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RESEARCH ARTICLE

Leaf production and quality of quinoa 'Titicaca' is enhanced under moderate salinity

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ABSTRACT

Quinoa (*Chenopodium quinoa* Willd.) is described as high nutritional value crop due to the quality and quantity of their beneficial compounds and antioxidant potential. We assess the influence of salt stress on the functional value of quinoa 'Titicaca' leaves and the best growth conditions to obtain both a high leaf production and optimal levels of bioactive metabolites. Thus, quinoa 'Titicaca' plants were grown under hydroponic greenhouse conditions with 0, 50, 100 and 200 mM NaCl. The results indicated that salinity levels of 200 mM NaCl decreased shoot biomass in a 45.1% and leaf area in a 69.1%, together with a depletion of the concentration of starch and total soluble sugars of 62.3% and 17.7%, respectively. Proline levels increased in conditions over 100 mM NaCl and total proteins decreased in all salt treatments. Furthermore, the greatest values of glutathione (368.57 mmol mg^{-1} dw) and ascorbic acid (0.86 mg 100 g^{-1} dw) were shown in plants subjected to 100 mM NaCl, and total phenolics and antioxidant enzymes activity rose in unison with the salinity severity. Results revel that quinoa 'Titicaca' leaves are of high nutritional value especially when cultured under moderate salinity conditions of 100 mM NaCl. Not only grains, also greens of this edible halophyte could be of interest to maximize food production in arid and saline zones.

Key words: Antioxidants, *Chenopodium quinoa*, nutritional potential, phytochemicals, salt stress, yield.

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd.) grown for its edible grains, is from the Andean highlands in South America and belongs to the Amaranthaceae family. Is naturally gluten free, and the remarkable nutritional properties of its seeds confers worldwide attention, that include high protein content and essential amino acids, flavonoids, vitamins and minerals (Cañarejo-Antamba et al., 2021). Thus, the demand for quinoa seeds and its cultivation has been globally extended over the last decade (Bazile et al., 2016). Besides the seeds, leaves, young stems and inflorescences can be consumed in the human diet and as animal feed (Gawlik-Dziki et al., 2013). In this sense, quinoa leaves have been considered healthful vegetables with a better profile of minerals and vitamins than grains (Vazquez-Luna et al., 2019).

On the other hand, quinoa combines a high nutritional quality together with an inherent tolerance to various abiotic stress (Jacobsen et al., 2003) as salinity (Pulvento et al., 2012). In this sense, soil salinization is one of the main environmental factors affecting crop yield, especially in marginal lands with scarce water resources (Sanower, 2019). Thus, quinoa has been considered a future solution as a potential crop to provide food security worldwide (Bazile et al., 2016) and to become an important crop in arid areas and saline environments (Adolf et al., 2013). Despite several papers have addressed salt and drought tolerance in quinoa, less attention has been paid to new quinoa cultivars as Titicaca. 'Titicaca' is one of the registered in Europe and it has interesting properties as high antioxidant activity (Reguera et al., 2018). However, according to Bazile et al. (2016), 'Titicaca' genotype has a very heterogeneous yield potential depending on

the climatic conditions of the location. Despite it has been described that certain stresses as salinity are capable of enhancing the content of bioactive compounds in different plant species (Ahmadi and Souri, 2018), there are very few studies that analyze the behavior of this cultivar of quinoa under semi-controlled conditions, focusing on antioxidant and nutritional properties of its leaves.

For all this, the main aim of this work was to go deeper into quinoa 'Titicaca' by analyzing the antioxidant, polyphenol, protein and carbohydrate content of its leaves when subjected to saline stress. Both the optimal leaf production and high concentration of bioactive compounds were elucidated in quinoa grown under hydroponics and greenhouse conditions.

This study emphasizes the importance of the research, places it in a context, presents related literature, and gives enough information to understand the authors' hypothesis.

MATERIALS AND METHODS

Plant material and experimental design

Quinoa (*Chenopodium quinoa* Willd.) 'Titicaca' seeds (kindly provided by CEBAS-CSIC, Murcia, Spain) were surface disinfected during 2 h in 0.5% NaClO and pre-hydrated in aerated, deionized water for 22 h. Then, seeds were germinated on vermiculite containers placed in a growth chamber with 26/20 °C day/night (D/N) and 60%/80% D/N of relative humidity. Seedlings received a photosynthetically active radiation of 400 µmol m^{-2} s^{-1} supplied by a combination of fluorescent tubes (TLD 36W/83, Philips, Würzburg, Germany, and F36W/GRO, Sylvania, Wilmington, Massachusetts, USA) and halogen lamps (HQI-T 400W, Osram, Munich, Germany) for a 16:8 h photoperiod. After 10 d, seedlings were transplanted to a hydroponic culture and transferred to a greenhouse under semi-controlled conditions of 25/18 °C D/N, 60%/80% D/N of relative humidity and natural daylight. When transplanted, homogeneous groups of 10 seedlings were moved to 5 L plastic hydroponic containers filled with aerated Hoagland nutrient solution. After 2 wk in hydroponics, four different saline treatments were applied: Control (0 mM), 50, 100 and 200 mM NaCl. Salinity conditions lasted 7 d, when plants were harvested and the different determinations were performed. The electrical conductivity (EC) of the nutrient solution of the containers reached 1.79, 3.21, 8.81 and 18.60 dS m^{-1} in non-saline, 50, 100 and 200 mM NaCl treatments, respectively.

Growth parameters

Shoot dry weight (dw) was determined after drying fresh matter at 80 °C in an oven for a minimum of 2 d or until reaching a constant weight. Yield of plant's edible part was based on leaf fresh weight (fw) measurements. Moreover, the free application Easy Leaf Area estimated leaf area per plant.

Mineral analysis

For sodium analysis, samples (0.5 g leaf dw) were dry-ashed and digested with HCl. Sodium concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Optima 4300, Perkin Elmer, Waltham, Massachusetts, USA), with three replicates per each sample.

Biochemical analysis

Leaf soluble proteins, total soluble sugars (TSS), starch and proline were measured in 0.1 g fw with potassium phosphate extraction buffer (KPB) (50 mM, pH 7.5). After filtration with cheese-cloth, leaf extracts were centrifuged at 28 710 g for 15 min at 4 °C. Starch determinations were performed in the pellet. The supernatants were stored in aliquots at -20 °C for further determinations of TSS, proline and proteins. Total soluble sugars were estimated by spectrophotometric analysis at 620 nm with the anthrone method. Free proline was analyzed spectrophotometrically with ninhydrin reaction and the Bradford assay was used to measure the concentration of total soluble proteins with bovine serum albumin as standard. These biochemical analyses were performed according to the methods specified in Rodríguez-Hernández and Garmendia (2022).

Ascorbic acid and total glutathione were extracted according to Ruiz and Blumwald (2002). Leaf fresh sample was extracted in 6% metaphosphoric acid (pH 2.8 with 1 mM EDTA) and centrifuged at 15 000 g for 10 min at 4 °C. The supernatant was divided into aliquots and stored at -20 ºC for further analysis. Total glutathione (measured as total thiol) was assayed in a 1.12 mL reaction mixture consisting of reagent A, reagent B, and 400 µL of the leaf extract 1:50 dilution in 5% Na_2HPO_4 (pH 7.5) (Ruiz and Blumwald, 2002). The reaction was started by adding NADPH and the absorbance changes at 412 nm were recorded.

Ascorbic acid was measured via HPLC (uHPLC 1260 Infinity Binary LC System, Agilent Technologies, Santa Clara, California, USA) (Gokmen et al., 2000). The sample (20 µL) was introduced in a stainless-steel column, 100 RP-18 (5 µm) (Lichrospher, Agilent Technologies), which was at 30 °C. The mobile phase was 0.2 M KH_2PO_4 and a flow-rate of 0.5 mL min^{-1} was used. The mobile phase was prepared with deionized water and its pH was adjusted to 2.4 with H_3PO_4. UV-vis detector measurements operated at 254 nm. An ascorbic acid calibration curve with concentrations between 10 to 100 mg L^{-1} and 1 mg mL^{-1} DTT was constructed.

Leaf carotenoids were determined in 20 mg fw sample and was extracted with 5 mL 96% ethanol for 10 min at 80 °C. The spectrophotometric analysis of extracts was made according the equations of Lichtenthaler (1987). This pigment analysis was performed according to the methods specified in Rodríguez-Hernández and Garmendia (2022).

The activity assays of catalase, ascorbate peroxidase and glutathione reductase enzymes were performed on 0.4 g frozen youngest fully mature leaves. For it, 8 mL 0.1 M KPB (pH 7.0) with 0.1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone (PVPP) was used for the extraction of the enzymes. Leaf extracts were filtered through four cheesecloth layers and centrifuged at 34 452 g and 4 °C for 10 min. The supernatants were divided into different aliquots, which were stored at -80 °C for subsequent analysis. It should be noted that the entire process mentioned was carried out at 5 °C. Catalase activity (CAT) (EC1.11.1.6) was determined using the protocol of Aebi (1984) with some modifications. For that, 100 µL enzyme extract was mixed with 50 mM KPB (pH 7.0) and 12 mM H_2O_2. The reaction started when H_2O_2 was added, and the H_2O_2 breakdown was recorded by the decrease in A240. Ascorbate peroxidase (APX) (EC 1.11.1.11) was analyzed according to Nakano and Asada (1981) with minor modifications. The reaction mixture consisted of 100 µL enzyme extract with 80 mM KPB (pH 7.0 and 0.1 mM EDTA), 0.75 mM ascorbate and 0.5 mM H_2O_2. The reaction started when H_2O_2 was added and the ascorbate oxidation was determined by the reduction of the A290. Glutathione reductase (GR) (EC 1.6.4.2) was determined according to Schaedle and Bassham (1977), where 200 µL enzyme extract was mixed with 50 mM tris–HCl (pH 7.5 and 3 mM $MgCl_2$), 10 mM oxidized glutathione (GSSG) and 3 mM $NADPH_2$. The $NADPH_2$ oxidation was determined at 340 nm.

The total content of phenolic compounds was determined as described by Chapuis-Lardy et al. (2002), with slight modifications. Leaf fresh samples of 1 g were homogenized in 20 mL 80% methanol for 1 min at room temperature. Then, samples were filtered and 0.5 mL of each extract was mixed with 0.17 M $FeCl_3$ and 10 mL distilled water. The content of total phenolics from aqueous solutions was analyzed spectrophotometrically using Folin-Ciocalteu reagent at 760 nm (Waterman and Mole, 1994). The results obtained were expressed as mg gallic acid g^{-1} dw.

All biochemical determinations were made on the youngest fully mature leaves, collected at midday in liquid nitrogen and stored at -20 °C for later analysis.

Statistics analysis

The results were evaluated using the statistical software SPSS v.26 (IBM, Armonk, New York, USA). A one-way ANOVA was applied. The means ± standard deviations (SD) were determined and when the F ratio was significant ($p < 0.05$), significant differences were analyzed with the Duncan test setting the significance levels at 5%.

RESULTS

When cultivated at high salinity conditions, quinoa 'Titicaca' showed significant differences in shoot dry weight, leaf area and leaf fresh weight (Figure 1). Regarding shoot biomass, 100 mM NaCl treatment was significantly detrimental, with a strongest depletion due to 200 mM conditions (Figure 1A). On the contrary, in both leaf area and leaf fresh weight parameters, only plants grown with 200 mM NaCl were affected (Figures 1B, 1C). On the other hand, Na concentration in leaves increased according to salt stress (Figure 2).

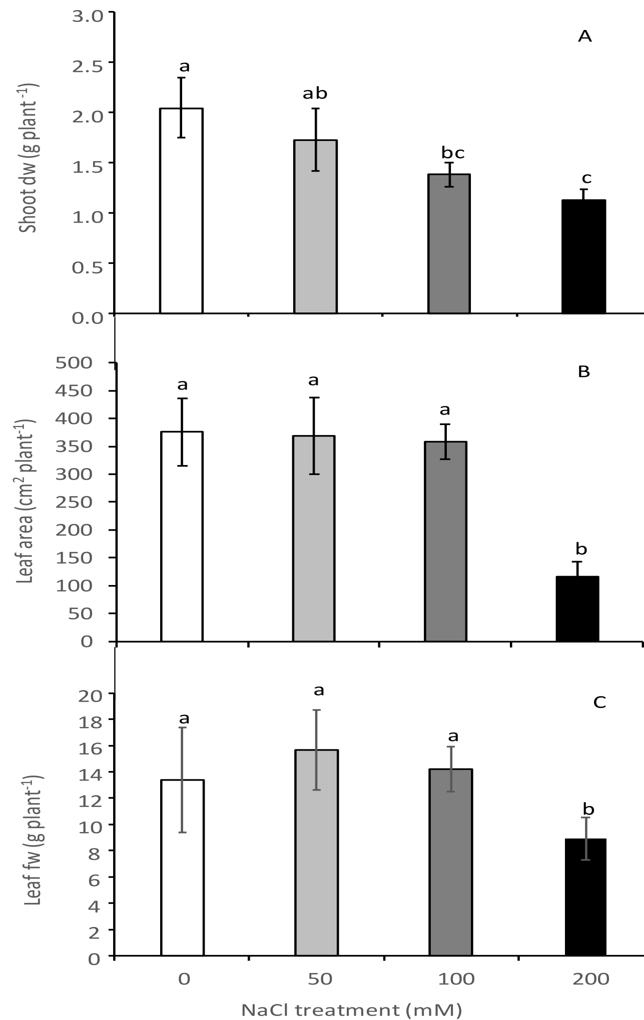

Figure 1. Shoot dry weight (dw) (A), leaf area (B) and leaf fresh weight (fw) (C) in quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Bars followed by a common letter are not significantly different ($p \leq 0.05$).

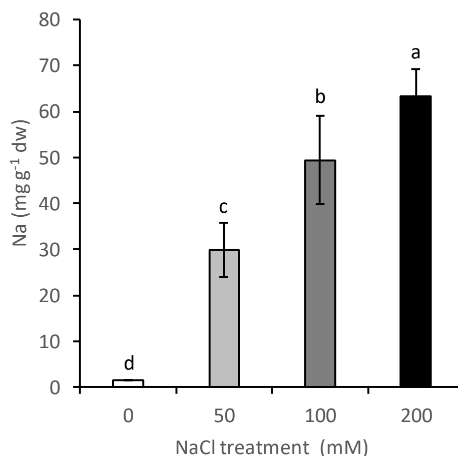

Figure 2. Leaf concentration of Na in quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Bars followed by a common letter are not significantly different ($p \leq 0.05$).

In relation to organic solutes, results in Table 1 revealed that starch content in leaves only declined in plants subjected to 200 mM NaCl. Moreover, there were no differences in TSS concentration, with the exception of significantly lower levels in 200 mM NaCl treatment than in 50 mM (Table 1). Related to proline, results showed that there was an increase under salt stress over 100 mM. Total soluble proteins in leaves decreased as a consequence of salinity, even at 50 mM NaCl treatment (Table 1).

Regarding glutathione concentration on Table 2, despite all saline treatments had a slight increase, significant differences were only shown at 100 mM NaCl treatment compared to the control. With respect to ascorbic acid in leaves, results reveal that the highest values were achieved in plants subjected to 100 mM NaCl, while the 200 mM NaCl treatment exhibited the lowest values (Table 2). On the other hand, despite the fact that treatments with 50 mM and 100 mM did not present differences with respect to control plants, plants grown with 200 mM NaCl decreased the concentration of leaf carotenoids (Table 2).

In this work, the possible enhancement of plant antioxidant enzymatic activities under saline environments was also analyzed (Table 3). Both glutathione reductase and catalase activities increased as salinity severity made. In the case of ascorbate peroxidase, all the salinity treatments enhanced its activity to the same level independently of the concentration of NaCl.

Table 1. Leaf concentrations of starch, total soluble sugars (TSS), proline and proteins in quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Within each column, values followed by a common letter are not significantly different ($p \leq 0.05$). dw: Dry weight.

NaCl treatment	Starch	TSS	Proline	Proteins
mM	mg g^{-1} dw		mmol g^{-1} dw	mg g^{-1} dw
0	8.67 ± 1.92^{a}	349.65 ± 60.54^{ab}	12.40 ± 2.76^{b}	111.69 ± 23.34^{a}
50	8.77 ± 1.70^{a}	402.89 ± 83.16^{a}	10.02 ± 2.90^{b}	61.24 ± 12.12^{c}
100	8.29 ± 1.35^{a}	366.59 ± 89.52^{ab}	42.58 ± 8.92^{a}	86.18 ± 24.61^{b}
200	3.27 ± 0.87^{b}	287.61 ± 53.81^{b}	35.04 ± 8.39^{a}	53.65 ± 11.77^{c}

Table 2. Total glutathione, ascorbic acid and carotenoids in leaves of quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Within each column, values followed by a common letter are not significantly different ($p \leq 0.05$). dw: Dry weight.

NaCl treatment	Glutathione	Ascorbic acid	Carotenoids
mM	mmol mg^{-1} dw	mg 100 g^{-1} dw	mg g^{-1} dw
0	153.73 ± 39.05[b]	0.66 ± 0.09[b]	4.38 ± 0.40[a]
50	180.44 ± 47.82[b]	0.56 ± 0.15[b]	4.31 ± 1.49[a]
100	368.57 ± 96.89[a]	0.86 ± 0.22[a]	3.27 ± 0.76[ab]
200	249.77 ± 36.62[b]	0.31 ± 0.06[c]	2.57 ± 0.47[b]

Table 3. Catalase, ascorbate peroxidase and glutathione reductase in leaves of quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Within each column, values followed by a common letter are not significantly different ($p \leq 0.05$).

NaCl treatment	Catalase	Ascorbate peroxidase	Glutathione reductase
mM	mmol H_2O_2 mg^{-1} protein min^{-1}	mmol ascorbate mg^{-1} protein min^{-1}	mmol NADPH mg^{-1} protein min^{-1}
0	40.80 ± 8.15[d]	0.45 ± 0.08[b]	0.08 ± 0.01[c]
50	64.60 ± 12.43[c]	0.86 ± 0.19[a]	0.32 ± 0.07[b]
100	100.71 ± 13.65[b]	0.75 ± 0.16[a]	0.32 ± 0.06[b]
200	124.42 ± 22.76[a]	0.72 ± 0.17[a]	0.41 ± 0.04[a]

Finally, results of total phenolics (Figure 3) revealed that salinity positively influenced the accumulation of these compounds.

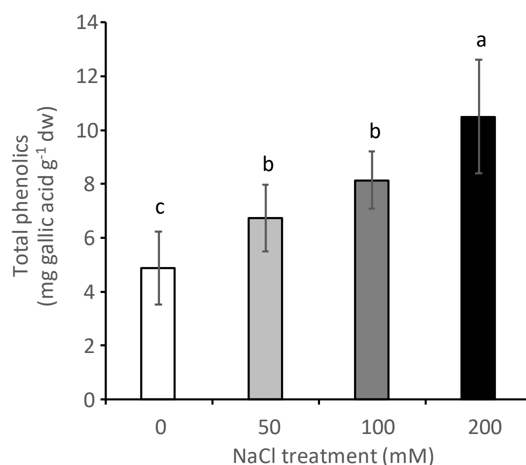

Figure 3. Total phenolics in leaves of quinoa 'Titicaca' subjected to different salt conditions. Means (n = 6) ± SD were compared with Duncan test. Bars followed by a common letter are not significantly different ($p \leq 0.05$).

DISCUSSION

In general, it is known that high salt concentrations negatively affect several physiological processes declining plant growth and production. However, sometimes salinity can increase the content of plant beneficial compounds (Ahmadi and Souri, 2018). In this sense, quinoa is particularly important not only due to high nutritional quality but also for its tolerance to salinity (Pulvento et al., 2012). Recently, the review of Pathan and Siddiqui (2022) conclude that quinoa greens are excellent sources of nutrients and health promoting compounds, and represent a promising value-added new vegetable. There is a large number of quinoa varieties with different agronomic characteristics. Moreover, it should be noted that there are varieties capable of growing in environments with a saline concentration similar to seawater (40 dS m^{-1}), or greater (Jacobsen et al., 2003). In this work, we demonstrated that in the case of the European-adapted 'Titicaca' under hydroponic glasshouse conditions, salinity declined the shoot growth when a rate of salinity above of 100 mM NaCl was applied for 1 wk. However, both the leaf area and leaf fresh weight were only affected with salt concentrations of 200 mM. These results are in accordance with those of Maestro-Gaitán et al. (2022) that defined 'Titicaca' within the growth habit 4, being more susceptible to water loss due to higher total leaf surface. Similarly, Talebnejad and Sepaskhah (2015) found a decrease of plant heigh of quinoa 'Titicaca' as salt intensity increased.

Halophyte plants as quinoa are capable of growing in environments with high salinity thanks to the osmotic adjustments caused by the accumulation of organic solutes (Wang et al., 2013). According to our results, quinoa 'Titicaca' showed a decrease of soluble sugars with high salinity conditions as it happens in other halophytes (Atzori et al., 2017) while proline content increased in parallel with salt stress. This fact indicates that proline acted as osmoregulatory metabolite despite the decrease in sugars, as in previous results of our group in the halophyte *Mesembryanthemum* (Rodríguez-Hernández and Garmendia, 2022). Thus, plants treated with 100 and 200 mM NaCl would present a greater osmotic adjustment due to the increase in proline concentration compared to the rest of the treatments. Although the influence of proline on salinity tolerance is not well known, its accumulation contributes to osmotic adjustment to regulate water potential (Dar et al., 2016). In this sense, Nadali et al. (2021) observed that the synthesis or accumulation of proline was detrimental to the plant growth because its synthesis needs about 41 moles ATP. This fact could affect to the content of starch, soluble sugars and proteins, which showed a decrease in plants subjected to 200 mM NaCl together with a reduction of the shoot biomass. Likewise, the carotenoids concentration diminished in plants grown above 100 mM NaCl. In this sense, it is known that carotenoids are critical for maintaining the stability of photosynthesis in response to environmental stress as salinity, because one of their function is the photoprotection of the photosystems (Yang et al., 2020). Thus, in this work, the decrease of their levels in plants subjected to high salinity could indicate that saline stress negatively affects the photosynthetic process and thus to the plant growth. Similar results were obtained before in quinoa, in which saline water caused gradual significant decreases in its contents of carotenoid and total pigments as compared with controls (Abdallah et al., 2020).

On the other hand, interest in quinoa has increased throughout the world, not only for the excellent nutritional properties of its seeds, but also the antioxidant and anticancer properties of its leaves (Gawlik-Dziki et al., 2013; Vazquez-Luna et al., 2019; Pathan and Siddiqui, 2022). In this work, it was shown that plants of quinoa 'Titicaca' cultivated with 100 mM NaCl had the highest content of ascorbic acid. In the same way, previous results in leaves of other halophytes revealed that ascorbic acid concentrations increase with salinity. Nevertheless, high level of saline stress leads to a decrease of this compound (Cao et al., 2015). In addition, the values of ascorbic acid found in 'Titicaca' leaves would indicate that this quinoa cultivar has lower concentration of this antioxidant compound compared with other varieties of quinoa (Villacrés et al., 2022).

Similarly, the glutathione concentrations in quinoa 'Titicaca' leaves increased also with 100 mM NaCl treatment. Kocsy et al. (2004) demonstrated that glutathione synthesized under conditions of salt stress was involved with tolerance response mechanism. Furthermore, salinity increased the antioxidant system with a higher activity of the enzymes CAT, APX and GR in order to maintain the cell's redox

homeostasis (Parida et al., 2004). This work shows that 'Titicaca' presents high CAT activity compared to previous works in other quinoa cultivars, while the opposite was detected in relation to GR (Derbali et al., 2021). However, the activities of APX found in this work are in accordance to previous results in 'Titicaca' (Turcios et al., 2021).

In relation to phenolic compounds, it is known that they serve as non-enzymatic antioxidants under salinity conditions (Kulbat, 2016). We found an increase of total phenolics as salinity rose, indicating that leaves of quinoa 'Titicaca' could be an important source of these metabolites, which can be used as food antioxidant. This would be in accordance with Stoleru et al. (2022), who evaluated the influence of the cultivar on the content of phenolic compounds in quinoa, showing that 'Titicaca' leaves are rich in phenolics.

CONCLUSIONS

Salinity influences quinoa 'Titicaca' cultivated under semi-controlled conditions differently depending on the stress severity. Salt stress increased the content of bioactive and antioxidant compounds in leaves. However, high salinity conditions induced a decrease in plant biomass and leaf fresh weight, which does not happen with moderate salt concentrations up to 100 mM NaCl. Thus, it would be particularly interesting to favor cultivation of quinoa greens under moderate salinity conditions to enhance or maintain crop yield and quality.

Author contribution

Conceptualization: M.C.R-H., I.G. Methodology: M.C.R-H., I.G. Investigation: M.C.R-H., I.G. Statistic analysis: M.C.R-H. Writing-original draft: M.C.R-H., I.G. Writing-review & editing: M.C.R-H., I.G. All co-authors reviewed the final version and approved the manuscript before submission.

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