Identification and Characterization of Δ^1-pyrroline-5-carboxylate Synthetase in *Lepidium draba* Plant and Investigating the Expression Level of this Gene and Some Biochemical Traits under Salt Stress

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ABSTRACT. *Lepidium draba*, a medicinal herb belongs to the Brassicaceae family is more considered because it contains high level of glucoraphanin, as a precursor of sulforaphane (an anti-carcinogenic and anticancer agent). Whilst there has been studies on plant response to salinity stress, to date the response of *L. draba* to salinity stress is poorly understood. The aims of this study were Identification of Δ^1- pyrroline-5-carboxylate synthetase (*P5CS*) gene and assessment of different concentrations of NaCl on *P5CS* gene expression level and some biochemical traits of *L. draba* treated seedlings. The five-day old *L. draba* seedlings were treated by different concentrations (0, 50, 100, 200

and 300 mM) of NaCl for 14 days in completely random design. Identification of the *P5CS* gene was done by purification of total RNA, synthesis of cDNA library and amplification of the cDNA in the presence of specific primers which followed by sequencing. The gene expression level of *P5CS* was done using real-time PCR methods. Thereafter the salts effects were analyzed on peroxidase and catalase activity and carotenoid, chlorophyll, total protein and proline contents were measured in the treated seedlings using spectrophotometer apparatus. Identification and characterization of *P5CS* mRNA were performed and its sequence partially registered in GeneBank. The data were shown that *P5CS* gene expression level and the proline content increased with increasing concentrations of NaCl up to 300 mM. Peroxidase and catalase activities as well as the total protein content were enhanced by increasing the concentrations of NaCl in all samples. Chlorophyll and carotenoid contents were significantly decreased by salinity stress. According to the results, a correlation was found between proline content and *P5CS* gene expression. Furthermore, these results also provide an insight into the response of this medicinal plant to salt stress.

Keywords: Biochemical traits; *Lepidium draba*; *P5CS* gene; Proline content; Salinity stress.

INTRODUCTION

Lepidium draba (*L. draba*), from the family Brassicaceae, is a deep-rooted perennial herb that grows to a height of 2 m. It is native to the Balkan Peninsula, Armenia, Turkey, Palestine and Iran and grows in gardens and vegetable fields (Rezvani and Zaefarian, 2016). Salinity affects physiological and biochemical traits of plants and decreases yield, significantly. Cells create many compatibility strategies in response to various stresses (Rejeb et al., 2014; Acosta-Motos et al., 2017), such as salinity. Salt stress causes increased construction of the reactive oxygen species (ROS), which causes lipid peroxidation (Chen et al., 2018). Therefore, cell membrane stability has been used widely to distinguish cultivars tolerant and sensitive to salinity (Jamali et al., 2015). The major ROS are superoxide radicals ($O2^{\cdot -}$), singlet oxygen ($1O_2$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) in plants (Phaniendra et al., 2015). The production of ROS is restricted or removed by antioxidant enzymes like superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) (Foyer and Noctor, 2003). These isozymes could be utilized as biochemical markers to investigate the plant tolerance to stress (Chunthaburee et al., 2016). It was specified that enzymatic antioxidant defense system including polyphenol oxidase, ascorbate peroxidase and catalase, non-enzymatic antioxidant defense system including total soluble carbohydrates and proline contents and oxidative stress parameters including malondialdehyde, other aldehydes, hydrogen peroxide and electrolyte leakage contents are extremely affected under drought and salinity stresses (alone or in combination) (Jamshidi Goharrizi et al., 2019a; Jamshidi Goharrizi et al., 2019c). In the same study, it was specified that in the more tolerant plants, enzymatic and non-enzymatic antioxidant defense systems are more active to reduce the oxidative stress indexes under drought and salinity stresses (alone or in combination) (Jamshidi Goharrizi et al., 2019a; Jamshidi Goharrizi et al., 2019c).

Salt stress reduces photosynthesis efficiency and affects the plant' leaves chlorophyll content (Hniličková et al., 2017). A decrease in δ-aminolevulinic acid dehydratase (ALAD) activity in stress conditions can cause reduced amount of chlorophyll synthesis (Killiny et al., 2018). However, it is unclear how this enzyme behaves in the leaf under salinity stress conditions.

Plants also show different changes in the pattern of synthesis and accumulation of proteins in a saline environment (Jamshidi Goharrizi et al., 2019a; Jamshidi Goharrizi et al., 2019c). One of the most prevalent responses to stress in higher plants is non-toxic solutes, called compatible solutes, osmoprotectants or osmolytes (Hossain et al., 2019). These solutes are mostly frequent carbohydrates, sugars, amino acids and proteins, which operate as osmolytes. Among a wide range of osmolytes, proline is the most extensive compound accumulated in many plant species under stress conditions. The role of proline and its metabolism under stress conditions have been studied in many plants. It is generally now accepted that proline acts as a multifunctional metabolite, as well as operating as a compatible osmolyte; it can also function in scavenging reactive oxygen species (ROS), fixing subcellular structures, adjusting cell redox homeostasis, providing energy, and acting as a signaling molecule to interact with other metabolic pathways under stress conditions (Per et al., 2017).

Proline in plants is synthesized from glutamate by the action of two enzymes: Δ^1- pyrroline-5-carboxylate synthetase or *P5CS* and pyrroline-5-carboxylate reductase or P5CR from glutamate (Hayat et al., 2012). *P5CS* has a main role in the control of proline biosynthesis, and increasing proline accumulation decreases osmotic stresses (Chun et al., 2018).

To our knowledge, no published study has been reported on the effect of salt stress on biochemical traits and *P5CS* gene expression in *L. draba* plant. The aims of this study are

[1]. To evaluate the comparative effects of 0, 50, 100, 200 and 300 mM NaCl on POD and CAT enzymes activity, chlorophyll and carotenoid biosynthesis, and total protein in addition to proline content
[2]. To identify and characterize the *P5CS* from *L. draba* plant
[3]. To measure the expression level of *P5CS* under different concentrations of NaCl including 0, 50, 100, 200 and 300 mM.

MATERIALS AND METHODS

Seed germination

In order to germinate, *L. draba* seeds were disinfected and were put on 1% agar (pH=7) in an incubator in complete darkness for 3 days at 28 ± 1°C and 55% relative humidity (Jamshidi Goharrizi et al., 2019e).

Plant culture

Hoagland solution (Hoagland and Arnon, 1950) with pH=6.2 was prepared to culture *L. draba* seeds in such a way that a lattice lace was put on the surface of a disposable container (bottom of the disposable container was cut) and germinated *L. draba* seeds were put on the surface of this lattice lace (Figure S1). This disposable container was placed inside another one that was containing 180 mL Hoagland solution (Figure S1). These disposable containers were put in the germinator for two days at 25°C with 16-h light and 8-h dark photoperiod (Jamshidi Goharrizi et al., 2019e). After two days, the Hoagland solution became empty, and then 0, 50, 100, 200 and 300 mM NaCl using of fresh Hoagland solution were prepared and 180 mL of each of these solutions was poured in the disposable containers. They were put in the germinator (at 26°C with 17-h light and 7-h dark photoperiod), and an aquarium pump was applied to aerate the Hoagland solution. After 14 days, in order to evaluate some biochemical traits and gene expression, sprouts were cut, immediately put in aluminum, flash frozen in liquid nitrogen and stored at -80°C.

Preparation of enzyme extract

Using liquid nitrogen, 0.5 g of *L. draba* sprouts were crushed and 5 mL of 50 mmol sodium phosphate buffer (pH 7.0), including 1% polyvinylpyrrolidone and 0.2 mM Ethylenediamine-Tetraacetic acid (EDTA). The homogenate solution was centrifuged at 20,000 g for 25 min, and the supernatant was used for measuring the enzyme activity.

Estimation of peroxidase (POD) enzyme activity

POD activity was performed using the Chance and Maehly method, with slight modification (Chance and Maehly, 1955). To this end, 4 mL of assay solution, including 20 mM guaiacol, 50 mM potassium phosphate (pH 7.0), 40 mM H_2O_2, and 0.3 mL enzyme extract were applied to measure POD activity. Furthermore, 470 nm wavelengths were used to record absorbance of reaction after every 30 sec until 5 min had passed. Extinction coefficient of tetraguaiacol was considered at 25.5 mM^{-1} cm^{-1}, and POD activity in mmol of H_2O_2 min^{-1} mg^{-1} was also measured. The POD enzyme activity was reported by unit per minute per mg protein.

Estimation of catalase (CAT) enzyme activity

The method described by Chandlee and Scandalios was used to measure CAT activity; for this purpose, 0.4 mL H_2O_2 (15 mM), 2.6 mL of potassium phosphate (50 mM, pH 7.0) and 0.04 mL of enzyme extract were mixed, and CAT enzyme activity was calculated by measuring absorbance at a wavelength of 240 nm (Chandlee and Scandalios, 1984). The catalysis of H_2O_2 was performed by the decline absorbance at 240 nm. The extinction coefficient of 0.036 mM^{-1} cm^{-1} was used to calculate the activity of the enzyme, and CAT enzyme activity was reported by unit per minute per mg protein.

Extraction and estimation of chlorophyll and carotenoid contents

The method described by Lichtenthaler (Lichtenthaler, 1987) was applied to extract chlorophyll, and for this purpose, 0.1 g of leaves with 3 mL 80% acetone were ground in presence of 0.1% $CaCO_3$ for prevention of chlorophyllase activities. After filtering samples, the final volume (25 mL) was transferred to tubes, and wavelengths of A470, A646.8 and A663.2 were used to record absorbance of reactions. Equation 1-4 was used to calculate the contents of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid.

Eq. 1 Chlorophyll a = (12/25 × OD663.2 nm) – (2/79 × OD 646.8 nm)

Eq. 2 Chlorophyll b = (21/5× OD 646.8 nm) – (5/1 OD 663.2 nm)

Eq. 3 Total chlorophyll = chlorophyll a + chlorophyll b

Eq. 4 Total carotenoid = [(1000 × OD 470 nm) – (1/82 × chlorophyll a) – (85/02 × chlorophyll b] / 198

Extraction and estimation of total protein content

For extraction of total protein, 0.5 g *L. draba* sprouts were homogenized in 6 mL of 10 mM potassium phosphate buffer (pH 7.0), including 4% (w/v) polyvinylpyrrolidone (PVP). The solution was centrifuged at 15,000 g at 4°C for 20 min, and the supernatants were used to determine protein content. The method described by Bradford (Bradford, 1976) was applied to determine protein content, and for this purpose, 20 µl of supernatant with 980 µL of Bradford reagent was mixed and 595 nm was used to record the absorbance of the reactions. Bovine serum albumin in different concentrations (0 (blank = No protein), 250, 500, 750 and 1500 µg/mL) was used to draw the standard curve, and protein concentrations by line slope formula of this standard curve were quantified.

Extraction and estimation of proline content

Proline content was extracted from 14-day-old *L. draba* seedlings using the method described by Bates et al. (Bates et al., 1973), with a slight modification; 100 mg of *L. draba* seedlings were homogenized in 10 mL of 3% aqueous sulfosalicylic acid and centrifuged at 10,000 g for 15 min. In a new microtube, the supernatant was mixed with equal volumes of glacial acetic acid and ninhydrin solution. Microtubes were incubated for 1 hour at 100°C, and then 6 mL of toluene was added to the microtubes. At 520 nm, absorbance of fraction with toluene

aspired from fluid phase was recorded using a spectrophotometer. By calibration curve, proline concentration was determined and was reported as µmol/g FW.

Total RNA extraction and cDNA synthesis

In presence of liquid nitrogen, 100 mg of *L. draba* sprouts were crushed and transferred to microtubes, and 1 mL RNXplus (Cinnagen, Tehran, Iran) was added. Total RNA was extracted according to the manufacturer's protocol as described previously (Jamshidi Goharrizi et al., 2018). 1% agarose gel electrophoresis was used to test the integrity of isolated RNA. NanoDrop-1000 spectrophotometer was utilized to check the purification and concentration of each sample. High-quality RNA (OD 260/280 and OD 260/230 >2) was used for first-strand cDNA synthesis. The mRNA (1 µg of total RNA) was reverse transcribed into cDNA as described by Nasiri-Bezenjani et al. (Nasiri-Bezenjani et al., 2014) by the use of oligo dt (18 primer) as a primer and MMuLV reverse transcriptase.

P5CS mRNA identification and sequences analysis

Arabidopsis thaliana, *Camelina sativa*, *Brassica napus* and *Arabidopsis lyrata* (GenBank accession No. Y09355.1, XM_010506118.1, XM_013800452.2 and XM_021024621.1) were used for *P5CS* primer design using the Gene Runner software (version 3.05). *P5CS* was amplified using cDNA in presence of specific primers, including forward primer: 5'-AGAAAGCTTCAGGCCTTATCTTCAG-3' and reverse primer: 5'-TTATTCCCACCTCAGCACCAAGTC-3', and 1029 base pair was considered as PCR product. PCR amplification was done in a 25 µL reaction mixture. 0.5 µl of the cDNA as template, 25 mM KCl, 1.25 mM $MgCl_2$, 10 mM TriseHCl pH 8.4, 62.5 µM dNTPs, 0.1 µM each primer and 1.25 U Taq polymerase (Invitrogen, Carlsbad, USA) were all applied to the purpose of amplification of *P5CS* gene with the following conditions: initial denaturation at 94°C for 5 min, 40 cycle amplification (94°C for 45 sec, 63°C for 45 sec and 72°C for 1 min) and a final extension for 5 min at 72°C. Observation of PCR product by 1% agarose gel electrophoresis was visualized by UV (gel documentation system EQ, Bio-Rad). A 100 bp ladder (Takara, Japan) was used to estimate the amplicon size, and a solid single-band was selected and sequenced (Macrogen) using specific primers (forward primer: 5'-AGAAAGCTTCAGGCCTTATCTTCAG-3' and reverse primer: 5'-TTATTCCCACCTCAGCACCAAGTC-3'). Using NCBI (protein and nucleotide blast online software), percentages of identity and similarity were extracted, and using the IBIVU website (http://ibi.vu.nl/programs/), alignment of *L. draba P5CS* protein with other *P5CS* protein was performed to identify the conserved sequences.

Dendrogram analyses and estimates of evolutionary divergence for *P5CS* mRNA

For computing pairwise distances and relationships of *L. draba P5CS* with other plants from a common ancestor, estimating evolutionary divergence and dendrogram plotting, MEGA7 software was used (Amirmahani and Jamshidi Goharrizi, 2018). The bootstrap consensus tree inferred from 100 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. All positions comprising gaps and missing data were eliminated. There were a total of 411 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Analysis of *P5CS* gene expression

In this study, GAPDH was selected as the internal control gene, and sequence of primers was designed using the registered sequences in NCBI. Desirable sequences were designed by alignment of reported sequences in NCBI, and conserved sequences including:

Forward *P5CS* 5′-CTTGTTCAGATAGCTTCGCTTG-3′

Reverse *P5CS* 5′-AGCAAATCAGGAATCTCCTCTC-3′

Forward GAPDH 5′-TAGACTCGAGAAAGCTGCGA -3′

Reverse GAPDH 5′-TTGTCACTCAACGCGATTCC -3 were chosen as primers. Gradient PCR was performed to get the best conditions, and 57°C was selected as the best annealing temperature for GAPDH and *P5CS* genes. Amplicon length for GAPDH and *P5CS*, were 177 and 178 base pairs, respectively. The Rotor-Gene 3000 real-time PCR machine was utilized to amplify the reactions and to detect dsDNA synthesis. Using the LinReg PCR program (http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LinRegPCR.zip&sub=LinRegPCR), the threshold line was plotted, and CT of GAPDH and *P5CS* genes were computed using the threshold line. The correctness of amplification was confirmed by negative control and delta Ct was calculated from the difference between Cts of the *P5CS* and GAPDH. 2-ΔCt was used to calculate *P5CS*/GAPDH mRNA ratio.

Statistical analysis

The completely randomized design with three replicates was the condition of this experiment, and one-way ANOVA (Duncan multiple comparison tests) was applied for calculating the data.

RESULTS

POD and CAT activities

In this experiment, by increasing concentration of NaCl, POD activity was enhanced in all samples. The increased amounts of 1.14 fold, 1.69 fold, 2.2 fold and 3.18 fold were calculated under 50, 100, 200 and 300 mM NaCl compared to 0 mM, respectively (Figure 1(A)). Also, CAT enzyme activity was evaluated under 5 different concentrations of NaCl including 0, 50, 100, 200 and 300 mM NaCl. With the increasing levels of NaCl, CAT enzyme activity was enhanced up to 300 mM. Increasing levels of 1.25 fold, 1.54 fold, 1.95 fold and 2.25 fold were calculated for 50, 100, 200 and 300 mM NaCl compared to 0 mM, respectively (Figure 1(B)).

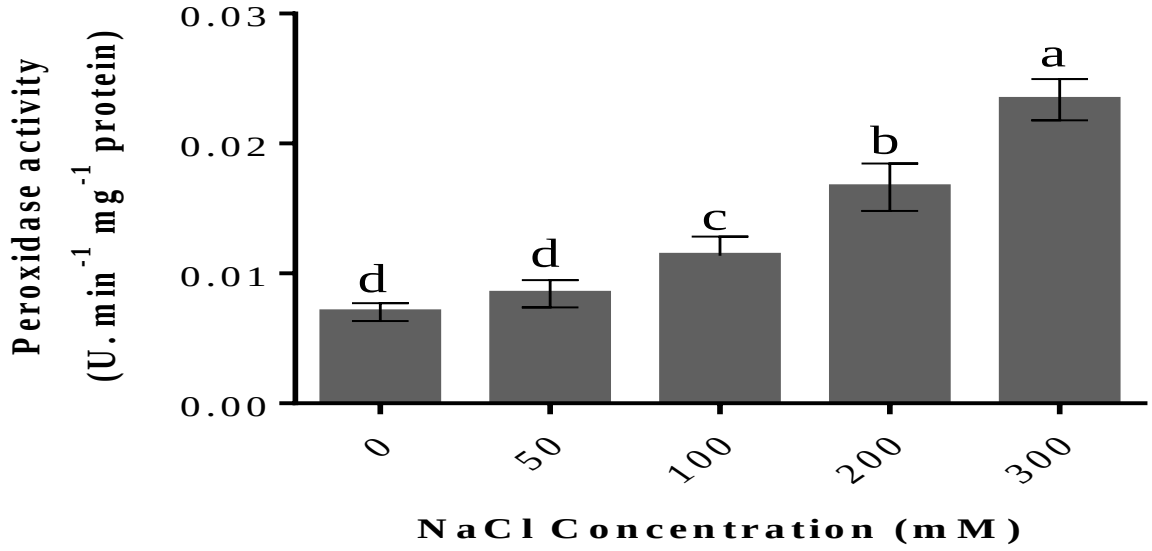

Figure 1(A). POD activity in different concentrations of NaCl in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

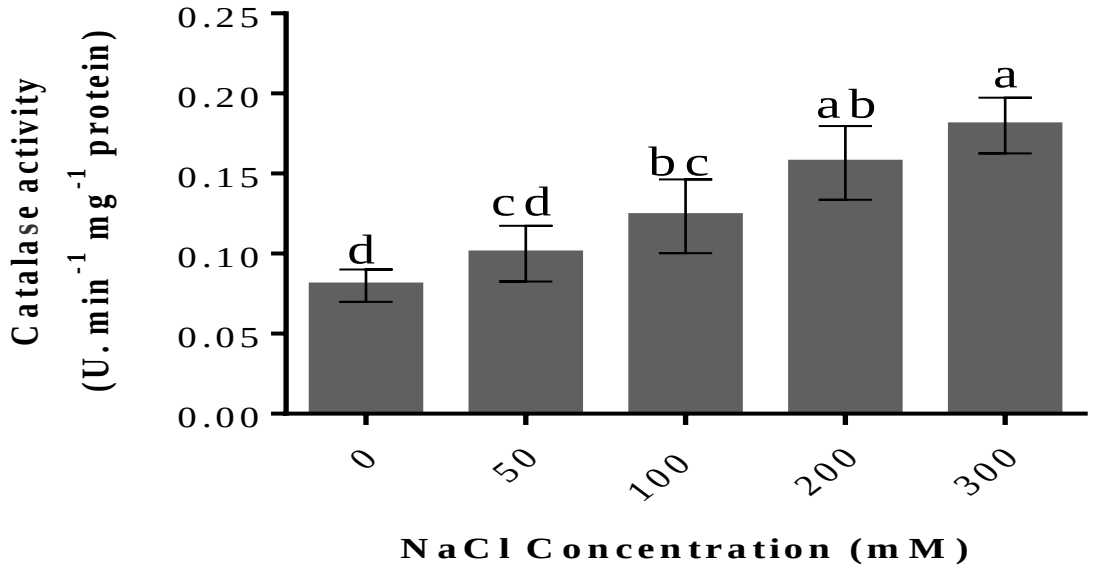

Figure 1(B). CAT activity in different concentrations of NaCl in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

Chlorophyll and carotenoid contents

In this research, with increasing concentration of NaCl from 0 to 300 mM, chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid decreased, significantly. The highest contents of them were observed at 0 mM and after that decreased (Table 1).

Table 1. Contents of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid in *L. draba* sprouts under different concentrations of salinity stress. Data are presented as the means ± SD (n=3). Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

100	0.36 ± 0.01^{b}	0.18 ± 0.04^{b}	0.54 ± 0.01^{b}	0.08 ± 0.03^{b}
200	0.27 ± 0.06^{b}	0.11 ± 0.01^{c}	0.38 ± 0.06^{c}	0.06 ± 0.01^{b}
300	0.13 ± 0.00^{c}	0.02 ± 0.01^{d}	0.16 ± 0.01^{d}	0.04 ± 0.01^{b}

Total protein content

Under 5 different levels of NaCl, it was observed that the total protein content was enhanced when NaCl increased (Figure 3). In addition, the highest content of protein was seen at 300 mM, No significant difference was observed between 100, 200 and 300 mM NaCl (Figure 2).

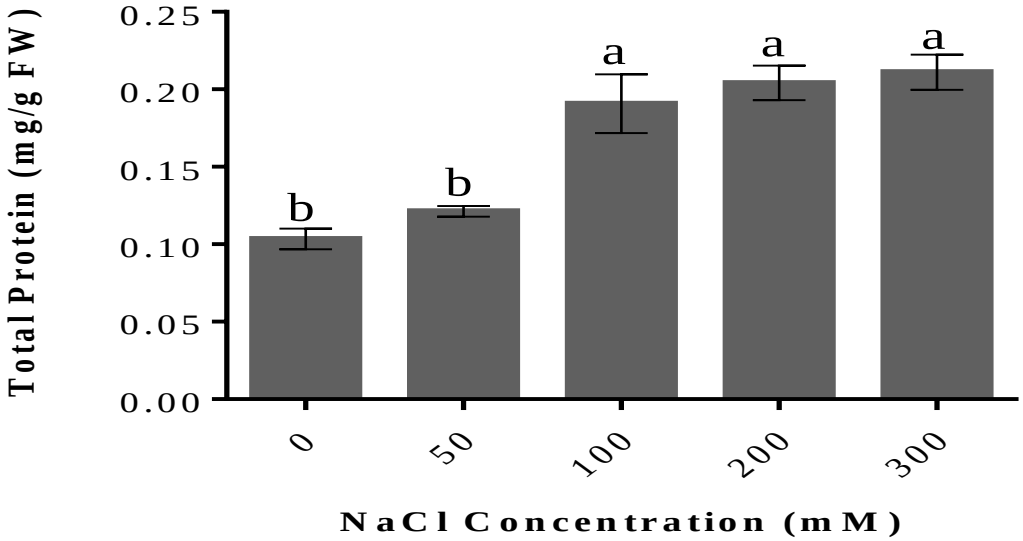

Figure 2. Content of total protein in *L. draba* sprouts under different concentrations of salinity stress. Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

Proline content

In this study, 5 different levels of NaCl including 0, 50, 100, 200 and 300 mM were used to measure proline content in *L. draba*. For proline content, analysis of variance showed a significant difference between all NaCl concentrations, and means comparison demonstrated that with increasing concentrations of NaCl, proline content increased in all samples. Comparison of 50, 100, 200 and 300 mM NaCl with 0 mM revealed the increased amounts of 1.99 fold, 4.53 fold, 6.13 fold and 15.62 fold in proline content, respectively (Figure 3).

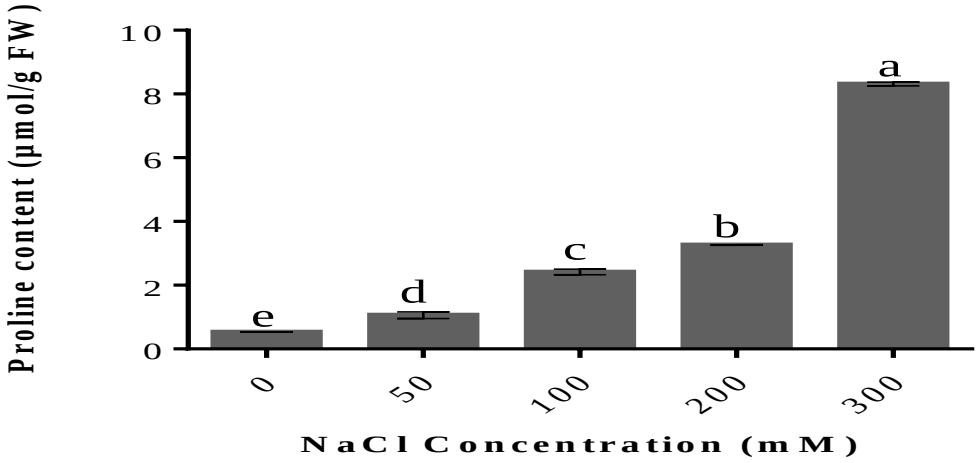

Figure 3. Proline content in different concentration of NaCl in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

P5CS gene amplification and sequences alignment

cDNA as a template was applied for *P5CS* gene amplification using specific primers, and a 1092 bp fragment was amplified (Figure S2). After sequencing, 382 nucleotides (Gene Bank Accesion number MK032798) (Text S1) and 127 amino acids (Gene Bank Accesion number QCP57269) (Text S2) belonging to the codon region of *P5CS* enzyme, were identified. The extracted sequence of *L. draba P5CS* mRNA represented 94%, 94%, 93%, 91% and 91% identity with *Camelina sativa*, *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Brassica napus* and *Brassica oleracea P5CS* mRNAs, respectively. In addition, 92%, 92% and 91% identities were observed between the extracted sequence of *L. draba P5CS* protein with Arabidopsis lyrata, Arabidopsis thaliana and *Camelina sativa P5CS* proteins, respectively.

The results of dendrogram analyses for *P5CS* mRNA suggested that *L. draba* and *Camelina sativa* have a common ancestor (Figure 4). The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The number of base substitutions per site between sequences was charted in Table 2.

Figure 4. Dendrogram analyses of *P5CS* mRNA.

Table 2. Estimates of evolutionary divergence between sequences (computed pairwise distances).

		1	2	3	4	5	6	7	8	9	10
1	**XM_010429044.1**										
2	**XM_021024621.1**	0.063									
3	**Y09355.1**	0.058	0.027								
4	**XM_006290587.2**	0.072	0.074	0.072							
5	**XM_013800452.2**	0.119	0.107	0.110	0.143						
6	**XM_013750087.1**	0.119	0.107	0.110	0.143	0.000					
7	**XM_006403379.2**	0.105	0.091	0.091	0.123	0.080	0.080				
8	**XM_009118014.2**	0.128	0.115	0.119	0.152	0.017	0.017	0.088			
9	**HM013941.1**	0.172	0.160	0.167	0.179	0.189	0.189	0.186	0.201		
10	**XM_006403379.2**	0.105	0.091	0.091	0.123	0.080	0.080	0.000	0.088	0.186	
11	***Lepidium_draba_P5CS***	0.069	0.077	0.077	0.086	0.111	0.111	0.114	0.128	0.196	0.114

P5CS gene expression

Variations in gene expression showed that NaCl affected *P5CS* gene expression significantly; *P5CS* expression was severely enhanced with increasing levels of NaCl. The increased amounts of 2.1 fold, 3.99 fold, 4.4 fold and 5.53 fold were estimated under 50, 100, 200 and 300 mM NaCl, compared to 0 (Figure 5). As shown in Fig. S3, Real-time quantitative RT-PCR amplification products for *P5CS* gene under 0, 50, 100, 200 and 300 mM NaCl were separated in 1% agarose gel electrophoresis.

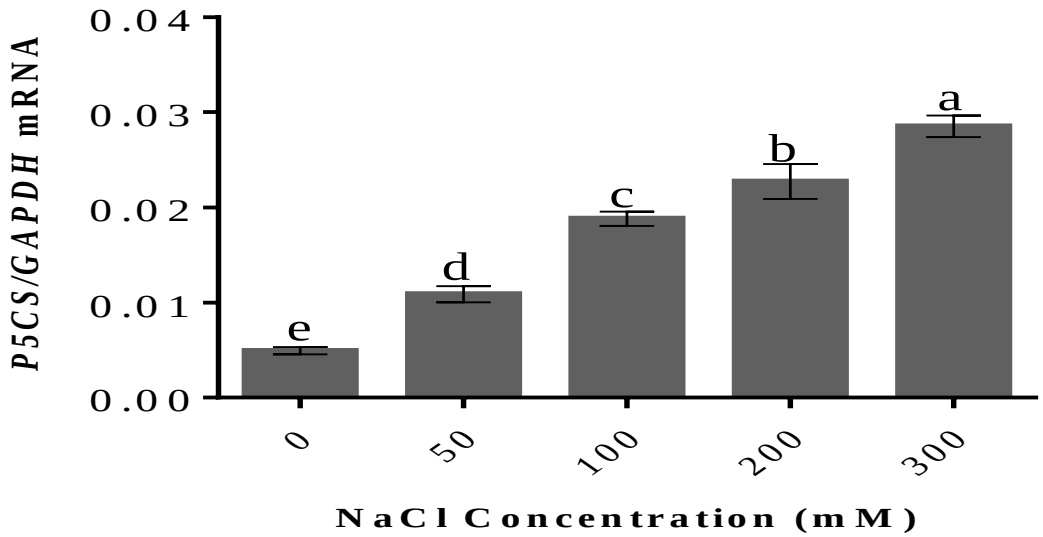

Figure 5. Expression of *P5CS* gene relative to GAPDH as internal control in different concentrations of NaCl in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at P<0.05, according to Duncan's multiple range tests.

DISCUSSION

Salinity is a great abiotic stress that can reduce water potential and cause nutrient imbalances in plants, and they affect plant growth, adversely (Acosta-Motos et al., 2017). Thus, the conception of a molecular basis for salt-stress tolerance mechanisms is necessary in breeding and genetic engineering programs in plants' tolerance to salinity stress (Hanin et al., 2016). In this research, the effects of different levels of salt stress on some biochemical traits and *P5CS* gene expression of *L. draba* were investigated.

It is specified that the defense of oxidative stress occurs through antioxidant enzymes' mechanisms, like POD and CAT (Chawla et al., 2013). Our results demonstrated that increasing NaCl concentrations induced POD and CAT activities. Generally, POD and CAT are considered as the key components of antioxidant protection of the plants (Yan-Feng, 2008). CAT and POD are the most effective antioxidant enzymes in cell damage preventing and have a major importance in regulating intracellular levels of H_2O_2 (Swathy Lekshmi and Jayadev, 2017). Increased activities of antioxidant enzymes in response to salt stress are related to the enhanced stress tolerance (Kusvuran et al., 2016). Similarly, these enzymes' activities increasing for ROS scavenging under salinity stress were reported in other cultivars (Abd Elgawad et al., 2016; Gharsallah et al., 2016; Jamshidi Goharrizi et al., 2019b).

The present study revealed that NaCl stress induced a decrease in the chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid contents. The results of our study are consistent with those of Grzeszczuket al.

(2018) on Salvia coccinea, where salt treatment reduced photosynthetic pigments (Grzeszczuk et al., 2018). Chlorophyll content reduction under salt stress could be due to chlorophyll pigments destruction. This is because of the elevated activity of chlorophyll-degrading enzymes, such as chlorophyllase and chlorophyll synthesis: 5-aminolevulinic acid (ALA) synthase inhibition in salinity stress conditions (Wu et al., 2018). Furthermore, the lack of chlorophylls in abiotic stresses is due to photoinhibition or reactive oxygen species formation (Pospíšil, 2016).

Carotenoids, as antioxidants, have the ability to detoxify the effects of reactive oxygen species (Verma and Mishra, 2005). A decline in carotenoid contents by enhanced salinity stress is in agreement with the results that reported by Akcin and Yalcin (2016) on Salicornia prostrata Pall. and Suaeda prostrata Pall. subsp. Prostrata (Akcin and Yalcin, 2016). The reduction in carotenoid contents demonstrated that the protection via carotenoid was not one of the most significant processes in response to salt stress. It has been specified that lipid peroxidation and reactive oxygen species (ROS) played a substantial role in damaging different photosynthetic membranes that photosynthetic pigments were bound (Sayyad-Amin et al., 2016). Moreover, the thylakoid membrane structure destruction was another cause for decreasing the affinity between chlorophylls and proteins of the chloroplast. This phenomenon reduced the activity of chlorophyll synthesis enzymes subsequently causing a reduction in chlorophyll stability and the chlorophyll breakdown without changing it by enzymatic synthesis (Sayyad-Amin et al., 2016).

Also, salinity stress induced an increase in total protein content by increasing salt stress. Because of some tolerance levels of *L. draba* at these NaCl concentrations, the total protein level was elevated. Similar finding was reported in *P. imperialis* and *P. fortune* (Ayala-Astorga and Alcaraz-Meléndez, 2010). Accumulation of proteins is important to cell survival against stress effects and makes membranes stabilized under salt stress conditions (Ayala-Astorga and Alcaraz-Meléndez, 2010). Protein accumulation may prepare nitrogen storage for further utilization when stress is over (Tegeder and Masclaux-Daubresse, 2018). These proteins can be synthesized in response to salt stress or the elevated level at present consecutive expression proteins when plants are under saline stress (Kaleem et al., 2018). Several proteins have been identified in Brassicaceae family in response to salt stress (Shokri-Gharelo and Noparvar, 2018).

Another interesting finding in the current work was the elevated proline content by increasing salinity stress. Proline has an adaptive role in mediation of osmotic adjustment and the sub-cellular structures protection in stress conditions. Proline is more effective in protecting against stress than other common osmolites, such as sugars and sugary alcohols (Hasanuzzaman et al., 2019). It has also been reported that proline content elevated as NaCl concentration increased in Plantago ovata Forsk (Golkar et al., 2017). Jamshidi Goharrizi et al. showed that proline content increased under drought and salinity stresses (alone or in combination) in UCB-1 rootstock, significantly (Jamshidi Goharrizi et al., 2019a; Jamshidi Goharrizi et al., 2019c). Also, Hmidi et al. (2018) reported proline accumulation due to soil salinity in *Cakile maritima* (Hmidi et al., 2018). It has been suggested that more proline accumulation occurs to protect chlorophyll level and cell turgor in order to maintain photosynthetic activity under salt stress (Acosta-Motos et al., 2017). Because proline level in plants is a mixture result of biosynthesis, catabolism, and transport activities, it is required to evaluate the expression profiles and functions of involved genes in these activities to comprehend the proline metabolism mechanism. There has been no report about genes involved in proline-metabolism in *L. draba* until now. *P5CS* is generally recognized to be the key enzyme of proline synthesis (Guan et al., 2018). Thus, we sequenced and aligned partial coding sequence of the *P5CS* gene with known *P5CS* genes in other plants. Bioinformatics analysis showed that the nucleotide sequences of it shared high similarities with *P5CS* genes in other plants and the highest similarity was observed with the *P5CS* gene of (94%).

In the present study, we analyzed the proline gene expression level in response to increasing salinity stress level. *P5CS* was overexpressed significantly by increasing stress level. *P5CS* gene overexpression causes increased proline accumulation in plants. Oxidative stresses produce free radicals, and free radicals increase proline accumulation, and proline accumulation is mediated by changing *P5CS* gene expression in Brassica napus L. seedlings under NaCl stress (Xiong et al., 2018). In previous studies, expression of *P5CS* gene was increased by different types of abiotic stress such as salt stress in Arabidopsis (Cui et al., 2015) and Brassica napus (Kubala et al., 2015). Also, Jamshidi Goharrizi et al. showed that *P5CS* and P5CR genes expression levels increased with increasing levels of PEG up to 12% (Jamshidi Goharrizi et al., 2019d).

CONCLUSION

Salinity is one of the most important abiotic stresses all over the world. This is the first study that investigates the biochemical and molecular responses of *L. draba* plant under salinity stress. Interestingly, salt stress has changed biochemical traits and *P5CS* gene expression, differently. According to the current study, by increasing NaCl concentrations, POD and CAT activity in addition to total protein and proline content improved, whereas the contents of photosynthetic pigments decreased. Increasing contents of proline, protein and antioxidant enzymes' activity in *L. draba* sprouts may act as a fundamental duty in protecting the plants under salinity stress. Also, the expression pattern of *P5CS* enhanced the view that it is involved in mechanisms related to the salt stress responses. Our results also proved that *P5CS* gene expression, content of proline, and NaCl concentration has a positive correlation together. Altogether, this study provides data on biochemical and molecular bases of salt stress on *L. draba* plant. They may improve our perception of the mechanisms by which salinity impacts on the growth and development of plants and may be applied as efficient methods to determine the tolerant and sensitive varieties of *L. draba* under salt stress.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Abd Elgawad H, Zinta G, Hegab MM, Pandey R, et al. (2016) High salinity induces different oxidative stress and antioxidant responses in Maize seedlings organs. Frontiers in Plant Science 7:276-276. https://doi.org/10.3389/fpls.2016.00276

Acosta-Motos J, Ortuño M, Bernal-Vicente A, Diaz-Vivancos P, et al. (2017) Plant responses to salt stress: adaptive mechanisms. Agronomy 7:18. https://doi.org/10.3390/agronomy7010018

Akcin A, Yalcin E (2016) Effect of salinity stress on chlorophyll, carotenoid content, and proline in Salicornia prostrata Pall. and Suaeda prostrata Pall. subsp. prostrata (Amaranthaceae). Brazilian Journal of Botany 39:101-106. https://doi.org/10.1007/s40415-015-0218-y

Amirmahani F, Goharrizi KJ (2018) Phylogenetic analysis of three long non-coding RNA Genes: AK082072, AK043754 and AK082467. Journal of Genetic Resources 4: 56-64. https://dx.doi.org/10.22080/jgr.2018.14224.1103

Ayala-Astorga GI, Alcaraz-Meléndez L (2010) Salinity effects on protein content, lipid peroxidation, pigments, and proline in *Paulownia imperialis* (Siebold & Zuccarini) and Paulownia fortunei (Seemann & Hemsley) grown *in vitro*. Electronic Journal of Biotechnology 13: 13-14. https://doi.org/10.2225/vol13-issue5-fulltext-13

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39: 205-207. https://doi.org/10.1007/bf00018060

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254. https://doi.org/10.1006/abio.1976.9999

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. Methods in Enzymology 2:764-775. https://doi.org/10.1016/s0076-6879(55)02300-8

Chandlee JM, Scandalios JG (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. Theor Appl Genet 69: 71-77. https://doi.org/10.1007/bf00262543

Chawla S, Jain S, Jain V (2013) Salinity induced oxidative stress and antioxidant system in salt-tolerant and salt-sensitive cultivars of rice (*Oryza sativa* L.). Journal of Plant Biochemistry and Biotechnology 22: 27-34. https://doi.org/10.1007/s13562-012-0107-4

Chen C, Wang C, Liu Z, Liu X, et al. (2018) Variations in physiology and multiple bioactive constituents under salt stress provide insight into the quality evaluation of *Apocyni veneti* Folium. International Journal of Molecular Sciences 19: 3042. https://doi.org/10.3390/ijms19103042

Chun SC, Paramasivan M, Chandrasekaran M (2018) Proline accumulation influenced by osmotic stress in Arbuscular Mycorrhizal symbiotic plants. Frontiers in Microbiology 9: 2525-2525. https://doi.org/10.3389/fmicb.2018.02525

Chunthaburee S, Dongsansuk A, Sanitchon J, Pattanagul W, et al. (2016) Physiological and biochemical parameters for evaluation and clustering of rice cultivars differing in salt tolerance at seedling stage. Saudi Journal of Biological Sciences 23: 467-477. https://doi.org/10.1016/j.sjbs.2015.05.013

Cui M, Lin Y, Zu Y, Efferth T, et al. (2015) Ethylene increases accumulation of compatible solutes and decreases oxidative stress to improve plant tolerance to water stress in Arabidopsis. Journal of Plant Biology 58: 193-201. https://doi.org/10.1007/s12374-014-0302-z

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiologia Plantarum 119: 355-364. https://doi.org/10.1034/j.1399-3054.2003.00223.x

Gharsallah C, Fakhfakh H, Grubb D, Gorsane F (2016) Effect of salt stress on ion concentration, proline content, antioxidant enzyme activities and gene expression in tomato cultivars. AoB Plants 8: plw055. https://doi.org/10.1093/aobpla/plw055

Grzeszczuk M, Salachna P, Meller E (2018) Changes in photosynthetic pigments, total phenolic content, and antioxidant activity of *Salvia coccinea* Buc'hoz Ex Etl. induced by exogenous salicylic acid and soil salinity. Molecules (Basel, Switzerland) 23: 1296. https://doi.org/10.3390/molecules23061296

Guan C, Huang YH, Cui X, Liu SJ, et al. (2018) Overexpression of gene encoding the key enzyme involved in proline-biosynthesis (Pu*P5CS*) to improve salt tolerance in switchgrass (*Panicum virgatum* L.). Plant cell reports 37: 1187-1199. https://doi.org/10.1007/s00299-018-2304-7

Hanin M, Ebel C, Ngom M, Laplaze L, et al. (2016) New insights on plant salt tolerance mechanisms and their potential use for breeding. Frontiers in Plant Science 7: 1787-1787. https://doi.org/10.3389/fpls.2016.01787

Hasanuzzaman M, Anee TI, Bhuiyan TF, Nahar K, et al. (2019) Emerging role of osmolytes in enhancing abiotic stress tolerance in rice. In: Advances in rice research for abiotic stress tolerance. Elsevier, pp 677-708. https://doi.org/10.1016/b978-0-12-814332-2.00033-2

Hayat S, Hayat Q, Alyemeni MN, Wani AS, et al. (2012) Role of proline under changing environments: A review. Plant Signal Behav 7: 1456-1466. https://doi.org/10.4161/psb.21949

Hmidi D, Abdelly C, Ashraf M, Messedi D (2018) Effect of salinity on osmotic adjustment, proline accumulation and possible role of ornithine-δ-aminotransferase in proline biosynthesis in Cakile maritima. Physiology and Molecular Biology of Plants 24: 1017-1033. https://doi.org/10.1007/s12298-018-0601-9

Hniličková H, Hnilička F, Martinkova J, Kraus K (2017) Effects of salt stress on water status, photosynthesis and chlorophyll fluorescence of rocket. Plant, Soil and Environment 63: 362-367. https://doi.org/10.17221/398/2017-pse

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circular California agricultural experiment station p. 347 (2nd edition)

Hossain MA, Kumar V, Burritt D, Fujita M, et al. (2019) Osmoprotectant-mediated abiotic stress tolerance: Recent advances and future perspectives.

Jamali SS, Borzouei A, Aghamirzaei M, Khosronejad HR, et al. (2015) Cell membrane stability and biochemical response of seven wheat cultivars under salinity stress. Brazilian Journal of Botany 38: 63-69. https://doi.org/10.1007/s40415-014-0123-9

Jamshidi Goharrizi K, Amirmahani F, Salehi F (2019a) Assessment of changes in physiological and biochemical traits in four pistachio rootstocks under drought, salinity and drought + salinity stresses. Physiol Plantarum. https://doi.org/10.1111/ppl.13042

Jamshidi Goharrizi K, Baghizadeh A, Kalantar M, Fatehi F (2019b) Assessment of changes in some biochemical traits and proteomic profile of UCB-1 pistachio rootstock leaf under salinity stress. Journal of Plant Growth Regulation. https://doi.org/10.1007/s00344-019-10004-3

Jamshidi Goharrizi K, Baghizadeh A, Kalantar M, Fatehi F (2019c) Combined effects of salinity and drought on physiological and biochemical characteristics of pistachio rootstocks. Scientia Horticulturae 108970. https://doi.org/10.1016/j.scienta.2019.108970

Jamshidi Goharrizi K, Dayton Wilde H, Amirmahani F, Mehdi Moemeni M, et al. (2018) Selection and validation of reference genes for normalization of qRT-PCR gene expression in wheat (*Triticum durum* L.) under drought and salt stresses. Journal of genetics 97: 1433-1444. https://doi.org/10.1007/s12041-018-1042-5

Jamshidi Goharrizi K, Moosavi S, Amirmahani F, Salehi F, et al. (2019d) Assessment of changes in growth traits, oxidative stress parameters and enzymatic and non-enzymatic anti-oxidant defense mechanisms in *Lepidium draba* plant under osmotic stress induced by polyethylene glycol. Protoplasma.

Jamshidi Goharrizi K, Riahi-Madvar A, Rezaee F, Pakzad R, et al. (2019e) Effect of salinity stress on enzymes' activity, ions concentration, oxidative stress parameters, biochemical traits, content of sulforaphane, and CYP79F1 gene expression level in *Lepidium draba* plant. Journal of Plant Growth Regulation. https://doi.org/10.1007/s00344-019-10047-6

Kaleem F, Shabir G, Aslam K, Rasul S, et al. (2018) An overview of the genetics of plant response to salt stress: present status and the way forward. Applied Biochemistry and Biotechnology 186: 306-334. https://doi.org/10.1007/s12010-018-2738-y

Killiny N, Hijaz F, Nehela Y, Hajeri S, et al. (2018) Effects of δ - aminolevulinic acid dehydratase silencing on the primary and secondary metabolisms of citrus. Plant Direct 2: e00072. https://doi.org/10.1002/pld3.72

Kubala S, Wojtyla Ł, Quinet M, Lechowska K, et al. (2015) Enhanced expression of the proline synthesis gene *P5CS*A in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. Journal of Plant Physiology 183:1-12. https://doi.org/10.1016/j.jplph.2015.04.009

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33: 1870-1874. https://doi.org/10.1093/molbev/msw054

Kusvuran S, Kiran S, Ellialtioglu SS (2016) Antioxidant enzyme activities and abiotic stress tolerance relationship in vegetable crops. In: Abiotic and biotic stress in plants-recent advances and future perspectives IntechOpen. https://doi.org/10.5772/62235

Lichtenthaler HK (1987) [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods in Enzymology 148: 350-382. https://doi.org/10.1016/0076-6879(87)48036-1

Nasiri-Bezenjani MA, Riahi-Madvar A, Baghizadeh A, Ahmadi AR (2014) Rosmarinic acid production and expression of tyrosine aminotransferase gene in *Melissa officinalis* seedlings in response to yeast extract. Journal of Agricultural Science and Technology 16: 921-930.

Per TS, Khan NA, Reddy PS, Masood A, et al. (2017) Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. Plant Physiol Biochem 115: 126-140. https://doi.org/10.1016/j.plaphy.2017.03.018

Phaniendra A, Jestadi DB, Periyasamy L (2015) Free radicals: Properties, sources, targets, and their implication in various diseases. Indian Journal of Clinical Biochemistry: IJCB 30: 11-26. https://doi.org/10.1007/s12291-014-0446-0

Pospíšil P (2016) Production of reactive oxygen species by photosystem II as a response to light and temperature stress. Frontiers in Plant Science 7: 1950-1950. https://doi.org/10.3389/fpls.2016.01950

Rejeb IB, Pastor V, Mauch-Mani B (2014) Plant responses to simultaneous biotic and abiotic stress: Molecular mechanisms. Plants (Basel, Switzerland) 3: 458-475. https://doi.org/10.3390/plants3040458

Rezvani M, Zaefarian F (2016) Hoary cress (*Cardaria draba* (L.) Desv.) seed germination ecology, longevity and seedling emergence. Plant Species Biology 31: 280-287. https://doi.org/10.1111/1442-1984.12113

Sayyad Amin P, Jahansooz MR, Borzouei A, Ajili F (2016) Changes in photosynthetic pigments and chlorophyll-fluorescence attributes of sweet-forage and grain sorghum cultivars under salt stress. J Biol Phys 42: 601-620. https://doi.org/10.1007/s10867-016-9428-1

Shokri Gharelo R, Noparvar PM (2018) Molecular response of canola to salt stress: insights on tolerance mechanisms. PeerJ 6:e4822. https://doi.org/10.7717/peerj.4822

Swathy Lekshmi S, Jayadev A (2017) Influence of salt stress on the morphological physiological activity and anatomy of Cow pea plant (*Vigna unguiculata*). IJAR 3: 281-288.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tegeder M, Daubresse CM (2018) Source and sink mechanisms of nitrogen transport and use. New Phytologist 217: 35-53. https://doi.org/10.1111/nph.14876

Verma S, Mishra SN (2005) Putrescine alleviation of growth in salt stressed Brassica juncea by inducing antioxidative defense system. Journal of Plant Physiology 162: 669-677. https://doi.org/10.1016/j.jplph.2004.08.008

Wu Y, Jin X, Liao W, Hu L, et al. (2018) 5-Aminolevulinic acid (ALA) alleviated salinity stress in cucumber seedlings by enhancing chlorophyll synthesis pathway. Frontiers in Plant Science 9: 635-635. https://doi.org/10.3389/fpls.2018.00635

Xiong JL, Wang HC, Tan XY, Zhang CL, et al. (2018) 5-aminolevulinic acid improves salt tolerance mediated by regulation of tetrapyrrole and proline metabolism in *Brassica napus* L. seedlings under NaCl stress. Plant Physiology and Biochemistry 124: 88-99. https://doi.org/10.1016/j.plaphy.2018.01.001