

Antioxidant enzymes and molecular responses of purslane (*Portulaca oleracea* L.) subjected to mannitol under salt stress conditions

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Abstract

Salinity stress severely limits plant growth and productivity through oxidative damage and metabolic disturbances. This study investigated the effects of mannitol application on growth, antioxidant enzyme activities, lipid peroxidation, and expression of phenylpropanoid pathway genes in *Portulaca oleracea* under salinity stress. A factorial experiment based on a completely randomized design (CRD) with four replications was conducted using four mannitol concentrations (0, 10, 20, and 30 mM) and two salinity levels (0 and 120 mM NaCl). Salinity significantly reduced shoot dry weight (SDW), catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO) activities, while increasing superoxide dismutase (SOD), ascorbate peroxidase (APX), and malondialdehyde (MDA) content. Mannitol application effectively alleviated salinity-induced damage by enhancing antioxidant defense responses and reducing oxidative stress. The highest SDW was observed at 30 mM mannitol, with a 67.11% increase compared with untreated plants. Under saline conditions, 30 mM mannitol significantly increased SOD and CAT activities by 67.20% and 96.77%, respectively, while 20 mM mannitol produced the highest POX activity. In contrast, MDA accumulation decreased by 28.42% at 30 mM mannitol, indicating reduced membrane lipid peroxidation. Gene expression analysis revealed significant upregulation of *CHS* and *PAL* genes following mannitol treatment, with the highest expression levels recorded at 30 mM mannitol. A strong positive correlation ($r = 0.908$) between *CHS* and *PAL* expression indicated coordinated regulation of secondary metabolism under stress conditions. Overall, exogenous mannitol improved salinity tolerance in purslane by stimulating antioxidant enzyme activities, reducing oxidative stress, and enhancing the expression of stress-responsive genes associated with secondary metabolite biosynthesis.

1. Introduction

Portulaca oleracea L. (purslane), a succulent annual herb belonging to the Portulacaceae family, is widely distributed across tropical and subtropical regions and is recognized for its remarkable adaptability to adverse environmental conditions (Sultana and Rahman 2013; Talei et al. 2020). The plant is characterized by fleshy stems, smooth, oblong leaves, and small yellow flowers producing numerous tiny dark seeds (Sultana and Rahman 2013). Purslane has attracted increasing scientific and commercial interest due to its exceptional nutritional and medicinal value. It is considered an excellent source of proteins, vitamins (A, C, and E), essential minerals such as calcium, potassium, and magnesium, and polyunsaturated fatty acids, particularly omega-3 fatty acids, including α -linolenic acid (Talei et al. 2020; Zhou et al. 2015). In traditional medicine, purslane has long been used for its anti-inflammatory, antimicrobial, antioxidant, analgesic, and wound-healing properties, highlighting its importance as a medicinal and pharmaceutical plant (Zhou et al. 2015).

Salinity stress is among the most severe abiotic stresses limiting agricultural productivity worldwide. Excessive salt accumulation in soil adversely affects plant growth and metabolism through osmotic stress, ion toxicity, nutrient imbalance, and oxidative damage (Munns and Tester 2008). Salinity-induced oxidative stress leads to excessive generation of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, which disrupt membrane integrity, damage proteins

and nucleic acids, and impair photosynthetic efficiency (Pan et al. 2021). Consequently, physiological processes such as stomatal conductance, transpiration, nutrient uptake, and carbon assimilation are significantly reduced, ultimately limiting plant growth and yield (Chaves et al. 2003; Zörb et al. 2019). Although purslane is considered relatively tolerant to drought and salinity due to its succulent nature and efficient water-use mechanisms, severe salt stress can still negatively affect its physiological performance and biochemical stability (Weiss 2000).

Plants have evolved multiple adaptive mechanisms to cope with salinity stress, including osmotic adjustment, activation of antioxidant defense systems, and accumulation of compatible osmolytes. Osmoprotectants such as proline, soluble sugars, organic acids, and sugar alcohols contribute to cellular homeostasis by stabilizing proteins and membranes and maintaining water balance under stress conditions (Ashrafijou et al. 2010; Farouk 2011). Among these compounds, mannitol has received considerable attention due to its multifunctional role in stress tolerance. Mannitol acts not only as an osmotic regulator but also as a ROS scavenger, protecting cellular structures from oxidative injury (Mitoi et al. 2009; Nawaz and Ashraf 2010). Previous studies demonstrated that exogenous mannitol application can improve plant performance under salinity by enhancing biomass accumulation, photosynthetic activity, stomatal conductance, and antioxidant enzyme activities (Afzal et al. 2023; Dawood and El-Awadi 2022).

The antioxidant defense system represents one of the primary protective mechanisms against ROS-induced oxidative stress. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), and ascorbate peroxidase (APX) play critical roles in detoxifying ROS and preserving cellular redox homeostasis (Parvaiz and Satyawati 2008). In addition to enzymatic responses, salinity stress can induce major alterations in secondary metabolism through the regulation of phenylpropanoid biosynthesis pathways. Genes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) are key regulatory components involved in the synthesis of phenolic and flavonoid compounds that contribute to antioxidant capacity and stress adaptation. However, information regarding the influence of mannitol on antioxidant metabolism and stress-responsive gene expression in purslane under saline conditions remains limited.

Although several studies have reported the beneficial effects of mannitol in improving salinity tolerance in crop species, little is known about its physiological and molecular roles in medicinal plants such as purslane. Therefore, the present study aimed to investigate the effects of different mannitol concentrations on growth characteristics, antioxidant enzyme activities, lipid peroxidation, and expression of PAL and CHS genes in *Portulaca oleracea* under salinity stress. Understanding these responses may provide valuable insights into the mechanisms underlying mannitol-mediated salt tolerance and contribute to the development of sustainable strategies for improving the cultivation and medicinal quality of purslane in saline environments.

2. Materials and methods

2.1. Plant material and Experimental design

Seeds of purslane (*Portulaca oleracea* L.) were obtained from the Medicinal Plants Research Center of Shahed University, Tehran, Iran. The experiment was conducted under greenhouse conditions using a factorial arrangement based on a completely randomized design (CRD) with four replications.

Experimental factors included four concentrations of mannitol (0, 10, 20, and 30 mM) and two salinity levels (0 and 120 mM NaCl).

Seeds were sown in plastic pots (25 cm diameter × 30 cm height) filled with sterilized sand medium, with 20 seeds per pot. After seed germination and establishment, seedlings were thinned to five uniform plants per pot to ensure consistent growth conditions. The pots were maintained in a controlled greenhouse environment at $28 \pm 2^{\circ}\text{C}$, relative humidity of 60–75%, and a photoperiod of 14 h light/10 h dark.

Salinity and mannitol treatments were initiated 35 days after germination and continued for 30 days. Plants were treated daily through irrigation and foliar application using the designated NaCl and mannitol solutions according to each treatment combination. To prevent nutrient deficiency and maintain optimal plant growth, Hoagland's nutrient solution was applied after every three consecutive treatment applications. Control plants received distilled water without NaCl or mannitol supplementation. At the end of the experimental period, plants were harvested for the assessment of growth attributes, antioxidant enzyme activities, lipid peroxidation, and gene expression analyses.

2.2. Shoot Dry Weight Determination

To determine shoot dry weight (SDW), aerial parts of purslane plants were harvested and thoroughly washed with distilled water. Samples were dried in a forced-air oven at 65°C for 72 h until constant weight was achieved. The dried tissues were weighed using a digital analytical balance, and the obtained values were expressed as grams per plant. Dried samples were subsequently ground into a fine powder and stored for further biochemical and molecular analyses.

2.3. Enzyme Extraction

Fresh leaf samples (0.5 g) were homogenized in 3 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.8) containing 0.5% polyethylene glycol (PEG), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), and 2 mM dithiothreitol (DTT). Homogenization was carried out under chilled conditions to preserve enzyme activity. The homogenate was centrifuged at $18,000 \times g$ for 15 min at 4°C , and the resulting supernatant was filtered through Whatman No. 1 filter paper. The clear supernatant was immediately used for the determination of antioxidant enzyme activities (Giannopolitis and Ries 1977b).

2.4. Estimation of Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was assayed according to the method described by Giannopolitis and Ries (1977a), based on the enzyme's ability to inhibit the photochemical reduction of nitroblue

tetrazolium (NBT). The reaction mixture (2.5 mL total volume) consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μ M NBT, 0.1 mM EDTA, and 50 μ M riboflavin. Subsequently, 50 μ L of enzyme extract was added to the reaction mixture. The reaction was initiated by exposing the tubes to fluorescent light for 15 min at a distance of approximately 15 cm from the light source, whereas control tubes were kept in darkness to prevent photochemical reduction. After incubation, absorbance was measured at 560 nm using a UV–Vis spectrophotometer (Perkin Elmer Lambda 25, USA). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction under the assay conditions. Enzyme activity was expressed as units mg^{-1} protein min^{-1} .

2.5. Estimation of Catalase (CAT) Activity

Catalase (CAT) activity was determined according to the method described by Bailly et al. (1996), based on the decomposition rate of hydrogen peroxide (H_2O_2). The reaction mixture contained 2.5 mL of 50 mM potassium phosphate buffer (pH 7.2) and 20 μ L of 3% H_2O_2 solution. The mixture was maintained on ice before assay initiation. The enzymatic reaction was initiated by adding 50 μ L of enzyme extract, and the decrease in absorbance resulting from H_2O_2 decomposition was monitored at 240 nm using a UV–Vis spectrophotometer (PerkinElmer Lambda 25, USA).

Catalase activity was calculated as the amount of H_2O_2 decomposed per minute per mg of protein using the following equation:

$$\text{CAT activity (mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}) = (\Delta A_{240} \times V_{\text{mix}}) / (\Delta t \times \epsilon \times d \times V_s \times P)$$

where ΔA_{240} represents the change in absorbance at 240 nm, Δt is the reaction time (min), V_{mix} is the total reaction volume (L), ϵ is the extinction coefficient of H_2O_2 at 240 nm ($40 \text{ mM}^{-1} \text{ cm}^{-1}$), d is the optical path length of the cuvette (cm), V_s is the volume of enzyme extract used (mL), and P is the protein concentration of the enzyme extract (mg mL^{-1}).

2.6. Estimation of Peroxidase (POX) Activity

Peroxidase (POX) activity was measured according to the method of Ghanati et al. (2002) by monitoring guaiacol oxidation in the presence of hydrogen peroxide. The reaction mixture consisted of 100 μ L of enzyme extract, 500 μ L of 5 mM H_2O_2 , 500 μ L of 28 mM guaiacol, and 1900 μ L of 60 mM potassium phosphate buffer (pH 6.1). The reaction was initiated by adding the enzyme extract, and the increase in absorbance due to tetraguaiacol formation was recorded at 470 nm for 1 min using a UV–Vis spectrophotometer (PerkinElmer Lambda 25, USA). Peroxidase activity was expressed as the change in absorbance per minute per milligram of protein ($\Delta A_{470} \text{ mg}^{-1} \text{ protein min}^{-1}$).

2.7. Estimation of Polyphenol Oxidase (PPO) Activity

Polyphenol oxidase (PPO) activity was determined according to the method described by Ghanati et al. (2002), based on the oxidation of catechol as the substrate. The reaction mixture contained enzyme extract and catechol solution prepared in phosphate buffer under appropriate assay conditions. The enzymatic reaction was initiated by adding the enzyme extract to the reaction mixture, and the increase

in absorbance resulting from catechol oxidation was monitored at 410 nm for 1 min using a UV–Vis spectrophotometer (PerkinElmer Lambda 25, USA). Polyphenol oxidase activity was expressed as the change in absorbance per minute per mg of protein ($\Delta A_{410} \text{ mg}^{-1} \text{ protein min}^{-1}$).

2.8. Estimation of Ascorbate Peroxidase (APX) Activity

Ascorbate peroxidase (APX) activity was assayed following the method of Amako et al. (1994). The reaction mixture consisted of 1 mL enzyme extract supplemented with 0.5 mM ascorbic acid and 0.1 mM EDTA in phosphate buffer. To minimize possible interference from guaiacol peroxidases, parallel assays were conducted in the presence of 50 μM p-chloromercuribenzoate. The enzymatic activity was determined by monitoring the decrease in absorbance due to ascorbate oxidation at 265 nm for 1 min using a UV–Vis spectrophotometer (PerkinElmer Lambda 25, USA). The extinction coefficient of ascorbate ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for activity calculation. APX activity was expressed as units g^{-1} fresh weight (FW) min^{-1} .

2.9. Estimation of Malondialdehyde content (level of lipid peroxidation)

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) concentration according to the thiobarbituric acid (TBA) reaction method described by Hodges et al. (1999). Fresh leaf tissue (1 g) was homogenized in 5 mL of 20% trichloroacetic acid (TCA) under chilled conditions. The homogenate was centrifuged at 16,000 $\times g$ for 10 min (Beckman Coulter, Germany), and 1 mL of the resulting supernatant was mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The reaction mixture was incubated in a water bath at 95°C for 30 min and then immediately cooled in an ice bath to terminate the reaction. Samples were subsequently maintained at room temperature for 10 min and centrifuged at 10,000 $\times g$ for 5 min to remove precipitated proteins and cellular debris. The absorbance of the supernatant was measured at 532 nm, and nonspecific absorbance was corrected at 600 nm using a UV–Vis spectrophotometer (PerkinElmer Lambda 25, USA). Malondialdehyde concentration was calculated using the extinction coefficient of 155 $\text{mM}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1}$ fresh weight (FW), representing the extent of membrane lipid peroxidation under salinity stress conditions.

2.10. RNA extraction, cDNA synthesis, and Quantitative Real-Time PCR Analysis

Total RNA was extracted from 100 mg of fresh purslane tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of isolated RNA were evaluated using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA), while RNA integrity was confirmed by electrophoresis on 1% agarose gel. First-strand cDNA synthesis was performed using the ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, UK; Cat# E6300S) following the manufacturer's protocol. For gene expression analysis, quantitative real-time PCR (qRT-PCR) was carried out using SYBR Green PCR Master Mix on an AB StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The expression profiles of chalcone synthase (CHS)

and phenylalanine ammonia-lyase (PAL) genes were evaluated under different salinity and mannitol treatments. The 18S rRNA gene was used as the internal reference gene to normalize transcript abundance.

PCR amplification conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 35 amplification cycles of denaturation at 95°C for 10–15 s, annealing at 58–60°C for 20–30 s, and extension at 72°C for 30 s. Each reaction was performed using three biological replicates and two technical replicates to ensure reproducibility and accuracy of gene expression analysis. Relative transcript levels were calculated using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method. Primer pairs were designed using Primer Premier 6 software and synthesized commercially. The sequences and melting temperatures (T_m) of the primers used for qRT-PCR analysis are presented in Table 1.

Table 1
Primer sequences used for quantitative real-time PCR analysis of CHS and PAL genes in *Portulaca oleracea*.

Gene	Accession No.	Primer Sequence (5'-3')	T_m (°C)
CHS	NM-121396.4	F: CGCATCACCAACAGTGAACAC	63.40
		R: TCCTCCGTCAGATGCATGTG	63.89
PAL	AY07936.1	F: GCAGTGCTACCGAAAGAAGTG	64.07
		R: CGACCTACATTCCTTGATCCTG	63.61

2.11. Statistical Analysis

Before statistical analysis, all experimental data were tested for normality and homogeneity of variance using SPSS software version 27 (SPSS Inc., Chicago, IL, USA). Data were analyzed using analysis of variance (ANOVA) appropriate for a factorial experiment based on a completely randomized design (CRD). Mean comparisons among treatments were performed using Duncan’s multiple range test at a significance level of $P \leq 0.01$. Correlation coefficients among measured traits were also calculated to determine relationships between physiological, biochemical, and molecular parameters. Graphical representations of the data were generated using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Effects of Mannitol on Growth and Antioxidant Responses of *Portulaca oleracea* under Salinity Stress

Analysis of variance (ANOVA) revealed that salinity stress significantly affected shoot dry weight (SDW), superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and malondialdehyde (MDA) content in *Portulaca oleracea* ($P \leq 0.01$), whereas the effect of salinity on

polyphenol oxidase (PPO) activity was not significant (Table 1). In addition, mannitol treatments and the interaction between salinity and mannitol (S × M) significantly influenced all measured parameters, including SDW, SOD, CAT, POX, PPO, APX, and MDA content, indicating that the physiological responses of purslane to salinity were strongly modulated by exogenous mannitol application (Table 2).

Table 2

Analysis of variance (ANOVA) for growth characteristics, antioxidant enzyme activities, and lipid peroxidation in *Portulaca oleracea* subjected to salinity and mannitol treatments.

S. O. V	df	SDW	SOD	CAT	POX	PPO	APX	MDA
Salinity	1	11.88 ^{**}	28195.47 ^{**}	43.60 ^{**}	152.26 ^{**}	0.001 ^{ns}	877.76 ^{**}	0.027 ^{**}
Mannitol	3	3.23 ^{**}	33405.71 ^{**}	154.19 ^{**}	37.64 ^{**}	11.46 ^{**}	129.19 ^{**}	0.081 ^{**}
S×M	3	1.28 [*]	41250.71 ^{**}	27.62 ^{**}	21.01 ^{**}	2.57 ^{**}	1068.65 ^{**}	0.001 ^{**}
Error	24	0.35	3184.84	1.12	0.15	0.07	23.31	0.000
CV (%)		19.21	16.48	18.82	21.45	22.32	21.64	21.25

^{**}, ^{*} and ^{ns} indicate significance at $P \leq 0.01$, $P \leq 0.05$, and non-significant effects, respectively.

3.2. Shoot Dry Weight

Shoot dry weight was significantly affected by both salinity stress and mannitol application (Fig. 1a). Salinity stress caused a marked reduction in plant biomass, decreasing SDW by 33.71% compared with the non-saline control treatment. However, exogenous application of mannitol alleviated the inhibitory effects of salinity and significantly improved plant growth. Among the tested concentrations, 30 mM mannitol produced the highest SDW (3.81 ± 0.42 g), corresponding to a 67.11% increase relative to untreated plants (2.28 ± 0.27 g). These findings suggest that mannitol supplementation effectively enhances biomass accumulation and improves salinity tolerance in purslane.

3.3. Superoxide Dismutase (SOD) Activity

Salinity stress, mannitol concentration, and their interaction significantly influenced SOD activity in purslane plants ($P \leq 0.01$; Table 1). Exposure to salinity alone increased SOD activity by 13.52% compared with the control, indicating activation of the antioxidant defense system in response to oxidative stress. Furthermore, SOD activity progressively increased with rising mannitol concentrations under saline conditions. The highest SOD activity (505.28 ± 24.72 mmol H₂O₂ min⁻¹ mg protein⁻¹) was observed in plants treated with 30 mM mannitol under salinity stress, whereas the lowest value (345.56 ± 32.59 mmol H₂O₂ min⁻¹ mg protein⁻¹) was recorded in salt-stressed plants without mannitol application (Fig. 1b). Overall, treatment with 30 mM mannitol enhanced SOD activity by 67.20% compared with untreated stressed plants, indicating that mannitol plays an important role in strengthening the enzymatic antioxidant defense system and improving ROS detoxification under saline conditions.

3.4. Catalase Activity

Catalase activity was significantly influenced by salinity stress, mannitol treatment, and their interaction ($P \leq 0.01$; Table 1). Exposure to 120 mM NaCl markedly reduced CAT activity by 38.48% compared with non-stressed plants, indicating that salinity adversely affected the hydrogen peroxide detoxification system in purslane. Nevertheless, exogenous application of mannitol effectively alleviated this inhibitory effect and promoted CAT activity under saline conditions. CAT activity increased progressively with increasing mannitol concentrations, reaching its maximum value ($20.19 \pm 0.81 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) at 30 mM mannitol, whereas the lowest activity ($10.26 \pm 0.35 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) was observed in salt-stressed plants without mannitol treatment (Fig. 1c). Overall, application of 30 mM mannitol under salinity stress enhanced CAT activity by 96.77% compared with untreated stressed plants. These findings demonstrate that mannitol supplementation substantially improves antioxidant defense capacity by stimulating CAT-mediated scavenging of reactive oxygen species under saline conditions.

3.5. Peroxidase Activity

Peroxidase activity was significantly affected by salinity, mannitol concentration, and their interaction ($P \leq 0.01$; Table 1). Salinity stress alone reduced POX activity by 40.76% relative to the control treatment, suggesting impairment of enzymatic antioxidant function under high salt conditions. However, exogenous mannitol application effectively mitigated this reduction and promoted POX activity in salt-stressed plants. The stimulatory effect of mannitol was concentration-dependent, with POX activity increasing up to 20 mM mannitol. The highest POX activity ($12.92 \pm 0.29 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) was recorded in plants treated with 20 mM mannitol under salinity stress, whereas the lowest value ($6.28 \pm 0.17 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) was observed in salt-stressed plants without mannitol application (Fig. 1d). Compared with untreated stressed plants, application of 20 mM mannitol increased POX activity by 105.68%, indicating that moderate concentrations of mannitol strongly stimulate POX-mediated antioxidant responses and enhance the capacity of purslane plants to cope with oxidative stress.

3.6. Polyphenol Oxidase Activity

Analysis of variance indicated that PPO activity was significantly affected by salinity stress and the interaction between salinity and mannitol, whereas the main effect of mannitol alone was not statistically significant ($P \leq 0.01$; Table 1). Salinity stress reduced PPO activity by 19.44% compared with the control treatment, indicating partial inhibition of phenolic oxidation processes under saline conditions. Application of mannitol at lower concentrations partially alleviated this reduction. PPO activity increased with mannitol application up to 10 mM, after which a substantial decline was observed at higher concentrations. The highest PPO activity ($4.16 \pm 0.09 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) was obtained at 10 mM mannitol under salinity stress, whereas the lowest value ($1.27 \pm 0.05 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) was recorded at 30 mM mannitol (Fig. 1e). Overall, treatment with 10 mM mannitol enhanced PPO activity by 74.87% compared with untreated stressed plants, suggesting that moderate

mannitol concentrations may contribute to phenolic metabolism and antioxidant protection under salt stress.

3.7. Ascorbate Peroxidase Activity

Ascorbate peroxidase activity was significantly affected by salinity stress, mannitol treatment, and their interaction ($P \leq 0.01$; Table 1). In contrast to CAT and POX responses, salinity stress alone induced a considerable increase in APX activity, which rose by 37.41% compared with the control treatment. This elevation indicates activation of the ascorbate-glutathione cycle as a protective mechanism against salt-induced oxidative stress. Mannitol application exerted a differential effect on APX activity depending on concentration. Application of mannitol up to 10 mM enhanced APX activity, whereas higher concentrations (20 and 30 mM) caused a gradual decline. Under salinity stress, the highest APX activity ($62.90 \pm 2.01 \mu\text{mol g}^{-1} \text{FW}$) was observed in plants exposed to 120 mM NaCl without mannitol supplementation, while the lowest activity ($35.68 \pm 0.32 \mu\text{mol g}^{-1} \text{FW}$) occurred in plants treated with 30 mM mannitol under salinity stress (Fig. 1f). Overall, application of 30 mM mannitol reduced APX activity by 43.28% relative to untreated salt-stressed plants. The reduction in APX activity at higher mannitol concentrations may indicate decreased oxidative pressure resulting from improved ROS scavenging efficiency mediated by other antioxidant enzymes.

3.8. Malondialdehyde Content

Malondialdehyde content, an indicator of membrane lipid peroxidation and oxidative damage, was significantly affected by salinity stress, mannitol application, and their interaction ($P \leq 0.01$; Table 1). Exposure to salinity stress markedly increased MDA accumulation in purslane plants, with a 70.52% increase compared with the non-saline control treatment, indicating enhanced membrane deterioration and oxidative injury under high salt conditions. Exogenous application of mannitol significantly reduced MDA accumulation under salinity stress, demonstrating its protective role against oxidative membrane damage. The highest MDA content ($0.24 \pm 0.006 \mu\text{mol g}^{-1} \text{FW}$) was observed in salt-stressed plants without mannitol treatment, whereas the lowest value ($0.17 \pm 0.004 \mu\text{mol g}^{-1} \text{FW}$) was recorded in plants treated with 30 mM mannitol under saline conditions (Fig. 1g). Overall, application of 30 mM mannitol reduced MDA content by 28.42% relative to untreated salt-stressed plants. These findings indicate that mannitol effectively alleviates salinity-induced oxidative stress by limiting lipid peroxidation and preserving membrane integrity in purslane plants.

3.9. Effects of Mannitol on CHS and PAL Gene Expression under Salinity Stress

Analysis of variance demonstrated that salinity stress, mannitol treatment, and their interaction significantly affected the expression levels of chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) genes in *Portulaca oleracea* ($P \leq 0.01$; Table 3). The results indicated that exogenous mannitol application markedly modulated the transcriptional responses of genes involved in the phenylpropanoid biosynthesis pathway under saline conditions. Increasing mannitol concentrations

progressively enhanced CHS gene expression, with the highest transcript accumulation (8.63-fold change) observed at 30 mM mannitol treatment. In contrast, the lowest CHS expression level (0.26-fold change) was detected in plants exposed to salinity stress without mannitol supplementation, indicating severe downregulation under salt stress conditions (Fig. 2a).

A similar trend was observed for PAL gene expression. Mannitol application significantly stimulated PAL transcription, and the highest expression level (9.64-fold change) was recorded at 30 mM mannitol. Conversely, the lowest PAL expression level (0.90-fold change) occurred under salinity stress in the absence of mannitol treatment (Fig. 2b). These findings demonstrate that exogenous mannitol effectively activates key genes associated with secondary metabolite biosynthesis and antioxidant defense pathways in purslane under saline conditions. Furthermore, increasing mannitol concentrations under salinity stress consistently promoted the expression of both CHS and PAL genes, suggesting that mannitol plays a regulatory role in stress-responsive molecular pathways. Fold-change values between 0 and 1 indicated downregulation relative to the control treatment, values greater than 1 represented gene upregulation, and values close to 1 indicated no substantial difference compared with the control.

Table 3
Analysis of variance (ANOVA) for CHS and PAL gene expression in *Portulaca oleracea* under different mannitol concentrations and salinity conditions.

Gene	Source of Variation	Sum of Squares	Mean Square	F- value	P-value
CHS	Treatments	64.38	6.48	28.17	0.000
	Residuals	1.56	0.23		
PAL	Treatments	124.14	12.41	70.46	0.000
	Residuals	1.32	0.14		

3.10. Correlation Analysis of Physiological, Biochemical, and Molecular Traits

The correlation analysis among measured traits revealed significant relationships between physiological, biochemical, and molecular parameters in purslane plants subjected to salinity and mannitol treatments (Table 4). Shoot dry weight (SDW) exhibited significant negative correlations with APX activity ($r = -0.477^{**}$) and MDA content ($r = -0.517^{**}$), indicating that increased oxidative stress and membrane lipid peroxidation were associated with growth inhibition under saline conditions. Moreover, SDW showed a strong negative correlation with CHS expression ($r = -0.606^{**}$), suggesting that activation of secondary metabolic pathways was intensified under severe stress conditions where plant growth was suppressed.

Catalase (CAT) activity showed a significant positive correlation with APX activity ($r = 0.450^{**}$) and POX activity ($r = 0.381^{*}$), indicating coordinated regulation among antioxidant enzymes involved in hydrogen peroxide detoxification. Similarly, SOD activity was positively correlated with APX ($r = 0.405^{*}$) and POX activities ($r = 0.440^{*}$), reflecting the integrated function of enzymatic antioxidant defense systems in

scavenging reactive oxygen species generated under salinity stress. Peroxidase (POX) activity displayed a strong positive correlation with PPO activity ($r = 0.567^{**}$), suggesting close functional interactions between oxidative enzymes involved in phenolic metabolism and stress defense responses. In contrast, POX activity exhibited a significant negative correlation with MDA content ($r = -0.604^{**}$), demonstrating that increased POX activity contributed to reduced lipid peroxidation and enhanced membrane stability under stress conditions.

Malondialdehyde (MDA), a key indicator of oxidative membrane damage, showed a significant positive correlation with CHS expression ($r = 0.586^{**}$), suggesting that oxidative stress stimulated activation of the phenylpropanoid pathway and flavonoid biosynthesis as protective mechanisms against ROS-induced injury. In addition, APX activity was positively correlated with CHS expression ($r = 0.556^{**}$), further supporting the close interaction between antioxidant defense systems and secondary metabolism during salinity adaptation. Although PAL expression showed mostly weak and non-significant relationships with physiological traits and antioxidant enzymes, a strong positive correlation was observed between PAL and CHS expression ($r = 0.908^{**}$). This finding indicates coordinated regulation of these two key genes involved in phenylpropanoid biosynthesis and suggests synchronized activation of secondary metabolic pathways in response to salinity stress and mannitol treatment. Furthermore, the moderate positive correlation between PAL and SOD activity ($r = 0.374$) may imply a functional relationship between oxidative stress signaling and phenolic compound biosynthesis. Overall, the correlation analysis demonstrated that antioxidant enzyme activities, oxidative stress markers, and phenylpropanoid pathway-related genes are closely interconnected in the salinity stress response of purslane. Enhanced antioxidant defense and activation of secondary metabolism appear to play central roles in reducing oxidative damage and improving stress tolerance following exogenous mannitol application.

Table 4

Correlation coefficients (r) among growth, antioxidant, oxidative stress, and gene expression traits in *Portulaca oleracea* under salinity and mannitol treatments.

SDW	SDW	CAT	APX	PPO	POX	SOD	MDA	CHS	PAL
	1								
CAT	-0.180	1							
APX	-0.477**	0.450**	1						
PPO	-0.288	-0.247	-0.144	1					
POX	0.062	0.381*	-0.098	0.567**	1				
SOD	-0.123	0.292	0.405*	0.121	0.440*	1			
MDA	-0.517**	-0.210	0.107	-0.007	-0.604**	-0.240	1		
CHS	-0.606**	0.364	0.556**	-0.108	-0.288	0.127	0.586**	1	
PAL	-0.323	0.232	0.285	0.136	0.290	0.374	0.265	0.908**	1

*, ** indicate significance at $P \leq 0.05$ and $P \leq 0.01$, respectively.

4. Discussion

Salinity stress is recognized as one of the major abiotic stresses limiting plant growth and productivity worldwide by disrupting water relations, nutrient uptake, photosynthesis, and cellular homeostasis while simultaneously enhancing oxidative stress through excessive accumulation of reactive oxygen species (ROS) (Munns and Tester 2008; Zörb et al. 2019). In the present study, salinity significantly reduced shoot dry weight (SDW) in *Portulaca oleracea*, indicating suppression of vegetative growth and biomass accumulation under saline conditions. This reduction in biomass may be associated with impaired water uptake, nutritional imbalance, reduced photosynthetic efficiency, and enhanced oxidative damage, as previously reported for salt-stressed medicinal plants (Munns and Tester 2008; Zörb et al. 2019). However, exogenous application of mannitol markedly alleviated the inhibitory effects of salinity and significantly improved SDW, particularly at 30 mM mannitol. These findings suggest that mannitol contributes to osmotic adjustment, stabilization of cellular structures, and maintenance of water balance under saline conditions. Similar growth-promoting effects of mannitol under salinity stress have been reported in maize, rice, wheat, and tomato plants (Afzal et al. 2023; Dawood and El-Awadi 2022; Kaya et al. 2013).

Salinity-induced oxidative stress is commonly associated with enhanced production of ROS such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), which can damage lipids, proteins, nucleic acids, and membrane systems (Gill and Tuteja 2010). To counteract oxidative

stress, plants activate enzymatic antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), and ascorbate peroxidase (APX). In the current study, salinity stress significantly increased SOD and APX activities, whereas CAT, POX, and PPO activities declined under saline conditions. The increase in SOD activity under salinity may represent an adaptive response to enhanced superoxide radical production, converting $O_2^{\bullet-}$ into H_2O_2 . However, the reduction in CAT and POX activities under severe salinity suggests a limited capacity for H_2O_2 detoxification, leading to oxidative injury and membrane damage and elevated MDA accumulation.

Exogenous mannitol application markedly enhanced antioxidant enzyme activities in purslane plants subjected to salinity stress. The highest SOD and CAT activities were observed at 30 mM mannitol, while POX activity reached its maximum at 20 mM mannitol. These findings indicate that mannitol effectively stimulates antioxidant defense systems and enhances ROS scavenging capacity under saline conditions. Mannitol acts not only as an osmoprotectant but also as a hydroxyl radical scavenger capable of stabilizing proteins, membranes, and enzyme structures during oxidative stress (Smirnoff and Cumbes 1989). Furthermore, mannitol may regulate stress-responsive signaling pathways and induce transcriptional activation of antioxidant-related genes, thereby improving stress adaptation (Abebe et al. 2003). Similar enhancements of antioxidant enzyme activities following mannitol application have been reported in wheat, maize, and fenugreek under osmotic and salt stress conditions (Afzal et al. 2023; Mitoi et al. 2009).

Catalase is considered one of the most critical antioxidant enzymes involved in H_2O_2 detoxification because it rapidly converts hydrogen peroxide into water and oxygen without requiring reducing substrates (Gill and Tuteja 2010). In the present study, the significant increase in CAT activity following mannitol application indicates improved cellular redox regulation and enhanced oxidative stress tolerance. Previous studies have similarly reported that osmoprotectants such as mannitol and glycine betaine improve CAT activity under salt stress by maintaining enzyme integrity and cellular homeostasis (Ashraf and Foolad 2007). Enhanced CAT activity likely contributed to lower oxidative injury and reduced membrane lipid peroxidation observed in mannitol-treated plants.

Peroxidase activity also increased considerably following mannitol supplementation, particularly at moderate concentrations. POX enzymes participate in multiple defense-related processes, including H_2O_2 detoxification, phenolic oxidation, lignification, and strengthening of cell walls under abiotic stress conditions (Asada 2006). The significant enhancement of POX activity observed in the present study suggests improved antioxidant protection and enhanced stress tolerance in mannitol-treated plants. The positive correlations observed between POX and SOD activities further indicate coordinated regulation of enzymatic antioxidant systems in response to salinity stress. Such coordinated antioxidant responses are considered essential for maintaining ROS homeostasis and preventing oxidative damage under stressful environments.

Polyphenol oxidase (PPO) activity exhibited a moderate increase at lower mannitol concentrations but declined at higher concentrations. PPO enzymes are involved in the oxidation of phenolic compounds

and may contribute to antioxidant defense through secondary metabolism activation (Mayer 2006). Increased PPO activity at moderate mannitol concentrations may therefore reflect activation of phenolic defense pathways under stress conditions. However, excessive mannitol concentrations may alter substrate availability or cellular redox conditions, resulting in reduced PPO activity. Similar biphasic responses of PPO activity under osmotic treatments have been reported in medicinal and aromatic plants subjected to abiotic stress (Anjum et al. 2011).

Interestingly, APX activity showed a different trend compared with CAT and POX. Salinity stress alone strongly induced APX activity, whereas increasing mannitol concentrations reduced APX activity under saline conditions. APX is an important component of the ascorbate–glutathione cycle and is generally activated under elevated oxidative pressure (Shigeoka et al. 2002). Therefore, the decline in APX activity following mannitol application may indicate reduced ROS accumulation and alleviation of oxidative burden rather than suppression of antioxidant defense. This interpretation is further supported by the reduction in MDA content under mannitol treatment.

Malondialdehyde (MDA) is widely used as a biochemical indicator of lipid peroxidation and oxidative membrane damage under abiotic stress conditions (Hernández et al. 2001). In the current study, salinity stress markedly increased MDA accumulation, confirming enhanced membrane deterioration and oxidative injury in purslane plants. However, exogenous mannitol significantly reduced MDA content, particularly at 30 mM concentration. The reduction in lipid peroxidation may be attributed to enhanced antioxidant defense capacity and more efficient ROS detoxification in mannitol-treated plants. The strong negative correlation between MDA and POX activity further highlights the protective role of antioxidant enzymes in preserving membrane integrity under saline conditions. Similar reductions in MDA accumulation following mannitol application have been reported in wheat, rice, and maize under salt stress (Afzal et al. 2023; Dawood and El-Awadi 2022).

At the molecular level, salinity stress significantly affected the expression patterns of CHS and PAL genes, which are key regulatory genes in the phenylpropanoid biosynthesis pathway. In the absence of mannitol, salinity stress suppressed the expression of both genes, whereas exogenous mannitol strongly upregulated CHS and PAL transcription. The highest expression levels of both genes were observed at 30 mM mannitol, suggesting activation of secondary metabolic pathways associated with stress adaptation and antioxidant protection. PAL catalyzes the deamination of phenylalanine into cinnamic acid, the first committed step in phenylpropanoid biosynthesis, whereas CHS is a key enzyme regulating flavonoid production (Dixon and Paiva 1995). Increased expression of these genes likely promotes biosynthesis of phenolic and flavonoid compounds with potent antioxidant activities capable of scavenging ROS and protecting cellular structures under stress conditions.

The strong positive correlation between CHS and PAL expression ($r = 0.908$) indicates coordinated regulation of phenylpropanoid metabolism under mannitol treatment. Furthermore, the positive relationship between CHS expression and APX activity suggests close interactions between enzymatic and non-enzymatic antioxidant systems during stress adaptation. The negative correlation between SDW

and CHS expression may indicate allocation of metabolic resources toward defense-related secondary metabolism under stress conditions. Similar activation of phenylpropanoid pathway genes under osmoprotectant treatments has recently been reported in basil, thyme, and medicinal halophytes exposed to abiotic stress (Hasanuzzaman et al. 2021; Sharma et al. 2019).

Overall, the present study demonstrates that exogenous mannitol effectively mitigates the detrimental effects of salinity stress in *Portulaca oleracea* through integrated morphological, physiological, biochemical, antioxidant, and molecular mechanisms. Mannitol improved plant growth, enhanced antioxidant enzyme activities, reduced lipid peroxidation, and stimulated expression of stress-responsive genes associated with secondary metabolite biosynthesis. Among the tested concentrations, 30 mM mannitol proved to be the most effective treatment for enhancing salinity tolerance in purslane plants. These findings highlight the potential application of mannitol as an environmentally sustainable osmoprotectant for improving productivity and stress resilience in medicinal plants cultivated under saline environments.

5. Conclusion

The present study demonstrated that salinity stress significantly impaired growth performance and induced oxidative damage in *Portulaca oleracea* through disruption of antioxidant defense systems and enhancement of membrane lipid peroxidation. However, Exogenous application of mannitol effectively alleviated the adverse effects of salinity by enhancing physiological performance, strengthening antioxidant defense systems, and regulating stress-responsive molecular pathways. Mannitol treatment significantly improved shoot dry weight and enhanced the activities of key antioxidant enzymes, including SOD, CAT, and POX, while reducing MDA accumulation, indicating improved membrane stability and oxidative stress tolerance under saline conditions. At the molecular level, mannitol strongly upregulated the expression of *CHS* and *PAL* genes, suggesting activation of the phenylpropanoid biosynthesis pathway and stimulation of secondary metabolism associated with antioxidant protection. The strong positive correlation between *CHS* and *PAL* expression further confirmed the coordinated regulation of stress-adaptive metabolic pathways in response to mannitol treatment. Among the tested treatments, 30 mM mannitol produced the greatest improvement in shoot dry weight and significantly enhanced SOD and CAT activities, while also promoting strong upregulation of *CHS* and *PAL* genes. The coordinated activation of antioxidant enzymes and secondary metabolism-related genes indicates that mannitol improves salinity tolerance through integrated physiological and molecular defense mechanisms. Overall, the findings suggest that exogenous mannitol can be considered an effective and environmentally friendly strategy for improving salinity tolerance and maintaining growth performance in purslane and potentially other medicinal plants cultivated under salt-affected conditions. Further studies focusing on ion homeostasis, metabolite profiling, and long-term field evaluations would provide deeper insights into the regulatory mechanisms underlying mannitol-mediated stress tolerance.

Abbreviations

Catalase; CAT, dithiothreitol; DTT, ethylenediaminetetraacetic acid; EDTA, Malondialdehyde; MDA, polyethylene glycol; PEG, polyvinylpyrrolidone; PVP, peroxidase; POX, reactive oxygen species; ROS, shoot dry weight; SDW, superoxide dismutase; SOD.

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.

Author contribution

Daryush Talei developed the theory, designed and performed the experiments, analyzed data, and wrote the manuscript.

Ayatollah Rezaei encouraged Daryush Talei to investigate and supervise the findings of this work and edited the manuscript.

All authors discussed and reviewed the results and approved the final version of the manuscript.

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Figures

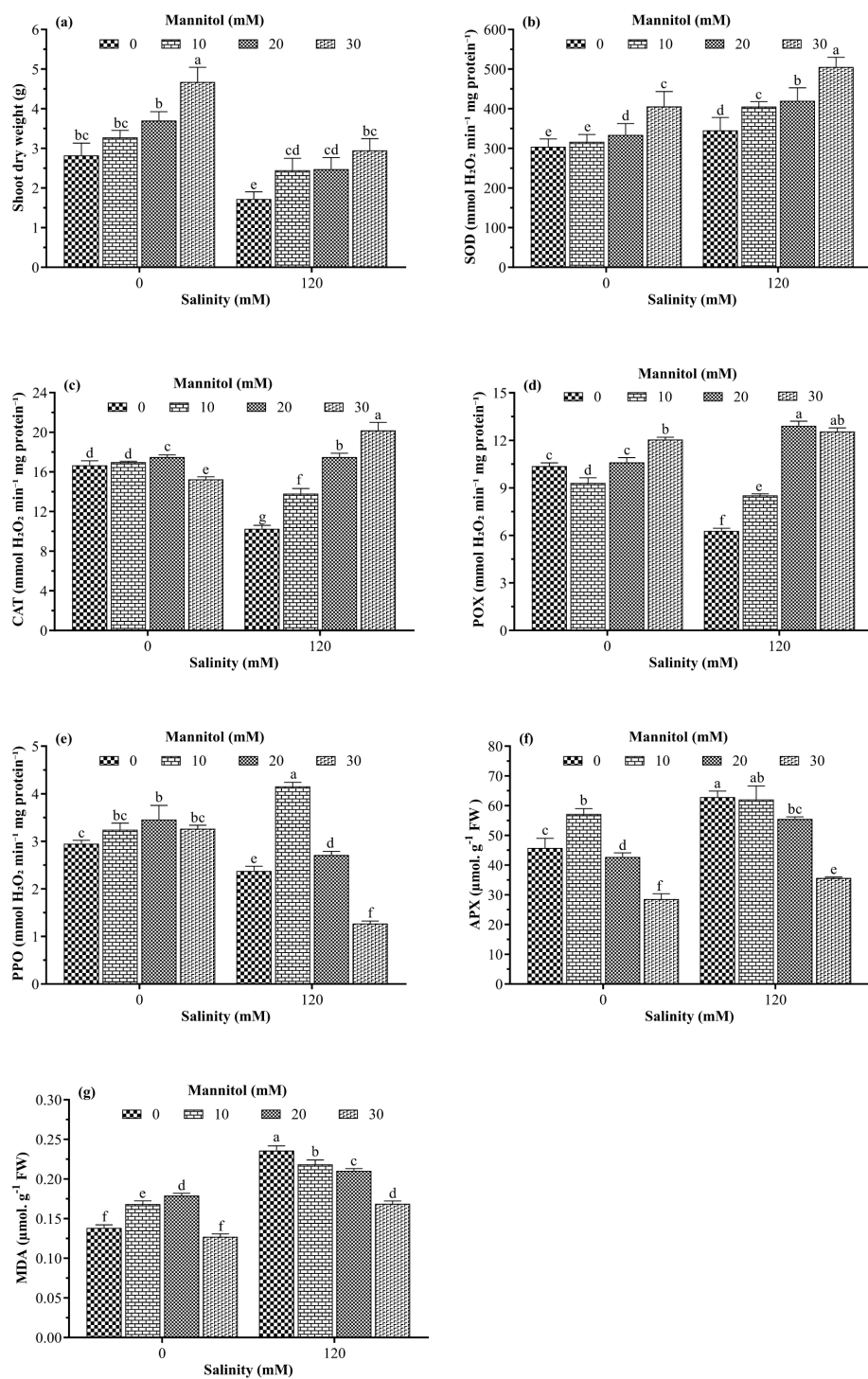


Figure 1

Antioxidant and growth responses of *Portulaca oleracea* subjected to different salinity and mannitol treatments. Measured parameters included shoot dry weight (SDW) (a), superoxide dismutase (SOD) activity (b), catalase (CAT) activity (c), peroxidase (POX) activity (d), polyphenol oxidase (PPO) activity (e), ascorbate peroxidase (APX) activity (f), and malondialdehyde (MDA) content (g). Values represent

mean $\pm$ standard error (SE) of four replicates. Different letters indicate statistically significant differences among treatments according to Duncan's multiple range test at $P \leq 0.01$.

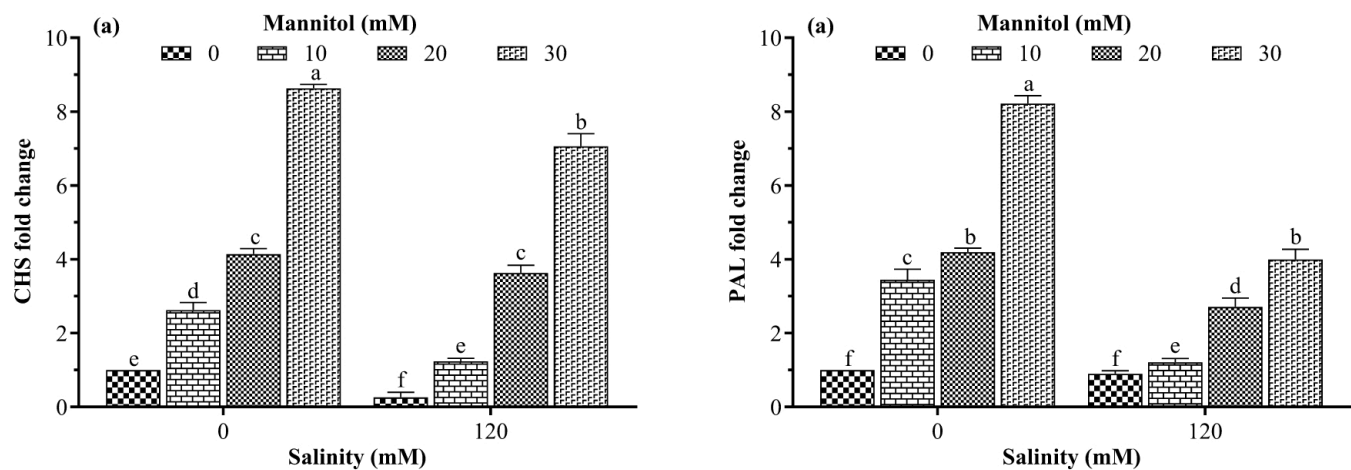


Figure 2

Relative expression levels of CHS and PAL genes in *Portulaca oleracea* under different salinity and mannitol treatments. (a) CHS gene expression; (b) PAL gene expression. Gene expression levels were normalized relative to the control treatment (0 mM mannitol), which was used as the reference. Values represent mean $\pm$ standard error of the mean (SEM) based on three biological replicates.