Exploring tolerance mechanisms and root morphological development of New Zealand spinach and quinoa across salinity levels

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A B S T R A C T

Soil salinity, root sodium concentration, and transpiration rate are important environmental factors impacting plant growth and productivity. This study investigates the salt-coping strategies of two plant species under varying salinity levels: New Zealand spinach species grown at three salinity levels (i.e., 0, 100, and 200 mM NaCl) and three varieties of quinoa, i.e., "Vikinga", "Dave 407" and "Red Head", grown with or without salt (i.e., 0 and 100 mM NaCl). The plants were grown on glass fiber sheets (rhizoslides) under controlled laboratory conditions. The relationship between transpiration rate, salinity, and root architecture was analyzed for each species. The root apparatus of New Zealand spinach was significantly more developed in plants grown at 100 mM NaCl compared to both 200 mM NaCl and control. The quinoa varieties responded differently to the increasing salt concentrations, with salt-treated "Red Head" plants significantly reducing their total root length and salt-treated "Vikinga" significantly reducing its lateral roots compared to the controls. Salt concentration near New Zealand spinach roots was lower compared to areas without roots, suggesting active salt uptake. In contrast, quinoa rhizoslides exhibited higher salt concentration in proximity to the roots, indicative of salt exclusion. The study provides insights into the adaptation and tolerance mechanisms of these two species to salinity, elucidating the mechanisms by which plants regulate their sodium uptake and root growth. The obtained results suggest that not only the selection of appropriate plant species (halophytes), but also varieties, plays a crucial role in improving crop productivity in salt-affected areas. Elucidating the salt-coping mechanisms of halophytes in relation to soil and climatic conditions is important to better predict their behavior in various saline environments.

1 Introduction

A large part of the world population is living in coastal areas, that are significantly affected by climate change: salinity in agricultural soils is increasing through natural processes like seawater intrusion and human activities such as irrigation with saline or brackish water and inappropriate leaching. For this reason, salinization is currently one of the challenges of modern agriculture. It is expected to further aggravate in the future mainly because of persistent drought and sea level rise (Hassani et al., 2021; Negacz et al., 2022), putting food production at risk (Ahmad et al., 2019).

Several scenarios predict a substantial decline in crop yields due to salinity, particularly in low-lying coastal and irrigated dryland agricultural regions (Hassani et al., 2021). Soil salinization represents a form of land degradation characterized by the accumulation of soluble salts in the soil. The main worldwide produced crops (e.g., wheat, maize, sugarcane) are sensitive to salinity and cannot grow on saline soils (Katerji et al., 2000). Elevated salinity levels can consequently lead to the loss of soil resources, commodities, and services, thereby exerting adverse effects on both agricultural productivity and environmental well-being. Current and future agricultural practices and techniques should diligently address these concerns.

The cultivation of salt-tolerant crops represents a valid option for sustaining food production in regions affected by salinity (Atzori, 2021; Rozema et al., 2013). Salt stress in plants can be simplified as a dual challenge involving osmotic and ionic stress components. Firstly,

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salt ions act as osmotic solutes and generate an osmotic pressure on plants' root surroundings (i.e., the osmotic effect). This osmotic salt stress acts as a form of drought stress, impairing plants' capacity to absorb water from the soil (Munns and Tester, 2008). On the other hand, certain salt ions can exhibit phytotoxic effects on plants (i.e., the ionic effect). Predominantly Na^+ ions can disrupt the balance of K^+ homeostasis and have several negative effects on plants' fundamental processes such as photosynthetic activity (Hao et al., 2021; Kronzucker et al., 2013). The ionic aspect of salt stress is primarily linked to nutrient imbalance and typically emerges later in time than osmotic stress, as toxic ions accumulate within plant cells. Salt stress exerts both short-term and long-term effects on plant physiology, affecting plant species from a whole-plant perspective down to cellular dynamics, through the interplay of these osmotic and ionic forces (Hameed et al., 2021; Johnson and Puthur, 2021; Murphy et al., 2003; Sudhir and Murthy, 2004).

Salt-tolerant plants have developed several mechanisms to deal with the effects of salt stress. Among these, we recall: growth only in favorable seasons or sites; selectivity against Na^+ and Cl^-; leaching of salts from shoots; sequestering of Na^+ and Cl^- ions in vacuoles or other compartments to prevent cytoplasmic toxicity (Böhm et al., 2018; Colin et al., 2023; Dschida et al., 1992); synthesis of organic solutes; re-translocation of salt to roots and re-secretion; re-secretion by gland-like structures on shoots as salt glands or salt bladders; increasing leaf and stem-succulence. One set of these mechanisms can be present in one group of halophytes whereas others prevail in another group (Breckle, 1990, 2002). Moreover, tolerance levels and strategies vary across species, from sensitive glycophytes to halophytes, using these approaches in various and mixed ways to cope with saline environments (Flowers and Colmer, 2015; Gong, 2021; Zhao et al., 2021). Identification of plant species and varieties capable of withstanding and maintaining productivity in environments characterized by elevated salinity levels is of crucial importance to maintain food production in salt-affected lands. This necessitates an in-depth comprehension of the underlying mechanisms behind salt tolerance.

In pursuit of this objective, we selected two halophyte species: New Zealand spinach (*Tetragonia tetragonioides* (Pall.) Kuntze) and quinoa (*Chenopodium quinoa* Willd.), serving as case study plants. We chose Tetragonia because showing in previous experiments an interesting crop potential in saline environments because of *i*) its salt tolerance; *ii*) its salt uptake capacity; and *iii*) the accumulation of mineral elements and the production of secondary metabolites under mild salinity stress (Atzori et al., 2020). Alike, quinoa is already widely cultivated as a crop and was defined by FAO as the "most nutritionally balanced crop in the world", together with its salinity tolerance as well as the ability to accumulate significant amounts of salt in its aboveground parts (Panta et al., 2014).New Zealand spinach is native to cool sandy and rocky seacoasts, notably in New Zealand, Japan, Argentina, and Chile, now widely distributed throughout the world (Taylor, 1994). Given its potential as a sodium-accumulating halophyte, it has been suggested as a candidate for the phytoremediation of salinized soils (Atzori et al., 2020; Hasanuzzaman et al., 2014; Neves et al., 2008). Quinoa cultivation was historically concentrated in South America (Alandia et al., 2021). In recent years, it has gained global recognition owing to its nutritional and functional attributes, as well as its capacity to grow under challenging climatic conditions, including salinity, acidity, drought, and frost (Ahmed et al., 2021; Angeli et al., 2020; Schmidt et al., 2021).

This study explores physiological and morphological features related to the two halophytes' ability to cope with salt-induced stress. The research aims to elucidate the interactions between salinity and root architecture, transpiration rate, and root-sodium concentrations within these two plant species. Root development, soil salinity, and transpiration rates are fundamental agronomic factors, capable of substantially impacting plant growth and productivity. Consequently,

the outcomes of this study hold significant implications, offering valuable insights for the efficient screening of salt-tolerant plant species and the formulation of agricultural strategies, particularly in salt-affected soils or in areas at risk of salinization. These findings may, in turn, contribute to the optimization of crop productivity in such challenging environments.

2. Material and methods

2.1. Plant material and germination

We used two salt-tolerant species for this trial: the New Zealand spinach and three varieties of quinoa, namely "Vikinga", "Dave 407" and "Red Head". Experiments on New Zealand spinach were conducted at the DAGRI Department facilities of the University of Florence, Italy. The seeds were purchased from Fratelli Ingegnoli (Milano, Italy) and left in water for 48 h to synchronize germination. After that, seeds were sown in pots with standard soil and kept constantly watered in a dark growth chamber at 19 °C. After plants emerged, pots were moved to a growth chamber set at 25 °C with a light/dark cycle of long day (14/10). Experiments on quinoa were conducted at the Flemish Research Institute for Agriculture, Fisheries and Food (ILVO) in Melle, Belgium. Seeds for varieties "Vikinga", "Dave 407" and "Red Head" were purchased, respectively, from Quinoa Quality (Regstrup, Denmark), Association Kokopelli (Le Mas d'Azil, France), and Wild Garden Seeds (Philomath, Oregon, USA). Seeds were sown in pots with a 1:1 perlite-vermiculite mix in a greenhouse and irrigated with tap water containing 2 g L^{-1} Hakaphos Spezial 16-8-22 (+3) fertilizer (Compo Expert, Germany).
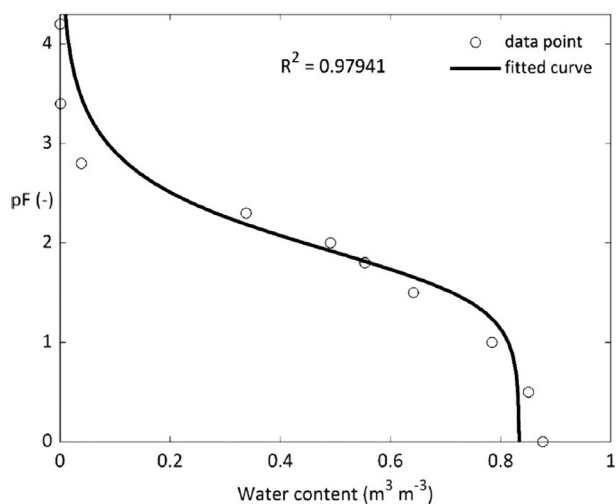
2.2. Rhizoslides set-up

The rhizoslide system was developed as a 2D soil substitute with known hydraulic characteristics to monitor salt accumulation, root architecture, and transpiration rate in young plants. The study was conducted under controlled laboratory conditions to eliminate other variables that could affect plant growth.

For both species, we gently removed the plant from the pot by washing the soil after plants reached the stage of six to eight leaves. Plantlets were then left for two days in the dark in an aerated tap water hydroponics system under a short-day regime (12/12) and at a temperature of 25 °C to let the root apparatus recover after the soil removal stress.

Using a similar methodology to that described by Perelman et al. (2020), we anchored the root apparatus of the plants with waterproof micropore tape on glass fiber filter sheets (10 × 14 cm for New Zealand spinach and 14.5 × 23 cm for quinoa; Fipa MN GF-4, fibers diameter ≤ 3.5 μm &≥ 10 μm in length, MACHEREY-NAGEL GmbH and Co. KG, Germany). This setup was used to determine the effects of saline irrigation water on the Na^+ distribution around the plant roots and along filter paper. Since the materials used are identical to those used in Perelman et al. (2020), it was possible to use the same water retention curve determined in that study (Fig. 1).

Each rhizoslide, i.e., a glass fiber sheet containing the root system of one plant, was positioned inside a plastic bag (15 × 18 cm for New Zealand spinach and 26.5 × 25 cm for quinoa). The bag was sealed on the top on both sides of the plant shoot. The shoot grew outside the plastic bag at a light intensity of 160 ± 20 μmol m^{-2} s^{-1} for New Zealand spinach and 260±1 μmol m^{-2} s^{-1} for quinoa. The plastic bag had an opening near the bottom (at a height of 5 cm for New Zealand spinach and at a height of 3 cm for quinoa) for irrigation by using a pipette without touching the filter paper (Fig. 2). As roots were never in direct contact with the solution, plant nutrient uptake was only possible through a capillary rise in the paper and subsequent root water uptake.

The treatments used in the experiments were: *i*) full-strength Hoagland solution (Hoagland and Arnon, 1938) for the control treatment (for both New Zealand spinach and quinoa experiments); *ii*)

Fig. 1. Water retention curve, i.e., the relation between the volumetric water content of filter paper used in the rhizoslides set-up and the pressure head expressed as pF ($\log_{10}$(|pressure head in cm|). Experimental data is represented by dots, while the fitted curve to the van Genuchten function is represented by a thick black line. Obtained values for the van Genuchten parameters were θs=0.83396, θr=0.001, α = 0.01982 cm^{-1} and n = 1.7622. (Reprinted with permission from Springer Nature from Perelman et al. (2020) "Tracing root-felt sodium concentrations under different transpiration rates and salinity levels", Plant and Soil Journal).

full-strength Hoagland solution added with a 100 mM NaCl solution (for both New Zealand spinach and quinoa experiments); *iii*) full-strength Hoagland solution added with a 200 mM NaCl solution (for New Zealand spinach experiment only). Quinoa plants were initially subjected also to the 200 mM NaCl salinity treatment but the tested concentration proved to be lethal.

Plastic bags were covered in aluminum foil (New Zealand spinach) or kept in a dark box (quinoa) to prevent algae formation and avoid the light stress on the roots (Yokawa et al., 2014). Plants were grown inside these systems in a growth chamber at 25 °C with a light/dark cycle of 12 h/12 h and relative humidity ranging from 60 % to 70 %. Experiments lasted 10 days for New Zealand spinach and 14 days for quinoa. For both species, irrigation (treatments) was supplied when
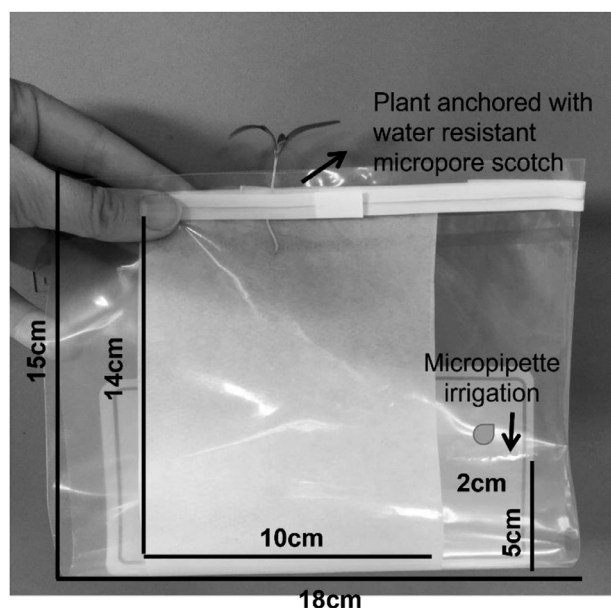
Fig. 2. The rhizoslide set-up for New Zealand spinach, serving as a 2D soil surrogate for tracking salt accumulation, is shown along with its dimensions.

needed, i.e., conducting punctual observations every 48 h and adding the solution when the bottom of the plastic bag was dry. Every irrigation amount was recorded.

Each salinity treatment had an extra reference sheet without plants. The plastic bag containing the filter paper without plant was kept in the same conditions as all other rhizoslides and irrigated when needed following the same procedure as for bags hosting plants. The reference bag was used to determine the evaporation of the solution (treatment). For the New Zealand spinach experiment, each treatment had 10 replicates + 1 reference bag. For quinoa, 5 replicates + 1 reference bag per treatment for each variety were used.

2.3. Transpiration of plants

The total amount (mL) of solution given during the trial was recorded to determine plant transpiration. At the end of the experiments, the residual solution in each bag was subtracted from the total amount of irrigation provided. The amount in mL of the evaporated solution obtained through the reference bag per each treatment was thus subtracted from the plants' water consumption to obtain the transpiration values. In the New Zealand spinach experiment, the dry weight of the plant's shoot (6 replicates per treatment) was measured after air-drying until constant weight. Plants' water use efficiency (WUE) was then calculated as follows:

WUE = DW Shoot / T where DW Shoot is the plant shoot dry weight (g) and T is the crop transpiration (L).

2.4. Determination of sodium accumulation on the rhizoslides

To determine the concentration of sodium accumulated around the root apparatus and on the glass filter paper, paper discs of 0.7 cm diameter were cut at the end of the experiments using a hole-puncher at different heights on the rhizoslide and at different distances from the root apparatus (i.e., close versus far from the roots) after drying the glass filter paper. For New Zealand spinach the cutouts were done at a regular spacing of 3 cm along the principal root and the first two secondary roots, and at comparable heights on the side of the paper. The heights for the collection of discs are named: "top", "middle-top", "middle-bottom", and "bottom". For the quinoa rhizoslides, the cutouts were taken at three distinct vertical heights, named "top," "middle," and "bottom," with an approximate separation of 6 cm between each level. As for New Zealand spinach, discs were collected at the described heights both close and far from the roots on the rhizoslide. Fig. 3 depicts this sampling protocol for both species.

Each cutout disc was then inserted in a tube filled with 1 mL Milli-Q water and shaken for 2 h to extract the accumulated salts: the volume of the disc was around 0.02 mL, thus the water volume in the disc can be neglected compared to the added water volume. The salt level of the solution was measured with the LAQUAtwin Na-11 electrode (B-722 LAQUAtwin Compact Sodium Ion Meter, Horiba, Japan). As the volume of the cutout disks was around 0.02 mL, the salt was diluted 50x during the extraction process. The LAQUAtwin electrode displays Na^+ concentration in ppm (23 ppm Na = 1 mM Na). Factoring in the 50x dilution, the observed $[Na^+]$ (ppm) on the electrode was converted to the real $[Na^+]$ (mM) by multiplying by 2.174 (= 50 / 23).

$[Na^+]$ (mM) = 2.174 x $[Na^+]$ (measured ppm)

We used 6 rhizoslides for each treatment to determine the Na-accumulation on the rhizoslides in the New Zealand spinach. Discs collected at the same height and distance from the roots on the 6 rhizoslides of the same treatment are indicated as replicates. This resulted in six discs collected in the "top" region under the roots, six discs collected in the "top" region far from the root, six discs collected in the "middle top" region under the roots, etc. for each treatment with NZ spinach. For quinoa, an average number of 10 replicates/treatment was used following the same description provided for the New Zealand spinach and the regions provided in Fig. 3: "top", "middle", and "bottom".
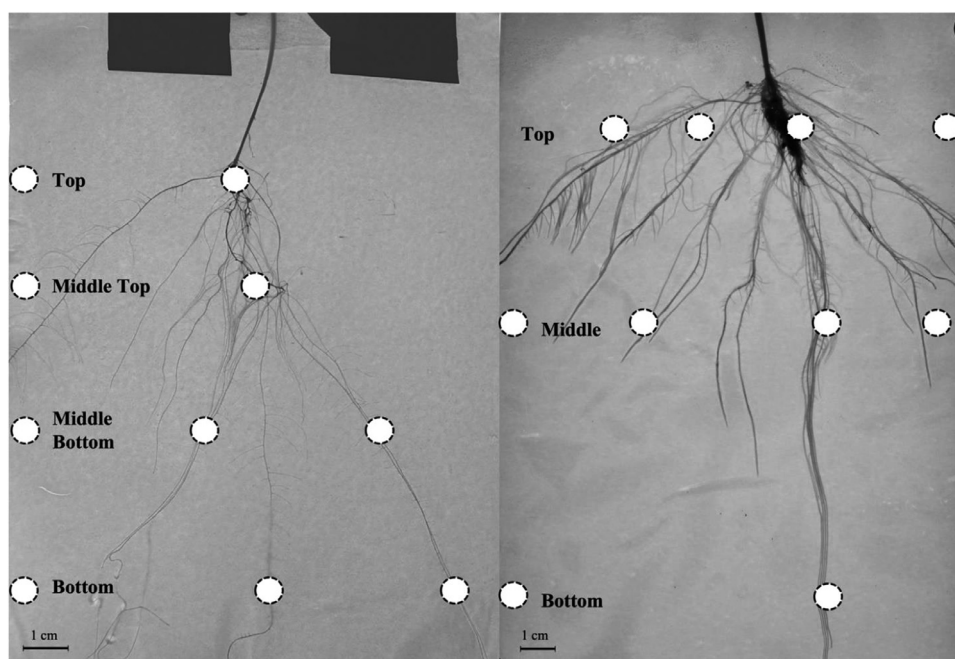
Fig. 3. Spatial arrangement of sampling cutouts designed for quantifying sodium accumulation in New Zealand spinach (left) and quinoa (right) rootzone.

2.5. Root apparatus imaging collection and analyses

We collected pictures of the growing root apparatus for both species at the end of the trial, once the main root had reached the bottom of the rhizoslide. The software ImageJ (Schneider et al., 2012) and its plugin SmartRoot (Lobet et al., 2011) were used to track the roots and to measure the parameters shown in Tables 1 and 2: total root length, median length, main root length, average insertion angle, total amount laterals, Q3 number laterals, Q3 lateral density, median lateral density, median lateral length, root system angle, the fraction with laterals. The plugin operates on an 8-bit grayscale version of the original image and allows for the tracing of different root types to form a complete topology of the root system. Despite its usefulness, manual annotation using SmartRoot can be time-consuming and challenging, especially for complex root systems, as was the case with New Zealand spinach. For this reason, the images of New Zealand spinach have been rebuilt using image editing software with a white background and all roots in clear black at a fixed mark thickness before each analysis (Fig. 4). Thus, the thickness of the roots could not be used. Once the root architecture was completed and the properties of each individual root were obtained, the root system angle was calculated as the difference between the root angle of the first quartile (the 25 % of roots with the lowest angles) and the third quartile (Q3, i.e., 25 % of roots with the highest angle). This calculation provides an estimate of the overall angle at which the entire root system extends, with a greater angle indicating a larger lateral extension of the root system.

2.6. Statistical analysis

All statistical analyses were performed using the software Graph-Pad Prism for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. The effects of salt concentrations (0, 100 mM NaCl, and 200 mM NaCl) on the different parameters in the case of the New Zealand spinach were analyzed through a one-way analysis of variance (ANOVA). In the case of quinoa, the effects and interaction of the two main factors - salt treatments and varieties - on the investigated parameters were analyzed through a two-way ANOVA. A post-hoc Tukey multiple comparison test was conducted for all cases to determine significant differences among means at $P \leq 0.05$.

3. Results

3.1. Root apparatus development

3.1.1. New Zealand spinach. Table 1 shows the results obtained from the analysis of the root architecture of New Zealand spinach grown using the rhizoslides experimental set-up.

Fig. 5 zooms in on the parameters significantly affected by the salinity treatments. Total root length increased significantly in plants grown at the 100 mM NaCl salt treatment ($RL_{tot,100mM}$=114.35 $\pm$ 20.41 cm), as compared to the 200 mM NaCl ($RL_{tot,200mM}$=68.56 $\pm$ 16.70 cm) and the control group

Table 1

Comprehensive parameters of the New Zealand spinach root architecture at different salinity levels (0, 100 mM NaCl,200 mM NaCl) obtained through the rhizoslide system. One-Way ANOVA and Tukey's Test (n = 5) was performed among treatments, different letters in the same column indicate statistically significant difference at $P <$ 0.05.

	Total Root Length (cm)	Median Length	Main root length	Avg insertion angle (°)	Total amount laterals	Q3 Number Laterals	Q3 Lateral Density (1/cm)	Median lateral density (1/cm)	System angle (°)
Control	58,35 ± 18,66 a	1,41 ± 0,49	8,5 ± 3,91 ab	56,9 ± 12,5	26,6 ± 11,01 ab	11,4 ± 7,9	4,77 ± 2,54	4,6 ± 2,54	40,65 ± 21,89
100 mM NaCl	114,35 ± 20,41 b	1,67 ± 1,19	11,71 ± 3,19 a	66,03 ± 8,33	46,4 ± 20,94 a	8,2 ± 5,9	5,39 ± 2,40	4,01 ± 1,78	75,81 ± 17,96
200 mM NaCl	43,57 ± 20,34 a	1,44 ± 0,64	4 ± 1,32 b	59,37 ± 19,76	18 ± 6,20 b	10 ± 1,49	4,97 ± 2,39	4,41 ± 1,79	41,612 ± 24,04

Table 2
Comprehensive parameters of the root architecture of the three varieties of quinoa at 0 and 100 mM NaCl obtained through the rhizoslide system. Two-way ANOVA and Tukey's Test ($P < 0.05$, $n = 5$), with asterisks representing statistically significant differences (* $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

	Total length (cm)	Median length (cm)	Main root length (cm)	Average insertion angle (°)	Total amount laterals	Q3 laterals per root	Q3 Lateral density (1/cm)	Median lateral density (1/cm)	Median lateral length (cm)	Root system angle (°)	Fraction with laterals (%)
DAVE											
Control	355,01 ± 187,27	355,01 ± 187,27	6,5 ± 5,13	59,8 ± 6,88	293,2 ± 134,99	14,4 ± 7,37	8,06 ± 2,15	4,86 ± 1,26	0,4 ± 0,17	74,06 ± 14,3	0,1 ± 0,04
100 mM NaCl	233,83 ± 119,46	233,83 ± 119,46	3,8 ± 1,52	56,8 ± 3,87	157,4 ± 67,61	7,4 ± 2,13	7,37 ± 1,95	4,96 ± 0,71	0,6 ± 0,34	66,33 ± 8,13	0,14 ± 0,03
RED HEAD											
Control	709,99 ± 231,96	0,96 ± 0,85	3,67 ± 2,35	60,83 ± 5,12	483 ± 171,2	11,4 ± 5,55	9,05 ± 6,04	4,14 ± 2,31	0,59 ± 0,26	81,22 ± 19,4	0,10 ± 0,06
100 mM NaCl	280,57 ± 50,61	0,58 ± 0,25	4,52 ± 3,71	65,01 ± 4,29	251,8 ± 50,78	9,45 ± 3,96	8,44 ± 2,71	5,37 ± 2,23	0,58 ± 0,25	84,89 ± 7,06	0,11 ± 0,04
VIKINGA											
Control	364,74 ± 139,17	0,41 ± 0,22	2,57 ± 1,11	67,48 ± 5,63	417 ± 243,4	34,75 ± 11,59	8,13 ± 1,10	5,78 ± 0,49	0,41 ± 0,22	96,73 ± 16,8	0,05 ± 0,02
100 mM NaCl	147,11 ± 107,71	0,30 ± 0,15	2,94 ± 1,51	64,02 ± 7,89	179,6 ± 95,55	13,6 ± 3,49	7 ± 1,57	4,87 ± 0,53	0,3 ± 0,15	103,99 ± 26,03	0,09 ± 0,01
Interaction	8325 ns	9713 ns	7133 ns	8161 ns	2006 ns	17,23 **	0,1392 ns	8729 ns	9846 ns	2632 ns	2364 ns
Varieties	22,13 **	13,85 ns	9927 ns	23,50 *	13,21 ns	41,79 ****	3252 ns	2,51 ns	13,44 ns	36,64 **	23,46 *
Salt treatments	32,94 ***	0,6872 ns	0,6289 ns	0,3685 ns	35,84 ***	28,48 ***	1894 ns	0,2419 ns	0,7775 ns	0,07148 ns	13,54 *
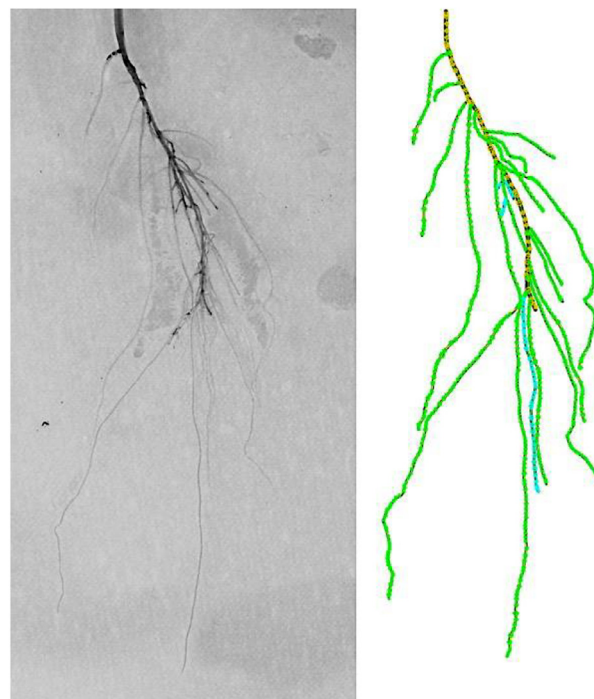
Fig. 4. Root system of New Zealand spinach and its analysis through the ImageJ software and its plugin SmartRoot (Lobet et al. 2011).

($RL_{tot,0mM}$=43.57 ± 20.34 cm). Furthermore, the number of lateral roots increased notably in the 100 mM NaCl treatment, with a total lateral root count of 46.4 ± 20.94, in contrast to the 200 mM NaCl treatment, which exhibited the lowest count of only 18 ± 6.20 roots. Similarly, the length of the primary root exhibited a consistent pattern, with the longest measurement observed in the 100 mM NaCl treatment (11.71 ± 3.19 cm), followed by the control (8.5 ± 3.91 cm) and the 100 mM NaCl treatment (4 ± 1.32 cm). All the other parameters did not show any statistical difference among treatments.

3.1.2. Quinoa. Salt treatments induced significant changes in the root architecture and in the investigated parameters on the quinoa varieties. Results on the parameters significantly affected by salinity are shown in Fig. 6.

All other parameters investigated on quinoa are shown in Table 2.

The obtained results show a different behavior of all three varieties in the total root length, even under control conditions, as shown in Table 2. The "Red Head" variety significantly reduced its root length in saline conditions compared to the control ($RL_{tot,100mM}$=280,57 ± 50,61 cm, and $RL_{tot,0mM}$=709,99 ± 231,96 cm), whereas, for the other varieties, no significant differences were assessed. The variety "Vikinga" is the only one for which the number of laterals per root is significantly affected by salinity (Fig. 5). Moreover, both variety and salt treatment are significant factors for the number of laterals per root. On the opposite, the root system angle is not affected by the salt treatment, whereas the variety factor is significant with a $P < 0,01$.

3.2. Transpiration

Fig. 7 shows the total plant transpiration averaged by treatment.

For New Zealand spinach, transpiration rates declined with increasing salinity in the nutrient solution. Specifically, the 100 mM and 200 mM NaCl treatments showed transpiration rates of 7.15± 3.94 mL and 5.75±5.38 mL, respectively, whereas the control group exhibited a transpiration rate of 31±10.58 mL. On the other hand, plant shoot dry weight (DW), shown in Fig. 8, increased in the
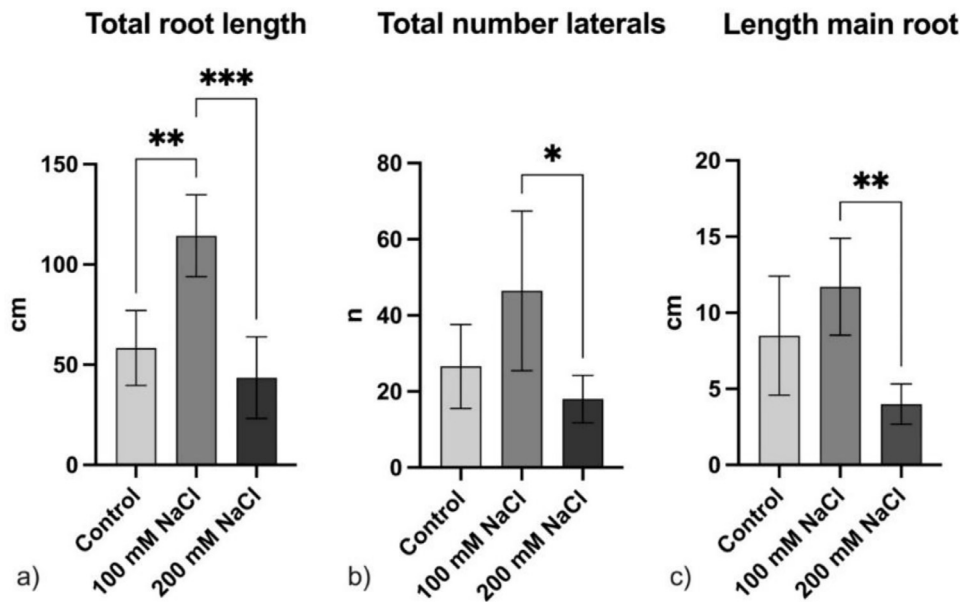
Fig. 5. Total root length a), total number of laterals b), and main root length c) of New Zealand spinach grown under three salt treatments. Values are mean (n=5) ± standard deviation (SD). Asterisks denote statistically significant differences at $P < 0.05$ (one-way ANOVA and Tukey's Test).
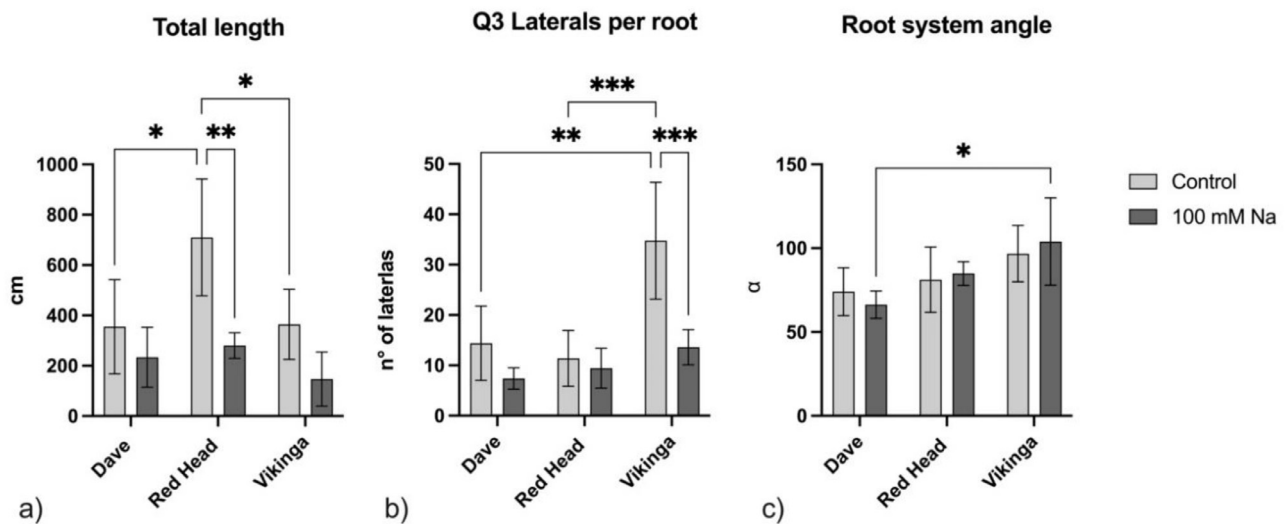

Fig. 6. Total root length a), third quartile of number of laterals per root b) and root system angle of quinoa c). Values are means (n=5) ± standard deviation (SD) . Asterisks denote statistically significant differences at $P < 0.05$ (one-way ANOVA and Tukey's Test).

100 mM NaCl treatment (0.049±0.009 g) in comparison to the 0.027±0.007 g observed in the 200 mM NaCl treatment and the 0.026±0.005 g observed in the control group. Such an increase in shoot production and a decrease in transpiration observed in the 100 mM NaCl translates into a significantly increased WUE compared to the control (Fig. 8).

Quinoa behaved differently. "Dave" and "Vikinga" varieties did not have significantly different transpiration rates among treatments (Fig. 7). The "Redhead" variety decreased its transpiration rate significantly under saline conditions as compared to the control. Unfortunately, no data on the shoot production are available to indicate if a related decrease in plant growth performance or an increase in WUE occurred. The leaves were very brittle and at the end of the experiment, we were unable to accurately assess the shoot production for quinoa, since many leaves had fallen already during the manipulation of the rhizoslides.

3.3. Na accumulation in the rhizoslides

3.3.1. New Zealand spinach. Fig. 9 shows the sodium ion concentration [Na⁺] along the rhizoslide paper. Results show the salt concentration within discs gathered both in proximity to the root apparatus and at the furthest point on the paper, but at an equivalent vertical height, following the scheme described in Fig. 3. As expected, no difference in sodium accumulation was observed in the control group across all the vertical heights. On the opposite, our findings revealed noteworthy distinctions within the 100 mM NaCl treatment, with a significantly higher [Na⁺] in the bottom region, where no roots were present (321±64 mM NaCl) compared to the comparable height close to the roots (159 ± 46 mM NaCl). Moreover, we observed a gradient when focusing on discs collected at all heights far from the root apparatus, with the bottom region assessing the highest [Na⁺] compared to the three other analyzed heights. In contrast, when focusing on
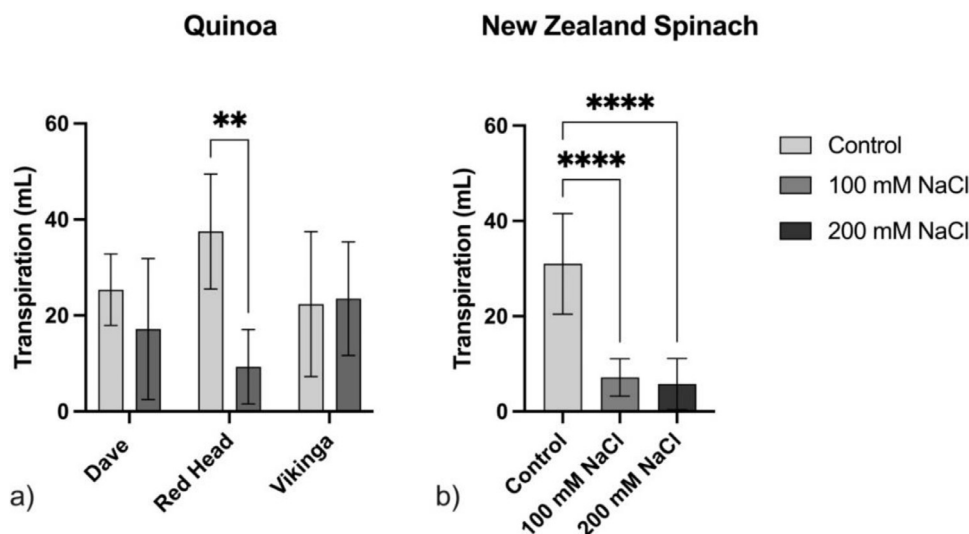
Fig. 7. Transpiration in quinoa a) and New Zealand spinach b) grown under increasing NaCl concentrations. For New Zealand spinach statistical analysis was conducted using One-Way ANOVA and Tukey's Test ($P < 0.05$, $n = 10$). For quinoa, using Two-Way ANOVA and Tukey's Test ($P < 0.05$, $n = 5$).
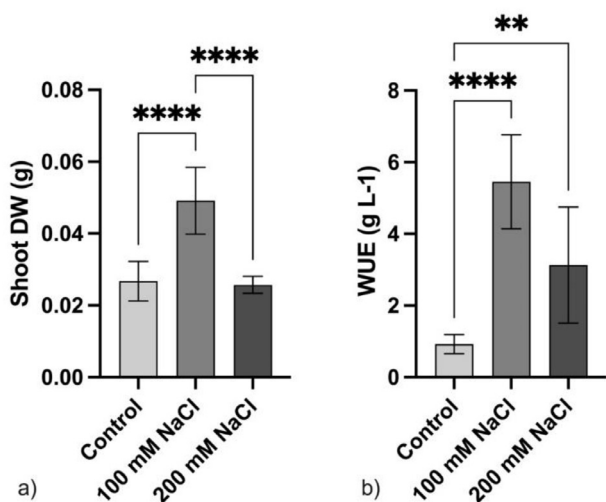

Fig. 8. Shoot dry weight a) and water use efficiency b) of New Zealand spinach plants exposed to various salt treatments (0, 100, 200 mM NaCl). Mean values ($n=10$) ± standard deviation (SD) are reported. Asterisks denote significant differences at $P < 0.05$ determined using One-way ANOVA and Tukey's Test.

the discs collected close to the root apparatus, no gradient was observed and all tested heights exhibited comparable salt concentrations. This suggests a role for roots in sodium sequestration, which would also explain the diminished salt levels in the vicinity of the root apparatus growth zone compared to the furthest points on the paper. Such root sodium uptake activity is furthermore suggested by the results obtained in the 200 mM NaCl treatment: even if the observed differences were not statistically significant, the [Na$^+$] was always higher in the disc collected far from the roots compared to the disc collected at the same height close to the root. Furthermore, we noticed an accumulation of salt on the rhizoslides compared to the given solution concentration, on average by a factor of about 2. This can be explained by multiple additions of new solution in time (not alternated by any rinse of the paper) and to the evaporation process. The amount of evaporation was comparable in all treatments, as expected. In contrast, its share of the total evapotranspiration changed significantly among treatments, with evaporation accounting for 45 %, 74 %, and 81 % of total evapotranspiration (data not shown) in the control, 100 mM NaCl and 200 mM NaCl treated plants, respectively.

3.3.2. Quinoa. As reported in Fig. 10, results on [Na$^+$] obtained from the quinoa variety "Dave" and "Vikinga" show no statistically
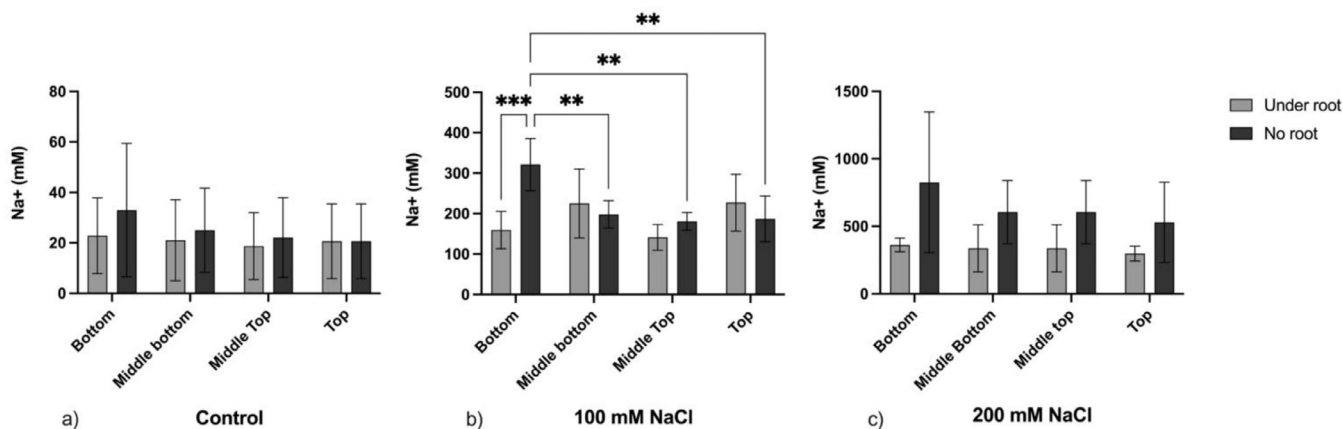
Fig. 9. Comparison of Na$^+$ accumulation on rhizoslides disks collected at various points for the three different salt concentrations (0 mM, 100 mM, 200 mM NaCl) of New Zealand spinach. Mean values (n=6) ± standard deviation (SD) are provided, with asterisks indicating statistically significant differences at $P < 0.05$ (determined using Two-way ANOVA and Tukey's Test).
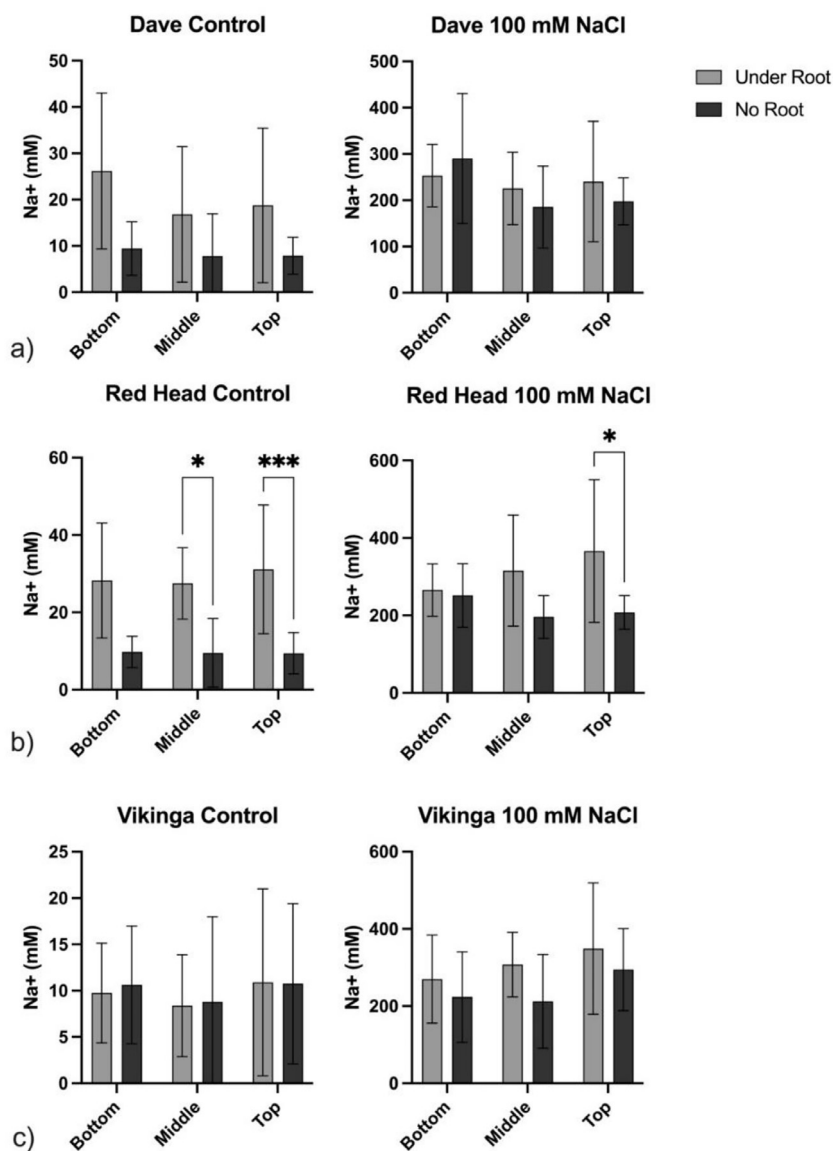
Fig. 10. Comparison of Na⁺ accumulation on quinoa rhizoslides disks collected at various points for the two different salt concentrations (0, 100 mM NaCl). Mean values ($n \approx 10$) ± standard deviation (SD) are provided, with asterisks indicating statistically significant differences at $P < 0.05$ (determined using Two-way ANOVA and Tukey's Test).

significant differences between samples taken in the proximity of the root system and those taken at the same height far from the roots for both treatments. Nevertheless, an opposite trend compared to that shown by New Zealand spinach is observed in rhizoslides treated at 100 mM NaCl: in both varieties, the [Na⁺] of discs collected close to the root is higher compared to the [Na⁺] of discs collected at a comparable height but far from the root, suggesting salt exclusion by roots. These observations are significant in "Red Head" variety results, with the under-root concentration showing significantly higher values in the "top" point (366,14 ± 183,99 mM) compared to that of discs collected at a comparable height far from the roots (207.9 ± 43.53 mM). As was also shown for New Zealand spinach, we observed an accumulation in [Na⁺] compared to the given concentration in the irrigation water on the rhizoslides of quinoa. Also here, we noted an increase by a factor of about 2, reinforcing the hypothesis of the role of the multiple irrigation treatments in time not alternated by any rinse of the paper and the evaporation process. Evaporation was comparable among treatments. Its share in the total ET increased with increasing salinity since transpiration decreased with salinity level. Evaporation represented in control rhizoslides 53 % of total ET and 62 % in salt-treated rhizoslides (for both treatments, the reported

percentages are an average among the three varieties, data not shown).

3. Discussion

3.1. Distinct root system responses under saline conditions between species

The tested halophytes behaved differently in their response to salt treatments. Many definitions are currently available on halophytes, which - from a more physiological or a more ecological point of view - identify this group of plants as capable of growing in saline environments. The lack of consensus on a unique definition is linked to halophytes' taxonomical and ecological complexity, as much as to the differences in what is defined as a saline environment (Grigore, 2021). Among these definitions (Flowers and Colmer, 2008; Grigore, 2021), in 1974, Walter identified two subgroups (Grigore and Toma, 2017): on one side the euhalophytes, which are those plants that accumulate in their organs large amounts of salts, without being damaged. They are stimulated at this "not too high" concentration. On the other side, other categories of salt-tolerant plants that have species-specific thresholds of tolerance (Atzori et al., 2022). A similar

distinction was highlighted in 1939 by Eijk (1939). He used a classification based on plant distribution and several plant responses, dividing halophytes into plants that have optimal development in saline habitats and plants that tolerate salts, but whose optimal development is in non-saline habitats (Eijk, 1939; Grigore, 2021). Such distinction is consistent with the behavior observed in the species of the present experiment, with New Zealand spinach belonging to the first and the tested varieties of quinoa to the second group, at least when considering the tested concentration of 100 mM NaCl.

The root apparatus development of New Zealand spinach reflected the species' salt tolerance threshold previously assessed in hydroponic conditions (Atzori et al., 2020; Guidi Nissim et al., 2021). Plants' growth both at the shoot and at the root level was not negatively affected by seawater addition to the nutrient solution up to an EC of 18 dS m^{-1} (Atzori et al., 2020) nor by treating plants with a 150 mM NaCl solution (Guidi Nissim et al., 2021). A significant growth reduction compared to the control was instead assessed when treating New Zealand spinach with a 280 mM NaCl solution, which exceeds the treatments used in the present trial. Both in the cited papers and the present experiment, New Zealand spinach growth was not only not impaired by salinity, it was even enhanced. For the present experiment, results on both shoot DW and total root length support this statement.

The quinoa species collects a wide number of varieties which are characterized by different levels of salinity tolerance (Atzori, 2022; Bazihizina et al., 2022a; 2022b; Hussin et al., 2023; Kiani-Pouya et al., 2019). Our results on the tested varieties also showed different responses to salinity. Even if the tested varieties, according to scientific literature, were expected to show salt tolerance characteristics, growth was restrained under saline conditions as compared to the control, suggesting an optimal development in non-saline habitats for the tested varieties. On the opposite, in a pot experiment testing the "Dave" variety (that is also found in literature as Colorado 407 D or CO407D) NaCl began to show a detrimental impact on yield at EC levels of 16 dS m^{-1} and above (Peterson and Murphy, 2015). This roughly corresponds to 160 mM NaCl and thus exceeds the treatment used in the present trial. In a field trial the yield of "Vikinga" did not change between saline (irrigation water reaching a conductivity of 22 dS m^{-1}) and non-saline conditions (Pulvento et al., 2022), again exceeding the 100 mM NaCl given as a treatment in the present experiment. Lastly, for "Red Head" the fresh forage yield (t ha^{-1}) and dry matter ratio of quinoa cultivars grown in non-saline and saline-alkaline soils (with an EC of 9.69 dS m^{-1}, comparable with this trial salinity treatment) were comparable (Keskin et al., 2023). Nevertheless, yield evaluation of quinoa was realized mainly under field or pot conditions, which did not lead to the possibility of evaluating root architecture and plasticity. We cannot thus directly compare the root apparatus development studied in the present experiment with the salinity thresholds assessed in literature through yield production determination. This said, the obtained results suggest further experimentations to correlate the changes in root apparatus development and yield production under saline conditions to be able to understand the role of root plasticity in quinoa species' salt tolerance. In fact, the tested varieties had a different root allocation pattern even without salt presence. Root development and plasticity are variety-dependent, but further studies are required to assess if other factors might induce these differences (i.e., growing on the glass fiber, oxygen availability, light conditions, etc.) or if a correlation with the salt tolerance of a specific variety can be determined.

3.2. Sodium includer, recretohalophytes, and sodium excluder halophytes

New Zealand spinach and quinoa are classified as halophytes (Flowers et al., 1986). According to Breckle (2002), the mechanisms of halophytes to thrive on saline sites include many different mechanisms as listed in the introduction. Based on the presence of certain

salinity tolerance mechanisms, salt-tolerant plants can be categorized as follows: i) recretohalophytes, which can excrete salt from the plant to the outside; ii) euhalophytes, already described in previous paragraph, including leaf succulent and stem succulent halophytes that accumulate salt in the vacuoles of succulent green tissues of leaves or stem, respectively; iii) salt excluding halophytes (Breckle et al., 1995), that achieve salt tolerance through salt exclusion by either excluding most of the Na$^+$ and Cl$^-$ into the soil solution or by accumulating the salt ions in the roots, e.g. monocotyledons halophytes (Chen et al., 2018; Flowers and Colmer, 2008).

In the current experiment, we observed that the New Zealand spinach showed both euhalophytes/sodium-includer characteristics and recretohalophytes traits. Quinoa, known to be part of the recretohalophytes due to its salt bladders on the leaves' surface (Yuan et al., 2016), also presented salt excluder characteristics in the current experiment. The includer behavior of the New Zealand spinach is supported by the lower [Na$^+$] on the rhizoslide paper collected close to the root system compared to that present in the farthest areas on the rhizoslides at both salinity levels. This phenomenon was already observed in prior publications (Atzori et al., 2020; Neves et al., 2007), highlighting the salt-uptake capacity of New Zealand spinach roots. Neves and collaborators (2007, 2008) indeed classified New Zealand spinach as a sodium-including species and emphasized its potential as a salt-tolerant crop in the context of saline agriculture, confirming such capability also by the accumulation of sodium in the leaves and stem tissues. In addition to that includer capability, its recretohalophyte traits are supported by the presence of bladder cells on its leaves surface, increasing their density accordingly with increasing salinity and accumulating [Na+] (Atzori et al., 2020).

Quinoa exhibited instead a behavior more aligned with the sodium excluder halophytes characteristics. The general trend of higher sodium concentration in the proximity of the roots compared to far from it at comparable heights on the rhizoslide suggests the exclusion of salts from the root system. This difference between [Na$^+$] in proximity versus far from the roots was observed in all tested varieties and was statistically significant for the "Red Head" variety. This last variety was previously found to tolerate a salinity level comparable to the treatment administered in the present treatment (Keskin et al., 2023) and its ability to exclude salt might be connected to its salt resistance. A wide range of studies identified recretohalophytes characteristics for quinoa linked to the exclusion of salts from its leaves' salt glands (Bazihizina et al., 2022a; 2022b; Otterbach et al., 2021; Yuan et al., 2016). Nevertheless, recent studies have highlighted the fact that our understanding of salt tolerance mechanisms in quinoa is still limited and remains an open question. A recent paper by Moog et al. (2022) investigated the role of quinoa epidermal bladder cells (EBCs), known to confer to this species salt tolerance in the frame of the recretohalophytes sets of mechanisms. Yet, when wild-type quinoa plants were exposed to saline conditions, EBCs accumulated potassium as the major cation, in quantities far exceeding those of sodium. Moreover, emerging leaves densely packed with EBCs had the lowest Na$^+$ content, whereas old leaves with deflated EBCs served as Na$^+$ sinks. Such results challenge the understanding of quinoa salinity tolerance. Moreover, little literature addresses the role of roots in quinoa's ability to salt exclusion and/or re-secretion. The findings of our current study on quinoa salt excluder behavior add more questions to such challenge. Our results suggest that quinoa salt tolerance can only partly be explained by its ability to excrete salts from its EBCs as recretohalophyte and that the role of roots in excluding salts should be further explored.

Importantly, even if both species are characterized by recretohalophyte traits, they behave in an opposite way when coping with salinity at the root level. Both tested species had to deal with Na at a cost. Understanding the "economics" of salt tolerance and the limits of the different strategies still leads to open questions and challenges. New Zealand spinach proved to take up salts into its shoot, thanks to its

ability to increase leaf succulence under saline conditions (Atzori et al., 2020), compartmentalizing the excessive salt ions both into the vacuole and on leaves bladder cells. On the one hand, this reduces the water potential of the plant and helps it absorb water from the saline soil. On the other hand, this reduces the ion content in the cytoplasm and avoids damage to enzymes and biological substances in the cytoplasm. The observed decrease in transpiration in saline versus control conditions also plays an important role. The decrease is correlated to a reduced stomatal conductance in saline versus non-saline conditions previously reported in literature (Atzori et al., 2020), which at a certain point comes at a cost for photosynthetic activity. Although increases in organic dry matter did occur in 100 mM NaCl conditions, the increases in the content of water and ions were higher. Flowers et al. (1977) argued that the increased turgor might be brought about by the increase in ion content, causing the stimulation of organic dry matter production through the control of growth by turgor. Quinoa, on the other hand, proved to be less tolerant compared to New Zealand spinach, suggesting that its sets of mechanisms in the frame of recretohalophytes and sodium excluder halophytes were less effective to deal with the administered salt treatment. Among the three tested varieties, "Red Head" seemed to possess the strongest mechanisms in salinity coping, such as a significant decrease in transpiration and the strongest sodium exclusion activity at the root level.

Salinity influences the capacity of plants to take up water and nutrients. In our study, the transpiration rate of both New Zealand spinach and the quinoa "Red Head" variety were indeed profoundly affected by salinity. This can partly be explained by osmotic stress, resulting in reduced transpiration rates under saline conditions. Nevertheless, the increase in dry matter production assessed for New Zealand spinach also makes evident a growth stimulation linked to salinity conditions. The obtained findings suggest that the transpiration rate plays a crucial role in regulating the amount of sodium absorbed by plant roots as already reported (Perelman et al., 2020) through the control of ion uptake and osmotic adjustment. Features such as the capability of stocking Na into the vacuoles of the cells and in external bladder cells allowed New Zealand spinach to cope with the administered levels of NaCl solution, in addition to ion relations and the capability of taking up Na. A linked mechanism is probably the impact of salt on the potential closure of stomata that has been already documented in New Zealand spinach (Atzori et al., 2020; Guidi Nissim et al., 2021) and that should be further explored in the quinoa "Red Head" variety.

4. Conclusions

This study investigated the effects of salinity on the growth and root development of two salt-tolerant plant species: New Zealand spinach and quinoa. We made use of paper as a porous grow-medium, to standardize the transport of water and solutes in the medium and isolate the effects of salt-coping mechanisms of the plant in the growing medium-root interactions of both species.

New Zealand spinach showed enhanced growth under 100 mM NaCl conditions. It exhibited both euhalophyte and recretohalophyte traits, uptaking salt into its roots and vacuoles while also excreting salt through bladder cells. This allowed the tested species to maintain productivity under salinity conditions.

The quinoa varieties were less tolerant, with growth restricted under salt exposure. They displayed sodium excluder characteristics, excluding salt from their roots. The "Red Head" variety showed the strongest response, with significant decreases in transpiration and higher salt exclusion compared to the "Vikinga" and "Dave 407" varieties.

Salinity influenced transpiration rates in both species. New Zealand spinach increase in dry matter under salt stress indicated a growth stimulation linked to salinity. Its mechanisms for storing

sodium in vacuoles and bladder cells helped this species to cope with the salt levels.

The study highlights different salt tolerance mechanisms between the two halophyte species. Further investigations under field conditions are needed to validate these strategies and identify new salt-tolerant plant candidates to cope with saline soils.

Declaration of competing interest

The authors have no competing interests to declare.

CRediT authorship contribution statement

D. Comparini: Formal analysis, Methodology, Software, Writing – original draft. **G. Mozzo:** Data curation, Visualization, Formal analysis, Methodology, Software, Writing – original draft. **L. Thiers:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **J. Vanderborght:** Conceptualization, Supervision, Validation, Writing – review & editing. **T. De Swaef:** Conceptualization, Supervision, Validation, Writing – review & editing. **S. Mancuso:** Funding acquisition, Project administration, Supervision. **S. Garré:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **G. Atzori:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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