

Improving biochemical and phytochemical compounds in purslane (*Portulaca oleracea* L.) by mannitol under salt stress conditions

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Abstract

This study aimed to investigate the impact of mannitol on the biochemical and phytochemical responses in purslane plants subjected to salinity stress conditions. For this purpose, a factorial experiment was carried out using a completely randomized design (CRD) with two factors and four replicates. The factors were four concentrations of mannitol (0, 10, 20, and 30 mM) and two salinity levels (control and 120 mM of NaCl). During the vegetative stage, the biochemical components, soluble sugar, relative water contents, and phytochemical compounds were measured. The results indicated a significant difference between salinity and mannitol levels regarding the most studied traits. The results demonstrate that the interaction effects of salinity and mannitol levels affected total leaf soluble proteins, proline, soluble carbohydrate amount, relative water content (RWC), and phytochemical compounds. According to our findings, the foliar application of mannitol significantly increased the amount of soluble carbohydrates (glucose, xylose, and mannose), leaf protein content, and phytochemical compounds under salinity conditions. Additionally, the proline and RWC showed a positive response to the application of mannitol. In conclusion, the foliar application of mannitol under salinity stress may counteract the negative effects of such stress and improve *Portulaca oleracea* tolerance to salinity by increasing some biochemical and plant secondary metabolites.

Keywords: Antioxidant enzymes, biochemical, phytochemical compounds, *Portulaca oleracea*, soluble sugar.

Abbreviations: DW, dry weight; FW, fresh weight; RWC, relative water content.

Introduction

Portulaca oleracea L., commonly known as purslane, is an herbaceous annual in the family of Portulacaceae. The plant is distributed worldwide, including Europe, America, Canada, India, New Zealand, Australia, China, and Japan (Talei et al. 2020; Zhou et al. 2015). The plant has a round, smooth, and succulent stem, with small, oblong, alternate, or sub-opposite dark-green leaves. The flowers are small and yellow, and the seeds are reddish brown to black, oval, and tiny (Sultana and Rahman 2013).

The plant is rich in fatty acids, proteins, vitamins, dietary minerals, and about 70% of its fatty acids are unsaturated, and about 50% contain only omega-3 fatty acids. According to Iranian traditional medicine sources, purslane is an antinociceptive, anesthetic, antiseptic, anti-ascorbate, anti-inflammatory, anti-inflammatory, blood purifier, anti-fungal, and reduces swelling and abscesses, insect bites, and scorpion stings (Zhou et al. 2015). This plant is considered to be a drought and salinity-tolerant crop, capable of obtaining moisture from levels that are not available to the majority of crops (Weiss 2000). The reaction of plants to salinity stress differs significantly at various organizational levels, depending upon intensity and duration of stress, as well as plant species and stage of development (Chaves et al. 2003).

All these factors cause impairment in different physiological and biochemical processes, which ultimately cause reduced plant growth (Shaddad 2010). One of the premier responses of plants to salinity is the synthesis and accumulation of compatible organic substances, including amino acids, especially proline (Ashrafijou et al. 2010), organic acids (Farouk 2011), and polyols such as sorbitol and mannitol (Mitoi et al. 2009). All these osmotically active organic solutes play a key role in osmotic adjustment, a phenomenon which has been widely reported to play an active role in maintaining cell turgor (Siringam et al. 2011).

Plant stress tolerance has been widely reported to be improved with the exogenous application of a variety of regulator chemicals (Nawaz and Ashraf 2010). Mannitol, an important osmolyte that plays a key role in oxidative stress response, is normally synthesized in large amounts in many plant species (Mitoi et al. 2009). Although mannitol plays an important role in osmotic adjustment, it acts as an antioxidant to scavenge hydroxyl radicals (OH) (Srivastava et al. 2010). However, little

Table 1. Analysis of variance on measured characteristics of purslane plants under mannitol and salinity stress.

S. O. V	df	RWC	Glucose	Xylose	Mannose	Protein	Proline
Salinity	1	550.008**	2814.169**	764.112**	1148.235**	0.001**	0.001**
Mannitol	3	209.798**	308.514**	81.045**	61.905**	0.005**	0.001**
S×M	3	287.909**	1417.441**	427.203**	346.462**	0.001**	0.002**
Error	24	3.06	12.18	13.61	14.50	0.0002	0.0001
CV (%)		2.37	3.10	5.44	4.75	3.71	22.37

** , * and ns, refer to 1%, 5% and not significant, respectively. RWC: relative water content.

information is available in the literature on the role of mannitol in plant stress tolerance. The main objective of the present study was to investigate the impact of mannitol on biochemical, soluble sugars, and phytochemical responses in purslane plants under salt stress conditions.

Results

Relative water content

The analysis of variance indicated a significant difference among mannitol and salinity levels in terms of relative water content in the plants. Variation due to their interaction was highly significant ($P \leq 0.01$) (Table 1). In the salinity level, relative water content decreased (10.73%) in comparison to the control condition. Increasing the mannitol levels under the control condition increased relative water content (6.98%), and the highest relative water content (84.36%) was obtained in 30 mM application of mannitol. In addition, by increasing the mannitol levels under the salinity stress condition, relative water content increased, and the highest value of relative water content was obtained in 20 mM application of mannitol (Figure 1).

Soluble sugars (glucose, mannose, and xylose) content

The application of mannitol and salinity affected the soluble sugars (glucose, mannose, and xylose) content in the plant (Table 1). In the salinity levels, the glucose, mannose, and xylose contents increased (18.15, 15.52, and 16.14%) in comparison to the control condition. Elevating mannitol levels in the control condition resulted in increased concentrations of glucose, mannose, and xylose, with peak values recorded at 124.75% for glucose, 85.16% for mannose, and 74.70% for xylose at a 30 mM mannitol application. Conversely, under salinity stress, higher mannitol levels led to a reduction in glucose, mannose, and xylose contents, with the maximum concentrations observed at 10 mM for glucose, 0 mM for mannose, and 10 mM for xylose, respectively (Figure 1).

Total leaf soluble protein content

The analysis of variance indicated a significant difference among mannitol (M) and salinity (S) levels, and their interaction (S×M) in terms of protein content in the plants ($P \leq 0.01$) (Table 1). Variation due to mannitol and its interaction with salinity was highly significant. By increasing the mannitol levels under the salinity condition, protein content increased, and the highest (0.418 mg g⁻¹ FW) and lowest (0.290 mg g⁻¹ FW) values of protein content were obtained in 30 mM and 0 mM application of mannitol under the salinity level, respectively. The protein content in the high concentration of mannitol (30 mM) increased (44.14%) in comparison to the control (0 mM of mannitol). The results showed an increase in leaf protein content in salinity levels by increasing mannitol levels (Figure 1).

Proline content

The application of mannitol and salinity affected the proline accumulation in the plant (Table 1). Under salinity stress, proline accumulation increased by 27.69% compared to the control condition. However, as mannitol levels increased, proline accumulation decreased. The highest proline accumulation (64.30 μg g⁻¹ FW) was observed with 0 mM mannitol, while the lowest value (36.73 μg g⁻¹ FW) was recorded with 10 mM mannitol under salinity conditions (Figure 1).

Phytochemical compounds analysis

The GC-MS results indicated that application of mannitol led to an increase in the number of phytochemical compounds and induced some phytochemical compounds like 9,12,15-Octadecatrienoic acid (69.86%) in comparison to the control condition, while salinity led to a decrease in the number of phytochemical compounds and some of phytochemical compounds like 9,12,15-Octadecatrienoic acid to be repressed (Table 2). In both treatment with mannitol and salinity, some phytochemical compounds were induced, some repressed, some upregulated, and some downregulated. Interestingly, the induced compounds in the treated plant with mannitol were more than the induced compounds in the treated plant with salinity, while the repressed compounds in the treated plant with salinity were more than the repressed compounds in the treated plant with mannitol. Using mannitol under salinity conditions induced most of the repressed compounds. The main compound in the control and salinity was 9,12-Octadecatrienoic acid, with the amount of 66.47% and 77.33%, respectively, while the main compound in the plant treated with mannitol and mannitol under salinity was 9,12,15-Octadecatrienoic acid, with the amount of 69.86% and 39.56%, respectively (Table 2).

Table 2. The phytochemical compounds of purslane plants under mannitol and salinity stress conditions using GC-MS analysis.

Phytochemical compound	Control	30 mM mannitol	120 mM salinity	30 mM mannitol+ 120 mM salinity
Compound number	20	23	13	19
9,12,15-Octadecatrienoic acid	-	69.86%	-	39.56%
9,12-Octadecadienoic acid	66.47%	-	77.33%	31.15%
Hexadecanoic acid	19.99%	12.91	17.83%	12.59%
9-Hexadecenoic acid	0.32%	0.12%	0.19%	-
Octadecanoic acid	4.88%	2.77%	2.25%	3.02%
Eicosanoic acid	1.30%	0.54%	0.52%	0.69%
Dodecane	1.10%	3.52	0.41%	3.10%
Tetradecane	0.98%	2.22%	0.44%	1.49%
Cyclohexane	-	0.37%	-	0.47%
Docosanoic acid	0.92%	-	0.28%	0.47%
Tetracosanoic acid	0.73%	-	-	0.24%
Undecane	-	0.29%	-	0.22%
Decane	0.71%	4.13%	0.19%	5.09%
Hexadecane	0.47%	0.86%	0.24%	0.59%
3-Methylnonane	-	0.28%	-	-
Cyclopentane	-	0.23%	-	0.23%
5-Methylnonane	-	0.19%	-	-
5-Methylundecane	-	0.14%	-	0.29%
3-Methylundecane	-	0.13%	-	0.30%
Heptadecanoic acid	0.41%	-	-	-
2-octanoic acid	0.33%	-	-	-
Eicosane	0.17%	0.18%	-	-
9-Octadecenoic acid	0.29%	-	-	-
11-Eicosenoic acid	0.25%	-	0.12%	-
Octadecane	0.21%	0.33%	0.08%	0.20%
Octane	-	0.10%	-	-
Phenol	0.18%	-	-	0.25%
Cyclododecyne	0.16%	-	-	-
Tridecane	0.14%	0.60%	0.11%	0.37%
Pentadecane	-	0.10%	-	-
Tetradecanoic acid	-	0.09%	-	-
Naphthalene	-	0.08%	-	-

Nevertheless, spraying the mannitol improved the phytochemical compounds. Salinity stress had negative effects on the phytochemical compounds. The largest variation under mannitol, salinity, and interaction of mannitol with salinity was obtained for 9,12-Octadecatrienoic acid (Table 2). A total of 13 to 23 phytochemical compounds were obtained in the GC-MS analysis results under different conditions (Table 2). The highest (69.76%) and the lowest (39.56%) 9,12,15-Octadecatrienoic acid percentages were obtained in 30 mM mannitol spraying and application of 30 mM mannitol under 120 mM salinity, respectively. The highest (77.33%) and the lowest (31.15%) 9,12-Octadecatrienoic acid percentages were obtained under 120 mM salinity and 30 mM mannitol under 120 mM salinity, respectively (Table 2). Figures 2 show the chromatograms of the phytochemical compounds under different conditions using GC-MS analysis.

Discussion

The interactive effects of salinity and mannitol on the leaf soluble proteins indicated that the foliar application of mannitol increased the leaf soluble proteins under salinity stress in comparison with the control treatments, which matched up with the results of Kaya et al. (2013) who studied the effect of exogenous application of mannitol and thiourea on maize under salinity conditions. The results indicated that the proline accumulation was affected by salinity stress and mannitol, and the highest proline accumulation was obtained with a 120 mM salinity level with no application of mannitol. Proline may have different roles in salinity-resistance mechanisms, such as scavenging free radicals and thereby protecting cellular structures against oxidative damage and denaturation (Girija et al. 2002), proline can serve as a carbon and nitrogen reservoir for growth after stress relief (Silveira et al. 2003). Moreover, Phutela et al. (2000) suggested that proline accumulates in tissues of stressed plants due to the increased rate of its synthesis by pyrroline-5-carboxylate synthetase, and then it decreases as it is degraded by proline oxidase enzyme, with concomitant increases in both cell turgidity and its activity. Likewise, proline had a negative and significant correlation with RWC. Thus, higher RWC content in leaves under non-stress conditions was accompanied by lower proline. In *Aeluropus lagopoides*, leaf RWC was significantly correlated with chlorophyll, proline, and soluble sugar contents (Mohsenzadeh et al. 2006).

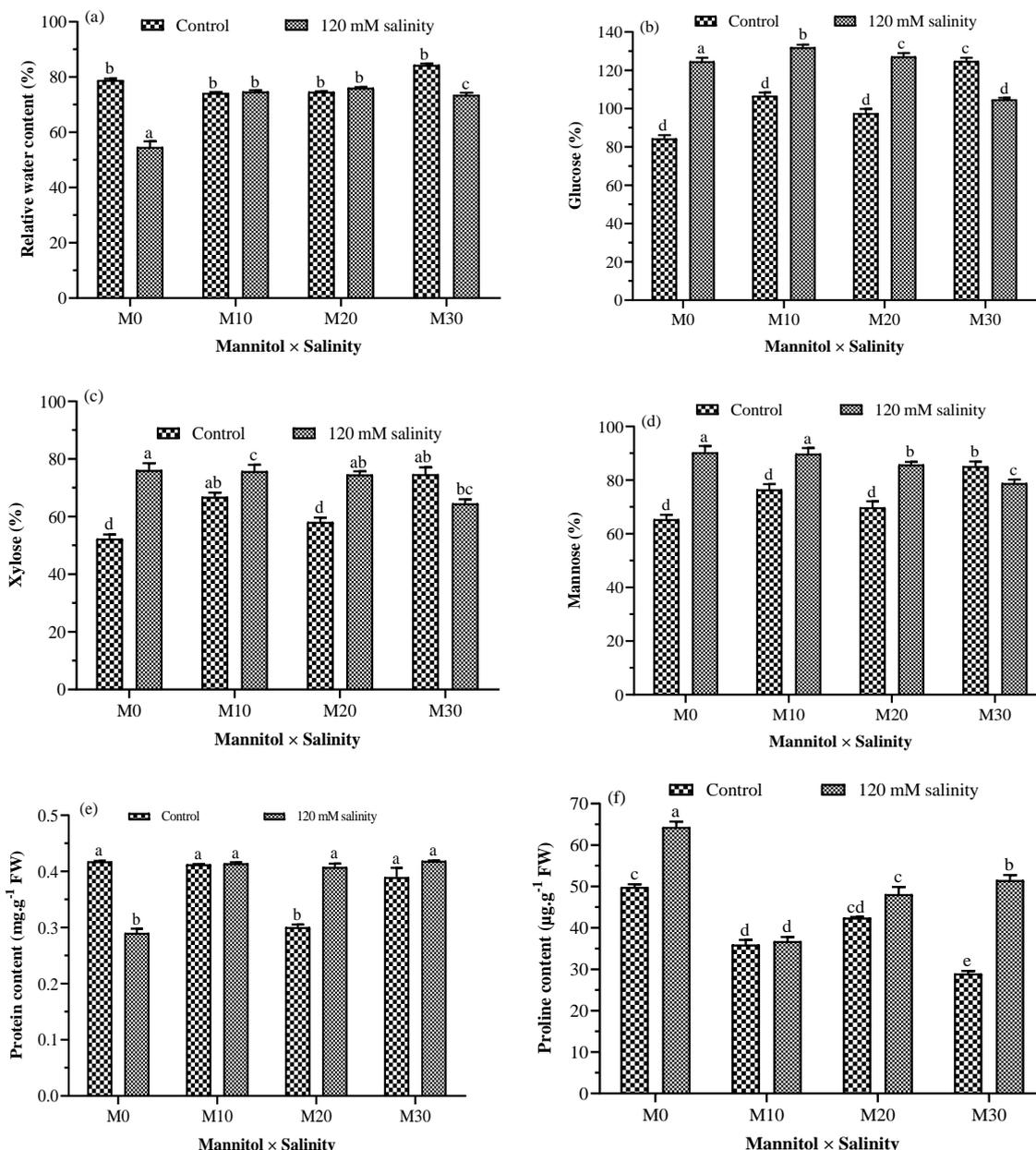


Fig 1. The interaction effects of salinity and mannitol on relative water content (a), glucose (b), xylose (c) and mannose (d), protein (e), and proline contents (f) in purslane plants using Duncan's multiple comparison test at $P \leq 0.01$. Vertical bars represent SEM for four samples.

The results showed that soluble sugars (glucose, xylose, and mannose) were significantly affected by the mannitol application under salinity stress, and the highest soluble sugar content was obtained in the salinity level with the application of mannitol. The accumulation of sugars could help maintain turgor of stressed tissues through osmoregulation (Correia et al. 2006). The nutritional status of plants has a great influence on the tolerance of plants to environmental stresses such as drought, salinity, and high light intensity (Marschner 1995). Similarly, in addition to the known beneficial effects of micronutrients on plants, they are involved in other processes such as carbohydrate and nitrogen metabolism as well as resistance to diseases and adverse environmental conditions. Mannitol regulates plant growth and oxidative stress responses in salt-stressed plants and is essential for the organization and rapid alternation of nutritional compounds within a plant (Massoud et al. 2005). Therefore, the mannitol application increased the purslane tolerance against salinity stress by improving the soluble carbohydrate content. The leaf RWC is a commonly used indicator of plant water status and plant salinity tolerance (Lawlor and Cornic 2002). The RWC was affected by both salinity stress and mannitol, and the highest and lowest RWCs were obtained with 30 mM application of mannitol. Kaya et al. (2013) reported that with increasing salinity, RWC decreased in maize plants. Therefore, in this experiment, the application of mannitol led to an increase in RWC.

Saline stress is a factor that triggers signaling pathways of secondary metabolite synthesis to increase plant resistance to stress conditions (Zhou et al. 2024), but this is crucially dependent on the salinity sensitivity of specific plant species (Volkov and Beilby 2017). Generally, plants produce secondary metabolites in nature as components of nonenzymatic antioxidant defense, which play an important role in protecting plants under salt stress conditions (Ashraf et al. 2019). In this regard,

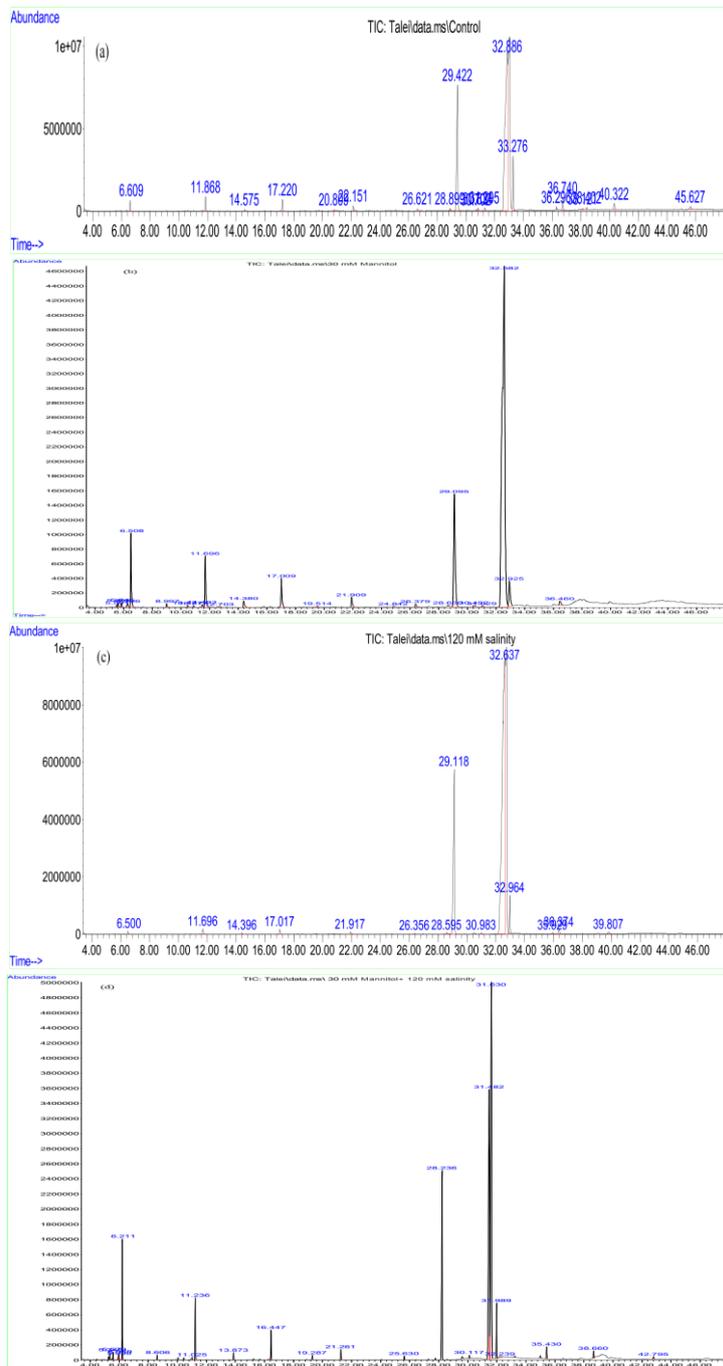


Fig 2. Chromatograms of the phytochemical compounds under control (a), 30 mM Mannitol (b), 120 mM salinity (c), and 30 mM Mannitol+120 mM salinity treatments using GC-MS analysis.

our results indicated that some induced phytochemical compounds, like 9,12,15-Octadecatrienoic acid, were produced under salinity conditions. Our results are in agreement with the concept of a close relationship between the availability of secondary metabolites and self-defense systems of the plant species against salinity stress. Interestingly, the induced compounds in the treated plant with mannitol were more than the induced compounds in the treated plant with salinity, while the repressed compounds in the treated plant with salinity were more than the repressed compounds in the treated plant with mannitol. Using mannitol under salinity conditions induced most of the repressed compounds. Likewise, many researchers suggested that proline and secondary metabolites are involved in osmotic adjustment, by accumulation in the vacuole, and play a significant role by increasing the oxidative stress tolerance through protecting the chloroplast and photosynthetic systems from solar radiation by absorbing UV (Koca et al. 2007; Talei et al. 2013a). Consequently, the results recommended that increasing the biosynthesis of proline in the plant could protect the cellular structures from oxidative damage and osmotic stress. This event complies with the hypothesis that the *Portulaca oleracea* may activate different cellular mechanisms in response to salinity stress in their tissues, so that they produce high amounts of proline and the phytochemical compounds, which might be related to tolerance abilities, indicating physiological performances. This finding matched up with the results of Chutipajit et al. (2009), who detected a positive correlation between flavonoid and proline accumulations and relative water content (RWC) in rice (*Oryza sativa* L. spp. Indica).

In agreement with the findings of other researchers, who reported a significant increase in polyphenolic compounds in *Spergularia marina* and *Glaux maritima* (Pungin et al. 2023), flavonoids in *Andrographis paniculata* (Talei et al. 2013a), bioactive compounds in *Azadirachta indica* (Omar et al. 2023), menthone in *Mentha pulegium* (Karray-Bouraoui et al. 2009), and phytochemical accumulation in the *Centella asiatica* (Gupta and Wao 2022), our results indicated that salinity could lead to a significant increase in phytochemical compounds. Enhancement of phenolic and flavonoid compounds in onion plants under salinity stress has been reported to improve the deleterious effects of salinity stress (Mohamed and Aly 2008). Accumulation of compatible solutes in the cytoplasm can contribute to decreasing the water potential in the cytoplasm. These neutral organic compounds can also improve the inhibitory effects of high ion concentrations on enzymatic activity without interfering with protein structure and function (Hare et al. 1998).

Materials and Methods

Plant material

The seeds of purslane were sourced by the Medicinal Plants Research Center at Shahed University in Tehran, Iran.

Experimental design

The experimental design was factorial based on a completely randomized design (CRD) with two factors and 4 replicates. The factors were four concentrations of mannitol (0, 10, 20, and 30 mM) and two salinity levels (control and 120 mM of NaCl). Twenty seeds were sown in each pot containing a sand medium. After germination, the weak seedlings were removed, and five healthy seedlings were maintained in each pot, and then placed in a controlled greenhouse for a further 30 days at 28°C with a light/dark regime of 14/10h and relative humidity of 60- 75%. After 35 days of culturing, the plants were exposed to mannitol levels (0, 10, 20, and 30 mM) and two salinity levels (control and 120 mM of NaCl) for one month. Each plant was sprayed and irrigated once a day with four levels of mannitol and two levels of saline water, respectively. After every three mannitol and salinity applications, the plants were irrigated with Hoagland's nutrient solution. After 30 days of mannitol and salinity exposure, all plants were harvested, and data on soluble sugar traits and phytochemical compounds were measured.

Relative water content

The leaf relative water content (RWC) was measured in the second youngest fully expanded leaves, which were harvested weekly in the morning as follows: Schonfeld et al., (1988) formula.

RWC (%): $100 \times (\text{FW}-\text{DW}) / (\text{TW}-\text{DW})$.

Turgid weight (TW) was obtained after soaking leaves in distilled water for 12 h at room temperature (approximately 25 °C). Then, leaves were quickly and carefully blotted dry with tissue paper in preparation for determining TW. The Dry weights of leaves were measured after oven drying at 65 °C for 48 h.

Determination of soluble sugars (glucose, mannose, and xylose)

Two g frozen samples were extracted in 3 mL of distilled water, and then the homogenous solution was filtered through filter paper. Afterwards, 0.5 mL of phenol and 2.5 mL of 98 % sulfuric acid were added to 50 μL of filtered solution to measure the carbohydrate concentration of the sample. After adding sulfuric acid, an exothermic reaction and an orange color were observed, and then the mixture was kept at room temperature for 10 minutes to cool down. A standard curve was generated using various concentrations of glucose, xylose, and mannose ranging from 0 to 20 $\mu\text{g mL}^{-1}$. The absorbance of both standards and samples was measured using a spectrophotometer (Perkin Elmer Lambda 25; UV/VIS, USA) at wavelengths of 480, 485, and 490 nm. The carbohydrate content of the samples was expressed as $\mu\text{g g}^{-1}$ FW (Masoudi-Sadaghiani et al. 2011).

Determination of proline and protein contents

Proline was determined by the ninhydrin method described by Bates et al. (Bates et al. 1973). In this method, proline was extracted from 0.5 g of fresh leaf tissue into 10 ml of 3% sulfosalicylic acid and filtered through Whatman No. 42 filter papers and determined in a Shimadzu UV-1201 model spectrophotometer. To determine the leaf protein content, 0.5 grams of collected leaf was ground in liquid nitrogen using a pre-cooled mortar and pestle to obtain a fine powder and then homogenized with 2 mL of the HEPES/KOH buffer according to the method of Talei et al. (2013b). Finally, the total protein concentration was determined by the Bradford method (1976) at 595 nm, using a spectrophotometer (Lambda 25, UV/VIS).

Phytochemical compounds assay

To assay phytochemical compounds, 20 g of seed from each sample was extracted in 50 mL hexane by the Soxhlet method, and then 100 μL of sodium methoxide (0.5 M) was added to 50 μL sample in one mL of hexane (Dinari et al. 2013). The mixture was shaken vigorously for 15 min, allowed to stand. The gas chromatographic analysis (Agilent 7890B GC System-5977 MSD model) was performed on a Helium gas chromatograph (99.999%) equipped with a PIF detector. We used an HP-5ms column (30 m \times 0.32 mm \times 0.25 μm). The temperatures of the injector, detector, and oven were 250 °C, 280 °C, and 180 °C, respectively. FAMES were identified based on the comparison of their relative RF (retention times) values with those of authentic standards.

Statistical analysis

All statistical analyses, including the raw data normality, two-way ANOVA, and Duncan's multiple range tests, were performed using SPSS 26 software (SPSS Inc., Chicago, Illinois, USA), at the significance level of $P \leq 0.05$. The results were expressed as the Mean \pm Standard error of mean (SEM), at the significance level of $P \leq 0.05$. The GraphPad Prism No. 8 software (GraphPad Prism Software Inc., La Jolla, California, USA) was used to draw the graphs.

Conclusions

According to our findings, the foliar application of mannitol significantly increased the amount of soluble carbohydrates, protein content in the leaves under salinity conditions. Similarly, the proline and phytochemical compounds contents indicated a positive response to the application of mannitol. Therefore, foliar application of mannitol could be useful in the sustainable cultivation of pharmaceutical plants like *Portulaca oleracea*, and it is highly recommended to minimize the possible damage in purslane plants due to salinity stress.

Statements and Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Competing Interests and Funding

The authors did not receive support from any organization for the submitted work.

Author contribution

All authors contributed to the study conception and design. Daryush Talei, Ayatollah Rezaei, Mojtaba Khayam Nekouei, and Saeid Kadkhodaei performed material preparation, data collection, and analysis. Daryush Talei wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and discussed the results and contributed to the final manuscript and approved the final version of the manuscript.

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