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Comparative Salt-Stress Responses in Salt-Tolerant (Vikinga) and Salt-Sensitive (Regalona) Quinoa Varieties. Physiological, Anatomical and Biochemical Perspectives

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Abstract: Soil salinization is an important stress factor that limits plant growth and yield. Increased salinization is projected to affect more than 50% of all arable land by 2050. In addition, the growing demand for food, together with the increase in the world population, forces the need to seek salt-tolerant crops. Quinoa (*Chenopodium quinoa* Willd.) is an Andean crop of high importance, due to its nutritional characteristics and high tolerance to different abiotic stresses. The aim of this work is to determine the physiological, anatomical, and biochemical salt-tolerance mechanisms of a salt-tolerant (Vikinga) and a salt-sensitive (Regalona) quinoa variety. Plants were subjected to salinity stress for 15 days, starting at 100 mM NaCl until progressively reaching 400 mM NaCl. Physiological, anatomical, and biochemical parameters including growth, chlorophyll content, quantum yield of PSII (ϕ_{PSII}), gas exchange, stomatal density, size, and lipid peroxidation (via malondialdehyde, MDA) were measured. Results show that chlorophyll content, ϕ_{PSII}, and MDA were not significantly reduced under saline stress in both varieties. The most stress-affected process was the CO_2 net assimilation, with an up to 60% reduction in both varieties, yet Vikinga produced higher dry weight than Regalona due to the number of leaves. The stomatal densities increased under salinity for both varieties, with Regalona the one showing higher values. The averaged stomatal size was also reduced under salinity in both varieties. The capacity of Vikinga to generate higher dry weight is a function of the capacity to generate greater amounts of leaves and roots in any condition. The stomatal control is a key mechanism in quinoa's salinity tolerance, acquiring higher densities with smaller sizes for efficient management of water loss and carbon assimilation. These findings highlight the potential of Vikinga for cultivation in temperate salinized environments during winter, such as Deltas and lowlands where rice is grown during summer.

Keywords: quinoa; abiotic stress; halophyte; salinity

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1. Introduction

Soil salinization is one of the worst and most ancient environmental problems [1]. Although salinization takes place in all climatic regions, it is more likely to occur in drier areas where potential evapotranspiration exceeds rainfall. Globally, 10% of the world's land surface is affected by the excess of salts, and this figure is persistently rising at a rate of two million hectares (Mha) per year [2] due to human-induced deforestation, inadequate watering, and other factors related to climate change, such as the seawater intrusion in coastal areas as a result of the sea level increase and the higher frequency of strong tempests [3,4].

By 2050, salinization is expected to affect 50% of the arable land [5], significantly compromising food security [6]. The growing global population is driving increased food demand. It is also likely that the predicted world population increase and the competition

between domestic and agricultural freshwater use will further increase this food shortage [6]. Thus, agriculture is now focusing on the search for alternative crops and cultivation methods to keep food security, and thus, renewed interest has arisen in stress-tolerant crop species [7].

Quinoa (*Chenopodium quinoa* Willd.) belongs to the Amaranthaceae family, and it was traditionally cultivated in the South American Andes between 5000 and 7000 years ago in different ecologic areas ranging from the sea level in the Chilean north-east region to 4000 m above sea level in the Bolivian–Peruvian highlands [8,9]. This marginal crop has become important in recent years due to its highly nutritious seed, which are rich in essential amino acids and fatty acids. Recently, quinoa cultivation has expanded to several countries across the world [9], and knowledge on quinoa production techniques and its response to different environments are necessary to adapt this crop to new farming areas.

Quinoa plants can tolerate extreme environmental conditions such as salinity, drought, and frost, being suitable for crop rotations with rice or other crops cultivated in salinized areas. Salinity stress impacts plant growth through two primary mechanisms: osmotic stress, which limits water uptake and leads to dehydration, and ionic stress, which results from toxic levels of salt accumulation, particularly sodium, disrupting cellular processes [10,11]. In this context, quinoa is considered a facultative halophyte species. Many quinoa varieties can recover after suffering salinities as high as seawater (approximately 500 mM de NaCl) [12–15] thanks to different mechanisms such as a good osmotic adjustment, osmo-protection, sodium exclusion and xylem load, potassium retention, gas-exchange adjustment, salt excretion through epidermal bladder cells, and stomata control and water use efficiency [12].

Quinoa leaves exhibit efficient osmotic adjustment, with old leaves reaching 95% and young leaves achieving 80–85%, primarily due to Na^+, K^+, and Cl^- accumulation [15]. An efficient Na^+ arrest is present in quinoa leaf vacuoles, as reported by Hariadi et al. [15], having the younger leaves higher K^+ and lower Na^+ concentrations than the old leaves under different salinity levels. Furthermore, this higher vacuole Na^+ concentration necessary to maintain the cell turgor is coupled to a simultaneous cytosolic osmolarity increase through the accumulation of soluble sugars, proline, and glycine betaine [15,16].

The osmotically induced stomata closing and the cytosolic toxic levels of accumulated Na^+ reduce the plant's capacity to completely use the absorbed light to be used for the photochemical processes, leading to the formation of reactive oxygen species (ROS) [17,18]. These ROS must be rapidly and efficiently removed to avoid lipid peroxidation, DNA mutations, protein denaturation, carbohydrate oxidation, pigment degradation, and enzymatic activity damage [19]. Hydrophilic (e.g., phenolics, betacyanins) and lipophilic properties (e.g., fatty acids, tocopherols, and carotenoids) are highly present in Quinoa leaves and seeds, contributing not only to the plant's ROS remediation but also increasing the health benefits of its consumption [20].

The potassium cellular retention and homeostasis are essential for salt tolerance in plants [21]. In plants, intracellular K^+ and Na^+ homeostasis is important for the activities of many cytosolic enzymes and for maintaining membrane potential and an appropriate osmoticum for cell turgor/expansion [22]. Nevertheless, massive cytosolic K^+ leakage often occurs in root and leaf tissues under salinity [23,24]. A high and efficient K^+ retention in roots can be one of the main quinoa's salt tolerance mechanisms [15,16].

Salinity tolerance can be related to three main mechanisms of Na^+ homeostasis: Na^+ exclusion from the shoot, Na^+ tissue tolerance, and osmotic tolerance [25,26]. High Na^+ exclusion is considered a common trait in glycophytic plant species [10]. In *Arabidopsis*, a Na^+/H^+ exchanger placed in the plasma membrane of root epidermal cells [27] and coded by SOS1 is primarily responsible for Na^+ exclusion [28]. Maughan et al. [29] have cloned and characterized SOS1 homologs in quinoa, whose expression rates are three to four times higher in the roots than in the leaf tissues. Ruiz-Carrasco et al. (2011) [22] further suggested that the cytosolic Na^+ concentration and the ionic homeostasis are fine-tuned in quinoa thanks to a precise SOS1 and NHX Na^+/H^+ exchangers activity coordination.

Under salinity stress, the photosynthetic activity reduction is mainly caused by a restricted stomata conductance which reduces both, the transpiration and the CO_2 uptake [30], also stated in quinoa plants [12,31]. Nevertheless, in quinoa, the PSII maximum photochemical efficiency measured as Fv/Fm was not affected by salinity in different studies [15,32]. More recent studies have highlighted the importance of the stomata density reduction strongly displayed by quinoa plants to control transpiration and water use efficiency (WUE) under salinity, an interesting mechanism to be further studied [33,34].

Contrary, Boyer et al. [35] reported that a substantial amount of water (up to 28%) is evaporated from the quinoa leaves surface directly from the cuticle, skipping the stomata. This cuticular transpiration is almost exclusively based on the leaf surface's passive hydraulic permeability, and thus, it cannot be adjusted as fast as stomata conductance which is a dynamic and fast response process through ion flows in and out of the guard cells [36].

One of the quinoa leaves' distinctive anatomical characteristics is the presence of epidermal bladder cells in the adaxial and abaxial leave surfaces. It is accepted that these epidermal bladder cells store the excess of Na^+, Cl^-, and K^+ [34,37]. Nevertheless, Orsini et al. [34] reported that the salt excretion through quinoa epidermal bladder cells is relatively low (<20%) when compared to other halophyte species. These bladder cells can also store water and different metabolic compounds, such as malate, flavonoids, cysteine, pinitol, inositol, and crystalized calcium oxalate salt [37,38]. The epidermal bladder cells can play an important secondary epidermal role in order to reduce the water loss and prevent plants from excessive UV damage. The accumulation of organic osmolytes in the bladder epidermal cells could also function as ROS scavenge.

Salt-tolerant quinoa varieties adapted to grow at sea level in the Mediterranean climate need to be improved in order to substantially produce quinoa in European rice. This European quinoa production would not compete for land use since no other crops can be produced in salinized rice-growing areas during winter. In a previous assay, we screened eight different quinoa varieties for salt tolerance in hydroponic conditions and Mediterranean salinized field conditions (unpublished results). The most salt-tolerant (Vikinga) and the most salt-sensitive (Regalona) varieties were selected for further studies. Other assessed varieties in this previous screening were Monegrina, Titicaca, ICBA-Q2, ICBA-Q3, ICBA-Q4, and ICBA-Q5, showing intermediate results.

This study aims to compare the physiological, anatomical, and biochemical mechanisms of salt tolerance in the Vikinga (salt-tolerant) and Regalona (salt-sensitive) quinoa varieties, with the goal of identifying genotypes suitable for cultivation in salinized Mediterranean rice fields.

2. Materials and Methods

2.1. Plant Material

Two quinoa varieties were used: Regalona and Vikinga. Regalona is an improved variety registered in the Chilean national system for the protection of new varieties [39] and was provided by Semillas Batlle S.L. (Barcelona, Spain). This variety is known to obtain high yields around parallel 40° north and south latitudes. Vikinga variety was first registered in 2015 in Denmark and was provided by Sven-Erik Jacobsen from Quinoa Quality ApS (Regstrup, Denmark). This variety has short stature, compact panicles, and early maturation [40].

These varieties were previously selected for being suitable for cultivation in rice fields being Regalona the most salt-sensitive and Vikinga the most salt-tolerant variety from a hydroponic varietal salt-tolerance screening under Mediterranean climate conditions where other varieties were included, such as Monegrina, Titicaca, ICBA-Q2, ICBA-Q3, ICBA-Q4, and ICBA-Q5, showing intermediate results. Monegrina is a variety selected in Monegros desert (Spain) and provided by Agroserveis.cat (Tarragona, Spain). ICBA-Q2, ICBA-Q3, ICBA-Q4, and ICBA-Q5 salt-tolerant varieties were provided by the International Center for Biosaline Agriculture (Dubai, United Arab Emirates). Titicaca was also provided by Quinoa

Quality Ltd. (Denmark), being a commercial variety resulting from breeding different Chilean highland and lowland varieties with for high productions under Danish climate.

2.2. Growth Measurements

The experiment was performed in a greenhouse in the Experimental Fields Service at the University of Barcelona with natural light, 20–28 °C, and 60–90% RH in May (12 h photoperiod). Plants were germinated for 9 days using a turf-perlite (1:9) substrate in 17 × 25 × 2 cm polystyrene multi-well trays. From them, 6 homogeneous plants of each variety were transplanted and placed in each of the eight 26.5 × 36.5 × 17 cm containers filled with 10 L of UB nutritive solution (Table S1), which is a modification of the one reported by Schlick and Bubenheim [41].

This acclimatization to hydroponic conditions lasted 10 days and the pH and electrical conductivity were adjusted every two days. Then, plantlets were subjected to a 14-day salinity treatment: 200 mL of 5 M NaCl was added in four of the containers to obtain 100 mM NaCl final concentration. After 4 days, an extra 200 mL 5 M NaCl was added to increase salinity from 100 mM to 200 mM, and finally, 4 days after, 400 mL 5 M NaCl was added to each salinized container to reach a final 400 mM NaCl concentration for 7 more days until the end of the experiment (Figure 1). The rationale of this stepwise salinity increase is to avoid the death of all plantlets observed when a sudden increase from 0 mM to 400 mM was applied under these hydroponics conditions.

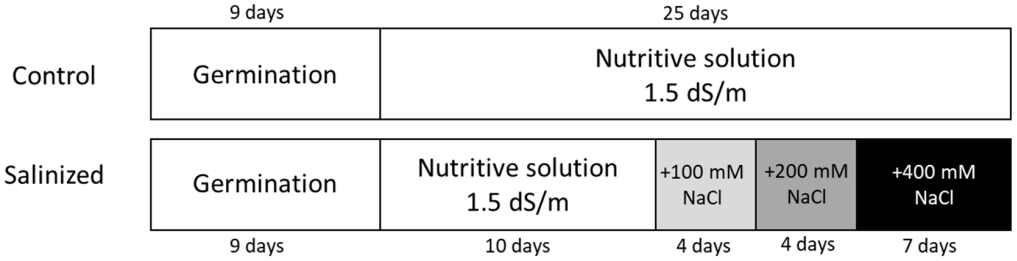

Figure 1. Scheme summarizing the experiment. Grey to black colors represent low (100 mM) to high (400 mM) NaCl concentration in the nutritive solutions.

Finally, plants were harvested fourteen days after the beginning of the saline treatment. First, plant shoot height was measured, then root and shoot fresh weights were scored, and plants were dried at 80 °C for 72 h in a Thermocenter TC40 (SalvisLab, CH-6343 Rotkreuz, Switzerland) oven to obtain dry weights.

2.3. Photosynthetic Parameters

The relative chlorophyll content, gas exchange, and chlorophyll fluorescence data were compared to determine each variety's salt-induced photosynthetic efficiency reductions. The relative chlorophyll content was obtained by measuring fully developed young leaves using a SPAD 502 (Minolta Camera Co., Ltd., Tokyo, Japan) on the 14th day after starting the salinity treatment. Gas exchange data were obtained by using a LI-6800 (LICOR Inc., Lincoln, NE, USA) on the 14th day after starting the salinity treatment under the following conditions: reference CO_2 concentration adjusted to 400 ppm, 25 °C, 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, and 2 cm^2 chamber area. Net assimilation (A_n) ($\mu mol \cdot m^{-2} \cdot s^{-1}$), stomata conductance ($g_s$) (mmol $m^{-2} \cdot s^{-1}$), intracellular CO_2 concentration (C_i) (µmol $CO_2 \cdot mol^{-1}$), and leaf temperature (°C) were obtained from each plant, treatment, and replicate. Chlorophyll fluorescence was measured in representative fully expanded young leaves on the 15th day under salinity. A MINI-PAM-II (Walz, Effeltrich, Germany) fluorometer was used to obtain quantum yield of photosystem II (ϕ_{PSII}), using a 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR.

To obtain the stomatal parameters data, the standard nail polish imprinting method was used, since it instantly fixes the status of stomatal guard cells and provides clear, stable, and almost permanent slides of epidermal impressions for measurement of stomatal aperture size. One leaf per plant was sampled on day 15 and covered with nitrocellulose-

based nail polish on adaxial and abaxial surfaces and let dry for 15 min before obtaining an adhesive tape print [42]. Tapes were pasted in microscope slides. Samples were analyzed using an Olympus CH (Olympus Optical, Tokyo, Japan) microscope. The field of view was 0.15904 mm^2 (40×).

The stomata size quantification was conducted using pictures taken by the Olympus DP70 digital camera (Olympus Optical, Tokyo, Japan) and captured by the Olympus DP controller software (Electron Microscopy Service (TEM/SEM), Scientific and Technological Centers of the University of Barcelona). ImageJ software (V.1.53a) was finally used to measure the stomata width and lengths.

2.4. Biochemical Analysis

The effects of salinity on photosynthesis may involve inhibition of electron transport and inactivation of the photosystem II (PSII) reaction centers, destroying the oxygen-evolving complex (OEC), impairing the electron transfer capacity on the donor side of PSII, while the associated osmotic stress impairs the ability of plant cells to detoxify reactive oxygen species (ROS). This ROS produces lipid peroxidation, which is a common salt-stress-measuring parameter. It was measured following a modification of the malondialdehyde (MDA) peroxidation quantification protocol described by Hodges et al. [43]:

- The first MDA extraction started by adding 350 µL ethanol/water at 80:20 (*v*/*v*) supplemented with 0.01% of butylhydroxitoluene (BTH) to 50 mg of ground tissue.
- Samples were then vortexed 15 s using an LBX V05 series vortex (Labmaterials, Lonay, Switzerland) and introduced in an ultra-sound emitter Branson 2800 (Marshall Scientific, Hampton, NH, EEUU) for 30 min at 4 °C.
- Then, samples were vortexed again for 15 s and sonicated again for 15 min at 4 °C. Then, samples were vortexed again and centrifuged for 8 min 9000 rpm 4 °C in a Hettich 32R (Hettich Universal 32R, Tuttlingen, Germany) centrifuge.
- The supernatant (300 µL) was recovered and maintained at 4 °C and dark. This process was repeated twice until reaching 900 µL MDA extraction per sample.
- Then, 400 µL of the MDA extract was mixed with 400 µL 20% (*w*/*v*) TCA and 0.01% (*w*/*v*) BHT (from now TBA solution). Another 400 µL of the MDA extract was mixed with 400 µL of 20% (*w*/*v*) TCA, 0.01% (*w*/*v*) BHT, and 0.65% (*w*/*v*) thiobarbituric acid (TBA) solution (from now TBA + solution).
- Samples were incubated for 25 min at 95 °C in an oven (Salvis Thermocenter; SalvisLab, Renggli AG, Schötz, Switzerland).
- Then, samples were cooled down to 4 °C and centrifuged at the same temperature and 9000 rpm for 8 min. The absorbance of the supernatants was measured using a spectrophotometer (xMark™ Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 440, 532, and 600 nm.
- The MDA equivalents were calculated according to the Hodges methodology [43].

2.5. Statistical Analysis

One-way ANOVA analysis was applied as long as the normality of the residuals and the homoscedasticity of variances were met using the Shapiro–Wilk and Bartlett tests, respectively. All the means and the standard errors of the anatomical, physiological, and biochemical parameters were obtained from four replicates. Statistically significant differences were considered when the *p*-value was lower than 0.05 ($p < 0.05$) when using Tukey's honest significance test. STATGRAPHICS Centurion 18 software (18.1.01 version) was used for all statistical tests.

3. Results

3.1. Growth Measurements

The salinity treatment reduced the plants' height in both varieties similarly (51% Vikinga and 52% Regalona), showing statistically significant differences between salinized and non-salinized treatments (Figure 2A), although no differences were found between

the varieties (p value = 0.1206). Under 400 mM of NaCl, Vikinga and Regalona varieties reduced the shoot/root ratio by 48% and 59% fresh weight, respectively, compared to the 0 mM NaCl control conditions (Figure 2B). Thus, both varieties reduced their aerial parts as a common strategy to face salinity. In our case, Regalona showed a significantly stronger reduction in the shoot/root index than Vikinga (p-value = 0.005). Regalona's aerial part fresh weight (76% with respect to control FW) and dry weight (70% control DW) reductions under salinity were higher than Vikinga's FW and DW reductions under salinity (62% and 48%, respectively) (Figure 2B,D). Again, Vikinga seems to better keep growth than Regalona. Similarly, leaf FW and DW reductions in salinized conditions were stronger in Regalona (62% FW and 81% DW) than in Vikinga (48% FW and 72% DW) with respect to their non-salinized controls (Figure 2B,D). Leafless steams FW and DW reductions also showed similar results, being stronger in Regalona (80% FW and 77% DW) than in Vikinga (66% FW and 58% DW). Finally, roots FW and DW also showed a significant reduction under saline treatment, with a reduction in the FW of 50 and 43% for Regalona and Vikinga, respectively (Figure 2B), and a reduction in the DW of 22 and 11% for the Regalona and Vikinga, respectively (Figure 2D). Aside from the salinity treatment, the variety factor also influenced the FW and DW parameters according to a two-way ANOVA analysis, but to a lesser extent, and no two-factors interaction was detected in all the above FW and DW analyses (p-values < 0.0001).

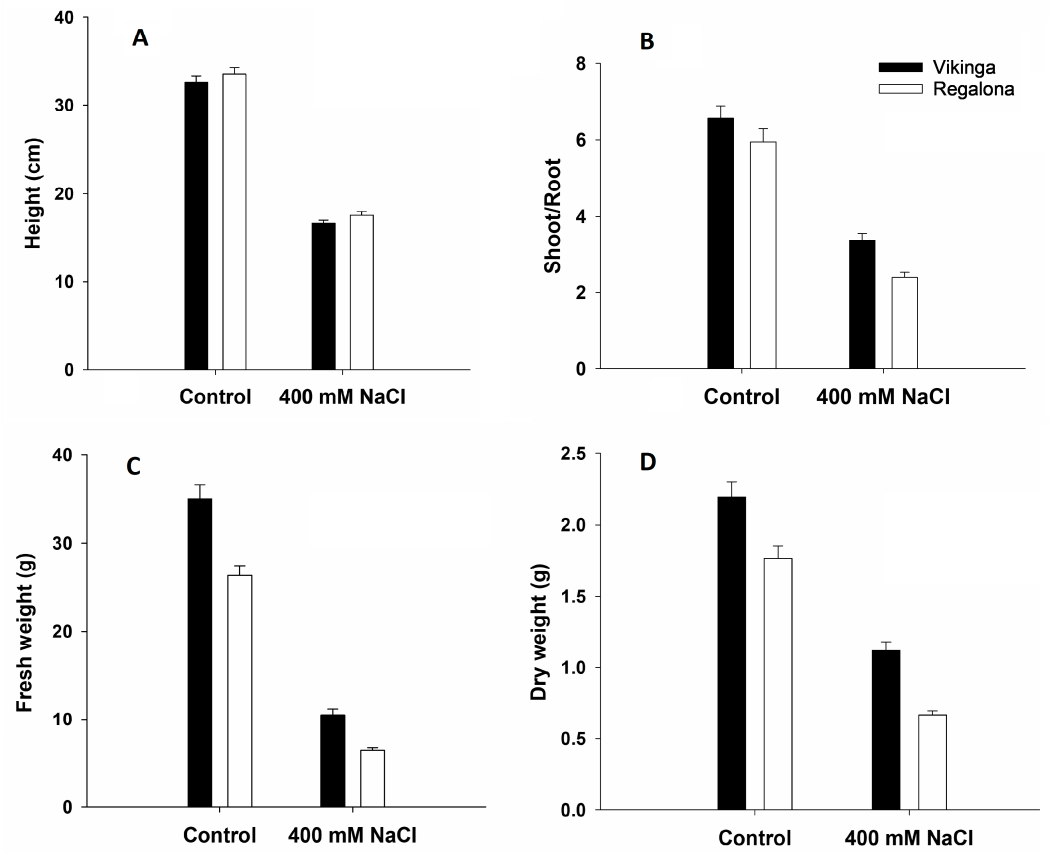

Figure 2. Growth measurements of Vikinga and Regalona varieties under salinity conditions (400 mM NaCl): (**A**) plant height (cm); (**B**) fresh weight of the whole plant (g) and fresh weight of the leaves (dark grey), stem (pale grey), and root (black); (**C**) shoot/root ratio; (**D**) dry weight (g) of the whole plant and dry weight of the leaves (dark grey), stem (pale grey), and root (black). Data are means of twenty-four repetitions (n = 24) ± standard error. According to Tukey's multiple-comparisons test, significant differences with a $p < 0.05$ have been represented with different letters.

3.2. Photosynthetic Parameters

No significant differences in relative chlorophyll content were detected between treatments, except for the Regalona variety, which had an increase of 16% compared to the mock control treatment (Figure 3A).

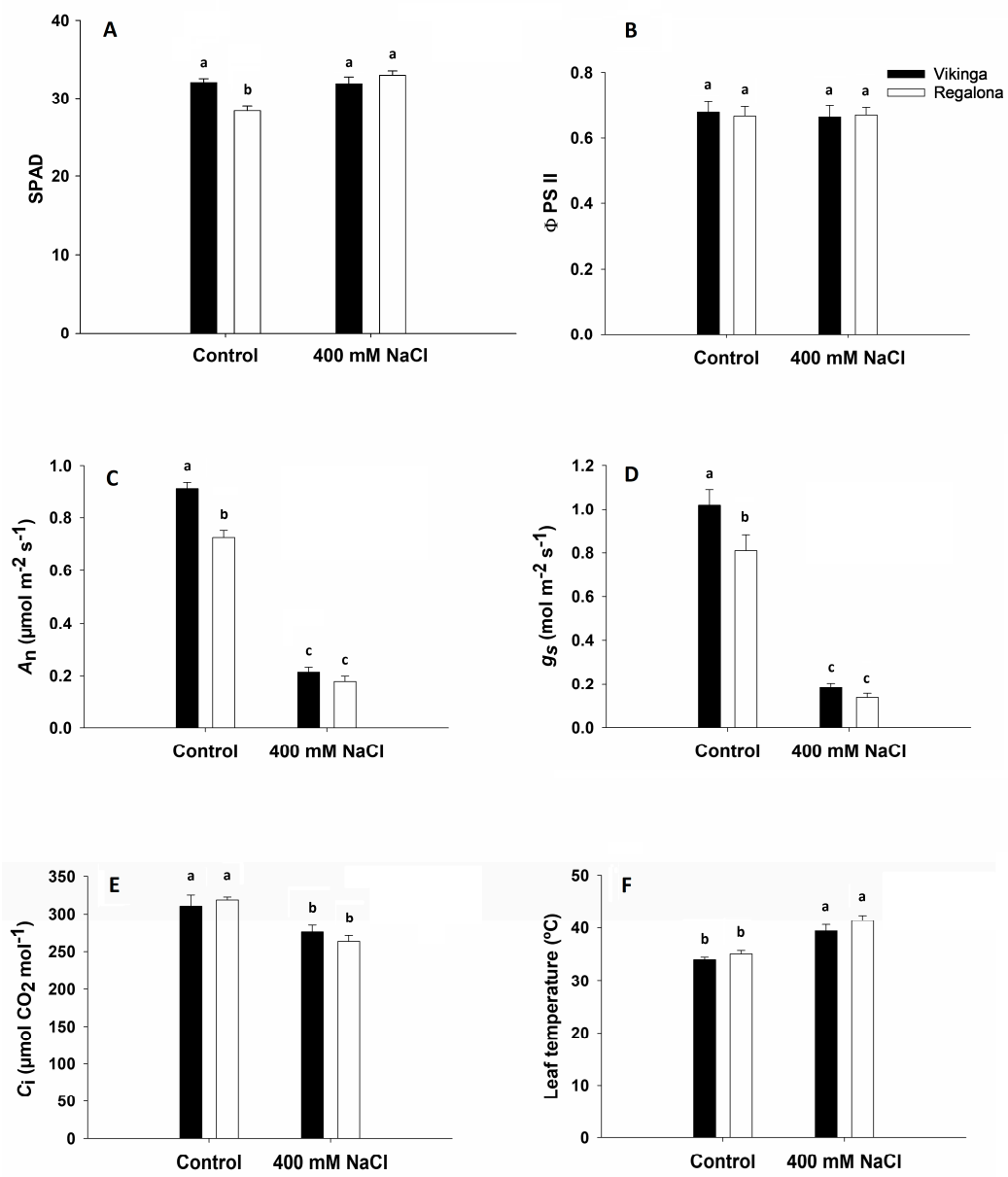

Figure 3. (**A**) SPAD, (**B**) quantum yield of photosystem II, (**C**) net CO_2 assimilation (A_n), (**D**) stomatal conductance (g_s), (**E**) intercellular concentration of CO_2 (C_i), and (**F**) leaf temperature for Vikinga (closed bars) and Regalona (open bars) varieties. Data are means of twenty-four repetitions (n = 24) ±standard error. According to Tukey's multiple-comparisons test, significant differences with a $p < 0.05$ have been represented with different letters.

The quantum yield values of photosystem II (ϕ_{PSII}) were not significantly different in both treatments (Figure 3B). In both varieties, net CO_2 assimilation was significantly reduced in the saline treatment compared to the control, with a reduction of 65 and 7% for the Regalona and Vikinga varieties, respectively (Figure 3C). The stomatal conductance values also showed a significant reduction in the saline treatment with 75 and 76% for the Regalona and Vikinga varieties, respectively (Figure 3D), although the variety factor also affected the stomatal conductance, as found by a two-way ANOVA analysis (p-value = 0.0007) with no

two-factors interaction. The intercellular CO_2 concentration values also showed a 7 and 4% reduction for the Regalona and Vikinga varieties, respectively (Figure 3E), while the leaf temperature obtained a significant increase in the saline treatment compared to the control treatment with an 11 and 12% increase for Regalona and Vikinga, respectively (Figure 3F).

The 75% stomatal conductance decrease in both varieties also resulted in a decreased carbon assimilation between 66 and 70%, which finally resulted in lower dry matter accumulation (62% less in Regalona and 48% less in Vikinga); this difference between both varieties could be due to a greater development of leaves in Vikinga (Figure 2D).

The stomatal closure generated by the action of NaCl also affected the CO_2 uptake capacity and increased the leaf temperature. This can be observed in the intracellular concentration of CO_2 (Figure 3E), which is significantly lower in both varieties (7% and 4% less in Regalona and Vikinga, respectively. The stomata density obtained a significant increase in saline conditions for both varieties (Figure 4A,B). For the adaxial surface of the leaves, an increase of 56 and 38% is observed for the Regalona and Vikinga varieties, respectively; in the abaxial surface of the leaves, an increase of 50 and 29% is observed for the Regalona and Vikinga varieties, respectively. As observed in Figure 3D, stomatal conductance is reduced because of osmotic stress.

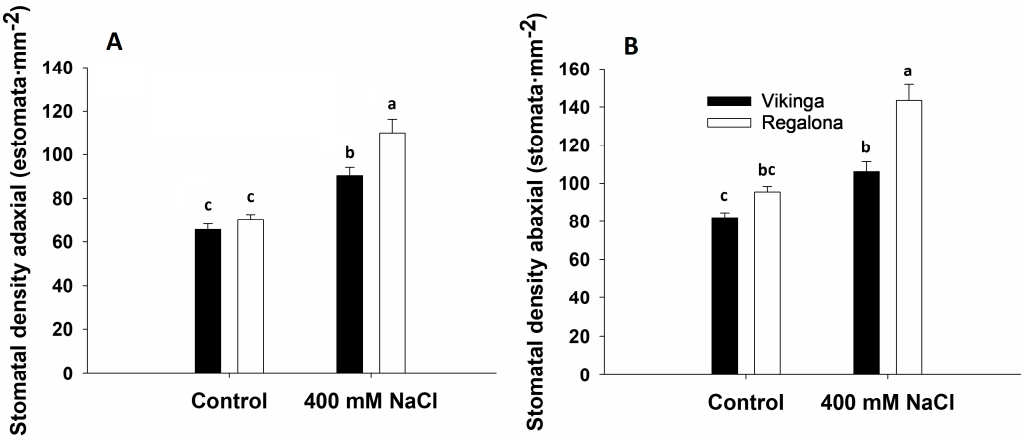

Figure 4. (**A**) Stomatal density of the adaxial surface of the leaves and (**B**) Stomatal density of the abaxial surface of the leaves for Vikinga (black bars) and Regalona (white bars) varieties. According to Tukey's multiple—comparisons test, significant differences with a p <0.05.

Microscopy images of the adaxial and abaxial surface of the Vikinga variety in the control treatment are shown in Figure 5A,B and of the Regalona variety in Figure 5C,D. The microscopy images of the adaxial and abaxial surface of the Vikinga variety in the saline treatment (400 mM NaCl) are shown in Figure 5E,F and of the Regalona variety in the same treatment in Figure 5G,H.

The stomata length and width evaluation indicated a decrease in both sizes, either in the stomata of the adaxial or abaxial surface. The adaxial was 26% for both varieties, while the abaxial stomatal length reduction was 23 and 16% for the Regalona and Vikinga varieties, respectively. Similarly, the adaxial stomatal width reduction was 25 and 27% for the Regalona and Vikinga, respectively, while the abaxial surface stomatal reduction was 27 and 24% for the Regalona and Vikinga varieties, respectively (Figure 6). Our results show an increase in stomatal density in saline treatment (Figure 4A,B) and a decrease in its size (length and width) (Figure 6).

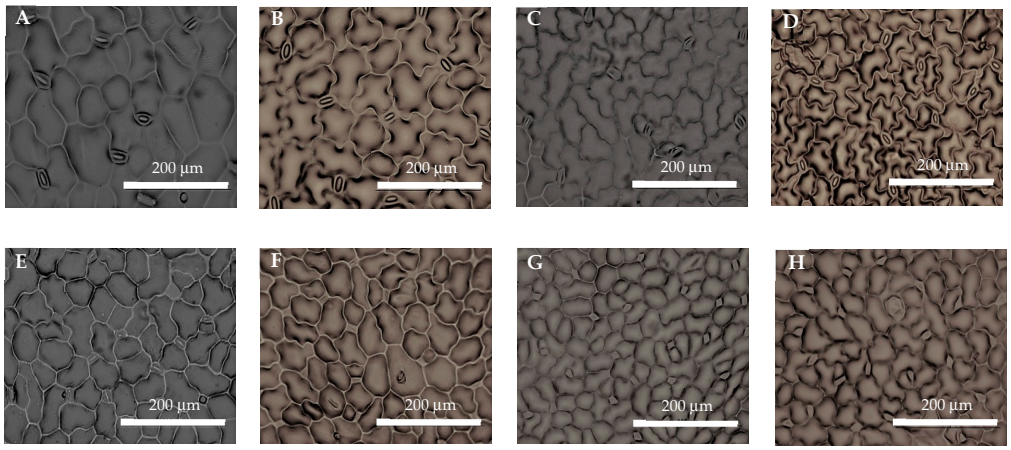

Figure 5. (**A**,**B**) Microscopic images of the adaxial and abaxial surface of the Vikinga variety in the control treatment. (**C**,**D**) Microscopic images of the adaxial and abaxial surface of the Regalona variety under control treatment. (**E**,**F**) Microscopic images of the adaxial and abaxial surface, respectively, of the Vikinga variety in the saline treatment (400 mM NaCl). (**G**,**H**) Microscopic images of the adaxial and abaxial surface, respectively, of the Regalona variety in the saline treatment (400 mM NaCl).

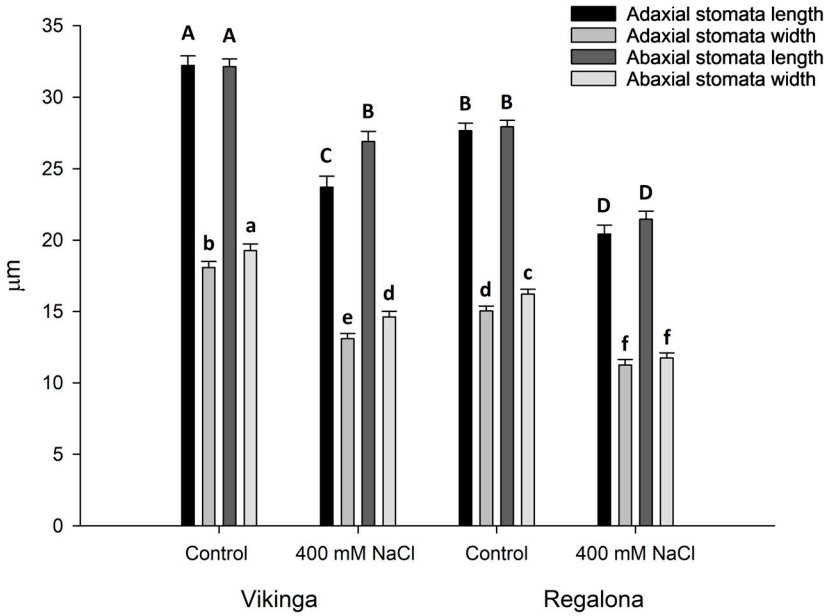

Figure 6. Length and width of adaxial and abaxial stomata comparing Vikinga and Regalona under salinity (400 mM NaCl) and control conditions (0 mM NaCl). Data means of thirty-two repetitions (n = 32) ± standard error. According to Tukey's multiple-comparisons test, significant differences with a $p < 0.05$ have been represented with capital letters when comparing stomata lengths and lower-case letters when comparing widths.

3.3. Malondialdehyde (MDA)

The MDA values have obtained a non-significant increase in both varieties, with 32% and 11% for the Regalona and Vikinga varieties, respectively (Figure 7).

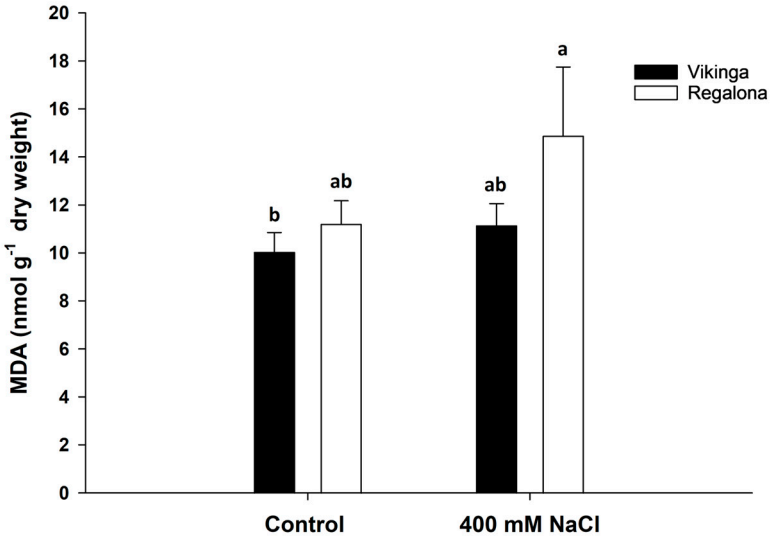

Figure 7. MDA values (nm g^{-1} PF) for Vikinga (black bars) and Regalona (white bars). The MDA data are means of four repetitions (n = 4). According to Tukey's multiple-comparisons test, significant differences with a $p < 0.05$ have been represented with different letters.

4. Discussion

4.1. Growth Measurements

The plants' height was similarly reduced when grown under salinity in both varieties (51% Vikinga and 52% Regalona) (Figure 2). Hariadi et al. [15] also reported 50% quinoa growth reductions when subjected to 500 mM NaCl concentration. Cai and Gao [44] reported a negative association between plant salinity tolerance and quinoa plant size. Indeed, growth and yield reduction are common effects derived from plant salinity stress as far as the osmotic potential reduces water absorption by roots leading to inner dehydration and cellular turgor loss [4,10]. Nevertheless, height has been proved to be a salt-tolerance characteristic by Gomez-Pando et al., since they found cultivars even increasing height in salinized treatments [45]. However, this is not the case with Vikinga's salt tolerance. The aerial mass dry weight, wet weight, and the shoot/root ratio were reduced under salinity treatments in both varieties as a common strategy to face salinity, although Regalona showed a significantly stronger reduction in the shoot/root index than Vikinga (Figure 2). Similarly, Cai and Gao reported a shoot/root ratio decrease with increasing salinities, although no genotype-specific shoot/root ratio variations were detected in response to salt stress [44]. Munns and Tester [10] indicate that a good root system can be maintained by avoiding the loss of water by evapotranspiration under saline stress.

It is worth noting that the decrease in fresh weight (FW) and dry weight (DW) in the aerial parts, leaves, leafless stems, and roots under salinity was higher in the Regalona variety compared to the Vikinga variety (see Figure 2B,D). Indeed, Cai and Gao also reported a decrease in biomass production ranging from 38.9 to 64.4% among different quinoa cultivars under 400 mM salinity [44]. It is well established that the ratio of belowground biomass to aboveground biomass (root/shoot ratio) decreases when light is limited and increases when soil water or nutrients are limited [10]. Salt stress affects crucial processes of cell function, including cell division, differentiation, and expansion, all of which have a substantial impact on plant growth and development [46]. Due to the osmotic stress generated by NaCl, plants reduce their ability to absorb water, which in turn reduces the rate of cell expansion in growing tissues. This can result in a slower formation of the photosynthetic leaf area, leading to reduced flow of dry matter or assimilates to the productive tissues of the plant. These effects are common in both roots and shoots, resulting in reduced growth [10,46,47]. Some authors, such as Koyro et al. [48], suggest that growth reduction induced by NaCl may be caused by an imbalance of nutrients in the quinoa tissues. Therefore, the ability to generate compounds such as soluble sugars and proline

could result in better tolerance to the saline environment [12]. Some reports have suggested that quinoa would cope with salinity through unique and as yet undescribed mechanisms involving salt-ion accumulation in specialized tissues, as well as adjustment of leaf water potential [49].

Our results show that under salinity, Vikinga has a lower FW and DW reduction when compared to the Regalona. Vikinga's growth maintenance under salinity could be subjected to two outstanding parameters. On one hand, we have observed that the reduction in the DW of the leaves is 51% with respect to the control, and on the other hand, the reduction in DW of the roots is only 11% with respect to the control. This could be in accordance with Gómez-Pando et al. [45], who evaluated 15 varieties of quinoa under saline stress (25–30 $dS \cdot m^{-1}$) and found common characteristics within the salt-tolerant varieties: low plant height, decreased life cycle duration, and high dry matter of roots and leaves.

4.2. Photosynthetic Parameters

Saline stress did not have a negative influence on the chlorophyll content or the quantum yield of PSII in both studied quinoa varieties, likely due to their efficient photoprotective mechanisms, which prevented damage to the photosynthetic apparatus under salinity conditions. Further, it is well established that in the first stages of abiotic stresses decreases in the light-saturated rate of CO_2 assimilation were accompanied by decreases in the maximum carboxylation velocity and the capacity for regeneration of ribulose 1,5-bisphosphate in the absence of any significant photodamage to photosystem II [50]. The relative chlorophyll content did not change with salinity in Vikinga (Figure 3A), as reported by many other authors under similar salinity treatments 300–400 mM NaCl [12,44,51], being attributed to quinoa's ability to dissipate excessive excitation energy. Contrary, in the case of Regalona, a significant increase in relative chlorophyll content was detected under salinity, which can be attributed to less cell expansion as also observed in other species [52,53]. Interestingly, the salinity treatment did not affect the quantum yield values of photosystem II (ϕ_{PSII}) (Figure 3B). This could be attributed to the aforementioned chlorophyll content, the ability to maintain good communication between the antenna complex and the PSII reaction centre, together with their high stability of the oxygen-generating complex (OEC) (PsbQ and PsbO proteins) [51]. Bonales-Alatorre et al. [54] also found that 400 mM NaCl saline treatment did not harm PSII of Titicaca and Huallata quinoa varieties.

The net CO_2 assimilation was significantly reduced in Vikinga and Regalona when subjected to the saline treatment (Figure 3C). The stomatal conductance and intracellular CO_2 concentration also significantly decreased in the saline treatment, while the leaf temperature obtained a significant increase in the saline treatment compared to the control (Figure 3D,F) as a consequence of the osmotic stress. In many crops, studies have showed that engineering plants to reduce stomatal number may be an effective tool to improve plant WUE and drought tolerance without yield reductions [55]. Other studies point out that 400 mM NaCl concentration reduces the number of stomata per leaf area in Titicaca variety [56], the Chilean variety 'BO78' [34], and 14 other different quinoa varieties [33]. However, our results show an opposite trend: we obtained, in both varieties, an increase between 29 and 56% in the saline treatment; this was also reported by Becker et al. [57], who observed that the variety Achachino increased the stomatal density around 18% when the plants were cultivated at 250 mM NaCl. Critically, the increase in stomatal density could be a consequence of the short duration of the present experiment. This could explain the inconsistency between the findings in this study and other larger and long-term salinity studies [33,34]. However, other long-term studies also point to a stomatal density increase related to a stomatal size reduction which will be discussed later. Small stomata with high density might allow plants to react faster to environmental changes, providing better control over water loss while maintaining gas exchange.

Salinity creates a so-called physiological drought in plants. Consequently, the water absorption capacity, cell expansion, and leaf expansion rate are reduced, leading to stomata closure, which ultimately results in a photosynthesis reduction [58]. According to Ahmad

and Prasad [58], as new leaves are continually being produced faster than old leaves die, there are enough leaves with photosynthetic capacity to produce more photoassimilates, although in small quantities. Stomatal behaviour plays an important role in the fixation of CO_2, which can limit the flow of assimilates towards the meristematic tissues in full growth, either in leaves or roots [10].

The 75% stomatal conductance decrease in both varieties also resulted in a decreased carbon assimilation between 66 and 70%, which finally resulted in lower dry matter accumulation (62% less in Regalona and 48% less in Vikinga); this difference between both varieties could be due to a greater development of leaves in Vikinga (Figure 2D). Furthermore, the stomata increased in density (Figure 4A,B) and decreased in length and width under salinity (Figures 5 and 6). This could be related to what was reported by Hetherington and Woodward (2003), who indicate that small stomata can open and close more quickly, and their general association with high densities provides the ability to rapidly increase the stomatal conductance, maximizing the diffusion of CO_2 in the leaf during favourable conditions for photosynthesis. The reduction is noticeable in both varieties, and this could be due to a decrease in cell size, exerted by osmotic stress [10]. Franks and Farquhar [59] suggest that a strategy to maintain acceptable levels of stomatal conductance is to multiply the number of stomata and reduce their size. Experimental evidence has shown that stomatal density is negatively correlated with stomatal size [60,61].

4.3. Malondialdehyde (MDA)

The MDA content shows an increased, but not significant, tendency in salinity. The non-significant increase in MDA both varieties could be interpreted as an indication of the successful activation of antioxidant defenses. Further experiments are needed to confirm this possibility. Cai and Gao also reported a non-significant, but genotype-dependent, increase in MDA in response to salinity [44]. It is observed that there is a noticeable difference in MDA values between varieties, this difference could affect the development of metabolic processes [12]. The osmotically induced stomata closing and the cytosolic toxic levels of accumulated Na^+, reduce the plant's capacity to completely use the absorbed light to be used for the photochemical processes, leading to the formation of reactive oxygen species (ROS) [17,18]. These ROS must be rapidly and efficiently removed to avoid lipid peroxidation, DNA mutations, protein denaturation, carbohydrate oxidation, pigments degradation, and enzymatic activity damage [19]. Two main antioxidative systems operate in plants: the enzymatic and non-enzymatic systems [19]. Non-enzymatic compatible solutes such as mannitol and proline have demonstrated their ROS elimination capacity [62], both being present in quinoa tissues [16,63]. In general, ROS production under saline conditions is attributed to the disruption of two key metabolic processes: photosynthesis and respiration [36]. The decrease in stomatal conductance in response to salinity stress may have a consequence on the amount of light absorbed since it can exceed the demand for photosynthesis [64]. This affects the rate of electron transport through photosystems and results in increased ROS production (mainly O_2 and $1O_2$) [65]; our results have not compromised the photosystem II (Figure 3B), nor the chlorophyll content (Figure 3A), so we could see this reflected in the MDA levels, which are not significantly different from the control.

5. Conclusions

Saline stress did not have a negative influence on the chlorophyll content or on the quantum yield of PSII, at least in both studied quinoa varieties, likely due to their efficient photoprotective mechanisms, which prevented damage to the photosynthetic apparatus under salinity conditions. In contrast, salinity significantly impacted the carbon assimilation processes by reducing both the photosynthetic rate and stomatal conductance. This reduction in stomatal aperture may have contributed to a limitation in CO_2 uptake, thereby affecting overall photosynthesis. The Vikinga variety produced a higher dry weight yield than Regalona, likely due to its greater growth vigor. This enhanced vigor may be attributed

to its ability to maintain larger leaf and root masses under saline conditions, suggesting that Vikinga is better equipped to manage ion compartmentalization and water uptake efficiency. Salinity also influenced stomatal control, leading to higher stomatal densities with smaller sizes. This adaptation may enhance water-use efficiency and improve carbon assimilation by allowing finer regulation of gas exchange under salt stress. Unfortunately, MDA values alone did not provide sufficient insights into the biochemical mechanisms of quinoa's salt tolerance. Further investigation into antioxidant enzyme activities and osmoprotectant synthesis is necessary to elucidate the precise mechanisms involved. Finally, Vikinga could be considered a vigorous, salt-tolerant quinoa variety with potential for breeding programs in saline regions such as river deltas, allowing for crop rotations with rice. However, further field testing under various environmental conditions is essential to validate Vikinga's performance and stability before its incorporation into breeding programs or cultivation on saline soils.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14123003/s1; Table S1: Modified Schlick & Bubenheim solution.

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