nitrogen quenched fresh biomass before being lyophilized.

2.3. Leaf Area, Fresh Yield and Leaf Biomass Determination

The red and green pigmented lettuce were harvested twice: 26 and 47 DAS, when the number of leaves reached approximately 5–6 leaves. In both harvest dates, the red and green pigmented lettuce were directly weighed, and total leaf area was quantified with an electronic area meter (LiCor 3100C model, LI-COR Biosciences, Lincoln, NE, USA). The lettuce fresh yield was recorded for each

experimental unit and expressed in kg per square meter. The lettuce leaf tissues of the two varieties were dried in a forced-air oven at 70 °C for 72 h until constant weight to determine the dry leaf biomass. Leaf dry matter (DM) percentage was calculated using the following formula: 100 × dry leaf biomass/fresh weight.

2.4. Physiological Parameters: SPAD Index and Leaf Gas Exchange

Before the two harvests, the soil plant analysis development (SPAD) index and leaf gas exchange were determined. The SPAD index was quantified on 15 entirely (i.e., healthy) expanded lettuce leaves per experimental unit, by using a portable SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan). The following physiological measurements were determined by an LCA-4; ADC portable gas exchange analyzer (BioScientific Ltd., Hoddesdon, UK) equipped with a 6.25 cm^2 broadleaf chamber: net carbon dioxide assimilation rate, stomatal resistance, and transpiration. The leaf gas exchange parameters were quantified on 12 entirely expanded lettuce leaves per treatment. Physiological (i.e., intrinsic) water use efficiency (WUE) was calculated as the ratio between net photosynthetic CO_2 rate and transpiration.

2.5. Macro and Micro Mineral Content Analysis

The lettuce leaf tissues were analyzed for the following macroelements sodium and chloride: nitrogen (N), nitrate (NO_3), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and chloride (Cl). One gram of lettuce ground material was used for the determination of total N using the micro-Kjeldahl method [38]. Moreover, 0.25 g ground material was also analyzed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) to determine the other minerals content according to the method proposed by Rouphael et al. [39]. The result of nitrate was expressed as mg kg^{-1} fresh weight (fw), while the other macrominerals, sodium and chloride were expressed as g kg^{-1} dry weight (dw).

2.6. Bioactive Compounds and Antioxidant Activity Assays Analysis

The bioactive compounds (total ascorbic acid and total phenols) as well as antioxidant activity assays were assessed by spectrophotometric detection (model Hach DR 2000, Hach Co., Loveland, CO, USA). Total ascorbic acid and total phenols were assessed based on the method proposed by Sarker et al. [40] and Sarker and Oba [41] (i.e., Folin-Ciocalteau procedure), respectively. The absorbance of the solution was read at 525 and 765 nm for total ascorbic acid and total phenols, respectively.

For the determination of the hydrophilic and lipophilic antioxidant activity assays, N, N-dimethyl-p-phenylenediamine (DMPD) method [42] and 2,20-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method [43], respectively, were adopted. Antioxidant capacities were measured by UV-Vis spectrophotometry, at 505 nm, for the hydrophilic fraction, and at 734 nm for lipophilic fraction.

2.7. Statistics

The experimental data were analyzed by the three-way analysis of variance (Cultivar, Salinity and Harvest) using the SPSS software version 21, and the Duncan multiple range test was performed for the mean separations at 0.05 probability level. The loading plot and score plot of all agronomical, physiological and qualitative parameters of red and green pigmented baby lettuce were determined after principal component analysis (PCA) by using Minitab® 18 statistical software (Minitab LLC, State College, PA, USA) [44].

3. Results

3.1. Biometric Measurements

With respect to the biometric variables, significant differences were observed between cultivars for fresh yield, leaf dry biomass and leaf dry matter (DM) content but not for leaf area (Table 1).

Superior crop productivity, dry biomass and leaf DM content were observed in green lettuce (Table 1). NaCl treatments had significant effect on all the morphological parameters. The high NaCl treatment (30 mM NaCl) suppressed both leaf area and fresh yield, while an opposite trend was observed for leaf DM content. Moreover, the 1 and 10 mM NaCl treatments did not differ significantly from each other for leaf area, dry biomass, and leaf DM content (Table 1). Except for leaf area, cut order significantly affected all biometric variables examined: dry biomass and leaf DM content were higher at cut 2, at the expense of marketable fresh yield (Table 1). Finally, irrespective of salt stress treatment (cultivar × cut interaction), the percentage of leaf DM increase caused by cut order was significantly higher in green lettuce (by 14.9%), compared to red lettuce (11.7%; Table 1).

Table 1. Significance of the main factors (cultivar, salinity and cut order) and their interactions on leaf area, total yield, leaf dry biomass and leaf dry matter percentage of *Lactuca sativa* L. var. *acephala* grown in a floating raft culture.

Source of Variance	Leaf Area (cm^2 $plant^{-1}$)	Fresh Yield (kg m^{-2})	Dry Biomass (g m^{-2})	Leaf Dry Matter (%)
Cultivar (CV)				
Green	92.10 ± 2.55	5.59 ± 0.14 a	271.7 ± 6.61 a	4.89 ± 0.10 a
Red	88.37 ± 2.02	4.98 ± 0.13 b	214.3 ± 4.96 b	4.33 ± 0.07 b
Salinity (S)				
1 mM NaCl	99.58 ± 3.16 a	5.82 ± 0.22 a	261.7 ± 14.6 a	4.48 ± 0.13 b
10 mM NaCl	94.92 ± 2.67 a	5.39 ± 0.16 b	247.2 ± 10.2 ab	4.58 ± 0.12 b
20 mM NaCl	88.25 ± 1.69 b	5.20 ± 0.19 b	235.1 ± 10.3 b	4.53 ± 0.16 b
30 mM NaCl	78.18 ± 1.40 c	4.73 ± 0.15 c	228.1 ± 10.1 b	4.83 ± 0.16 a
Cut (C)				
Cut 1	89.20 ± 1.80	5.49 ± 0.14 a	234.9 ± 7.15 b	4.27 ± 0.06 b
Cut 2	91.27 ± 2.74	5.08 ± 0.15 b	251.1 ± 9.12 a	4.94 ± 0.10 a
CV × S				
Green × 1 mM NaCl	101.65 ± 5.37	6.15 ± 0.34	297.0 ± 17.6	4.83 ± 0.13
Green × 10 mM NaCl	98.66 ± 4.55	5.72 ± 0.17	272.2 ± 9.05	4.77 ± 0.15
Green × 20 mM NaCl	89.82 ± 1.71	5.47 ± 0.30	261.0 ± 12.5	4.81 ± 0.25
Green × 30 mM NaCl	78.28 ± 0.91	5.02 ± 0.12	256.8 ± 7.61	5.14 ± 0.26
Red × 1 mM NaCl	97.51 ± 3.68	5.48 ± 0.21	226.4 ± 11.3	4.13 ± 0.10
Red × 10 mM NaCl	91.19 ± 2.25	5.06 ± 0.21	222.2 ± 11.3	4.40 ± 0.18
Red × 20 mM NaCl	86.68 ± 2.94	4.93 ± 0.20	209.2 ± 6.66	4.26 ± 0.15
Red × 30 mM NaCl	78.08 ± 2.79	4.43 ± 0.22	199.4 ± 7.92	4.51 ± 0.11
S × C				
1 mM NaCl × Cut 1	94.94 ± 2.95	5.86 ± 0.34	250.8 ± 19.7	4.26 ± 0.15
10 mM NaCl × Cut 1	92.51 ± 4.24	5.57 ± 0.19	236.5 ± 12.1	4.24 ± 0.10
20 mM NaCl × Cut 1	88.37 ± 3.00	5.49 ± 0.33	228.9 ± 15.4	4.16 ± 0.09
30 mM NaCl × Cut 1	80.98 ± 1.35	5.03 ± 0.13	223.4 ± 9.31	4.43 ± 0.10
1 mM NaCl × Cut 2	104.22 ± 5.17	5.77 ± 0.30	272.5 ± 22.4	4.70 ± 0.18
10 mM NaCl × Cut 2	97.34 ± 3.32	5.22 ± 0.26	257.8 ± 16.4	4.93 ± 0.10
20 mM NaCl × Cut 2	88.14 ± 1.87	4.91 ± 0.10	241.3 ± 14.7	4.90 ± 0.22
30 mM NaCl × Cut 2	75.38 ± 1.91	4.43 ± 0.21	232.8 ± 18.9	5.22 ± 0.22
CV × C				
Green × Cut 1	89.09 ± 3.00	5.81 ± 0.20	260.6 ± 8.31	4.49 ± 0.05 b
Green × Cut 2	95.11 ± 4.06	5.37 ± 0.19	282.8 ± 9.54	5.28 ± 0.11 a
Red × Cut 1	89.31 ± 2.15	5.17 ± 0.14	209.2 ± 4.98	4.06 ± 0.05 c
Red × Cut 2	87.43 ± 3.50	4.78 ± 0.20	219.4 ± 8.58	4.60 ± 0.07 b

Table 1. *Cont.*

Source of Variance	Leaf Area (cm^2 $plant^{-1}$)	Fresh Yield (kg m^{-2})	Dry Biomass (g m^{-2})	Leaf Dry Matter (%)
Significance				
Cultivar (CV)	ns	***	***	***
Salinity (S)	***	***	**	***
Cut (C)	ns	**	*	***
CV × S	ns	ns	ns	ns
S × C	ns	ns	ns	ns
CV × C	ns	ns	ns	*
CV × S × C	ns	ns	ns	ns

ns, *, **, *** Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters indicate statistically different groups ($p < 0.05$, Duncan's post hoc test following ANOVA; $n = 3$).

3.2. Leaf Mineral Profile

The cut order was the only factor with significant effect on all analyzed minerals (Table 2). Cultivar and cut were the only factors with a significant effect on leaf total N, which was higher in the red cultivar than in the green and in the first cut than in the second one (Table 2). Similarly, to total N, phosphorus content was also higher in red lettuce than in green cultivar and in the first cut than in the second one. Increasing the NaCl concentration from 1 to 30 mM in the nutrient solution incurred a significant decrease in the phosphorus content with no significant differences between 1 and 10 mM NaCl treatments (Table 2). Potassium was significantly affected by cultivar × salinity and cultivar × cut interactions. In particular, in green lettuce the concentration of K in leaf tissue decreased as the salinity in the nutrient solution increased from 10 to 30 mM NaCl with the lowest values recorded at 20 and 30 mM; whereas, the concentration of K remained unchanged in red pigmented lettuce under salt stress conditions. Averaged over salt treatment levels, red lettuce was able to accumulate more potassium than the green lettuce (+91% and +30% in cut 1 and cut 2, respectively) (Table 2). The concentrations of bivalent cations Ca and Mg in leaf tissue were significantly affected by cultivar × salinity and cultivar × cut interactions (Table 2). When averaged over cut order (cultivar × salinity interaction), the percentage of Ca and Mg reduction in leaf tissue caused by salinity was significantly lower (17.0–35.1% and 17.1–29.5% for Ca and Mg, respectively) in the red lettuce cultivar compared to those recorded in the green pigmented variety (13.8–53.8% and 18.4–44.0% for Ca and Mg, respectively; Table 2). Moreover, irrespective of lettuce cultivar (salinity × cut interaction), the highest concentrations of Ca and Mg were recorded in the 1 mM cut 2 treatment followed by lettuce plants fertigated with 10 mM NaCl and harvested 47 DAS (cut 2; Table 2).

The concentrations of both toxic ions (Na and Cl) were not influenced by the cultivar × cut interaction, but by the cultivar × salinity and salinity × cut (only for Na) interactions (Table 2). The highest Na content was attained in response to the high NaCl treatment. The mean cultivar Na content increased by 45.1% at cut 2 relative to cut 1; however, a cultivar × cut interaction revealed that the increase incurred by the red lettuce was 266.7–687%. On the other hand, in green lettuce a significant increase (330.1–662.8%) was also noted depending on the NaCl concentration in the nutrient solution (Table 2). Similarly, to Na, the highest Cl concentration was attained in response to the high NaCl treatment (30 mM). The mean cultivar Cl content was higher in the red cultivar than in the green one; however a salinity × cut interaction revealed that the increase incurred by the red pigmented lettuce was significantly higher at cut 2 compared to cut 1 (Table 2).

Table 2. Significance of the main factors (cultivar, salinity and cut order) and their interactions on leaf mineral composition (total nitrogen [N], phosphorus [P], potassium [K], calcium [Ca], magnesium [Mg], sodium [Na], chloride [Cl]) of *Lactuca sativa* L. var. *acephala* grown in a floating raft culture.

Source of Variance	N (g kg^{-1} dw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)	Cl (g kg^{-1} dw)
Cultivar (CV)							
Green	40.51 ± 0.32 b	13.80 ± 0.37 b	38.11 ± 2.13 b	7.68 ± 0.61 a	3.02 ± 0.21	14.89 ± 1.78	22.82 ± 2.05 b
Red	41.54 ± 0.52 a	14.63 ± 0.24 a	59.61 ± 0.94 a	6.75 ± 0.37 b	3.02 ± 0.17	13.23 ± 1.67	26.54 ± 2.05 a
Salinity (S)							
1 mM NaCl	40.72 ± 0.52	15.27 ± 0.34 a	53.02 ± 2.78 a	9.56 ± 0.63 a	3.87 ± 0.32 a	2.98 ± 0.29 d	10.00 ± 0.88 d
10 mM NaCl	40.93 ± 0.52	14.56 ± 0.34 ab	52.31 ± 2.48 a	8.10 ± 0.52 b	3.18 ± 0.19 b	11.91 ± 1.05 c	23.43 ± 0.88 c
20 mM NaCl	40.94 ± 0.92	14.14 ± 0.43 b	45.91 ± 4.69 b	6.02 ± 0.55 c	2.60 ± 0.21 c	18.31 ± 1.34 b	30.01 ± 0.94 b
30 mM NaCl	41.53 ± 0.49	12.90 ± 0.46 c	44.21 ± 4.83 b	5.18 ± 0.39 d	2.42 ± 0.10 c	23.03 ± 1.24 a	35.28 ± 1.20 a
Cut (C)							
Cut 1	42.16 ± 0.31 a	15.00 ± 0.28 a	47.83 ± 3.60 b	5.89 ± 0.43 b	2.49 ± 0.13 b	11.47 ± 1.25 b	24.09 ± 1.71 b
Cut 2	39.90 ± 0.43 b	13.43 ± 0.29 b	49.90 ± 1.56 a	8.54 ± 0.43 a	3.54 ± 0.18 a	16.64 ± 1.96 a	25.27 ± 2.40 a
CV × S							
Green × 1 mM NaCl	39.55 ± 0.66	15.19 ± 0.58	44.48 ± 2.05 b	10.74 ± 0.73 a	4.12 ± 0.39 a	3.15 ± 0.52 e	8.45 ± 0.70
Green × 10 mM NaCl	40.72 ± 0.54	13.99 ± 0.51	45.26 ± 1.69 b	9.25 ± 0.48 ab	3.36 ± 0.24 abc	13.55 ± 1.17 cd	21.33 ± 0.90
Green × 20 mM NaCl	41.01 ± 0.67	14.01 ± 0.75	32.32 ± 4.43 c	5.77 ± 1.00 d	2.29 ± 0.37 d	18.82 ± 2.18 b	27.99 ± 1.42
Green × 30 mM NaCl	40.76 ± 0.68	12.02 ± 0.61	30.38 ± 4.44 c	4.96 ± 0.69 d	2.29 ± 0.14 d	24.03 ± 1.99 a	33.51 ± 2.03
Red × 1 mM NaCl	41.88 ± 0.46	15.34 ± 0.40	61.57 ± 0.85 a	8.38 ± 0.82 bc	3.62 ± 0.52 ab	2.80 ± 0.29 e	11.54 ± 1.39
Red × 10 mM NaCl	41.13 ± 0.93	15.13 ± 0.36	59.36 ± 2.08 a	6.95 ± 0.66 cd	3.00 ± 0.30 bcd	10.27 ± 1.55 d	25.53 ± 0.92
Red × 20 mM NaCl	40.86 ± 1.80	14.26 ± 0.47	59.49 ± 1.78 a	6.26 ± 0.55 d	2.90 ± 0.15 bcd	17.80 ± 1.76 bc	32.04 ± 0.52
Red × 30 mM NaCl	42.29 ± 0.60	13.77 ± 0.51	58.03 ± 2.57 a	5.40 ± 0.43 d	2.55 ± 0.13 cd	22.04 ± 1.55 ab	37.05 ± 0.96
S × C							
1 mM NaCl × Cut 1	41.36 ± 0.64	16.15 ± 0.28	52.91 ± 4.70	7.95 ± 0.70 bc	2.92 ± 0.26 c	3.56 ± 0.38 f	12.12 ± 1.13 e
10 mM NaCl × Cut 1	41.91 ± 0.47	15.26 ± 0.22	53.78 ± 4.36	6.96 ± 0.69 cd	2.62 ± 0.15 cd	9.06 ± 1.08 e	23.68 ± 1.66 d
20 mM NaCl × Cut 1	42.99 ± 0.75	14.91 ± 0.53	42.82 ± 9.09	4.32 ± 0.35 e	2.14 ± 0.32 d	14.07 ± 0.67 d	28.33 ± 1.51 c
30 mM NaCl × Cut 1	42.38 ± 0.53	13.66 ± 0.58	41.80 ± 9.47	4.32 ± 0.57 e	2.28 ± 0.17 d	19.19 ± 0.38 c	32.22 ± 1.51 b
1 mM NaCl × Cut 2	40.07 ± 0.78	14.38 ± 0.33	53.14 ± 3.47	11.16 ± 0.50 a	4.82 ± 0.12 a	2.39 ± 0.29 f	7.87 ± 0.53 f
10 mM NaCl × Cut 2	39.94 ± 0.75	13.87 ± 0.52	50.84 ± 2.68	9.24 ± 0.46 b	3.74 ± 0.09 b	14.75 ± 0.66 d	23.18 ± 0.81 d
20 mM NaCl × Cut 2	38.89 ± 1.20	13.36 ± 0.52	48.99 ± 3.18	7.71 ± 0.23 c	3.05 ± 0.10 c	22.55 ± 0.56 b	31.69 ± 0.70 b
30 mM NaCl × Cut 2	40.68 ± 0.70	12.13 ± 0.60	46.62 ± 3.27	6.05 ± 0.23 d	2.56 ± 0.08 cd	26.88 ± 0.85 a	38.34 ± 0.53 a

Table 2. *Cont.*

Source of Variance	N (g kg^{-1} dw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)	Cl (g kg^{-1} dw)
CV × C							
Green × Cut 1	41.43 ± 0.34	15.01 ± 0.40 a	32.84 ± 3.53 d	6.19 ± 0.82	2.48 ± 0.23	12.27 ± 1.70	21.20 ± 2.24
Green × Cut 2	39.60 ± 0.40	12.59 ± 0.40 b	43.39 ± 1.19 c	9.17 ± 0.69	3.55 ± 0.29	17.51 ± 3.01	24.43 ± 3.48
Red × Cut 1	42.89 ± 0.43	14.98 ± 0.40 a	62.81 ± 0.88 a	5.58 ± 0.32	2.50 ± 0.11	10.67 ± 1.88	26.98 ± 2.40
Red × Cut 2	40.20 ± 0.78	14.28 ± 0.26 a	56.41 ± 1.01 b	7.92 ± 0.48	3.54 ± 0.23	15.78 ± 2.63	26.10 ± 3.43
Significance							
Cultivar (CV)	*	**	***	***	ns	ns	***
Salinity (S)	ns	***	***	***	***	***	***
Cut (C)	***	***	*	***	***	***	*
CV × S	ns	ns	***	***	***	*	ns
S × C	ns	ns	ns	**	***	***	***
CV × C	ns	***	***	ns	ns	ns	ns
CV × S × C	ns	ns	***	**	ns	ns	ns

ns, *, **, *** Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters indicate statistically different groups ($p < 0.05$, Duncan's post hoc test following ANOVA; $n = 3$).

3.3. SPAD Index and Leaf Gas Exchange

The SPAD index was significantly affected by cultivar, salinity and cut order but not by their interactions (Table 3). The SPAD index values were higher in the red cultivar than in the green one and in the first cut than in the second one. Increasing the NaCl concentration in the nutrient solution reduced the SPAD index with the lowest values recorded with 30 mM NaCl (Table 3).

Table 3. Significance of the main factors (cultivar, salinity and cut order) and their interactions on SPAD index, net photosynthetic CO_2 assimilation rate[A_{CO2}], stomatal resistance [r_s], transpiration [T] and intrinsic water use efficiency [WUE_i] of *Lactuca sativa* L. var. *acephala* grown in a floating raft culture.

Source of Variance	SPAD Index	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	r_s (m^2 s^{-1} mol^{-1})	T (mol H_2O m^{-2} s^{-1})	WUEi (μmol CO_2 mol^{-1} H_2O)
Cultivar (CV)					
Green	16.53 ± 0.30 b	7.82 ± 0.25 a	3.96 ± 0.21 b	6.41 ± 0.16 a	1.24 ± 0.04 a
Red	18.66 ± 0.43 a	5.20 ± 0.21 b	4.83 ± 0.26 a	5.43 ± 0.12 b	0.95 ± 0.03 b
Salinity (S)					
1 mM NaCl	19.34 ± 0.51 a	7.97 ± 0.38 a	3.32 ± 0.22 c	6.33 ± 0.27 a	1.29 ± 0.07 a
10 mM NaCl	18.14 ± 0.55 b	6.88 ± 0.37 b	3.96 ± 0.25 bc	6.03 ± 0.20 a	1.14 ± 0.05 b
20 mM NaCl	17.31 ± 0.43 b	6.04 ± 0.38 c	4.61 ± 0.26 b	6.01 ± 0.20 a	0.99 ± 0.04 c
30 mM NaCl	15.60 ± 0.39 c	5.15 ± 0.33 d	5.83 ± 0.35 a	5.30 ± 0.17 b	0.97 ± 0.05 c
Cut (C)					
Cut 1	18.14 ± 0.44 a	6.19 ± 0.31 b	4.56 ± 0.21 a	5.47 ± 0.12 b	1.11 ± 0.05
Cut 2	17.06 ± 0.40 b	6.83 ± 0.27 a	4.20 ± 0.26 b	6.37 ± 0.17 a	1.08 ± 0.04
CV × S					
Green × 1 mM NaCl	18.05 ± 0.41	9.53 ± 0.24	3.12 ± 0.38 d	6.78 ± 0.46	1.47 ± 0.09
Green × 10 mM NaCl	16.83 ± 0.47	8.00 ± 0.49	3.65 ± 0.44 cd	6.46 ± 0.28	1.26 ± 0.08
Green × 20 mM NaCl	16.19 ± 0.27	7.31 ± 0.40	4.13 ± 0.31 bcd	6.43 ± 0.26	1.14 ± 0.04
Green × 30 mM NaCl	15.07 ± 0.53	6.46 ± 0.35	4.92 ± 0.33 bc	5.97 ± 0.18	1.10 ± 0.08
Red × 1 mM NaCl	20.64 ± 0.54	6.41 ± 0.33	3.48 ± 0.26 d	5.87 ± 0.24	1.10 ± 0.06
Red × 10 mM NaCl	19.46 ± 0.64	5.76 ± 0.33	4.27 ± 0.15 bcd	5.60 ± 0.22	1.03 ± 0.05
Red × 20 mM NaCl	18.42 ± 0.50	4.76 ± 0.37	5.38 ± 0.34 b	5.60 ± 0.26	0.83 ± 0.05
Red × 30 mM NaCl	16.12 ± 0.52	3.85 ± 0.18	7.25 ± 1.30 a	4.64 ± 0.09	0.83 ± 0.04
S × C					
1 mM NaCl × Cut 1	19.44 ± 0.94	7.75 ± 0.57 a	3.51 ± 0.18	5.95 ± 0.28	1.30 ± 0.07
10 mM NaCl × Cut 1	19.00 ± 0.80	6.56 ± 0.59 bc	4.08 ± 0.16	5.71 ± 0.25	1.16 ± 0.10
20 mM NaCl × Cut 1	18.08 ± 0.63	5.07 ± 0.50 d	5.38 ± 0.38	5.23 ± 0.16	0.95 ± 0.08
30 mM NaCl × Cut 1	16.03 ± 0.47	5.38 ± 0.57 cd	6.04 ± 0.47	4.99 ± 0.18	1.06 ± 0.09
1 mM NaCl × Cut 2	19.25 ± 0.50	8.19 ± 0.52 a	3.09 ± 0.45	6.71 ± 0.46	1.27 ± 0.12
10 mM NaCl × Cut 2	17.29 ± 0.63	7.20 ± 0.46 ab	3.84 ± 0.58	6.35 ± 0.28	1.13 ± 0.05
20 mM NaCl × Cut 2	16.54 ± 0.44	7.00 ± 0.41 ab	4.14 ± 0.58	6.79 ± 0.18	1.03 ± 0.04
30 mM NaCl × Cut 2	15.16 ± 0.60	4.93 ± 0.36 d	5.93 ± 0.55	5.62 ± 0.27	0.88 ± 0.05
CV × C					
Green × Cut 1	16.90 ± 0.26	7.71 ± 0.34	4.22 ± 0.20	5.97 ± 0.17	1.30 ± 0.06
Green × Cut 2	16.17 ± 0.54	7.94 ± 0.36	3.69 ± 0.37	6.85 ± 0.24	1.18 ± 0.06
Red × Cut 1	19.37 ± 0.67	4.67 ± 0.28	5.39 ± 0.40	4.97 ± 0.09	0.93 ± 0.04
Red × Cut 2	17.95 ± 0.48	5.73 ± 0.27	4.81 ± 0.35	5.89 ± 0.19	0.97 ± 0.04
Significance					
Cultivar (CV)	***	***	***	***	***
Salinity (S)	***	***	***	***	***
Cut (C)	***	**	**	***	ns
CV × S	ns	ns	*	ns	ns
S × C	ns	**	ns	ns	ns
CV × C	ns	ns	ns	ns	ns
CV × S × C	ns	ns	ns	ns	ns

ns, *, **, *** Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters indicate statistically different groups ($p < 0.05$, Duncan's post hoc test following ANOVA; $n = 3$).

The net CO_2 assimilation rate (A_{CO2}) content incurred a significant effect with respect to cultivar, as it was higher in green lettuce than in red one. Moreover, irrespective of cultivar and cut order, the A_{CO2} decreased linearly with increasing salinity in the nutrient solution, with the lowest values observed on plants treated with 30 mM NaCl (Table 3). Contrarily to A_{CO2}, the stomatal resistance (r_s) was significantly higher in red cultivar than in the green and in the first cut than in the second

one. In addition, the r_s increased linearly with increasing salinity in the nutrient solution, with a strong significant increase in the red cultivar at 20 and 30 mM NaCl compared to the green one at the same NaCl concentrations. However, no significant differences were found between cut 1 and cut 2, independently of salinity (Table 3). Moreover, a significant effect of the genetic material was observed on both transpiration (T) and intrinsic water use efficiency (WUE_i). Transpiration and WUE_i were higher by 18.4% and 30.5% in the green cultivar than in the red one (Table 3). Finally, increasing the salinity in the nutrient solution from 1 to 30 mM suppressed E only under severe salt stress (30 mM), while the reduction in WUE_i initiated directly after the addition of the mild salt stress (10 mM NaCl; Table 3).

3.4. Qualitative Characteristics

Except for the nitrate and hydrophilic antioxidant activity (HAA), the lipophilic antioxidant activity (LAA) and bioactive compounds such as total phenols (TP) and total ascorbic acid (TAA) were higher in red than in green baby lettuce (Table 4). Irrespective of the genetic material and cut order, reduced nitrate content was apparent only under severe stress conditions (30 mM NaCl). Both antioxidants molecules (TP and TAA) were significantly affected by cultivar × salinity, salinity × cut order and cultivar × cut order interactions (Table 4). For instance, the high significant interaction between cultivar and salinity was evident in our study, in particular the synthesis and accumulation of TP and TAA was not observed in green lettuce, while in red lettuce it peaked at 10 mM NaCl (for TP) and at 20 mM NaCl (for TAA; Table 4). Finally, the increase in TAA content was more pronounced in the red cultivar (Table 4).

Table 4. Significance of the main factors (cultivar, salinity and cut order) and their interactions on nitrate content, lipophilic antioxidant activity (LAA), hydrophilic antioxidant activity (HAA), total phenols (TP) and total ascorbic acid (TAA) of *Lactuca sativa* L. var. *acephala* grown in a floating raft culture.

Source of Variance	Nitrate (mg kg^{-1} fw)	LAA (mmol Trolox 100g^{-1} dw)	HAA (mmol Ascorbic ac. eq. kg^{-1} dw)	TP (mg eq. Gallic acid g^{-1} dw)	TAA (mg 100g^{-1} fw)
Cultivar (CV)					
Green	2019 ± 93.2	5.05 ± 0.22 b	1.44 ± 0.04	51.10 ± 1.99 b	21.72 ± 1.24 b
Red	1881 ± 67.4	6.73 ± 0.30 a	1.49 ± 0.03	66.50 ± 2.89 a	30.61 ± 2.54 a
Salinity (S)					
1 mM NaCl	2125 ± 142 a	5.81 ± 0.48	1.43 ± 0.05	58.88 ± 4.19	20.39 ± 1.32 c
10 mM NaCl	2048 ± 120 a	6.07 ± 0.48	1.44 ± 0.04	58.45 ± 5.59	27.28 ± 2.59 b
20 mM NaCl	1925 ± 91.4 a	5.82 ± 0.38	1.51 ± 0.05	59.88 ± 3.88	35.54 ± 3.91 a
30 mM NaCl	1703 ± 69.3 b	5.85 ± 0.47	1.47 ± 0.06	57.98 ± 2.99	21.44 ± 1.89 c
Cut (C)					
Cut 1	1679 ± 39.5 b	6.81 ± 0.25 a	1.58 ± 0.02 a	67.10 ± 2.80 a	24.43 ± 1.81 b
Cut 2	2221 ± 75.3 a	4.97 ± 0.25 b	1.34 ± 0.03 b	50.50 ± 1.90 b	27.89 ± 2.48 a
CV × S					
Green × 1 mM NaCl	2265 ± 253	4.71 ± 0.51	1.41 ± 0.08	54.11 ± 2.81 bcd	21.10 ± 2.02 c
Green × 10 mM NaCl	2154 ± 185	5.08 ± 0.48	1.45 ± 0.05	46.26 ± 5.09 d	22.32 ± 3.00 c
Green × 20 mM NaCl	1957 ± 115	5.25 ± 0.52	1.47 ± 0.08	51.07 ± 4.18 cd	23.72 ± 1.82 c
Green × 30 mM NaCl	1700 ± 108	5.16 ± 0.34	1.43 ± 0.09	52.97 ± 3.75 bcd	19.74 ± 3.19 c
Red × 1 mM NaCl	1985 ± 132	6.91 ± 0.54	1.45 ± 0.06	63.66 ± 7.76 ab	19.67 ± 1.84 c
Red × 10 mM NaCl	1943 ± 156	7.05 ± 0.6399	1.44 ± 0.07	70.65 ± 7.21 a	32.24 ± 3.28 b
Red × 20 mM NaCl	1892 ± 152	6.39 ± 0.49	1.54 ± 0.08	68.69 ± 4.22 a	47.36 ± 2.86 a
Red × 30 mM NaCl	1705 ± 97.7	6.55 ± 0.81	1.52 ± 0.08	63.00 ± 3.91 ab	23.14 ± 2.10 c

Table 4. *Cont.*

Source of Variance	Nitrate (mg kg^{-1} fw)	LAA (mmol Trolox $100g^{-1}$ dw)	HAA (mmol Ascorbic ac. eq. kg^{-1} dw)	TP (mg eq. Gallic acid g^{-1} dw)	TAA (mg $100g^{-1}$ fw)
S × C					
1 mM NaCl × Cut 1	1806 ± 66.2	6.75 ± 0.59	1.56 ± 0.03	68.56 ± 5.91 ab	19.02 ± 1.04 c
10 mM NaCl × Cut 1	1733 ± 91.8	6.87 ± 0.48	1.53 ± 0.06	70.96 ± 7.13 a	21.15 ± 2.53 c
20 mM NaCl × Cut 1	1682 ± 66.6	6.76 ± 0.38	1.63 ± 0.05	66.46 ± 5.54 ab	33.75 ± 4.69 ab
30 mM NaCl × Cut 1	1494 ± 28.2	6.85 ± 0.64	1.62 ± 0.04	62.42 ± 4.51 abc	23.81 ± 2.32 bc
1 mM NaCl × Cut 2	2443 ± 210	4.87 ± 0.57	1.30 ± 0.05	49.21 ± 2.23 cd	21.75 ± 2.41 c
10 mM NaCl × Cut 2	2364 ± 122	5.27 ± 0.72	1.36 ± 0.02	45.95 ± 4.90 d	33.41 ± 2.86 ab
20 mM NaCl × Cut 2	2167 ± 94.0	4.87 ± 0.38	1.39 ± 0.07	53.31 ± 4.29 bcd	37.33 ± 6.65 a
30 mM NaCl × Cut 2	1912 ± 52.9	4.86 ± 0.40	1.32 ± 0.07	53.55 ± 3.35 bcd	19.08 ± 2.84 c
CV × C					
Green × Cut 1	1737 ± 52.9	5.88 ± 0.20	1.56 ± 0.03	56.95 ± 2.07 b	21.42 ± 1.54 c
Green × Cut 2	2301 ± 138	4.21 ± 0.20	1.32 ± 0.04	45.25 ± 2.45 c	22.03 ± 2.00 c
Red × Cut 1	1620 ± 55.8	7.73 ± 0.25	1.61 ± 0.04	77.24 ± 3.14 a	27.45 ± 3.11 b
Red × Cut 2	2142 ± 59.1	5.72 ± 0.35	1.36 ± 0.03	55.76 ± 2.03 b	33.76 ± 3.93 a
Significance					
Cultivar (CV)	ns	***	ns	***	***
Salinity (S)	**	ns	ns	ns	***
Cut (C)	***	***	***	***	*
CV × S	ns	ns	ns	*	***
S × C	ns	ns	ns	*	***
CV × C	ns	ns	ns	*	*
CV × S × C	ns	ns	ns	ns	ns

ns, *, **, *** Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters indicate statistically different groups ($p < 0.05$, Duncan's post hoc test following ANOVA; $n = 3$).

3.5. Principal Component Analysis (PCA)

A principal component analysis was performed on all analyzed data, and the loading plot and scores are reported in Figure 1. The variables in the first three principal components (PCs) were highly correlated, with eigen values greater than 1, thus explaining for 86.3% of the total variance, with PC1, PC2, and PC3 accounting for 45.9%, 27.4%, and 13.0%, respectively. PC1 was positively correlated with r_s, LAA, TP, N, Cl and HAA; while it was negatively correlated with transpiration (T), A_{CO2}, dry biomass, Ca, nitrate, WUE, Mg and leaf area. Moreover, PC2 was positively correlated with P, SPAD, fresh yield, LAA, leaf area and TP, while it was negatively correlated with Na, DM, and Cl.

The two cultivars were well separated but not univocally clustered in respect to PC1 and PC2. In fact, they changed in dependence on harvests/cuts along PC1 and in dependence on salinity concentration along PC2. In particular, red and green lettuce coming from cut 1 were distributed in the positive side of PC1, while green and red lettuce obtained at cut 2 were distributed in the negative side of PC1. Moreover, 1 mM NaCl was positioned in the positive side of PC2, while 30 mM NaCl was distributed in the negative side of PC2 (Figure 1). Interestingly, the combination red cultivar-cut 1 under 10 and 20 mM NaCl, produced a higher premium quality lettuce (higher LAA, TP, HAA and N). While the green cultivar-cut 2 under 1 and 10 mM NaCl, accumulated more Ca and dry mass, but had also the highest contents of the antinutrient nitrate. Finally, in the higher left quadrant the red cultivar-cut 2 and green cultivar-cut 1, clustered together, showing lettuce characterized by the highest leaf area and fresh yield (Figure 1).

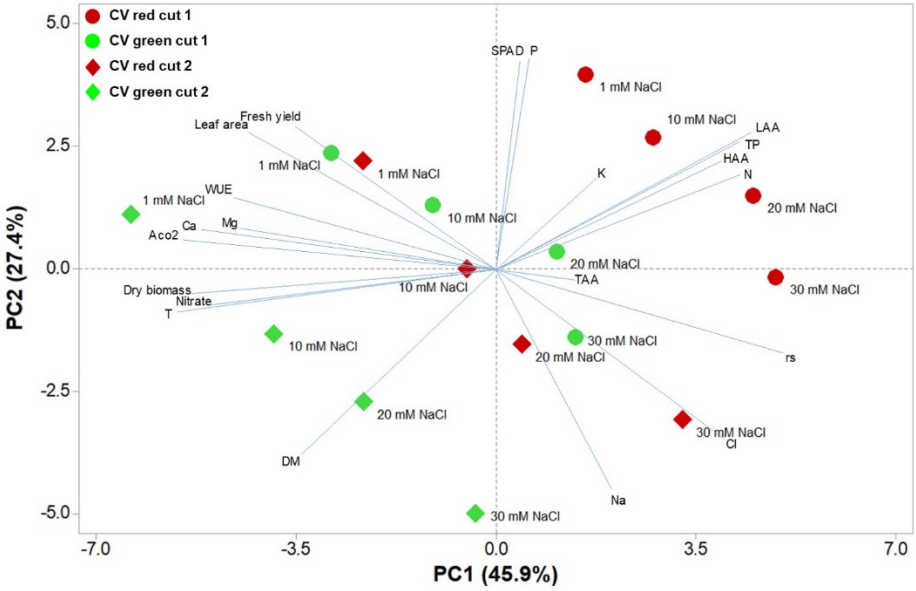

Figure 1. Principal component loading plot and scores of principal component analysis (PCA) of morphological parameters, mineral composition, leaf gas exchanges, bioactive compounds, and antioxidant activity of green and red pigmented lettuces under four salinity levels (1, 10, 20 and 30 mM NaCl) and two successive harvests. T: transpiration rate, DM: lead dry matter percentage, TAA: total ascorbic acid, r_s: stomatal resistance, HAA: hydrophilic antioxidant activity, LAA: Lipophilic antioxidant activity, SPAD: soil plant analysis development, WUE: water use efficiency, A_{CO2}: net CO_2 assimilation rate.

4. Discussion

A mild stress (eustress) can induce plants to reshuffle plant metabolism, by triggering beneficial changes on nutritional and functional quality of the final products [18,19,25]. However, the increase of beneficial metabolites may affect plant growth and yield if the right amount of stress is not expertly administered to plants [18]. Therefore, in order to identify the best possible treatment to improve the nutritional quality of green and red baby lettuces without affecting productivity, we tried to understand how to modulate their physiological and biochemical parameters by using four salinity concentrations in combination with two successive harvests.

Green lettuce was significantly more productive than red one in terms of fresh yield, dry biomass, and leaf dry matter content, particularly at 1 mM NaCl salinity. This feature was certainly related to its lower stomatal resistance, which enhanced the net CO_2 assimilation rate, notwithstanding the lower SPAD index present in the green cultivar, and consequently increased WUE_i, plant vegetative growth and productivity, as previously seen in green perilla [18]. This is in agreement with previous studies that showed that green lettuce cultivars generally grow faster than red ones [45]. However, green lettuce was more prone to the effect of salinity than the other cultivar, despite neither green lettuce nor red one were able to retain lower contents of Na, and in addition, the red cultivar significantly accumulated more Cl than the green one, and this increase was even higher at cut 2. Na and Cl are both toxic when present at high concentrations in cytosol and organelles [46]; however, Na toxicity does not depend on its absolute amount in the cytosol, but mainly on the K to Na ratio present in the cell and on the capacity of cell to compartmentalize this ion in the vacuole [47–49]. Indeed, the fact that only in the green cultivar, K decreased as the salinity in the nutrient solution increased, could certainly account for the higher sensitivity of this cultivar to salinity. Whereas, the ability of red lettuce to retain K at a constant level, independently of salinity, contributed to maintain higher K to Na ratio in the cytoplasm than green one, eventually also using stored K in the vacuole [46]. This latter mechanism could allow red lettuce to satisfy plant metabolic demand for K under salinity, by compartmentalizing the majority of K in the cytosol and Na in the vacuole [50]. It has been proved that even low concentrations of K

exclusively compartmentalized in the cytosol, which accounts for less than 10% of the cell volume, may generate a remarkable osmotic pressure able to counteract the osmotic potential of vacuole, minimizing stress damage and re-establishing growth [47,51].

On the contrary, in green lettuce, as salinity increased, K decreased making probably Na accumulating at higher concentration in the cytosol. This event probably determined an alteration in the uptake and translocation of K, which further decreased, and bivalent cations Ca and Mg, which decreased more in green than in red lettuce at 20 and 30 mM NaCl, due to the competitive interactions with Na [52]. Therefore, green but also red lettuce even at a lesser degree, became more sensitive to Na and/or Cl damages at 20 and 30 mM NaCl, resulting in biomass reduction and fresh yield loss.

To the decrease of these macro-nutrients could be indirectly ascribed the more pronounced increase of stomatal resistance and decrease of photosynthetic rate present in green lettuce in comparison to the other cultivar. The present study revealed that salinity treatment reduced stomatal conductance and, consequently, photosynthetic rate in lettuce because of a reduction in macro-nutrients. Whereas, in Amaranthus leafy vegetables a stress dependent reduction in photosynthetic efficiency was demonstrated due to the reduction in photosynthetic pigments [53–56]. In agreement with our results, Ridolfi et al. [57] demonstrated that a decrease of Ca concentration could affect stomatal opening during dark-light transitions, decreasing stomatal conductance under full light and net CO_2 assimilation rates. In addition, K is the main inorganic osmolyte modulating the guard cell turgor and stomatal movement, and its decrease exerts a negative influence on these physiological functions [58]. Moreover, since Mg is involved in the activation of RuBisCO, via carbamylation, and other redox regulated enzymes, its decrease may reduce the rate of Calvin cycle, consequently slowing down the rate of photosynthetic electron transport chain, therefore contributing to reduce photosynthetic rate and generating photo-oxidation [58]. Mg decrease can also affect the phloem export of sucrose from source leaves impairing photoassimilates partitioning between shoots and roots and determining a relevant increase in the leaf dry matter due to the accumulation of starch and sucrose [59]. The higher dry matter was in fact another effect of high NaCl salinity on green lettuce.

However, previous studies have demonstrated that salinity tolerance varies with cultivars/genotypes not only in dependence on the capacity of plants to exclude and/or compartmentalize toxic ions, but also on the ability to enact responses aimed at minimizing stress damages and re-establishing biochemical and physiological functions and, therefore growth [19,60–62]. In this view, the higher contents of LAA and antioxidant agents such as TP and TAA present in red than in green baby lettuce were genetically determined, and their increase at the onset of stress (starting from 10 mM NaCl) favored the tolerance response of red lettuce to stress. In addition, the increase of phenolics, among which the largest group is that of flavonoids, is also responsible for the leaf color of red lettuce that strongly influence consumer preference and choice making [2,10,18,25].

At cut 2 both cultivars were able to accumulate more Ca and Mg than at cut 1 except that at 30 mM NaCl, and only the green cultivar accumulated more K. The increase in macro-minerals can be associated with health-promoting functions because K is able to lower blood pressure and favor elimination of toxins, while Ca and Mg may contribute to improved skeletal health and prevent osteoporosis [63]. Moreover, this increase of macro-nutrients could be associated with the decreased r_s, slight increase in photosynthetic rate as well as dry biomass and leaf dry matter content in both cultivars, but neither WUE_i nor fresh weight or antioxidant activity increased, with the exception of TAA in red lettuce, compared to cut 1. Accordingly, Corrado et al. [64] found that both the lipid-soluble and water-soluble antioxidant activity of basil leaves from the second cut were 15% lower than those in leaves of the first cut. At cut 2 the photosynthates in lettuce plants were probably mostly used for increasing the number of leaves and root area (not shown), in addition to the leaf area indirectly enhancing transpiration and uptake of nutrients, among which not only the beneficial K, Ca and Mg but also the antinutrient nitrate, in particular in green lettuce, while the total N decreased. Anyway, the nitrate content remained within the limits established by the market according to EU regulation no. 1258/2011 (e.g., lower than 3000–5000 mg kg^{-1} fw). Accordingly, Corrado et al. [64]

found higher concentration of nitrate in basil leaves at the second cut but also a higher total nitrogen content. This means that lettuce plants were efficient in uptaking and transporting nitrate to leaves, although the nitrate assimilatory reducing pathway was not. This higher accumulation of nitrate in lettuce leaves at second harvest happened notwithstanding the hydroponically grown plants were supplied with equal and constant amount of nitrogen fertilization until the end of cultivation. This phenomenon can directly depend on the higher levels of Mg and ATP (probably produced by the higher photosynthetic activity) present in lettuce tissues at the second harvest. In fact, nitrate reductase (NR), the enzyme catalyzing the assimilatory reduction of nitrate into nitrite [65], undergoes a reversible protein phosphorylation on a serine residue in presence of Mg and ATP; the phosphorylated enzyme binds to 14–3–3 proteins, inhibiting enzyme activity and making it more susceptible to proteolytic degradation [66]. Such inhibiting effect of Mg and ATP on NR is widespread among higher plants [67], and could be therefore responsible for the higher accumulation of nitrate in both lettuce varieties at the second harvest. It is important to underline that in these two varieties of lettuce the positive effect of salinity on mitigating the accumulation of nitrate was evident only under 30 mM NaCl, a salinity concentration that highly affected plant fresh yield. Therefore, in this case the mechanism behind the decrease of nitrate more than an uptake inhibition due to the competition between NO_3^- and Cl^- [68], could be the reduction in plant growth and development, causing a down regulation of net nitrate uptake [35,69].

5. Conclusions

Salinity eustress in combination with successive harvests modulated the physiological and chemical composition of red and green pigmented genotypes of baby lettuce grown in floating raft system. Our study demonstrated the differential response of lettuce genotypes to salinity stress, with the green cultivar exhibiting higher sensitivity to salinity. Moreover, it was clearly shown that mild salinity can induce an increase of phytonutrients (total phenols and total ascorbic acid) and beneficial minerals (K, Ca, and Mg) in red lettuce, thus enhancing the nutraceutical and nutritional quality of the product. This improvement was incurred at the expense of an acceptable, moderate yield reduction, ranging between 8% and 10% at 10 and 20 mM NaCl, respectively. In particular, the increase in antioxidant metabolites in this cultivar was noteworthy since phenolics and ascorbic acid may contribute to the extension of the product's shelf-life and underpin its nutraceutical value by imparting anti-oxidant, anti-inflammatory and anti-proliferative properties, essential to prevent or treat cancer, cardiovascular and neurodegenerative diseases. As discussed above, the increase in macro-minerals can been associated also with pivotal health-promoting functions. Our results additionally demonstrated an improved leaf mineral status (K, Ca, and Mg) recorded in lettuce plants coming from the second cut, irrespective of the salt stress treatment and genotype effects. Total ascorbic acid content in red lettuce increased at cut2 whereas in green lettuce it was unaltered. Finally, these findings suggest that salinity eustress is a simple tool to exploit the quantitative variability of selected genotypes and could be valuable for accelerating breeding programs.

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