Article

Effect of salinity and nitrogen sources on leaf quality, biomass, and metabolic responses of two ecotypes of *Portulaca oleracea*

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Abstract: Halophytic plants are, by definition, well adapted to saline soils. However, even halophytes can face nutritional imbalance and accumulation of high levels of compounds such as oxalic acid (OA), and nitrate (NO_3^-). These compounds compromise the potential nutritional health benefits associated with salt tolerant plants such as *Portulaca oleracea*. Thus, preventing the accumulation of non-nutritional compounds will allow plants to be grown in saline conditions as crops. To this end, two ecotypes (ET and RN) of *Portulaca oleracea* plants were grown under growth room conditions with two levels of salinity (0, 50 mM NaCl) and three ratios of nitrate: ammonium (0:100%; 33:66%; 25:75% NO_3^-:NH_4^+). The results showed that both ecotypes exposed to elevated NO_3^-, showed severe leaf chlorosis, high levels of OA, citric acid, and malic acid, while plants of ecotype ET exposed to elevated NH_4^+ concentrations (33% and 75%) and 50 mM NaCl displayed a marked reduction in OA content, increased total chlorophyll and carotenoid contents, crude protein content, total fatty acid (TFA) and α-Linolenic acid (ALA) thus enhancing leaf quality. This opens the potential to grow high biomass, low OA *P. oleracae* crops. Lastly, our experiments suggest that ecotype ET copes with saline conditions and elevated NH_4^+ through shifts in leaf metabolites.

Keywords: halophyte; salt-tolerance; N-nutrition; *Portulaca oleracea*; oxalic acid; ammonium nutrition

1. Introduction

Soil salinity is increasing around the world, especially in arid and semi-arid ecosystems, due to high evaporation and insufficient ion leaching [1]. An estimated 45 million hectares (20%) of soils of irrigated agriculture is affected by salinity [2]. Salinity is a significant abiotic stress which can induce physiological, chemical, and molecular changes in plants [3]. For instance, plant physiological processes such as growth are affected by salinity by inhibiting root growth, which in turn limits the uptake of water and nutrients [4] thus leading to water deficiency and nutrient imbalances, causing a shift in the plant growth rate [5]. Therefore, salinized soil and saline water are generally unsuitable for agriculture. However, the use of halophyte species (salt-tolerant plants) could be a promising solution for increasing agricultural production in saline zones [6].

Halophyte plants can complete their life cycle in a saline environment rich in sodium chloride or sulphate salts [7, 8]. There are a few species of halophytes such *as Crithmum maritimum*, *Salicornia spp*, *Aster tripolium*, and *Portulaca oleracea*, which have been proposed to be introduced into saline water agriculture (reviewed in [9]). However, the utilization of halophytes for saline agriculture needs careful study because even halophytic plant species can be affected by salinity, for instance, salinity caused iron (Fe) deficiency in *Aster tripolium* leads to leaf chlorosis [10]. On the other hand, under saline conditions, nitrate (NO_3^-) uptake is affected due to the antagonistic effect between Cl^- and NO_3^-

[11]. This leads to nitrogen (N) deficiency affecting plant development and yield. Additional N fertilization can alleviate N deficiency. However, N surplus can also increase nitrate content increasing oxalic acid (OA) levels in plants [12 – 16].

Oxalic acid may play several roles in plants such as plant protection, tissue support, and heavy metal detoxification. Another vital function is its role in balancing excess inorganic cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}), over anions (NO_3^-, Cl^-, and SO_4^{2-}) [17, 18]. However, high levels of OA can have adverse health effects in vulnerable people and animals, such as those at risk from kidney stones or low plasma levels of iron and calcium [19, 20]. It has been reported that *P. oleracea* accumulates OA under high levels of nitrate [21]. On the other hand, it is well documented that salinity and ammonium can reduce OA content in *P. oleracea* [14, 20 – 22]. Unfortunately, ammonium fertilization lead to decreased biomass production in *P. oleracea* [14, 20, 21] and also, salinity has a negative effect in terms of visible leaf quality [10]. The study of the combined effects of high ammonium nutrition and salinity will help to understand which mechanism plants use to cope with different and combined stresses because the decrease of biomass caused by ammonium still is not well understood. Understating this mechanism could be a critical point to improve the yield and quality of *P. oleracea*.

In the current study, we elucidated the salt tolerance mechanism and ammonium affinity of two *Portulaca* ecotypes grown under three nitrate:ammonium ratios. This study sheds light on the mechanisms that one ecotype (ET) uses to tolerate salinity and high levels of ammonium, with a concomitant reduction in OA, while maintaining biomass production, through rapid assimilation of ammonium-N into the amino acid pool and sugar alcohols accumulation. This finding suggests that ecotype ET is a good candidate to introduce to agriculture of saline soils.

2. Materials and Methods

Seeds of two Israeli *P. oleracea* ecotypes were used. These were designated as ecotype ET and ecotype RN. Experiments were conducted under growth room conditions at the Ben-Gurion University Sde Boqer Campus, at 27°C, 16:8 hr light-dark with a photosynthetically active rediation (PAR) level of 150 μmol m^{-2} s^{-1}. Seeds of two ecotypes (ET and RN) of *P. oleracea* plants were surface sterilized and germinated in standard 90 mm Petri dishes filled with 0.5 x Murashige-Skoog medium without vitamins (MS) (2.15 g L^{-1}) in 1 % agar or 0.2% Gelrite. The pH of the medium was adjusted to 5.7 before the addition of Gelrite agar. After germination two day old seedlings (30 seedlings of each ecotype (60 total) per Petri dish) were aseptically transplanted to sterile 183 x 40 mm Petri dishes containing 200 mL of autoclaved 0.5 x MS in 1% agar or 0.2% Gelrite, pH 5.7. Treatments were a factorial combination of NO_3^-:NH_4^+ ratios (100:0, 66:33 and 25:75), and two levels of salinity (0, 50 mM NaCl), chosen based on the results of previous studies (data not shown).

2.1. Biomass

Nineteen days after the treatment onset, plants were harvested and separated into shoots and roots for fresh weight determination. The weights of the 30 fresh shoots were determined immediately, and the shoots were dried at 70°C for 72 hours. The dry biomass production was expressed as mg dry weight per shoot (DW mg shoot$^-$1). Shoots for biochemicals analysis were immediately frozen in liquid nitrogen and stored at - 80°C until required for analyses.

2.2. Measurement of Total Chlorophyll, Total Carotenoids, and Protein content

Total chlorophyll was extracted from 20 mg of the youngest leaves of three replicates per treatment. Samples were placed in 0.5 mL of 80% ethanol and incubated in the dark for 48 h at 4°C. The extracts were centrifuged at 18,400 x *g* for 15 min, the supernatant collected, and centrifuged for a further 15 min. Chlorophyll content was measured in 200 μL samples with a spectrophotometer at 649, 665, and 652 nm (Epoch Microplate Spectrophotometer, BioTek) with Gen5 2.05 software. The total carotenoids were measured at 470 nm using the same sample. The values obtained from chlorophyll a, b, were used to calculate the total chlorophyll using the Wintermans and de Mots equation [23]. The total chlorophyll concentration was expressed as mg total chlorophyll g^{-1} FW. Total carotenoids were

expressed as mg total carotenoids g^{-1} FW. The extraction for protein content was performed according to Elavarthi and Martin (2010). Approximately 100mg fresh tissue leaf was ground to a fine powder in liquid nitrogen using a mortar and pestle 1 mL of 200 mM potassium phosphate buffer (pH 7.8) containing 0.1mM EDTA and 4% polyvinylpyrrolidone was added. The samples were centrifuged at 18,400 x *g* for 20 minutes at 4^0C, supernatant collected. Protein content was determined using the [24] assay adapted for microplate reader using bovine serum albumin (BSA) as the protein standard. Total protein content was measured in 200 µL samples with a spectrophotometer at 595 nm (Epoch Microplate Spectrophotometer, BioTek) with Gen5 2.05 software.

2.3. Metabolite Analysis by GC-MS (Gas Chromatography-Mass Spectrometry)

Lyophilized leaves (20 mg) of each sample were homogenized in 2 mL Eppendorf tubes with three 5 mm tungsten carbide beads for 10 seconds. The extraction solution was made following Lisec [25]. One thousand mL pre-chilled reaction mixture contending (25 mL methanol pre-cooled at -20°C with 380 µL of a stock of corticosterone 1 mg mL^{-1} in HPLC grade methanol), 10 mL chloroform and 10 mL Milli-Q water with 400 µL of ribitol (0.2 mg mL^{-1} ribitol in Milli-Q water), 200 µL sorbitol $6C^{13}$ (0.2 mg mL^{-1} sorbitol $6C^{13}$ in Milli-Q water) and 200 µL norleucine (0.2 mg mL^{-1} norleucine in Milli-Q water) were used as an internal quantitative standard and samples were vortexed. Homogenized tissue was incubated for 10 min at 25°C in an orbital shaker. Samples were then sonicated for 10 min in an ultra-sonication bath at room temperature. After that, the samples were centrifuged at 18,000 x *g* for 10 min at 4°C. After centrifugation, the supernatant was transferred to 2 mL Eppendorf tubes, and 300 µL Milli-Q water and 300 µL chloroform were added, then vortexed for 10 s; these samples were centrifuged at 18,000 x *g* for 10 min. After centrifugation, 150 µL of the polar phase (methanol and water) of the supernatant was dried under a vacuum for derivatization.

2.3.1. Derivatization

N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) diluted in chloroform to a final concentration of 0.4 mL $mL^{-1}$and methoxyamine hydrochloride dissolved in pyridine (20 mg ml^{-1} MSTFA in pyridine) were used for derivatization. Forty µL of methoxyamine hydrochloride was added to the samples, and the samples were shaken for 2 hours at 37°C. Then a mixture of 70 µL MSTFA and 7 µL of Alkan were added to the samples. Samples were shaken again for 30 min at 37°C. Aliquots were then transferred into glass vials suitable for GM-CS analysis. Samples were used to determine free organic acids and metabolites in the leaf tissue following Lisec [25].

2.3.2. Lipid analysis

Total polar lipids were quantified following Brychkova et al. [26] by Trace GC Ultra as the methyl esters of the constituent fatty acids after 2-D thin-layer chromatography. The first dimension was chlorophorm:methanol:water (Milli-Q) (65:25:4, v/v/v) followed by the second dimension of chlorophorm:methanol:ammonium hydroxide: isopropylamine (65:35:5:0.5, v/v/v/v). Visualization was performed under UV.

2.3.3. GC-MS conditions and analysis

The GC-MS system consisted of an AS 3000 autosampler, a Trace GC Ultra gas chromatograph, and a DSQII quadrupole mass spectrometer (Thermo-Fisher Ltd). GC was performed on a 30 m VF-5ms column with 0.25 mm i.d. and 0.25 m film thickness +10 m EZ-Guard (Agilent). A 1 µL sample was injected into an injection port liner (Split liner with Wool, Restek, USA). Programmed Temperature Vaporization (PTV) injection temperature was from 60°C to 300 °C in 14.5°C sec^{-1}, the Transfer line was 300°C, and the ion source adjusted to 250°C. The carrier gas used was helium set at a constant flow rate of 1 mL min^{-1}. The temperature program was 1 min isothermal heating at 70°C, followed by a 1°C min^{-1} oven temperature ramp to 76 °C, followed by a 6°C min^{-1} oven temperature ramp to 350°C, and a final 5 min heating at 350°C. The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). Mass spectra were

recorded at 8 scans per second with a mass-to-charge ratio of 70 to 700 scanning range with electron energy of 70eV. Spectral searching utilized the National Institute of Standards and Technology (NIST,Gaithersburg, USA) algorithm incorporated in the Xcalibur® data system (version 2.0.7) against Retention Index (RI) libraries downloadable from the Max-Planck Institute for Plant Physiology in Golm, Germany (http://www.mpimp-golm.mpg.de/mms-library/).

2.3.4. Statistical analysis

Statistical analyses of the data set consisted of two categorical variables (Nitrate:ammonium ratios, with three levels, 100:0, 66:33 and 25:75 respectively, and salt two levels (0, 50 mM NaCl), and 42 continuous metabolomic variables identified by libraries downloadable from the Max-Planck Institute for Plant Physiology in Golm, Germany (http://www.mpimp-golm.mpg.de/mms-library). To test for differences between nitrogen ratios and salinity, the data were subjected to a Two-Way ANOVA for all parameters measured. Where ANOVA indicated significant differences, means were compared by the Tukey-Kramer test. A data set of the two ecotypes was analysed by principal component analysis (PCA) to determine variability among treatments. Hierarchical cluster analysis was calculated among the metabolites. Metabolite spectra data were normalized by the internal standard sorbitol $6C^{13}$ as well as by the median of each metabolite across all samples, and finally, the resulting values transformed to its $\log_2$ for the PCA and heat map.

3. Results

3.1. Effect of nitrogen forms and salinity on leave quality of Portulaca plants and biomass production

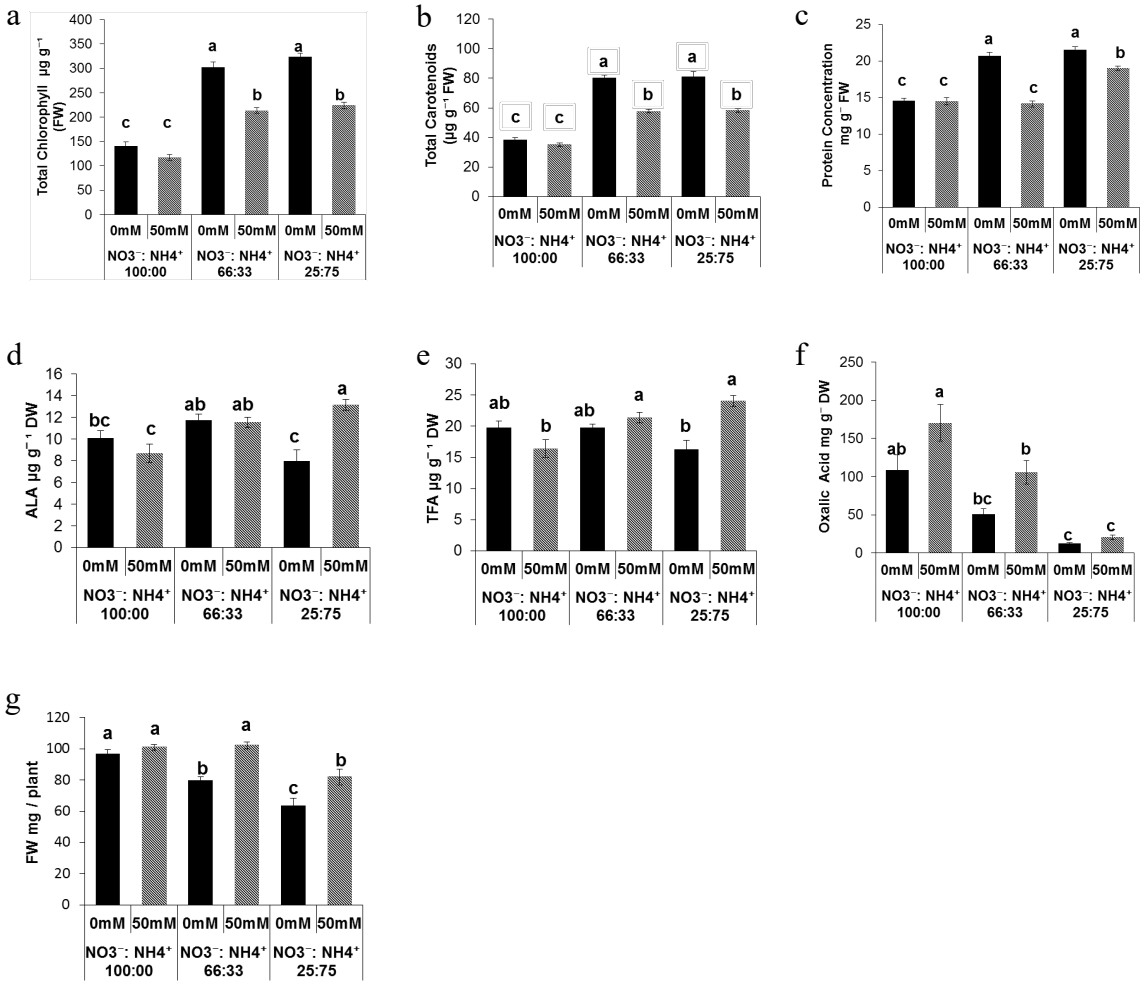
Different ratios nitrate: ammonium, significantly affected total leaf chlorophyll and total carotenoids content. Two-way ANOVA confirmed that there was a highly significant ($P < 0.001$) effect of the nitrate to ammonium ratios, salinity, and their interaction on total chlorophyll and total carotenoids accumulation in both ecotypes (Fig.1 Aa; Ba). The higher levels of total chlorophyll were found in plants grown either with 33 or 75% of ammonium without salinity compared with plants exposed to elevated NO_3^- in both ecotypes (Fig.1 Aa; Ba). Salinity in ammonium-grown plants leads to the drop of total chlorophyll; however, the levels of the total chlorophyll observed were still higher compared to the plants exposed to 100% NO_3^- (Fig.1 Aa). We observed a similar effect on the total carotenoids levels. The highest levels were found either with 33 or 75% of ammonium without salinity in both ecotypes (Fig.1 Ab; Bb). Salinity in ammonium-grown plants slightly affected total carotenoids, although the levels found under salinity conditions in ammonium treated plants were higher than the plants exposed to 100% NO_3^- (Fig.1 Ab; Bb). Ammonium nutrition had a positive effect in terms of protein content. As was confirmed by two-way ANOVA, the protein content was significantly ($P < 0.001$) affected by nitrate to ammonium ratio, salinity, and their interaction in both ecotypes (Fig.1 Ac; Bc). The highest levels of the protein content were found in plants exposed to high levels of ammonium nutrition in both ecotypes (Fig.1 Ac; Bc).

In plants exposed to high levels of ammonium and in saline conditions showed a decrease in protein content, but the levels found there were higher compared to the plants exposed to 100% NO_3^- nutrition (Fig.1 Ac). Significant differences ($P < 0.05$ in the levels of the α-Linolenic acid (ALA) (Fig.1 Ad; Bd) and total fatty acid (TFA) (Fig.1 Ae; Be) were found in both ecotypes. The highest levels of ammonium in ecotype ET grown under saline conditions induced the up-regulation of TFA and ALA (Fig.1 Ad, Ae). Lower levels of these compounds were found in plants exposed to high levels on ammonium without salinity and in plants exposed to 100% NO_3^- with salinity (Fig.1 Ad, Ae). In ecotype RN, the levels of TFA and ALA were affected by salinity in plants treated either with 100% or 66% NO_3^- (Fig.1 Bd, Be).

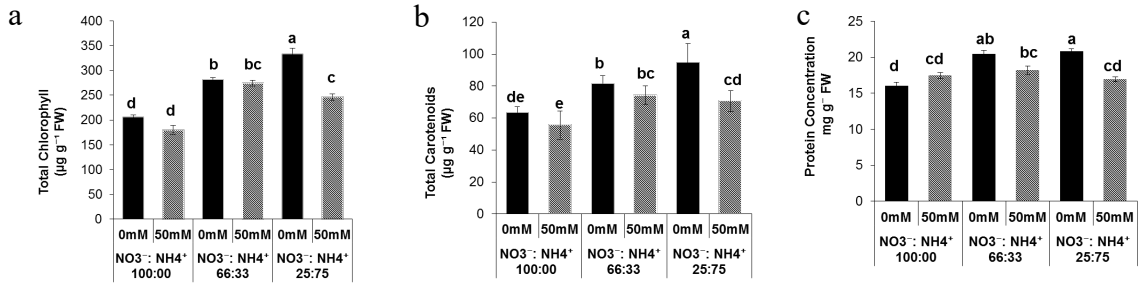
In contrast to ecotype ET, the TFA and ALA levels in RN were not affected by high levels of ammonium (Fig.1 Bd). OA was highly significantly ($P < 0.001$) affected by nitrate to ammonium ratios, and there was also a significant effect ($P < 0.01$) of salinity on OA content (Fig.1 Af; Bf). The combination of the salinity and nitrogen forms was not significant ($P > 0.05$) in terms of OA accumulation (Fig.1 Af). The lowest level of OA was observed in plants exposed to 75% of ammonium

while the highest levels were found in plants exposed to 100% $NO3^-$ in both ecotypes (Fig.1 Af; Bf). Salinity decreased levels of OA in plants grown either with 33 or 75% ammonium compared with plants grown with 100 % NO_3^- in both ecotypes (Fig.1 Af; Bf). Shoot FW was significantly ($P < 0.001$) affected by nitrogen form and salinity. Also, a significant ($P < 0.01$) effect of the interaction of salinity and nitrogen form was observed in both ecotypes (Fig.1 Ag; Bg). The highest shoot FW accumulation was found in plants exposed to 100% NO_3^- under saline and non-saline conditions (Fig.1 Ag; Bg). Ammonium affects shoot FW where the combination of ammonium with salinity enhanced shoot FW in ecotype ET (Fig.1 Ag); this effect was not observed in ecotype RN (Fig.1 Bg).

A: ecotype ET



B: ecotype RN



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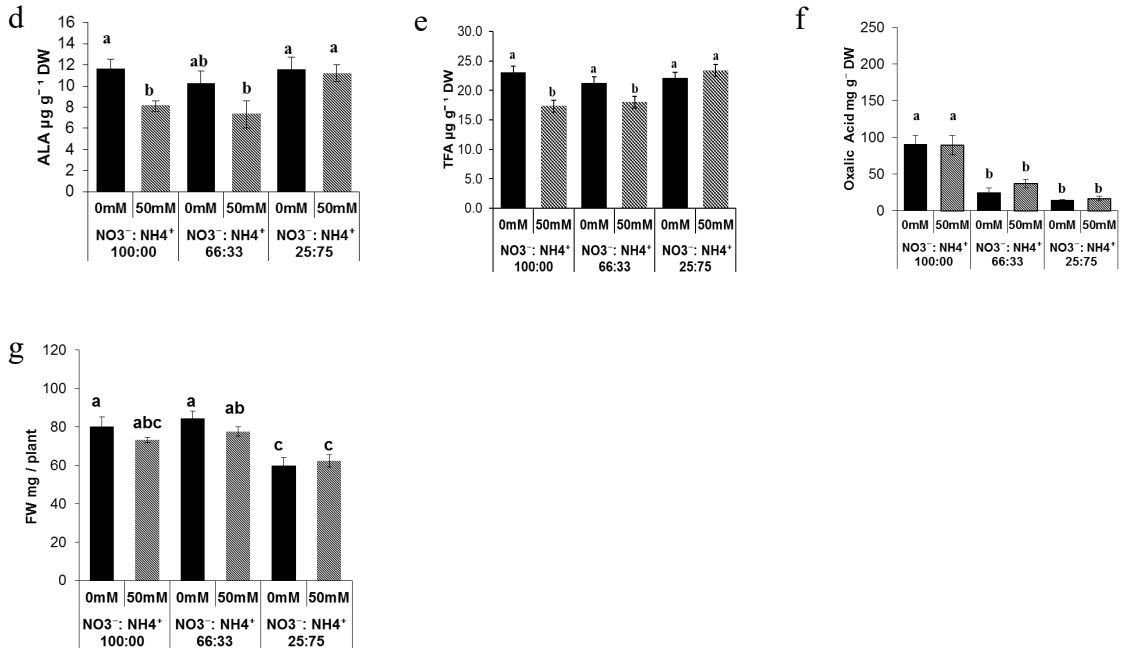

Figure 1. Effect of the nitrate to ammonium ratio (100:00, 66:33, 25:75) and salinity (0, 50 mM) on leave quality and biomass accumulation of *P. oleracea* leaves ecotypes ET (A) and RN (B). Values are means ± SE (n=6). Bars with different letters are significantly different between treatments, $P \leq 0.05$, as determined by Tukey-Kramer HSD.

3.2. Sugar metabolite changes in response to salt and nitrogen forms

The metabolomic profiling revealed leaf metabolomic variation between two ecopytes in response to the three nitrogen forms and salt stress. The principal component analysis (PCA) of leaf metabolites revealed a clear separation between nitrogen forms in both ecotypes (Supplementary. 1*A*, 2*A*). In the ecotype, ET, the first component explained 83.6% of the total metabolic variation among nitrate forms, and the second component separated salt stress plants from those control plants explained 5 % of the total metabolic variation (Supplementary. 2*A*). While in the ecopyte RN, the first component 65.4% of the total metabolic variation between nitrogen forms and the second component separated salt stress plants from the control plant. (Supplementary. 2*A*). The metabolites that relative abundance was significantly different between the treatments are presented below.

3.3. Sugar metabolite changes in response to salt and nitrogen forms

In response to salt stress, plants accumulated sugars; the metabolomics profiling revealed that salt stress leads to sugar accumulation in both ecopytes regardless of the nitrogen forms used (Supplementary 1B, 2B). Two-way ANOVA confirmed that there was a highly significant ($P < 0.001$) effect of the nitrate to ammonium ratios, salinity, and their interaction on fructose and galactinol accumulation (Fig. 2 Aa, b). Salt stress enhanced fructose content of ecotype RN plants grown with 75% of ammonium while in ecopyte ET, these levels were higher in 75%-ammonium grown plants (Fig.2 Aa; Ba) and the lowest levels in plants exposed to 100% NO_3^- (Fig. 2 Aa). Salinity rose leaves galactinol content of ecotype ET; however, this level was low in 66% NO_3^- plants. While in ecotype RN salinity increased leaf galactinol content, but nitrogen form did not affect (Fig. 2 Bb). The levels of raffinose, pinitol, and ononitol were significantly ($P < 0.001$) affected by salinity and nitrogen form but not by their interaction (Fig. 2 Ae, f). The levels of the pinitol and ononitol under saline conditions were approximately the same in the three forms of nitrogen tested, while levels of raffinose were different in plants exposed to 66% NO_3^- (Fig. 2 Ac, e, f). In ecotype, RN, these sugars accumulated in plants exposed to 100% NO_3^- under saline conditions (Fig. 2 Be, f). Raffinose levels were lower only in plants exposed to 100 and 66 % NO_3^- without salinity (Fig. 2 Bc). Myoinositol levels were affected by salinity

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but not nitrogen form in ecotype ET, but in ecotype, RN was affected by both salinity and nitrogen form (Fig. 2 Ad; Bd). The highest level of myoinositol in ecotype ET was found in plants exposed to 75% ammonium under saline conditions and the lowest levels in plants exposed to 66% NO_3^- without salinity (Fig. 2 Ad). A similar effect was observed in ecotype RN, wherein the lowest levels were found in both levels of NO_3^- (100 and 66%) (Fig. 2 Bd). These results suggested that Portulaca plants alleviate salt stress by sugar accumulation, which probably helps to keep the cellular osmotic balance.

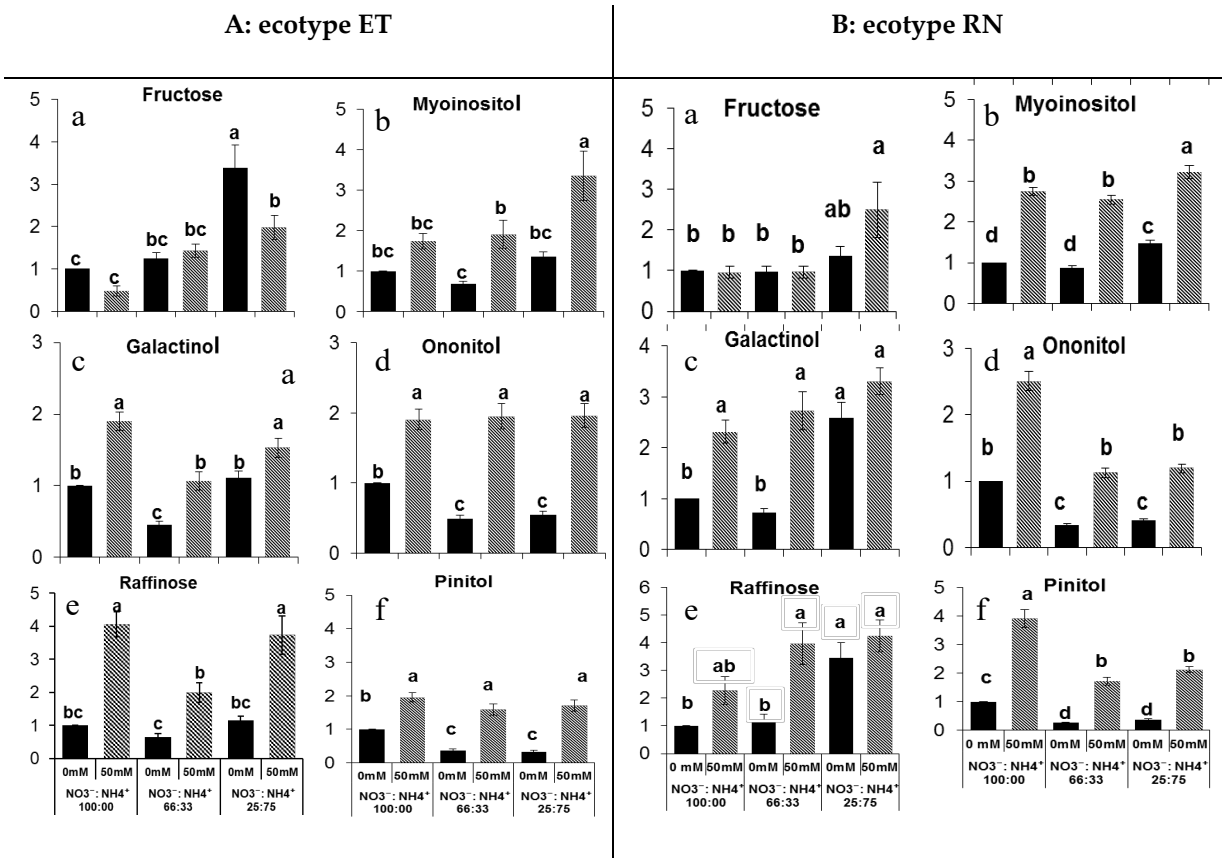

Figure 2. Effect of the nitrate to ammonium ratio (100:00, 66:33, 25:75) and salinity (0, 50 mM) on sugar accumulation of *P. oleracea* leaves ecotypes ET (A) and RN (B). Values are means ± SE (n=6). Bars with different letters are significantly different between treatments, $P \leq 0.05$, as determined by Tukey-Kramer HSD.

3.4. Free amino acid metabolite changes in response to salt and nitrogen forms

Leaf metabolomic profiling showed that the changes in the amino acid profiles in response to nitrogen form and salinity were noticeable in the ammonium-grown plants (Supplementary 1*B* and 2*B*). The 11 amino acids identified here were significantly ($P < 0.001$) affected by nitrogen form. Under high ammonium nutrition, some amino acids, including glutamate, glycine, ornithine, phenylalanine, proline, serine, and tyramine, showed increased accumulation (Fig. 3). Aspartate, ornithine, and phenylalanine metabolism was enhanced by salinity ($P < 0.001$) (Fig. 3b, g, h). Ornithine was the most abundant amino acid found in plants exposed to 75% ammonium under saline conditions wherein the enhancement was 10-fold more than in plants exposed elevated NO_3^- (Fig. 3g). Urea level also was affected by nitrogen form but not by salinity. In ecotype RN, the same 11 amino acids were detected (Fig. 4). However, the relative abundances were lower compared with ecotype ET. The levels of glutamate, glycine, leucine, ornithine, phenylalanine, proline, serine, and tyramine were significantly ($P < 0.001$) affected by nitrogen form. In this ecotype, ornithine was also the most abundant amino acid detected, although it was lower compared to ecotype ET. In addition, organic acids of the tricarboxylic acid (TCA) cycle were detected. Quantification was made using linear calibration curves of analytical standards derivatized in the same way as the leaf samples. In ecotype ET malic acid, citric acid and

ascorbic acid contents were elevated in plants exposed to 100% NO_3^-. (Fig. 5 Aa, b). Salinity leads enhancement of citric acid in nitrate-grown plants; this result was directly correlated with the leaf chlorosis observed (Fig. 5Aa). Ascorbic acid was significantly ($P < 0.001$) affected by the interaction of salinity with nitrogen forms (Fig.5Ac). The highest levels were found in 66% NO_3^- and ammonium-grown plants under saline conditions (Fig.5Ac). In ecotype RN, this organic acid was also affected by nitrogen forms; the highest levels of these three organic acids were observed in plants exposed to 100% NO_3^- (Fig. 5B). These results underline that ecopyte ET assimilated ammonium faster into the amino acids pool either under salt and no salt stress conditions, thus, avoid leaf ammonium toxicity.

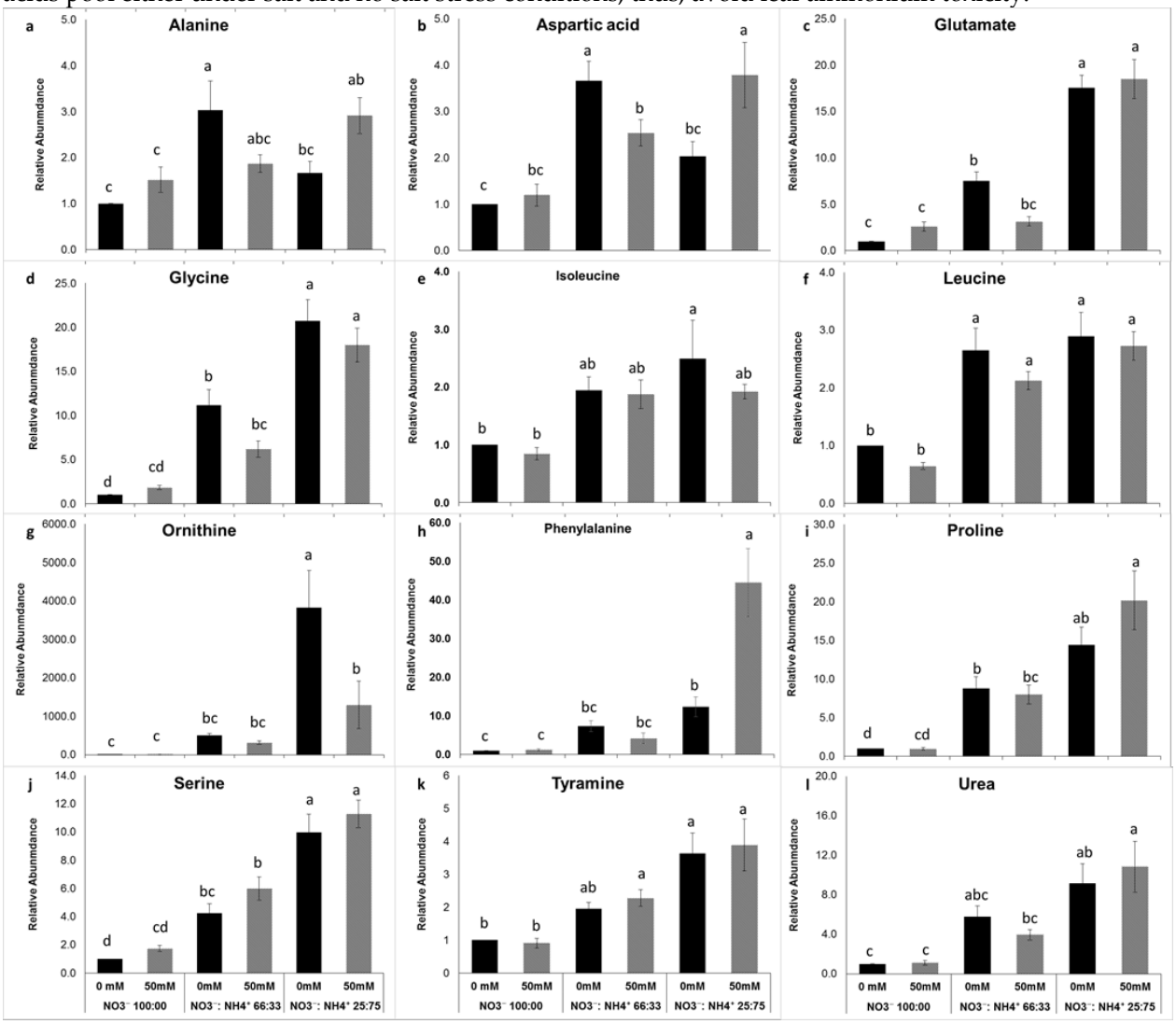

Figure 3. Effect of the nitrate to ammonium ratio (100:00, 66:33, 25:75) and salinity (0, 50 mM) on amino acid accumulation of *P. oleracea* leaves ecotype ET. Values are means ± SE (n=6). Bars with different letters are significantly different between treatments, $P \leq 0.05$ as determined by Tukey-Kramer HSD.

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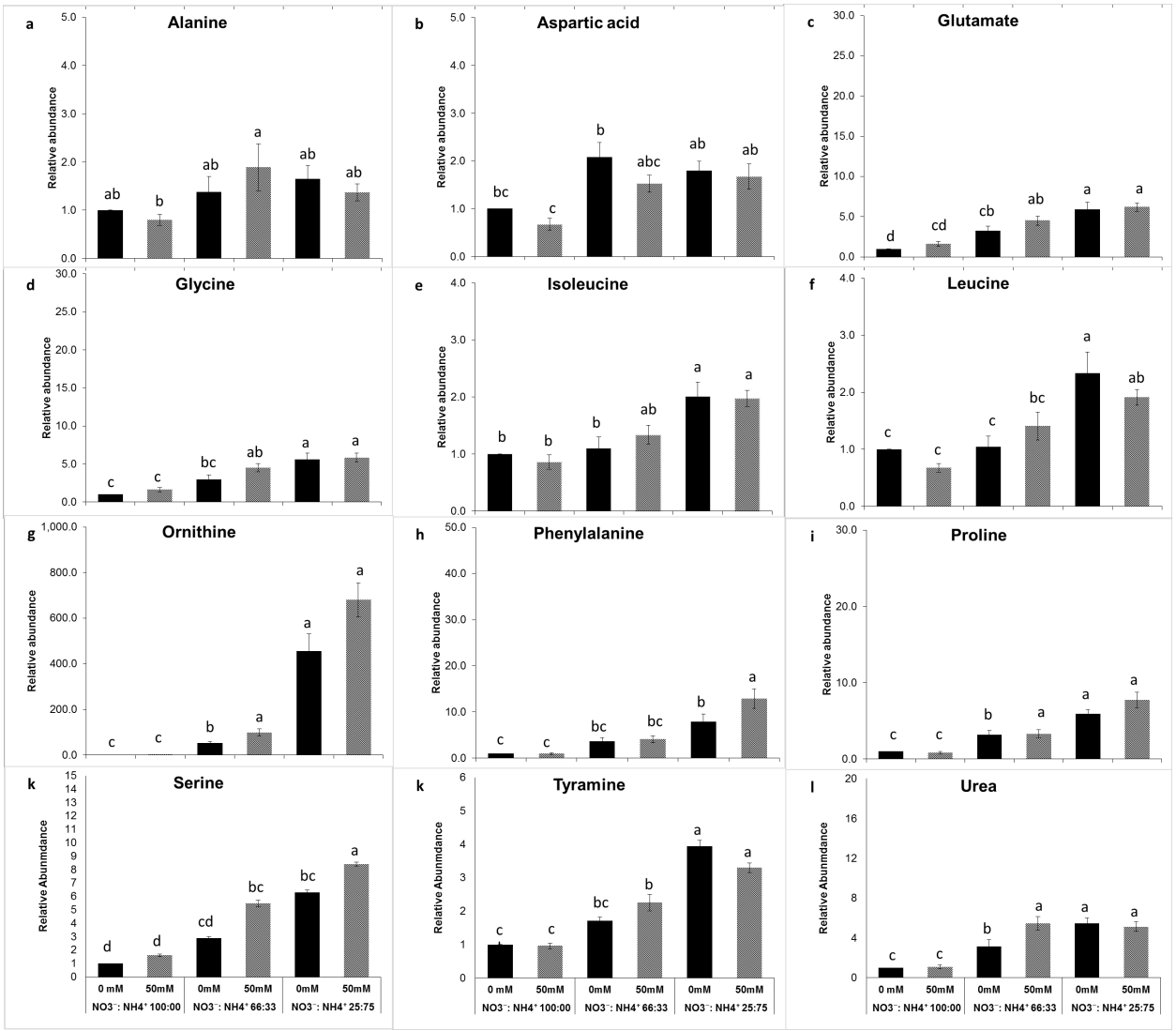

Figure 4. Effect of the nitrate to ammonium ratio (100:00, 66:33, 25:75) and salinity (0, 50 mM) on amino acid accumulation of *P. oleracea* leaves ecotype RN. Values are means ± SE (n=6). Bars with different letters are significantly different between treatments, $P \leq 0.05$ as determined by Tukey-Kramer HSD.

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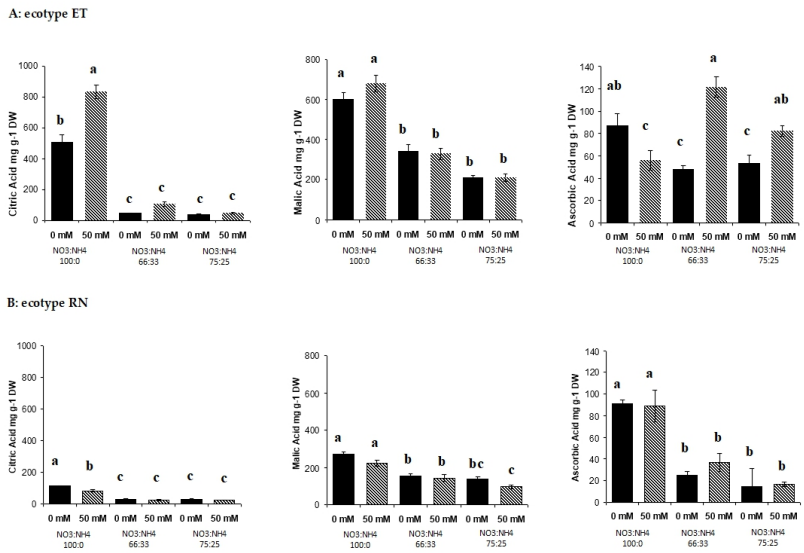

Figure 5. Effect of the nitrate to ammonium ratio (100:00, 66:33, 25:75) and salinity (0, 50 mM) on organic acid accumulation of *P. oleracea* leaves ecotypes ET (A) RN (B). Values are means ± SE (n=6). Bars with different letters are significantly different between treatments, $P \leq 0.05$, as determined by Tukey-Kramer HSD.

4. Discussion and conclusion

P. oleracea plants are well known to accumulate high levels of NO_3^- and OA, but although a nutritionally desirable decrease in OA, can be achieved by altering the NO_3^-:NH_4^+ ratio (in non-saline conditions) it has a negative effect on biomass [14, 20, 21]. Our results were in agreement this. However, applying 75% ammonium to the growth medium in saline conditions resulted in increased dry weight in ecotype ET. This enhancement suggests that under saline conditions ecotype ET can assimilate ammonium faster than ecotype RN as shown by the increase of the free amino acid pool and fatty acids. Furthermore, 75% ammonium in both saline and non-saline medium led to increased levels of total chlorophyll, carotenoid, crude protein, ALA, and TFA, enhancing leaf quality while, importantly, having reduced OA levels. The same general effect was found in ecotype RN, but leaf toxicity was observed. When plants were exposed to elevated NO_3^- (100%) leaf chlorosis, high levels of OA, malic acid, and citric acid were observed in both ecotypes. The high levels of citric acid-induced leaf chlorosis were observed in both ecotypes, since high levels of citric acid limit Fe translocation [27], inducing leaf chlorosis [28]. On the other hand, the high levels of malic acid found in plants exposed to 100% NO_3^- suggest that, under elevated NO_3^-, *P. oleracea* biosynthesizes malic acid in order to maintain ion balance over anionic NO_3^- (data not shown). Malic acid is one of the organic acids which plants accumulate to control osmotic balance [29]. Little is known about NO_3^-:NH_4^+ effects on ALA and TFA contents under saline conditions. The accumulation of free amino acids under high levels of ammonium may serve as N and C sources for metabolic pathways. Fatty acids could play an essential role in plant protection as enhancing protective compounds transport such as glycine betaine [30]. Therefore, the application of 75% ammonium in saline conditions can improve fatty acid production and acts as an energy source improving biomass production.

To cope with adverse environmental conditions, plants accumulate osmoprotectants to alleviate cellular hyperosmolarity and ion disequilibrium [31]. Sugars and polyols such as ononitol and pinitol act as compatible solutes resulting in increased cellular osmolarity and as scavengers of ROS [32 – 34];

these sugars have been associated with drought and salinity tolerance. Ononitol, pinitol, raffinose, and myoinositol, slightly accumulated under saline conditions in both ecotypes. Nevertheless, the accumulation of ononitol and pinitol under saline conditions in ecotype ET were significantly higher at all three nitrate-to-ammonium ratios (100%: 00; 66:33%; 25:75%) contrasting with ecotype RN where these sugars were higher only in the plants exposed to elevated NO_3^-. The current study suggests that the main strategies for tolerating salinity are by the up-accumulation of sugars and polyols. These findings are consistent with the results obtained by [31], where the combined effect of drought and heat leads to increased sugar synthesis.

Halophytes accumulate amino acids in response to salinity and drought [35]. These amino acids, including aspartic acid, phenylalanine, proline, and ornithine, showed an increase under saline conditions in ammonium growth plants in both ecotypes, but the relative abundance was higher in ecotype ET. Ornithine was by far the most abundant non-protein amino acid; this result confirms that the significant non-protein amino acid synthesized under saline conditions is ornithine. It is well known that decarboxylation of ornithine is the first step for polyamides formation, and polyamides are known as osmoprotective substances [36, 37]. The accumulation of this amino acid under saline conditions and high ammonium levels suggests cellular osmotic adjustments to maintain leaf turgidity. The up-regulation of glutamate, glycine, leucine, isoleucine, serine, tyramine, proline and ornithine in ammonium grown plants suggests faster assimilation and conversion of ammonium into free amino acids to avoid ammonium toxicity.

Besides sugar and amino acids, the involvement of organic acids in response to osmotic stress has also been demonstrated. Organic acids could play a central role in the regulation of intracellular pH by accumulation in vacuoles to neutralize excess cations [38]. Indeed, we observed that elevated NO_3^- lead to NO_3^- accumulation (data not shown) inducing malic and citric acid accumulation in both ecotypes. The accumulation of ascorbic acid is recognized as an effective reactive oxygen species (ROS) scavenger [39, 40]. The accumulation of ascorbic acid in transgenic potato plants was directly correlated with their ability to withstand abiotic stress [41]. Therefore higher accumulation of ascorbic acid in ecotype ET might contribute to ROS scavenging, thus avoiding leaf nitrate toxicity and aiding salt tolerance. In contrast, the higher ascorbic acid accumulation in ecotype RN was only found in plants exposed to elevated NO_3^-. We concluded that the damage observed on the leaves of ecotype RN grown under 33 and 75% of ammonium were as a result of ROS accumulation due to the suppression of ascorbic acid necessary for the reductive detoxification of ROS molecules.

In terms of crop potential ammonium fertilization generally results in low OA contents but this comes at the cost of low biomass production [14, 20]. Based on the results of these experiments, it can be concluded that the deleterious effect of high levels of NH_4^+ fertilization on biomass production can be minimized by moderate salinity. This effect seems to be correlated to the increased sugar biosynthesis together with high levels of ascorbic acid accumulation, supporting the idea that NH_4^+ under saline conditions reduced the energy requirements of growth. The results showed that combined stresses of salinity and high ammonium nutrition cause different results than saline or ammonium individual stresses, as evidenced by enhancing biomass production, TFA, and ascorbic acid accumulation in one ecotype. The current study also showed natural differences between the ecotypes as shown by ammonium and salinity toxicity in one ecotype (RN), and ammonium affinity together with salt-tolerance by another (ecotype ET). Although nitrogen fertilization improves yield, it seems clear that nitrate cannot be used as a sole nitrogen source for Portulaca plants as this form harms yield, quality, and the salt-tolerance threshold. Therefore 75% ammonium is recommended under moderate saline conditions. Further molecular study of the molecular mechanism will contribute to better explaining of salinity response tolerance.

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