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ORIGINAL PAPER

Effectiveness of fungal bacterial biofertilizers on agrobiochemical attributes of quinoa (*Chenopodium quinoa* willd.) under salinity stress

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Abstract

The effects of fungi and bacteria biofertilizers under salinity stress were studied on some morphological and physiological traits of quinoa. The experiment was conducted as a factorial based on a completely randomized design with three replications. The factors included NaCl (no salinity-150 mM NaCl and 300 mM NaCl), Trichoderma (no Trichoderma and Trichoderma), and bacterial biofertilizers (no biofertilizer-N biofertilizer and P biofertilizer). The highest shoot and root length and dry weight were observed in the treatment of no salinity + no Trichoderma + N biofertilizer. Salinity increased Na + and K + content and decreased K + /Na + ratio, whereas the application of Trichoderma alleviated the effect of salinity on these factors. Ca^{2+}/Na^{+} and Mg^{2+}/Na^{+} ratios were significantly decreased in high salinity level compared to the zero salinity level. The highest and lowest phenol content was observed in the treatments of 300 mM NaCl + Trichoderma + P biofertilizer and no salinity + no Trichoderma + N biofertilizer, respectively. Flavonoid content was higher in the treatments of no salinity + Trichoderma + N biofertilizer and no salinity + Trichoderma + P biofertilizer than in the other treatments, also The high-performance liquid chromatography analysis revealed that all phenolic and flavonoid compounds exhibited the greatest increase in treatments of no salinity + Trichoderma + N biofertilizer and no salinity + Trichoderma + P biofertilizer, which is consistent with the significant increase in total flavonoid content in these treatments. The results showed that using N biofertilizer can improve the growth and development of quinoa in saline soils.

Keywords Flavonoids · Growth · Halophyte · High-performance liquid chromatography · Phenols · Quinoa

Introduction

Quinoa (*Chenopodium quinoa willd*) is a plant rich in biologically and nutritionally active compounds with plenty of health benefits, which has drawn interest in recent years (Küster and Vila 2017). The high genetic diversity of this species allows it to adapt to and grow in extremely adverse environmental conditions, and therefore, it is highly resistant to abiotic stresses (Aziz et al. 2018). Plants exposed to salinity stress exhibit different responses, including changes in nutrient uptake, cell homeostasis, and metabolic pathways, which may change stem and root elongation (Patterson et al. 2009). Research on two quinoa genotypes showed that salinity stress did not influence the growth and biomass of either genotype at low levels, but high levels of salinity reduced their shoot/root ratio, growth, and biomass significantly (Parvez et al. 2020). Water deficit induced by salinity stress results in the over-accumulation of Na and Cl ions inside the cells, leading to ion unbalance (Gupta and Huang 2014). Salinity stress disrupts the K^{+}/Na^{+} ratio, which is important for plants (Wu 2018) so that it imbalances these ions and reduces the K^{+}/Na^{+} ratio (Reich et al. 2017). Na^{+} NSCC channels are a large family of non-selective cation channels in plasma and tonoplast membranes, which are usually permeable to a wide range of univalent cations (Demidchick and Maathuis 2007). The secretion of Na^{+} to vacuoles is a common and important mechanism of salinity tolerance in

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plants, which is mediated by Na^+/H^+ antiporters and reduces cytoplasm Na^+ content (Shi et al. 2000).

Abiotic stresses in plants, e.g., salinity, induce oxidative stress by producing reactive oxygen species (ROS). ROS are highly cytotoxic, which severely injure such molecules as proteins, lipids, carbohydrates, and nucleic acids and tear cell membrane, causing the leakage of cell content and the death of plant cells (Islam et al. 2015). To detoxify ROS, plants can activate antioxidant defensive systems by inducing enzymatic and non-enzymatic antioxidants (Zhang et al. 2019). Defensive mechanisms have evolved in plants to minimize detrimental ROS or protect the plants against their toxic effects and also protect the balance between ROS production and decomposition to keep cell redox homeostasis (Lin et al. 2011). Antioxidant systems reduce oxidative damage and improve resistance to environmental stress, which is often related to a regular antioxidant system (Suzuki et al. 2012). These systems in a plant are activated by inducing the phenylpropanoid biosynthesis pathway, which can lead to the activation of the genes related to salinity stress and the antioxidant system (Zhu et al. 2021). Flavonoid contents can be increased by biological fertilizers or a combination of biological and chemical fertilizers, such as nitrogen (Gendy et al. 2013). Fertilization sources are an effective factor of biological control, especially against abiotic stresses (Mahdavikia et al. 2019). On the other hand, the efficient application of soil microorganisms (plant growth-promoting rhizobacteria–PGPR) can be effective in increasing and improving plant growth in farming. Different genera of Pseudomonas and Azotobacter are plant growth-promoting microorganisms that improve plant growth and development in both normal and stressful conditions (Vimal et al. 2017). In addition to their role in nitrogen biofixation, siderophore generation, the dissolution of mineral phosphates, and the synthesis of plant hormones, rhizobacteria can influence plant development and performance and mitigate salinity-induced risks (Li et al. 2017). Previous studies have shown that biofertilizers that contain bacteria can be used in saline environments to biologically enhance plant resistance to stress (Ortuno et al. 2013). The fungal genus Trichoderma is another soil microorganism that safeguards plants against environmental stresses such as salinity and improves their growth and development (Brotman et al. 2013). Increased growth of quinoa was reported with the treatment of Trichoderma (Ortuno et al. 2017). Trichoderma produces fungal materials that can stimulate plants to produce their defensive metabolites (Vinale et al. 2012) and can increase plant tolerance against abiotic stresses, e.g., salinity, by inducing protection against oxidative damage (Zhang et al. 2019).

The interaction of plants and beneficial soil microbes, e.g., Trichoderma and bacteria, can improve plant growth under salinity stress and influence plant tolerance of this stress. The present study aimed to explore the effect of N and P biofertilizers and Trichoderma, applied alone or in combination, on morphological traits and leaf polyphenols of quinoa exposed to salinity stress. The results may contribute to provide practical recommendations on the use of these microorganisms to improve the growth and development of this species in highly saline environments. This research was done at the research greenhouse of the Faculty of Agriculture in Urmia University from August to October 2018.

Materials and methods

Cultivation and treatment

The study was conducted as a factorial experiment based on a completely randomized design with three replications under controlled conditions. The factors included salinity at three levels of 0 (S0), 150 (S150), and 300 (S300) millimolar (Mm) NaCl, Trichoderma at two levels of no fungi (T0) and fungi application (T), and bacterial biofertilizers at three levels of control (C), nitrogen (FN), and phosphorus (FP). The treatments included [no salinity + no Trichoderma + no biofertilizer (S_0T_0C), no salinity + no Trichoderma + N biofertilizer ($S_0T_0F_N$), no salinity + no Trichoderma + P biofertilizer ($S_0T_0F_P$), no salinity + Trichoderma + no biofertilizer (S_0TC), no salinity + Trichoderma + N biofertilizer (S_0TF_N), no salinity + Trichoderma + P biofertilizer (S_0TF_P), 150 mM salinity + no Trichoderma + no biofertilizer ($S_{150}T_0C$), 150 mM salinity + no Trichoderma + N biofertilizer ($S_{150}T_0F_N$), 150 mM salinity + no Trichoderma + P biofertilizer ($S_{150}T_0F_P$), 150 mM salinity + Trichoderma + no biofertilizer ($S_{150}TC$), 150 mM salinity + Trichoderma + N biofertilizer ($S_{150}TF_N$), 150 mM salinity + Trichoderma + P biofertilizer ($S_{150}TF_P$), 300 mM salinity + no Trichoderma + no biofertilizer ($S_{300}T_0C$), 300 mM salinity + no Trichoderma + N biofertilizer ($S_{300}T_0F_N$), 300 mM salinity + no Trichoderma + P biofertilizer ($S_{300}T_0F_P$), 300 mM salinity + Trichoderma + no biofertilizer ($S_{300}TC$), 300 mM salinity + Trichoderma + N biofertilizer ($S_{300}TF_N$), 300 mM salinity + Trichoderma + P biofertilizer ($S_{300}TF_P$)]. A total of 54 sterilized 6.5-kg pots containing soil and sand at a 3:1 ratio were prepared for the 18 treatments in three replications. Before initiation, the soil sample was tested for its compounds (Table 1). After performing the calculations, mineral N, P, and K fertilizers (urea, triple superphosphate, and potassium sulfate) were applied to the pot soil at half of the quinoa plant requirement. Trichoderma (*Trichoderma harzianum*) were applied to the pot soil so that it did not directly touch the seeds. The seeds of quinoa 'Titicaca' were surface-disinfected with sodium hypochlorite 5% for 10 min and washed with distilled water before sowing. The biofertilizer Azotobacter-1, which contained strain O_4 of *Azotobacter vinelandii*, was used as the N biofertilizer, and the

Table 1 The physicochemical characteristics of the soil at the study site

Measured parameter	Value
Salinity (dS/m)	0.65
Soil texture	Loam-clay
pH	6.7
Clay (%)	36
Silt (%)	55
Sand (%)	9
Organic carbon (%)	0.20
OM (%)	0.34
$CaCO_3$ (%)	27.5
P (mg/kg)	11
K (mg/kg)	290

biofertilizer PhosphoBARVAR®-2, which contained strain P_5 of Pantoea and strain P_{13} of *Pseudomonas putida*, was used as the P biofertilizer. The seeds that were intended to be treated with the biofertilizers were impregnated with the biofertilizers for 30 min. Each pot was sown with 20 seeds. After germination, they were thinned to keep 6 plants. When the plants were at the 6-leaf stage (one month), the salinity stress was applied at various rates. After 20 days, the pots were transferred to a laboratory to harvest the plants.

Measurement of morphological traits

Shoot and root length were determined with millimeter ruler. They were, then, oven-dried at 70 °C for 48 h. Shoot and root dry weight were determined with a precise 0.001-g scale.

Measurement of Na, K, Ca, and Mg

One gram of powdered dry matter of the leaves for each treatment was incinerated in a furnace at 550 °C for 5 h and was digested in 10 mL of 6 M HCI. The solution was filtered with a Whatman Paper No. 1 and the volume of the digested solution was, then, adjusted to 50 mL with distilled water. and its Na and K contents were measured with a PFP7 flame-photometer. Ca and Mg contents of digested solution were measured by EDTA titration.

Plant extract preparation to measure total phenol and total flavonoid

The extract was prepared by the protocol reconstruct Aysel and Munevver (2004). For this purpose, 1 g of leaf fresh tissue was homogenized in 25 mL of methanol on a magnetic shaker in darkness for 3 h. The solution was, then, filtered with a Whatman Paper No. 1 and centrifuged at 15,000 g for 30 min. Then, the supernatant was kept at − 80 °C until the experiment time.

The total phenol content was determined by Marinova et al. (2005) protocol with some modifications. According to this method, 1 mL of the extract was added with 9 mL of distilled water and 1 mL of the Folin–Ciocalteu reagent. After 5 min, 10 mL of 7% sodium carbonate was added to the solution, and it was incubated at the laboratory temperature for 90 min. Then, the absorbance of the samples was read at 750 nm with a spectrophotometer. The total phenol content was calculated by using the standard gallic acid curve in mg/100 g fresh weight (FW).

The total flavonoid content was determined by Chang et al. (2002) procedure based on aluminum colorimetry. Accordingly, 20 µL of the plant extract was diluted with 1 mL of distilled water and was added with 0.075 mL of 5% sodium nitrite. Five minutes after the reaction, 0.15 mL of 10% aluminum chloride and, 6 min later, 0.5 mL of 1 mol/L sodium hydroxide were added to the solution. Their absorbance was, then, read at 510 nm. The total flavonoid content was calculated by the standard quercetin curve in mg/100 g FW.

Isolation, detection, and quantification of polyphenol compounds

The polyphenol compounds were isolated, detected, and quantified using an Agilent 1100 Series high-performance liquid chromatography device (the USA) equipped with a 20-µL injection loop, four-solvent gradient pump, degasification system, column oven (set at 25 °C), and diode array detector set at 250, 272, and 310 nm. The isolation was performed with a Dr. Maincsch ZORBAX Eclipse XDB Octadecyl silane column (with a length of 25 cm, an internal diameter of 4.6 mm, and a particle size of 5 µm). Data were processed in the Chemstation software package.

A dilution plan was used for better isolation of the components. To this end, the moving phase was initiated with a ratio of 10% acetonitrile and 90% acetic acid 1% solution and was reached the acetonitrile/1% acetic acid ratio of 25/75% at a flow rate of 1 mL/min. Then, it was reached the ratio of 65/35% at a flow rate of 1 mL/min. The isolation time was 15 min.

Statistical analysis

The data were statistically analyzed in the SAS (ver. 9.4) software package. The means were compared by the PLSD test at the $P < 0.01$ level, and the graphs were drawn in MS-Excel.

Results and discussion

The results of variance analysis revealed that the morphological and antioxidant traits of the quinoa were influenced significantly at the $P < 0.01$ level (Table 2).

Morphological traits

Shoot length

The highest shoot length (cm) was obtained from the treatment of $S_0T_0F_N$, and the lowest was related to the treatments of $S_{300}T_0C$ and $S_{300}TF_N$, which did not differ from $S_{300}T_0F_P$ significantly. The shoots were significantly longer in the treatments of S_0TF_N and $S_{150}TF_N$ than in the treatments of S_0T_0C and $S_{150}T_0C$, respectively. The treatments of $S_{300}T_0F_N$, $S_{300}TC$, and $S_{300}TF_P$ also exhibited a significant increase in this trait versus the treatment of $S_{300}T_0C$ (Fig. 1a).

Shoot dry weight

The plants treated with $S_0T_0F_N$ exhibited the highest and those treated with S_0T_0C or $S_{300}T_0C$ exhibited the lowest shoot dry weight (g). A significant increase was observed in the shoot dry weight of the plants treated with S_0TF_N or $S_{150}TF_N$ versus those treated with S_0T_0C or $S_{150}T_0C$. The treatments of $S_{300}TC$ and $S_{300}T_0F_N$ also had significantly higher shoot dry weight than the treatments of $S_{300}T_0C$ (Fig. 1b).

Root length

The longest and shortest roots length (cm) were observed in the treatments of $S_{150}TF_N$ and $S_{300}TF_N$, respectively. The root length was significantly lower in the treatments of S_0TF_N and $S_{300}TF_N$ than in the treatments of S_0T_0C and $S_{300}T_0C$. The root length was increased remarkably in the treatment of $S_{150}TF_N$. However, a significant increase was observed in the root length of plants treated with $S_{300}T_0F_N$ and $S_{300}TF_P$ versus those treated with $S_{300}T_0C$ (Fig. 1c).

Root dry weight

The highest root dry weight (g) was obtained from the treatment of $S_0T_0F_N$, which was significant compared to the other treatments. The lowest root dry weight was related to the treatment of $S_{300}TF_N$. The treatments of S_0TF_N and $S_{300}TF_N$ had significantly lower root dry weight than the treatments of $S_0T_0F_N$ and $S_{300}T_0F_N$, respectively. The plants treated with $S_{150}TF_N$ had significantly higher root dry weight than those treated with $S_{150}T_0F_N$. The root weight was increased remarkably in the plants treated with $S_{300}T_0F_N$ versus those treated with $S_{300}T_0C$ (Fig. 1d).

Since the treatment of $S_0T_0F_N$ increased plant growth remarkably compared to the other treatments (Fig. 1), it seems that Azotobacter plays an effective role in increasing plant growth by synthesizing and secreting biologically active compounds, e.g., auxins, gibberellins, vitamins B, nicotinic acid, pantothenic acid, biotins, etc., in the root environment (Karthikeyan et al. 2008). On the other hand, the quinoa plants treated with S_0TF_N exhibited significantly higher growth than the zero salinity control (S_0T_0C) (Fig. 1) although the comparison of the plants treated with S_0TF_N with those treated with $S_0T_0F_N$ shows that Trichoderma had an antagonistic effect on the activity of Azotobacter and reduced plants growth. Perhaps, buffer acidification by Trichoderma and its perception by the plants influenced the interaction of Trichoderma with Azotobacter and even with the plants themselves, thereby disrupting nutrient uptake. Acidification induced by Trichoderma at initial interaction stages plays an essential role in plant interaction with Trichoderma (Pelagio-Flores et al. 2017). Apparently, NSCC cation channels are activated by Ca^{2+} signaling and through the effects of H^+ on inward-rectifying K^+ channels or by a specific pH sensor (Lager et al. 2010). The competition triggered between the ions during their uptake might have unbalanced the plants' ion composition, subsequently affecting their morphological traits.

The shoot and root length and dry weight were significantly increased in the treatment of $S_{150}TF_N$ versus the other treatments of this salinity level (150 mM NaCl) (Fig. 1). Seemingly, the simultaneous application of Trichoderma and Azotobacter-containing biofertilizers at the low salinity level, which is normal salinity for quinoa as a halophyte plant, inducing the growth of the shoots and roots, especially the root length, reflecting the synergistic effect of Trichoderma and Azotobacter on the plant growth at low salinity conditions. Trichoderma can increase the availability of soil nutrients by secreting organic acids and acidifying the root environment (Pelagio-Flores et al. 2017). It can also increase the K content of plants (Yildirim et al. 2006). On the other hand, the increase in K^+ uptake can alleviate the negative effects of salinity (Shabala and Cuin 2008). Potassium is an activator of many enzymes that are necessary for photosynthesis and respiration, so its deficiency suppresses photosynthesis and even reduces plant growth. Additionally, K^+ plays a vital role in keeping and creating turgor pressure and adjusting plant water balance. On the other hand, the abundance of Na^+ under salinity stress and its substitution with K^+ at the colloid level and soil solution phase, which results in its greater uptake by the roots, increases its concentration and reduces the concentration of other ions, especially K^+, in the plant. It was reported the effects of Na^+ accumulation

Table 2 The results of the variance analysis for the effect of salinity, biofertilizer, and Trichoderma on morphological traits and antioxidant of quinoa

Sources of variations	df	Shoot length (cm)	Shoot dry weight (g)	Root Length (cm)	Root dry weight (g)	Total phenols content (mg GAE 100 g^{-1} Fw)	Total flavonoids content (mg QE 100 g^{-1} Fw)	Leaf Na^+ content (%)	Leaf K^+ content (%)	Leaf K^+/Na^+
Replication	2	1.185 ^{ns}	0.014 ^{ns}	1.865 ^{ns}	0.003 ^{ns}	7.749 ^{ns}	0.106 ^{ns}	0.0007 ^{ns}	0.073 ^{ns}	24.445 ^{ns}
A	2	284.129**	1.753**	61.99**	0.067**	1267.38**	41.47**	0.0187**	29.56**	2031.9**
B	1	4.16 ^{ns}	0.698**	0.155 ^{ns}	0.006 ^{ns}	1615.18**	3.734**	0.0039**	0.038 ^{ns}	1099.4**
C	2	364.462**	14.878**	45.48**	0.329**	572.93**	12.90**	0.0001**	2.446**	539.3**
AB	2	6.16 ^{ns}	3.209**	31.28**	0.043**	1374.12**	0.4362 ^{ns}	0.0026**	0.571**	671.6**
AC	4	100.462**	5.889**	37.71**	0.145**	1625.97**	1.279**	0.0006**	2.176**	843.7**
BC	2	92.72**	3.587**	1.09 ^{ns}	0.053**	3345.46**	2.991**	0.0003**	3.931**	223.5**
ABC	4	72.38**	4.598**	178.55**	0.058**	365.43**	4.973**	0.0008**	0.276**	666.9**
Experimental error	34	2.518	0.083	1.76	0.002	59.52	9.514	0.0005	0.06	10.28
Coefficient of variation	3.698	8.468	6.09	11.66	11.81	7.039	3.18	2.97	4.56	

Sources of variations	df	Leaf Ca^{2+} content (%)	Leaf Mg^{2+}content (%)	Leaf Ca^{2+}/Na^+	Leaf Mg^{2+}/Na^+
Replication	2	0.009 ^{ns}	0.0005 ^{ns}	0.089 ^{ns}	0.050 ^{ns}
A	2	0.104**	0.150**	128.32**	99.84**
B	1	0.018*	0.002 ^{ns}	2.16*	5.47**
C	2	0.029**	0.006*	5.40**	1.25**
AB	2	0.153**	0.236**	5.96**	9.39**
AC	4	0.052**	0.106**	3.49**	6.85**
BC	2	0.068**	0.084**	11.02**	12.80**
ABC	4	0.035**	0.061**	16.23**	16.40**
Experimental error	34	0.009	0.001	0.308	0.151
Coefficient of variation	5.98	4.85	7.005	6.28	

ns: nonsignificant; * and **: significant at the $P < 0.05$ and $P < 0.01$ levels, respectively

(Salinity = A, Trichoderma fungi = B, Bacterial biofertilizers = C)

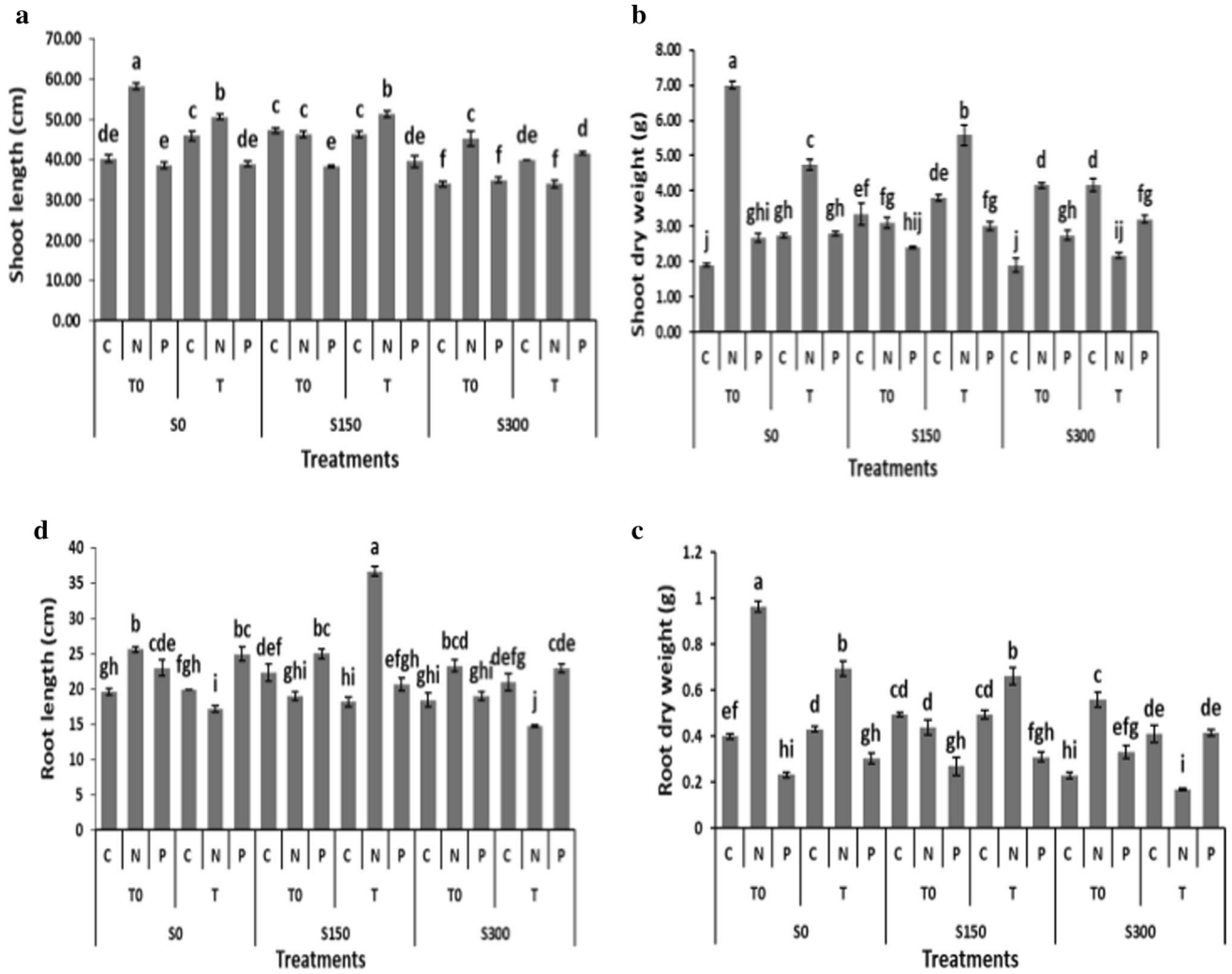


Fig. 1 Means comparison for **a** the shoot length, **b** shoot dry weight, **c** root length, and **d** root dry weight of quinoa plants affected by salinity stress, biofertilizers, and Trichoderma. Unsimilar letters show significant differences at the $P<0.01$ level. (S_0T_0C: no salinity × no Trichoderma × no biofertilizer; $S_0T_0F_N$: no salinity × no Trichoderma × N biofertilizer; $S_0T_0F_P$: no salinity × no Trichoderma × P biofertilizer; S_0TC: no salinity × Trichoderma × no biofertilizer; S_0TF_N: no salinity × Trichoderma × N biofertilizer; S_0TF_P: no salinity × Trichoderma × P biofertilizer; $S_{150}T_0C$: 150 mM salinity × no Trichoderma × no biofertilizer; $S_{150}T_0F_N$: 150 mM salinity × no Trichoderma × N biofertilizer; $S_{150}T_0F_P$: 150 mM salinity × no Trichoderma × P biofertilizer; $S_{150}TC$: 150 mM salinity × Trichoderma × no biofertilizer; $S_{150}TF_N$: 150 mM salinity × Trichoderma × N biofertilizer; $S_{150}TF_P$: 150 mM salinity × Trichoderma × P biofertilizer; $S_{300}T_0C$: 300 mM salinity × no Trichoderma × no biofertilizer; $S_{300}T_0F_N$: 300 mM salinity × no Trichoderma × N biofertilizer; $S_{300}T_0F_P$: 300 mM salinity × no Trichoderma × P biofertilizer; $S_{300}TC$: 300 mM salinity × Trichoderma × no biofertilizer; $S_{300}TF_N$: 300 mM salinity × Trichoderma × N biofertilizer; $S_{300}TF_P$: 300 mM salinity × Trichoderma × P biofertilizer)

and a change in the Na^+/K^+ ratio in cells on the biological processes of the plant (Sudhir and Murthy 2004). Yang et al. (2016) investigated the relationship between quinoa growth and two salt-resistant rhizobacteria in salinity stress and reported that both bacteria reduced Na^+ uptake and the negative effects of salinity on the plants. Given the functions of K in the physiological processes of plants, it is necessary for plant survival in stressful conditions to keep K content at a proper level. Quinoa plants seem to use this mechanism to reduce Na^+ accumulation and prevent the decline in the K^+ content in salinity stress, thereby securing their survival. Our results suggest that the simultaneous application of Trichoderma and Azotobacter in low salinity stress can provide suitable conditions for plant growth by increasing K^+/Na^+ content and maintaining leaf cell homeostasis.

In the treatment of ($S_{300}T_0F_N$) the length and dry weight of shoots and roots showed a significant increase compared to treatment ($S_{300}T_0C$). Because in this treatment the ratio of potassium to sodium did not show significant changes (Fig. 2c), Quinoa plants may use mechanisms such as increasing Proline content as an osmotic protector and increasing the activity of antioxidant enzymes to clear ROS

from salinity stress (Shabala et al. 2012). Therefore, they have provided suitable conditions for plant growth.

In the treatment of $S_{300}TF_N$, plant growth was significantly reduced compared to other treatments (Fig. 1). Trichoderma acidified the medium. This process, which is at least partially mediated by H^+-ATPases, influences root and shoot growth (Pelagio-Flores et al. 2017). The proton increase in the apoplast may induce pH stress and disrupt the process of nutrient uptake by channels and nutrient transporters. It has been reported that NRT1.1 and OsNRT2.3b nitrate transporters are involved in H^+ resistance in Arabidopsis and rice, respectively, depending on their nitrate uptake activity (Fan et al. 2016). Vos et al. (2015) reported the antagonistic capability of Trichoderma with bacteria. On the other hand, excessive acidification of buffer may cause excessive uptake of some nutrients and their toxicity (Shavrukov and Hirai 2016).

The growth factors were not changed significantly by the treatment of S_0TF_P versus the treatment of $S_0T_0F_P$ (Fig. 1). It seems that the application of Trichoderma at the zero salinity level had no effects on the activity of phosphate solubilizing bacteria. On the other hand, the shoot dry weight of the plants treated with $S_{150}TF_P$ was higher than those treated with $S_{150}T_0F_P$ whereas their roots were shorter. Plants are capable of acidifying the rhizosphere to improve the availability or uptake of such nutrients like phosphates and Fe, especially in alkaline limy soils. Trichoderma and Pseudomonas both acidify the soil. It has been reported that only low pH can be good for plant growth and development (Hinsinger et al. 2003). The reduction of root growth may be related to the loss of the root meristem functioning due to acidification. Acidification by Trichoderma causes the redistribution of auxins among root cap cells, the bending of root tips, and its growth suppression in Arabidopsis. Acidity perception by the roots is the mediator of the interaction between Trichoderma and the plants (Pelagio-Flores et al. 2017). While over-acidification adversely impacts plant growth, it should be perceived by the plant. Apparently, various factors, e.g., Na^+ content, are involved in this perception.

At the high salinity level (300 mM NaCl–S_{300}), the application of Trichoderma along with phosphate solubilizing bacteria ($S_{300}TF_P$) influenced plant growth significantly. These bacteria release such metabolites as organic acids containing hydroxyl (gluconic) and carboxyl (ketogluconic) groups to convert phosphates from insoluble forms to soluble forms. These organic acids chelate phosphate-attached cations and turn them into a soluble form that can be taken up by the plants. The secreted acids, also, reduce soil pH and make the attached phosphate available to the plants (Itelima et al. 2018). It seems that Trichoderma at high salinity level ($S_{300}TF_P$) had an increasing effect on the activity of phosphate solubilizing bacteria and finally improved plant growth at this salinity level. Both Pseudomonas and Trichoderma acidify the medium. On the one hand, the increased concentration of H^+ may affect the activity of antiporter SOS1 and NSCC cation channels and increase Na^+ outflow from the root cells. On the other hand, the increased concentration of intracellular and extracellular Na^+ at high salinity levels can suppress cation channels. Na^+ blocks K^+-selective outwardly rectifying channel (KOR), thereby hindering the loss of cell K^+ content (Demidchik and Maathuis 2007). Consequently, the increased ratio of K^+/Na^+ provides proper conditions for the cell.

Leaf Na^+ content

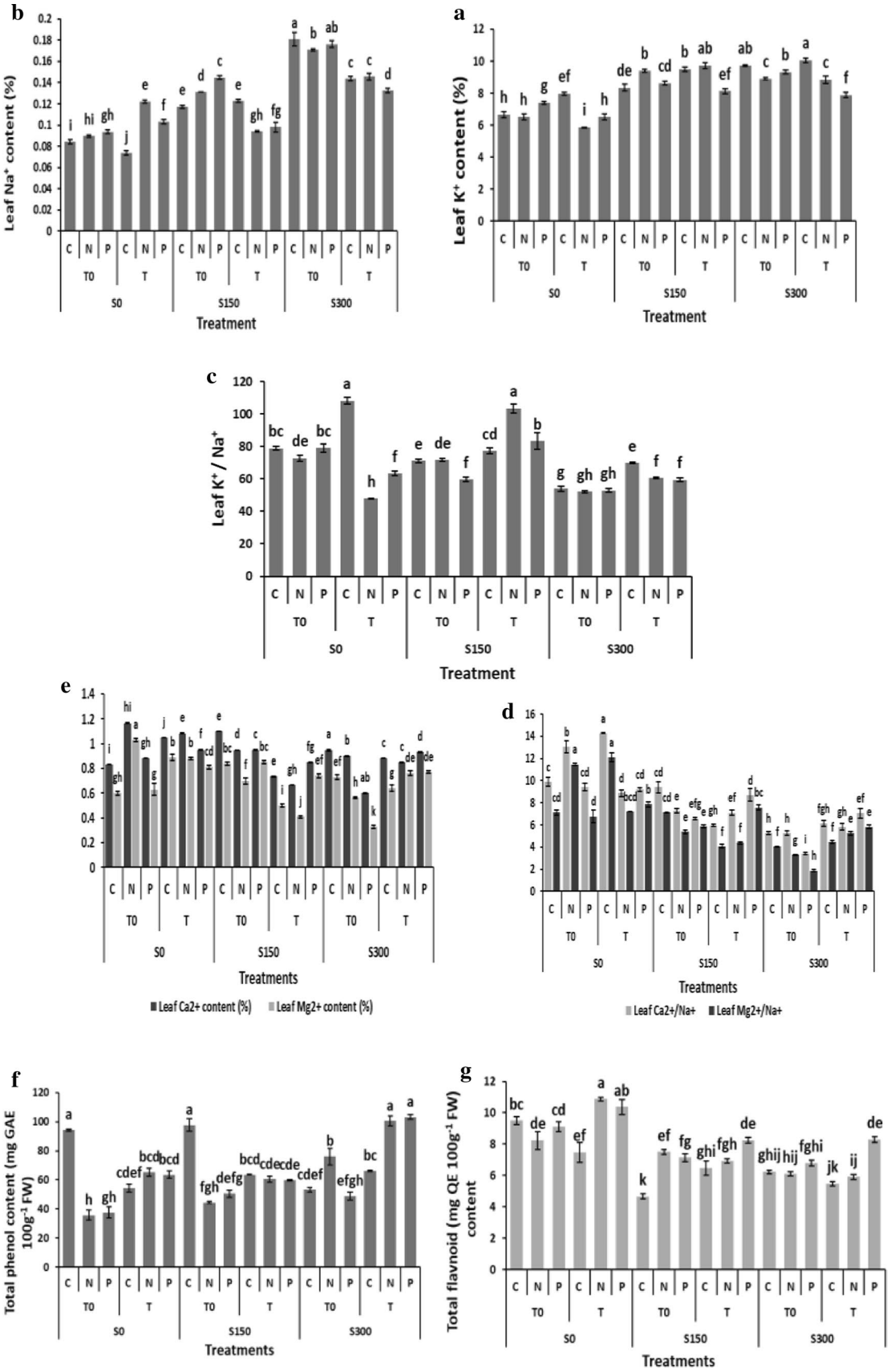
The highest and lowest leaf Na^+ contents (%) were observed in the treatments of $S_{300}T_OC$ and S_0TC, respectively. The treatment of S_0TC had higher Na^+ content than the other treatments of zero salinity level (S_0). This trait was increased in the treatment of S_0TF_N compared to the treatment of S_0T_0C significantly. The leaf Na^+ content in the treatment of $S_{150}T_0F_P$ was significantly increased when compared to the other low salinity treatments (S_{150}). It was found that at high salinity levels (S_{300}), Na^+ content was decreased in the treatments containing Trichoderma (T) than those lacking it (T_0) significantly (Fig. 2a).

Leaf K^+ content

The leaf K^+ content (%) was the highest in the treatment of $S_{300}TC$, but it had no significant difference from the treatments of $S_{300}T_0C$ and $S_{150}TF_N$. The lowest leaf K^+ content was observed in the treatment of S_0TF_N. This trait was significantly increased in low and high salinity levels (S_{150} and S_{300}) versus the zero salinity level (Fig. 2b).

Leaf K^+/Na^+ ratio

The highest leaf K^+/Na^+ ratio was obtained from the treatment of S_0TC, not differing from $S_{150}TF_N$ significantly. The lowest leaf K^+/Na^+ ratio was observed in the treatment of S_0TF_N. The K^+/Na^+ ratio was significantly higher in the treatment of S_0TC than the other zero salinity treatments (S_0). The leaf K^+/Na^+ ratio was significantly decreased in the treatment of S_0TF_N versus the other zero salinity treatments. The leaf K^+/Na^+ ratio was significantly higher in the treatment of $S_{150}TF_N$ than in the treatments containing 150 mM NaCl (S_{150}). The results revealed that at higher salinity levels (S_{300}), the Na^+ content was increased in the treatments applied with Trichoderma (T) than those lacking Trichoderma (T_0) significantly (Fig. 2c).


b
Leaf Na+ content (%)
a
Leaf K+ content (%)
c
Leaf K+ / Na+
Treatment
e
Treatments
Leaf Ca2+ content (%)
Leaf Mg2+ content (%)
d
Leaf Ca2+/Na+
Leaf Mg2+/Na+
f
Total phenol content (mg GAE 100g-1 FW)
g
Total flavnoid (mg QE 100g-1 FW) content
C N P C N P C N P C N P C N P C N P
T0 T S0 S150 S300

◂**Fig. 2** Means comparison for **a** Na^+ %, **b** K^+%, **c** K^+/Na^+, **d** Ca^{2+} and Mg^{2+}, **e** Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios, **f** total phenol, and **g** total flavonoid of quinoa leaf affected by salinity stress, biofertilizers, and Trichoderma. Unsimilar letters show a significant difference at the $P < 0.01$ level. (S_0T_0C: no salinity × no Trichoderma × no biofertilizer; $S_0T_0F_N$: no salinity × no Trichoderma × N biofertilizer; $S_0T_0F_P$: no salinity × no Trichoderma × P biofertilizer; S_0TC: no salinity × Trichoderma × no biofertilizer; S_0TF_N: no salinity × Trichoderma × N biofertilizer; S_0TF_P: no salinity × Trichoderma × P biofertilizer; $S_{150}T_0C$: 150 mM salinity × no Trichoderma × no biofertilizer; $S_{150}T_0F_N$: 150 mM salinity × no Trichoderma × N biofertilizer; $S_{150}T_0F_P$: 150 mM salinity × no Trichoderma × P biofertilizer; $S_{150}TC$: 150 mM salinity × Trichoderma × no biofertilizer; $S_{150}TF_N$: 150 mM salinity × Trichoderma × N biofertilizer; $S_{150}TF_P$: 150 mM salinity × Trichoderma × P biofertilizer; $S_{300}T_0C$: 300 mM salinity × no Trichoderma × no biofertilizer; $S_{300}T_0F_N$: 300 mM salinity × no Trichoderma × N biofertilizer; $S_{300}T_0F_P$: 300 mM salinity × no Trichoderma × P biofertilizer; $S_{300}TC$: 300 mM salinity × Trichoderma × no biofertilizer; $S_{300}TF_N$: 300 mM salinity × Trichoderma × N biofertilizer; $S_{300}TF_P$: 300 mM salinity × Trichoderma × P biofertilizer)

Leaf Ca^{2+} content

The leaf Ca^{2+}content (%) was the highest in the treatment of $S_0T_0F_N$ but it had no significant difference from the treatments of S_0TF_N and $S_{150}T_0C$. The lowest leaf Ca^{2+}content were observed in the treatment of $S_{300}T_0F_P$, which did not differ from $S_{150}TF_N$ significantly (Fig. 2d).

Leaf Mg^{2+} content

The highest and lowest leaf Mg^{2+} contents (%) were observed in the treatments of $S_0T_OF_N$ and $S_{300}T_0F_P$, respectively (Fig. 2d).

Leaf Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratio

The highest and lowest leaf Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios were observed in the treatments of S_0TC and $S_{300}T_0F_P$, respectively. These traits were significantly decreased in high salinity level (S_{300}) versus the zero salinity level (Fig. 2e).

Leaf antioxidants

Total phenol

The highest leaf total phenol content (mg GAE 100 g^{-1} FW) was observed in the plants treated with $S_{300}TF_P$, but it did not differ from the treatments of $S_{300}TF_N$, $S_{150}T_0C$, and S_0T_0C significantly. The lowest was observed in those treated with $S_0T_0F_N$ (Fig. 2f).

Total flavonoid

The treatments of S_0TF_N and $S_{150}T_0C$ were related to the highest and lowest leaf total flavonoid content (mg QE 100 g^{-1} FW), respectively (Fig. 2g).

Leaf K^+/Na^+, Ca^{2+}/Na^+, and Mg^{2+}/Na^+ ratio

We observed that Trichoderma increased the K^+/Na^+ ratio at the low (S_{150}) and high (S_{300}) salinity levels (Fig. 2c). Razzaghi et al. (2014) reported that salinity increased K^+ and Na^+ uptake in quinoa plants. The colonization by the endophyte fungi Trichoderma might contribute to protecting ion homeostasis in quinoa by modulating ion accumulation and improving nutrient uptake. It also increases the K^+/Na^+ ratio in the cytosol by limiting Na^+ translocation to the leaves. Morales et al. (2011) state that quinoa plants are capable of accumulating salts in their cells by mechanisms like sequestration of Na^+ and compatible minerals in vacuoles, and to avoid salt stress, they use the mechanisms related to SOS1 (Na^+/H^+ antiport) for the outflow of Na^+ from cell cytoplasms to vacuoles and apoplast. It has been reported that the expression of the gene SOS1 is adjusted in plants exposed to salinity stress (Quan et al. 2017) and NHX1 (Na^+ and K^+/H^+) exchanger involves in preserving cytosol K^+/Na^+ ratio and controlling the osmotic potential in salinity-affected plants. The over-expression of the gene NHX1 increases the salinity tolerance of plants (Wu 2018). Also, there are reports as to the participation of endophyte fungi in increasing the transcription of the genes encoding K transporter with high K^+ affinity (HKT1) and the internal KAT_1 and KAT_2 potassium channels, which play a key role in homeostasis adjustment of Na^+ and K^+ (Gupta et al. 2020). Therefore, it is likely that Trichoderma plays a role in adjusting gene expression or the activity of ion transporters and channels at different salinity levels by reducing pH and increasing H^+ content.

There is competition among magnesium, calcium, potassium and ammonium in adsorption sites on root cell membranes (Hosseinzad et al. 2014). Our results showed that the highest and lowest leaf Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios were observed in the treatments of S_0TC and $S_{300}T_0F_P$, respectively (Fig. 2e). The interaction among the Trichoderma, biofertilizer and plant at three salinity levels may alter the activity of some membrane ion transporters and channels. Subsequently, the competition triggered among the ions for their uptake disrupts the balance in the ion composition of the plant.

Leaf antioxidants

Quinoa leaves have been claimed to be a potential and rich source of phenol and flavonoid compounds (Ruiz Carrasco

et al. 2016). In the treatments of S_0T_0C and $S_{150}T_0C$, phenolic compounds were accumulated by the plants for various reasons. The no-salinity level in the absence of biofertilizers and/or Trichoderma (the control) provided stressful conditions for the halophyte species quinoa so that the plant spent a great part of its photosynthetic products on survival by various mechanisms like accumulating phenols. It has been reported that at low salinity levels, the plant's secondary metabolites can prevent oxidative stress-related destructive disorders by exhibiting antioxidant properties and indirectly modulating the functioning of several oxidative-related proteins (Hirose et al. 2010).

It was found in this study that at the salinity levels of 0 and 150 mM, the application of biofertilizers and Trichoderma, either individually or simultaneously, reduced total phenol content (Fig. 2f). When the salinity conditions change, quinoa plants may change the contents of these compounds and their uptake and mobilization as a means of enhancing their adaptation. Therefore, we investigated the Na^+ and K^+ contents of the leaves to better understand the mechanisms used by the quinoa plants at different salinity levels applied. The lowest total phenol content was observed in the treatment of $S_0T_0F_N$ (Fig. 2f). Since the plant growth was increased remarkably in this treatment, it can be said that most photosynthetic products were spent on plant growth. It has been reported that cytosol Na^+ is compartmentalized and mobilized into the vacuoles to prevent the detrimental effects of Na in plant cell cytosols (Gouiaa et al. 2012). In the treatments of S_0TF_N and S_0TF_P versus the zero-salinity control, the leaf Na^+ content was increased (Fig. 2a) and the K^+/Na^+ ratio was decreased significantly (Fig. 2c). Since quinoa is a halophyte plant, it can be drawn from the increase in the leaf Na^+ content of this plant that when Trichoderma and bacteria were applied at the zero salinity level, the plants took up soil Na^+ and accumulated it in their leaves. It is likely that the N and/or P biofertilizers applied with Trichoderma were involved in the increased uptake of Na^+ by the roots and its accumulation in the leaves (the salinity of the farm soil was 0.65 dS/m; Table 1). The nutrient uptake by cation channels is non-selective, so the interaction of Trichoderma and bacteria may have increased the activity of the NH_4^+-transporter cation channel and subsequently, Na^+ uptake (Niu et al. 2013). Given the increase in the Na^+ content and the decrease in the K^+/Na^+ ratio of the leaves in the treatments of S_0TF_N and S_0TF_P versus the control treatment (S_0TF_P), The significant increase in total phenol and flavonoid contents in this treatment (Fig. 2f, g) reflects that phenols and flavonoids might be involved in chelating toxic Na^+ and its sequestration in vacuoles and/or scavenging ROS synthesized by Na^+ stress, thereby mitigating ion-induced oxidative damages and protecting cytoplasm and chloroplast structures against the negative effects of salinity (Kanwal et al. 2013).

The total phenol and flavonoid contents were significantly increased in the treatment of $S_{300}TF_P$ (Fig. 2f g). Phenols act as the chelators of metal ions (Huang et al. 2005). It seems that the Na^+ in leaf cells is compartmentalized with the involvement of these compounds. When appropriate cell conditions are provided, Photosynthetic products are used to synthesize proteins and other material required for plant growth, thereby increasing plant growth in this treatment. In the treatment of $S_{300}TF_N$, no increases happened in the shoot and root length and biomass unlike the treatment of $S_{300}TF_P$, but the root growth was decreased significantly. In this treatment, nutrient uptake is seemingly disrupted due to soil acidification by Trichoderma on the one hand and the abundance of Na^+ on the other. A factor that can increase plant polyphenols is nutrient stress, especially N stress. In these conditions, plant growth decreases to a greater extent than photosynthesis. Consequently, the surplus carbon produced by photosynthesis is assigned to defensive carbon-based compounds, e.g., polyphenols. Hence, the plant's polyphenol content increases with decreasing its growth (Zhao et al. 2007). The increased content of phenols under osmotic stress in different tissues of many plants can be related to the role of total phenols in adjusting the mechanism of the main metabolic processes of the plants, which influence their growth (Allah et al. 2015). At the salinity levels of 150 and 300 mM, total flavonoids were decreased versus the salinity level of 0 mM significantly (Fig. 2e). Flavonoids are synthesized at the end of the phenylpropanoid biosynthesis pathway. In this pathway, the PAL enzyme l-phenylalanine is deaminized into trans-cinnamic acid and then, secondary metabolites, e.g., flavonoids, anthocyanins, lignins, and other phenolic compounds, are biosynthesized because cinnamic acid is the precursor of both lignins and flavonoids (Saufi 2007). It seems that the defensive system of quinoa in salinity stress conditions is mainly based on increasing other phenols and it is likely that it has consumed more cinnamic acid of the phenylpropanoid pathway than flavonoids for synthesizing secondary metabolites like lignins.

Leaf Polyphenolic profile (HPLC)

In the control treatment (S_0T_0C), coumaric and cinnamic acids were the most and least abundant among the measured phenolic compounds (mg Kg^{-1} DW), respectively. At the zero salinity levels, coumaric, rosmarinic, chlorogenic, caffeic, and cinnamic acids were higher in the treatments of S_0TF_N and S_0TF_P than in the control (S_0T_0C). Coumaric, chlorogenic, and caffeic acids were increased in the treatment of $S_{150}TF_N$ versus the control (S_0T_0C). Increases were also observed in rosmarinic, chlorogenic, and caffeic acids in the treatment of $S_{150}TF_P$. The chlorogenic acid content was slightly increased in the treatment of $S_{300}T_0$ versus the control (S_0T_0C) Fig. 3a, b).

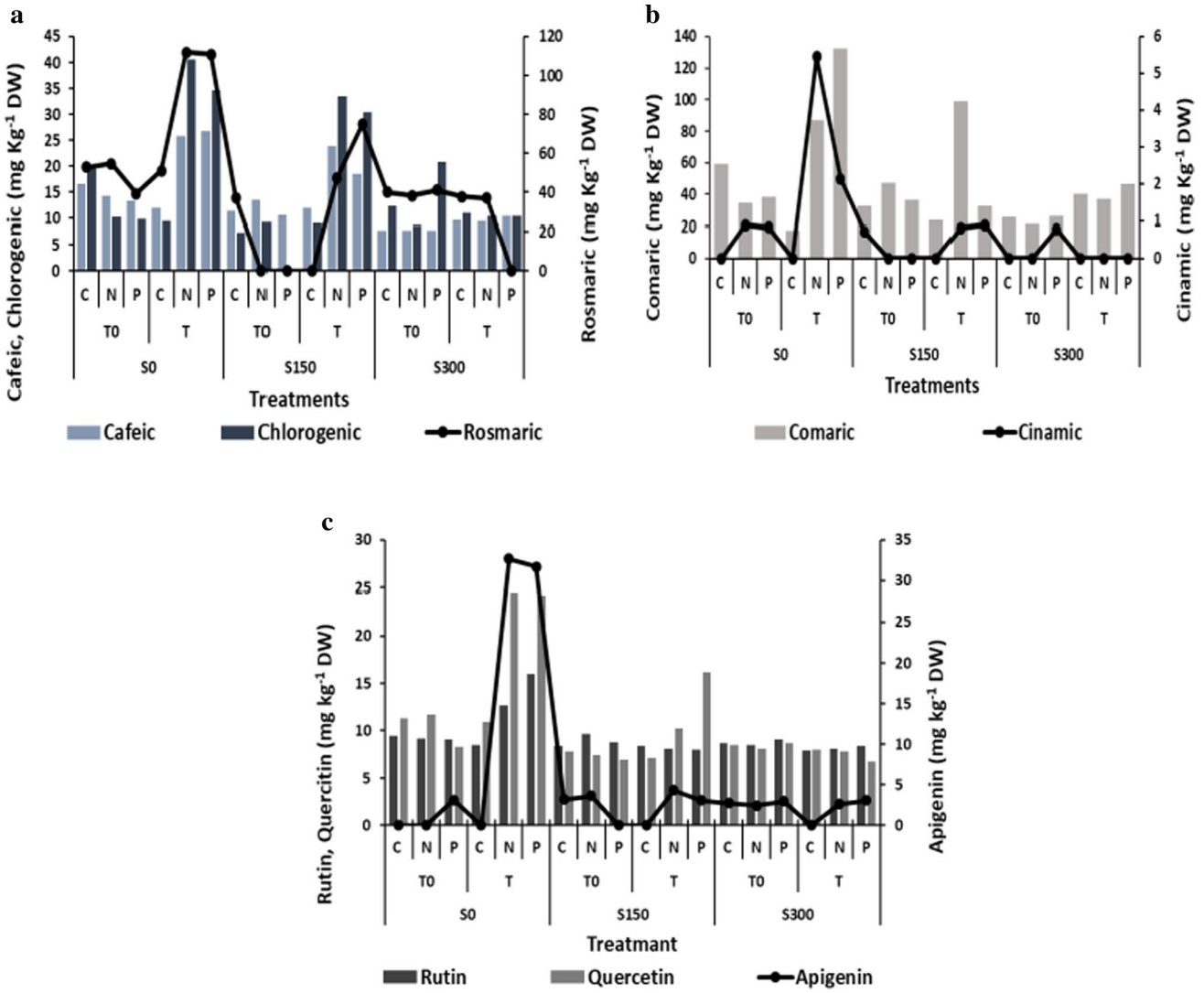


Fig. 3 HPLC for **a** caffeic acid, rosmarinic acid, and chlorogenic acid, **b** coumaric acid and cinnamic acid, **c** rutin, quercetin, and apigenin of quinoa leaf affected by salinity stress, biofertilizers, and Trichoderma. (S_0T_0C: no salinity × no Trichoderma × no biofertilizer; $S_0T_0F_N$: no salinity × no Trichoderma × N biofertilizer; $S_0T_0F_P$: no salinity × no Trichoderma × P biofertilizer; S_0TC: no salinity × Trichoderma × no biofertilizer; S_0TF_N: no salinity × Trichoderma × N biofertilizer; S_0TF_P: no salinity × Trichoderma × P biofertilizer; $S_{150}T_0C$: 150 mM salinity × no Trichoderma × no biofertilizer; $S_{150}T_0F_N$: 150 mM salinity × no Trichoderma × N biofertilizer; $S_{150}T_0F_P$: 150 mM salinity × no Trichoderma × P biofertilizer; $S_{150}TC$: 150 mM salinity × Trichoderma × no biofertilizer; $S_{150}TF_N$: 150 mM salinity × Trichoderma × N biofertilizer; $S_{150}TF_P$: 150 mM salinity × Trichoderma × P biofertilizer; $S_{300}T_0C$: 300 mM salinity × no Trichoderma × no biofertilizer; $S_{300}T_0F_N$: 300 mM salinity × no Trichoderma × N biofertilizer; $S_{300}T_0F_P$: 300 mM salinity × no Trichoderma × P biofertilizer; $S_{300}TC$: 300 mM salinity × Trichoderma × no biofertilizer; $S_{300}TF_N$: 300 mM salinity × Trichoderma × N biofertilizer; $S_{300}TF_P$: 300 mM salinity × Trichoderma × P biofertilizer)

Among the measured flavonoids, quercetin and apigenin had the highest and lowest amounts in the control treatment (S_0T_0C), respectively. Quercetin, rutin, and apigenin were increased in the treatments of S_0TF_N and S_0TF_P versus the control (S_0T_0C). Quercetin of the treatment of $S_{150}TF_P$ was slightly higher than that of the control (S_0T_0C) (Fig. 3c).

Leaf Polyphenolic profile (HPLC)

The research performed HPLC analysis on the quinoa leaves to measure caffeic, chlorogenic, coumaric, rosmarinic, and cinnamic acids and the flavonoids of rutin, quercetin, and apigenin. Among the phenolic compounds studied here, coumaric and rosmarinic acids were dominant in the quinoa

leaves. Chlorogenic and caffeic acids were observed in lower quantities. Cinnamic acid was in a tiny amount (Fig. 3a b). The amount of apigenin was increased in the treatments of S_0TF_N and S_0TF_P, considerably, but it was slight in other treatments (Fig. 3c).

The endogenous accumulation of phenolic acids has been reported as a mechanism of plant tolerance of some abiotic stresses, such as toxic metals, in many plant species (Mohammadi et al. 2021; Mousavi et al. 2021). All phenolic acids and flavonoids measured in this research were significantly increased in the treatments of S_0TF_N and S_0TF_P, which is consistent with high amounts of total flavonoids in these two treatments. Since the Na^+ content in these two treatments was increased versus the other zero salinity treatments and the K^+/Na^+ ratio was decreased (Fig. 2a, c), it can be drawn that the quinoa plants accumulated Na in their leaves and increased their polyphenols to chelate and compartmentalize detrimental ions and protect cell homeostasis. Lignin synthesis can be another reason for the increase in plant polyphenols. Hydroxycinnamic acids (including p-coumaric, sinapic, caffeic, and chlorogenic acids) are the precursor of lignin synthesis (Shahidi and Naczktuno 2003). The methylation of caffeic acid yields ferulic acid, which forms lignin precursors with p-coumaric acid. Finally, lignin is formed by the polymerization of p-coumaric, ferulic, and sinapic acids and their alcohols. Lignin accumulation and the early lignification of roots have been proven in metal stress conditions. Lignification has a protective role in plants and hinders the penetration of harmful metals (Zhou et al. 2018). Significant increases were recorded in caffeic, chlorogenic, and coumaric acids in the treatment of $S_{150}TF_N$ and chlorogenic, caffeic, and rosmarinic acids in the treatment of $S_{150}TF_P$. Lignin may be involved in the protection of the quinoa plants against salinity. Phenolic compounds cover a wide range of compounds, such as phenolic acids, flavonoids, and tannins (Kumaran and Karunakaran 2007). The increase in total phenol in the treatments of $S_{300}TF_N$ and $S_{300}TF_P$ can be related to other phenolic compounds, which were not measured in this research.

Conclusion

The results as to the plant morphology showed that the best treatment for plant growth under sever salinity was the application of only N biofertilizer with no Trichoderma. When Azotobacter was applied with Trichoderma under severe salinity the root growth was significantly decreased, resulting in a significant loss of plant growth. It seems that despite the impact of salinity and soil microorganisms on the amount of sodium and potassium ions, their final concentrations and the ratio of potassium to sodium of leaf are determined by the plant itself. Our results are related to not only the plant response to different levels of salt stress but also the interaction of antioxidants. Phenolic compounds can influence one another antagonistically or synergistically. The changes in the studied phenols can reflect the potential of different antioxidants in the face of salt stress and the interaction of microorganisms. Obviously these results need further studies.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Consent to Participate The authors have agreed to be listed and approved the submitted version of the manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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