



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

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Abstract

In the first step in this study, the effect of 50 mM NaCl was studied on germination percentage of five different *Lepidium draba* (*L. draba*) ecotypes, and Rafsanjan ecotype was selected as experimental material as it had the highest germination percentage. In the second step, some biochemical, physiological, and morphological traits along with content of sulforaphane (SFN) as well as the expression level of Cytochrome P450 79F1 (*CYP79F1*) were evaluated in 14-day-old *L. draba* sprouts that grew 9 days in the presence of various concentrations of NaCl including 0, 25, 50, 75, and 100 mM. According to the results of this study, germination percentage of Rafsanjan ecotype along with lengths of stem and root were declined with increasing concentrations of NaCl. Ascorbate peroxidase, guaiacol peroxidase, and superoxide dismutase enzymes activity increased up to 75 mM NaCl and then decreased. With increasing the doses of NaCl, concentrations of Na⁺ and Cl⁻ increased, whereas P, Ca²⁺, and K⁺ decreased. Also, accumulation of some oxidative stress parameters including electrolyte leakage, malondialdehyde, other aldehydes, and hydrogen peroxide increased with increasing NaCl concentrations in all samples. Furthermore, contents of total phenolic, total flavonoid, total anthocyanin, total free amino acids, and total soluble carbohydrate were induced with the induction of NaCl concentrations. In this study, SFN formation increased with increasing concentration of sodium chloride up to 75 mM and decreased at higher concentration. In the last step, a partial *CYP79F1* mRNA and its protein sequence were identified and registered in GenBank and then changes in the *CYP79F1* gene expression levels under 0, 25, 50, 75, and 100 mM NaCl were calculated. The gene expression levels of *CYP79F1* also showed the same pattern as was seen for SFN formation under salinity stress.

Keywords *L. draba* · Sulforaphane · *CYP79F1* · Oxidative stress · Biochemical traits · Ions concentration

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Introduction

Lepidium draba is a plant indigenous to Central Asia, the Black Sea, and native to Southern Europe which is now widespread throughout most of Europe (Mulligan and Frankton 1962). Wild and noxious *L. draba* weeds are extensively found along roadsides, in meadows, and in fields (Radonic et al. 2011). This weed, which has been shown to belong to the Brassica family, like the other Brassicaceae genus, contains glucosinolates, a unique group of secondary metabolites containing sulfur and nitrogen. This family has about 350 genders, with more than 2000 species. Some of them are commercial and have economic interest such as broccoli and watercress (Gautam et al. 2014). For humans, glucosinolates are very important as relish compounds, cancer-preventive agents, and biopesticides in agriculture (Mikkelsen et al. 2003). More than 100 different kinds of these metabolites have been identified (Fahey et al. 2001; Halkier and Gershenzon 2006), the number of which has reached more than 130 types up to now. Glucosinolates have a defensive role and are classified in two classes: phytoanticipins and phytoalexins (Pedras et al. 2009). Glucosinolates are accumulated in the cell vacuole and released in response to cell injuries or pathogens attack. At this time, when glucosinolates are exposed to myrosinase (β -thioglucoside glucohydrolase, EC. 3.2.3.1), they are hydrolysed by myrosinase and produce glucose and an unstable intermediate molecule (aglycone). According to the environmental conditions, aglycone can be converted to thiocyanates, isothiocyanates, and nitriles (Rask et al. 2000), of which isothiocyanates (ITC) can exhibit various pharmacological activities and antioxidant properties (Traka and Mithen 2009; Fahey et al. 2002). One of the most important ITCs in cruciferous vegetables is SFN (4 methylsulfonyl butyl isothiocyanate) which can be produced through glucoraphanin hydrolysis via myrosinase activity and has anti-cancer properties (Gu et al. 2012). SFN is able to remove carcinogens, stimulate detoxification, induce apoptotic cell death, and inhibit cell cycle progression and angiogenesis in diverse cancer cell types (Zhang et al. 1992; Clarke et al. 2008).

Lately, several studies have shown that salt stress increases the glucosinolate content in broccoli florets, significantly (López-Berenguer et al. 2008, 2009). Every year, salinity stress, which is one of the major abiotic stresses, extremely affects crop production worldwide (Kumar et al. 2007). Various events such as reactive oxygen species (ROSs) production, prevention of enzymatic activities, decreasing plant production and photosynthetic speed, and postponement of the plant growth occur when plants are exposed to salinity stress (Lee et al. 2013). For prevention of ROSs damage, plants use antioxidant defense

metabolites such as carotenoids, flavonoid, anthocyanin, and other secondary metabolites (Ahmad et al. 2010b; Núñez et al. 2003; Yasar et al. 2008; Gengmao et al. 2015).

Cytochromes P450, *CYP79F1*, and *CYP79F2* catalyze the short- and long-chain aliphatic glucosinolates from chain-elongated methionines (Chen et al. 2003a, b; Reintanz et al. 2001). *CYP79F1* can take both short- and long-chain methionine derivatives, while *CYP79F2* solely metabolizes the long-chain methionine derivatives (Penta- and hexahomomethionine) (Chen et al. 2001). Most probably, *CYP79F1* and *CYP79F2* play different roles in plant growth and extension (Hansen et al. 2001).

When plants face a variety of abiotic stresses such as salinity and drought, reactive oxygen species are produced in these conditions, and finally, normal growth of plants is disturbed in response to these stresses (Sharma et al. 2019a). Salinity stress in plants leads to hyperosmotic stress and ionic imbalance, and this condition is able to induce reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($OH\cdot$) which can expose oxidative stress (Bose et al. 2013). These reactions could extremely harm the lipids, photosynthetic pigments, nucleic acids, cell membranes, and proteins (Ahmad et al. 2010a). H_2O_2 , which is a diffusible molecule, can enter the cell membrane and harm the cells (Kordrostami et al. 2017). In 2013, Gil et al. proved that conformity to salinity stress is a complex process and requires the selective ion uptake to hold a suitable Na^+ , Cl^- , and K^+ balance (Gil et al. 2013).

It is specified that exposure to different stress conditions is able to disturb the plant metabolism, which results in an increase in the reactive oxygen species (ROS) levels (Foyer and Noctor 2005; Hussain et al. 2016). In plants, ROS scavenging happens using antioxidative defense system, which results in reducing negative effects of oxidative stress (Sharma et al. 2015; Tanveer et al. 2018). Using two ways including non-enzymatic antioxidants defense system (carotenoids, flavonoids, anthocyanin, and so on) and enzymatic antioxidant defense system [superoxide dismutase (*SOD*), peroxidase (*POD*), ascorbate peroxidase (*APX*), and guaiacol peroxidase (*GPX*)], plants are able to alleviate oxidative damage caused by ROS (Gengmao et al. 2015; Wei et al. 2015).

One of the flavonoids is anthocyanin which is also called the flavylium (2-phenylchromenylium) ion. A positive charge at the oxygen atom of the C-ring of basic flavonoid construction exists in anthocyanins. Osmotic stresses, such as salinity, light, pH, and temperature effect on the stability of anthocyanin considerably (Khoo et al. 2017). Purple, red, and blue pigments in fruits, flowers, and tubers are anthocyanin that in alkaline and acidic conditions emerge as blue and red pigment, respectively. (Laleh et al. 2006). Moreover, plants are able to increase their tolerance with changes in their level of secondary metabolites. One of the

main secondary metabolites in plants is total phenolic compounds, which act as plant advocacy mechanisms versus salinity stress and are able to reduce the destructive effect of salinity (Lim et al. 2012). The main objectives of the present study were to measure the activity of *APX*, *GPX*, and *SOD* enzymes, the concentrations of Na^+ , Cl^- , P, Ca^{2+} , and K^+ , the accumulation of electrolyte leakage (EL), malondialdehyde (MDA), other aldehydes, and hydrogen peroxide (H_2O_2), the contents of total phenolic, total flavonoid, total anthocyanin, total free amino acids, total soluble carbohydrate, and SFN along with expression level of *CYP79F1* gene.

Material and Methods

Germination Percentage

Matured *L. draba* seeds were gathered from different parts of Kerman province in Iran including: Lalezar, Baft, Rafsanjan, Kerman, and Zarand at the end of May and in early June 2018. For disinfection, seeds were immersed in 2/5% sodium hypochlorite for 15 min, followed by washing three times with distilled water. Next, the seeds were exposed to 70% ethanol for 1 min and again washed several times with distilled water. After disinfection of seeds, Whatman papers were put inside the Petri dishes and 100 *L. draba* seeds were placed in each Petri dish, and 5 ml of 50 mM sodium chloride solution was added to it. To determine the germination percentage, germinated seeds in each Petri dish till the 14th day were counted.

Quite similar to the previous method (effect of 50 mM NaCl on the germination percentage of different ecotypes of *L. draba*), after selection of the *L. draba* ecotype with the highest germination percentage, it was exposed to different concentrations of NaCl including 0, 25, 50, 75, and 100 mM to assess the germination percentage of this ecotype in response to the different concentrations of NaCl.

Seeds Germination

Lepidium draba ecotype which had the highest germination percentage was selected as the experimental material and after disinfection, it was gently placed on the surface of the 0.8% agar (pH 7) in a 90 mm Petri dish and transferred to the incubator. Seeds were cultured in complete darkness for 3 days at temperature of 28 ± 1 °C and 55% relative humidity in incubator.

Plant Culture

Germinated *L. draba* seeds were cultured in containers containing 1/2 Hoagland nutrient solution (Hoagland and

Arnon 1950) with pH 6.2, and these dishes were placed in the germinator at 25 °C with 16-h light and 8-h dark photoperiod ($450\text{--}550 \text{ lmol m}^{-2} \text{ s}^{-1}$ flux density) and 55% relative humidity for 2 days. After 2 days, Hoagland solution containers became empty and new Hoagland solutions with different concentrations of NaCl (0 as control, 25, 50, 75, and 100 mM) were prepared. The dishes were filled with 180 cc Hoagland nutrient solution and were put in the germinator with previous conditions. During this time, an aquarium pump was used to aerate the Hoagland nutrient solution. In order to measure the electrolyte leakage, lipid peroxidation, and hydrogen peroxide and contents of total phenolic, flavonoid, anthocyanin, and gene expression, some of the 14-day-old sprouts were immediately harvested and frozen in aluminum paper in liquid nitrogen and kept in polyethylene bags at -80 °C. The rest of the sprouts were used for the evaluation of ion concentrations and SFN content with the following procedure.

Measurement of Stem and Root Lengths

Lengths of stem and root in 14-day-old *L. draba* plant were evaluated by ruler and reported in cm.

Preparation of Enzyme Extract

Lepidium draba sprouts (0.5 g) in attendance of liquid nitrogen were eroded and by 5 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVPP) was homogenated. This solution for 20 min at $17,600 \times g$ (4 °C) was centrifuged and then its supernatant was applied to calculate the enzymes' activity.

Estimation of Ascorbate Peroxidase Enzyme Activity

Lepidium draba sprouts (1 g) were eroded in a mortar by using liquid nitrogen with 0.1 mM EDTA, 2% PVPP, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT) in 50 mM Na-P buffer, pH 7.5. Also, the extraction buffer contained 2 mM ascorbate for ascorbate peroxidase analysis. The activity of ascorbate peroxidase was measured by monitoring the decline in absorbance at the 290 nm wavelength (as for 3 min, ascorbate ($\epsilon = 2.8 \text{ M}^{-1} \text{ cm}^{-1}$) was oxidized) (Asada and Nakano 1981). The assay medium contained 200 μl of the sample, 200 μl ascorbate, 200 μl EDTA, 1200 μl of 50 mM potassium phosphate buffer (pH 7.0), and 200 μl of 0.1 mM H_2O_2 .

Estimation of Guaiacol Peroxidase Enzyme Activity

Guaiacol peroxidase enzyme activity was measured by a reaction medium containing 50 mM phosphate buffer (pH

7), 19 mM H₂O₂, and 9 mM guaiacol described by (Lin and Kao 1999). Wavelength of 470 nm was applied to measure the kinetic evolution of solution during 1 min. 26.6 mM⁻¹ cm⁻¹ extinction coefficient was used to measure peroxidase enzyme activity. One unit of peroxidase is the amount of enzyme that leads to the constitution of 1 mM of tetraguaiacol per 1 min.

Estimation of Superoxide Dismutase Enzyme Activity

McCord and Fridovich method by using Xanthine, Cytochrome C, and Xanthine Oxidase was employed to assess the SOD activity (McCord and Fridovich 1969) using the following assay medium: EDTA 0.1 mM, ferricytochrome C 1×10^{-5} M, 100 mM phosphate buffer pH 7.5, xanthine oxidase 0.004 U/assay, and xanthine 5×10^{-5} M. The velocity of absorbance variations at 550 nm was 0.025 Au min⁻¹ with the above reactants; enough plant extract was appended to the above to try to decrease the latter rate by 50%. Purified SOD (from human erythrocytes, Sigma) was diluted to give the equivalent of 1 U/assay (decreased the reference rate of O₂^{-•} production by 50%) and was used as control for this assay.

Measurement of the Mineral Nutrient

The concentrations of Na⁺, P, Ca²⁺, and K⁺ were determined under 0, 25, 50, 75, and 100 mM NaCl for 14-day-old sprouts, and for this intention, 1 g of frozen samples at 550 °C for 10 h was pulverized and ashed and then the ash was completely dissolved in HCl. Flame photometer was used to assess the concentrations of Na⁺, P, Ca²⁺, and K⁺ in the solution. In order to calculate the Cl⁻ concentrations under 0, 25, 50, 75, and 100 mM NaCl in 14-day-old sprouts of this plant, coulometric–amperometric titration with AgNO₃ was used (Kamiab et al. 2014).

Measurement of the Electrolyte Leakage

In this research, Hamed et al. method was applied to determine the amount of electrolyte leakage (Hamed et al. 2007), and for this purpose, 300 mg of plant samples was put in plastic test tubes in the presence of 15 ml of ultra-pure water. These plastic test tubes were incubated in a water bath at 25 °C for 3 h, and then, the elementary electrical conductivity was evaluated by an EC meter. In the further step, the plant samples were heated for 20 min at 96 °C to release all the electrolytes. The electrolyte leakage was calculated using the following formula:

$$\text{Electrolyte leakage} = (\text{EC elementary}/\text{EC final}) \times 100.$$

Measurement of Lipid Peroxidation and Other Aldehydes

In this study, Heath and Packer method with slight modification was used to measure the level of lipid peroxidation (Heath and Packer 1968), and to this aim, 0.2 g of sprouts was homogenized in 3 ml of 50 mM phosphate buffer with pH 7.0. The homogenate was centrifuged at 16,000×g for 20 min, and then, to 1.0 ml aliquot of the supernatant, 2.0 ml of 0.5% 2-thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was appended. The water bath was used to heat the mixture at 96 °C for 35 min, and then, the mixture was cooled in an ice bath. After centrifugation at 11,000 g for 15 min, 532 nm wavelength was applied to record the absorbance of the supernatant. Also, the quantity for non-specific absorption at 600 nm was recorded and this value was reduced from the absorbance recorded at 532 nm. The extinction coefficient of 155 mM⁻¹ cm⁻¹ was used to calculate the MDA concentration. MDA content was expressed as nmol g⁻¹ fresh weight tissue. Other aldehydes concentrations were calculated using the extinction coefficient of 457 mM⁻¹ cm⁻¹ (Meir et al. 1992).

Measurement of the Hydrogen Peroxide

Velikova et al. method with slight modification was used to determine the quantity of H₂O₂ (Velikova et al. 2000), and to this aim, 0.25 g of sprouts was ground in 3 ml of 5% TCA with 0.1 g activated charcoal, and 13,000×g for 16 min was applied to centrifuge the homogenate. To 0.5 ml of the supernatant, 0.75 ml of 1 M potassium iodide (KI) and 0.5 ml of 10 mM potassium phosphate buffer were appended. 390 nm wavelength was used to calculate the content of H₂O₂ using the extinction coefficient of 0.28 M⁻¹ cm⁻¹ and presented as μmol g⁻¹ fresh weight tissue.

Plant Extract Preparation

An aqueous extract of *L. draba* sprouts was obtained according to the Fattahi et al. method (Fattahi et al. 2013). For this intention and briefly, 15 g of plant sprouts was completely powdered and extracted with 300 ml of 5% ethanol by boiling for 16 min. After filtration of this homogenate, the filtrate was evaporated and kept at – 20 °C for subsequent analysis (Fattahi et al. 2014).

Measurement of the Total Phenolic Content

To measure the total phenolics content of *L. draba* sprouts, Folin–Ciocalteu reagent was applied (Chun et al. 2003). 0.5 ml of Folin–Ciocalteu reagent was mixed with 0.5 ml of the plant extract, and this solution was kept at 25 °C for 8 min. After 8 min, 2 ml of sodium carbonate solution

(7.5%) was added to this solution, and then, the volume of this solution was adjusted to 8 ml with water. After 2 h, 725 nm wavelength was used to measure the total phenolic content. Calibration curve was drawn using the gallic acid as standard. To express the total phenolic content, mg gallic acid equivalents per gram of sample was used.

Extraction and Measurement of Total Flavonoids

Measurement of total flavonoids was calculated according to the aluminum chloride method described by (Zhishen et al. 1999), using quercetin as reference standard developed by (Vattem et al. 2004). To this aim, 1 ml of extract or standard solution of quercetin (20, 40, 60, 80, and 100 $\mu\text{g ml}^{-1}$) was appended to a 10 ml volumetric flask including 4 ml of distilled water. 0.30 ml of 5% NaNO_2 was appended to the flask and after 5 min, 0.3 ml of 10% AlCl_3 was added. 2 ml of 1 M NaOH after 5 min was appended and the volume with distilled water was made up to 10 ml. 510 nm wavelength was used to measure the total flavonoids content. Amounts of total flavonoids were reported as mg quercetin equivalent (mg QE/100 g FW).

Extraction and Measurement of Total Anthocyanins

By using Wagner et al. protocol (Wagner 1979) and with a slight modification, 0.1 g of *L. draba* sprouts in 10 ml of acidic methanol (1% acidified methanol) was rubbed and centrifuged at $4000\times g$ for 10 min. The supernatant was placed in darkness for 24 h, then, its absorption was read at wavelength of 550 nm using Carry-50 spectrophotometer device. Anthocyanins concentration was calculated using extinction coefficient of $33,000 \text{ M}^{-1} \text{ cm}^{-1}$ ($\epsilon = 33,000 \text{ M}^{-1} \text{ cm}^{-1}$).

Measurement of Total Free Amino Acids

Yemm and Cocking method was used to calculate the total free amino acids using ninhydrin assay with some modifications (Yemm et al. 1955). Sprouts (500 mg) were extracted in 5 ml of 80% ethanol and centrifuged at $17,500\times g$ for 40 min. Total free amino acids were measured using 7.6 ml of ninhydrin reagents containing 1% ninhydrin in 0.5 M citrate buffer pH 5.5, glycerol (87%), and 0.5 M citrate buffer pH 5.5 in a ratio of 5:12:2 using 0.1 ml of the test extract. After 2 min of violent shaking, a water bath was used to heat the contents for 16 min at 100 °C. After this step, the contents were cooled, and then, 570 nm wavelength was applied to calculate the amounts of free amino acids using glycine standard curve.

Measurement of Total Soluble Carbohydrate

Wardlaw and Willenbrink method with slight modification was used to calculate the total soluble carbohydrates (Wardlaw and Willenbrink 1994). *L. draba* sprouts (0.5 g) were finely homogenized using 5 ml of 95% ethanol, and then, 0.1 ml of alcoholic extract was mixed with 3 ml of anthrone (150 mg anthrone, 100 ml of 72% sulphuric acid, W/W). After this step, a water bath at 100 °C for 15 min was used to heat the homogenate solution. After cooling, 625 nm was used to measure the quantity of total soluble carbohydrates using a glucose standard curve.

SFN Standard Solution Preparation and SFN Extraction and Quantification

SFN standard (Sigma-Aldrich) solution was made according to the (Liang et al. 2006) method, so 5 mg of SFN was solved in 10 ml acetonitrile and kept at $-80 \text{ }^\circ\text{C}$ until injection. Also, with some slight modification, extraction and determination of SFN were carried out according to the (Liang et al. 2006) and (Riahi-Madvar et al. 2014) methods; therefore, 0.3 g of the fresh tissue was eroded with a mortar and pestle and mixed with 1 ml of acidic water (pH 5). After 2.5-h incubation at 43 °C, 5 ml of acetonitrile was added to the mixture, and it was sonicated for 3 min. The resulting mixture was centrifuged at 11,000 rpm for 13 min at 4 °C. Finally, the supernatant was passed through 0.2 μm syringe filter and the SFN content was measured using high-performance liquid chromatography [HPLC, Agilent 1100 series, C18 column ($25\times 0.46 \text{ cm}$)]. Moving phase solvent including acetonitrile/ H_2O (60/40 v/v) and a flow rate of 1 ml min^{-1} under room temperature were used in order to separate SFN. The retention time was compared with authentic standard detected at 254 nm in order to identify the SFN peak.

Total RNA Extraction

In order to remove RNase enzyme, all microtubes, pipette tips, falcons, mortars, and utensils were put in 0.1% diethyl pyrocarbonate (DEPC) treated water for 24 h and then were dried in an oven at 80 °C. After that, they were sterilized two times in an autoclave. Also, DEPC-treated water was used for the preparation of all solutions. The following steps were performed for all treatments and replicates. Initially, 100 mg of plant tissue was crushed in the presence of liquid nitrogen and transferred to a 1.5 ml tube, and 1 ml cold RNX_ plus solution (Cinnagen, Tehran, Iran) was added to the tubes and vortexed for 30 s. Tubes were put on ice for 20 min, during this time the tubes were gently shaken every 5 min. 200 μl of chloroform was added to the tubes and they were shaken, strongly. Tubes were placed on ice for 5 min. Following

this step, the tubes were centrifuged (4 °C, 12,000 rpm) for 15 min. Supernatant liquid was discarded and transferred to a new microtube and the same amount of isopropyl alcohol was added. Tubes were gently shaken and were put on ice for 15 min and again centrifuged with previous conditions. The supernatant liquid was discarded, and 1000 ml of 75% ethanol was added to the pellet. Tubes were centrifuged (4 °C, 7500 rpm for 4 min), supernatant liquid was removed, and the tubes were placed in reverse on sterile paper until they were dried. Next, 30 ml DEPC solution was added to the tubes and they were kept at – 80 °C. In order to survey the quality and quantity of total RNA, 1% agarose gel (photography was done under UV light) and spectrophotometer (260 nm) were used, respectively.

CDNA Synthesis

For cDNA synthesis, 1 µg of total RNA, 1 µl 10× buffer, 1 µl DNase, and 1 µl deionized water were poured in 0.2 ml microtubes and they were kept at 37 °C for 30 min. 2 µl EDTA (50 mM) was added to the microtubes and they were kept at 65 °C for 10 min. In the next step, 0.5 µl oligo (dt) 18 primer and 0.5 µl random hexamer were appended to the microtubes and they were maintained at 65 °C for 5 min. Eventually, 1 µl RNase Inhibitor, 4 µl RT buffer (10×), 1 µl dNTP, 1 µl Reverse Transcriptase enzyme, and 2 µl deionized water were, respectively, added to the microtubes and they were kept at 42 °C for 60 min. In order to prevent reverse transcriptase enzyme activity, microtubes were kept at 85 °C for 5 min.

CYP79F1 Gene Identification and Sequences Analysis

Because of the unavailability of *L. draba CYP79F1* gene sequence in databases, its sequence was derived by PCR. For this purpose, amplification of cDNA was carried out in the presence of specific primers (forward and reverse) using *Taq* DNA polymerase. The forward (5'-TTACCATACCCCTTTTCACATCCTACTAGTC-3') and reverse (5'-TGCCGGATTATCAATCGCTGCTATAC-3') primers were designed using Gene Runner (version 3.05) software according to the *CYP79F1* gene sequence of *Arabidopsis thaliana*, *Brassica rapa*, *Brassica oleracea*, *Raphanus sativus*, and *Brassica napus* (GenBank accession No. 838,211, 103,872,429, 106,343,089, 108,812,420, 106,367, and 453, respectively), which belong to the Brassicaceae family like *L. draba*. 25 µl reaction mixture was considered for *CYP79F1* gene amplification. 0.5 µl of the cDNA, 1.25 mM MgCl₂, 10 mM Tris-HCl pH 8.4, 1.25 U *Taq* polymerase (Invitrogen, Carlsbad, USA), 0.1 µM each primer, 25 mM potassium chloride (KCl) and 62.5 µM dNTPs were used to extend *CYP79F1* gene. cDNA amplification of *CYP79F1* gene was done under the

following conditions: initial denaturation at 95 °C for 4 min, 35 cycle amplification (95 °C for 1 min, 68 °C for 30 s, and 72 °C for 1 min) and a final extension for 10 min at 72 °C. Observation and separation of amplified product was performed using 1% agarose gel electrophoresis. Agarose gel image was viewed using the UV Gel Document. The size of the bands was estimated based on 100-bp DNA marker. After electrophoresis, the product of the PCR in 1% agarose gel, a solid single-band PCR product, and non-elongation were sent to be sequenced by an automatic sequencer (23 ABI 3730XLs, Macrogen, Korea) according to the specific forward and reverse primers. After determining the sequence of the desired mRNA, files related to the sequenced fragment were changed to Fasta format using the Chromas v.1.95 software. Similarity search for nucleotide sequence was obtained using Blast online software in NCBI gene database. Also, similarity and identity percentages were extracted from protein and nucleotide blast in NCBI.

Dendrogram Analyses and Estimates of Evolutionary Divergence for CYP79F1 mRNA

In order to study the relationship and compute pairwise distances of *L. draba CYP79F1* mRNA with other plants *CYP79F1* mRNAs that were derived from a common ancestor, dendrogram was drawn and evolutionary divergence was estimated using MEGA7 software.

Analysis of CYP79F1 Gene Expression

The sequences of the primers related to *CYP79F1* as the target gene and *GAPDH* as internal control gene were designed from the registered sequences in NCBI. Goharrizi et al. showed that *GAPDH* is a stable internal control gene under salinity stress (Goharrizi et al. 2018). Through alignment of the reported sequences, conserved sequences were determined and primers were designed using Gene Runner software (Table 1). In order to achieve the best conditions, amplification was performed at different temperatures in gradient PCR and 57 °C was chosen as the best annealing temperature for *CYP79F1* and *GAPDH* genes. Amplicon length for *CYP79F1* and *GAPDH*, 180 and 177 base pairs were calculated, respectively.

Table 1 Sequence of primers for amplification of *CYP79 F1* and *GAPDH* genes

Primers name	Primers sequence
Forward <i>CYP79F1</i>	5'-AAGCACTCTATGTATAAACGGTC-3'
Reverse <i>CYP79F1</i>	5'-TTCCGCCTTTCCTAATCTTC-3'
Forward <i>GAPDH</i>	5'-TAGACTCGAGAAAGCTGCGA-3'
Reverse <i>GAPDH</i>	5'-TTGCACTCAACGCGATTCC-3'

PCRs were performed with a ROTOR GENE 3000 REAL-TIME PCR MACHINE using SYBR® Green to detect dsDNA synthesis. Reactions were done in 20 μ l volumes containing 0.6 μ l of each primer (0.1 μ M), 2 μ l cDNA, 10 μ l SYBR Green Master Mix Reagent (Takara), 0.4 μ l ROX, and 6.4 μ l distilled water. Reactions were run using the following conditions, 94 °C for 4 min, 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and finally 75 °C for 10 s. After seeing a single peak in real-time PCRs, the threshold line was drawn by LinRegPCR program (<https://www.hartfaalce ntrum.nl/index.php?main=files&fileName=LinRegPCR.zip&sub=LinRegPCR>) and Ct of *CYP79F1* and *GAPDH* genes was calculated by the threshold line. $2^{-\Delta CT}$ method was used for calculating *CYP79F1* gene expression. Delta Ct is gotten by calculating the difference between Ct of the *CYP79F1* gene and *GAPDH* gene. Negative control in all steps confirmed the accuracy of the amplification.

Statistical Analysis

Experiments were conducted in completely randomized design in three replicates. Data were analyzed by one-way ANOVA, followed by Duncan multiple comparison tests.

The values were reported as means with their standard error for all results. Also, statistical differences were considered significant at $P < 0.05$.

Results

Germination Percentage

Among the collected seeds (Lalezar, Baft, Rafsanjan, Kerman, and Zarand), the seeds that were gathered around Rafsanjan and Baft showed the highest and lowest germination percentage under 50 mM NaCl, respectively (Fig. 1a). The seeds that were collected around Rafsanjan were selected for other analyses. Also, Rafsanjan *L. draba* ecotype seeds that were exposed to the 5 different levels of NaCl showed a decreasing trend in their germination percentage with increasing concentrations of NaCl (Fig. 1b).

Stem and Root Lengths

According to our results, stem and root lengths were declined with induction of NaCl in all samples (Fig. 2).

Fig. 1 Germination percentage of different ecotypes of *L. draba* seeds under 50 mM NaCl (a) and germination percentage of Rafsanjan *L. draba* ecotype seeds under 0, 25, 50, 75, and 100 mM NaCl (b). Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests

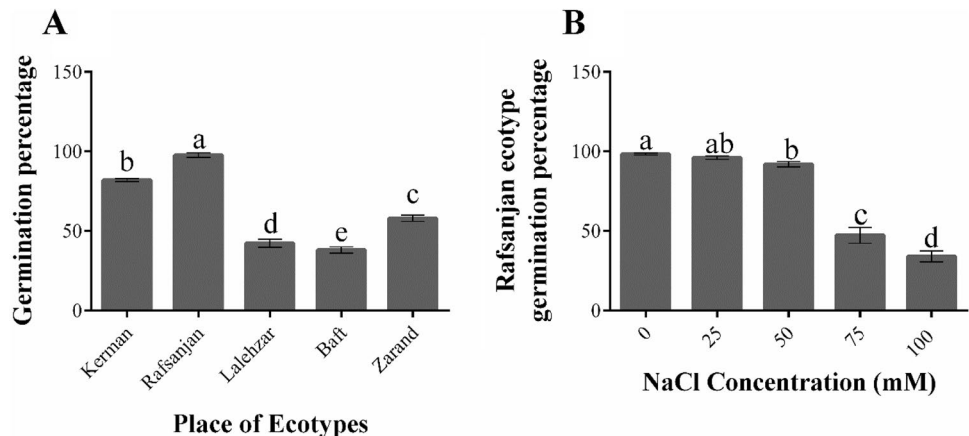
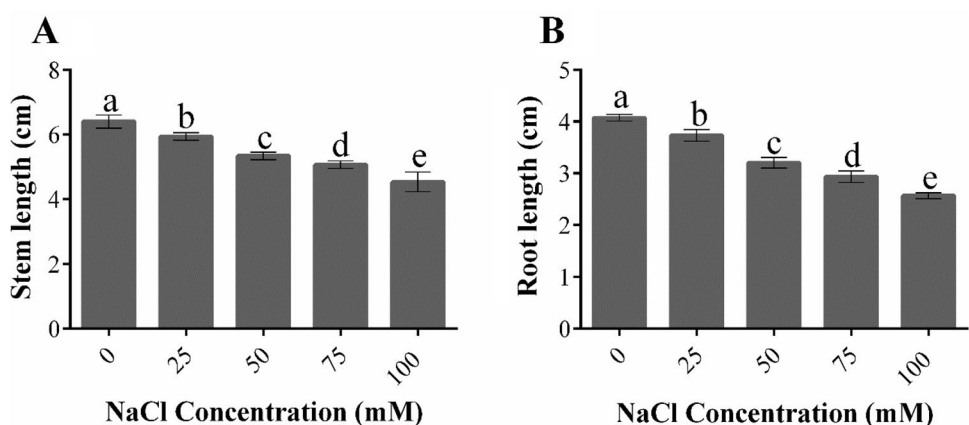


Fig. 2 Effect of different NaCl levels on the stem (a) and root (b) lengths in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests



For stem length, 0.92-, 0.83-, 0.79-, and 0.7-fold down-regulation and for root length, 0.91-, 0.87-, 0.72-, and 0.63-fold down-regulation were calculated under 25, 50, 75, and 100 mM NaCl compared to normal environment, respectively (Fig. 2a, b).

Ascorbate Peroxidase, Guaiacol Peroxidase, and Superoxide Dismutase Enzymes Activities

Ascorbate peroxidase enzyme activity was generated by inducing the NaCl concentrations up to 75 mM and then decreased. 1.09-, 1.37-, 1.9-, and 0.65-fold change were calculated under 25, 50, 75, and 100 mM NaCl compared to normal environment, respectively (Fig. 3a). According to the results of this study, guaiacol peroxidase activity increased with increasing concentrations of NaCl up to 75 mM and after that decreased (Fig. 3b). 1.67-, 2.4-, 2.62-, and 0.54-fold change were assessed under 25, 50, 75, and 100 mM NaCl in comparison with 0 mM environment, respectively (Fig. 3b). Our results showed that the amount of superoxide dismutase enzyme activity was elevated by elevating the NaCl concentrations up to 75 mM and then decreased (Fig. 3c). 1.18-, 1.33-, 1.42-, and 1.14-fold variations were evaluated for 25, 50, 75, and 100 mM NaCl compared to 0 mM, respectively (Fig. 3c).

Measurement of the Mineral Nutrient Content

The amounts of Na^+ , Cl^- , P, Ca^{2+} , and K^+ accumulation under different doses of NaCl in the sprouts of this plant were assessed to recognize the impact of this stress on plants' physiology. As shown in Fig. 4, the results of this study showed that the Na^+ and Cl^- concentrations with an increase in NaCl concentrations increase, while the

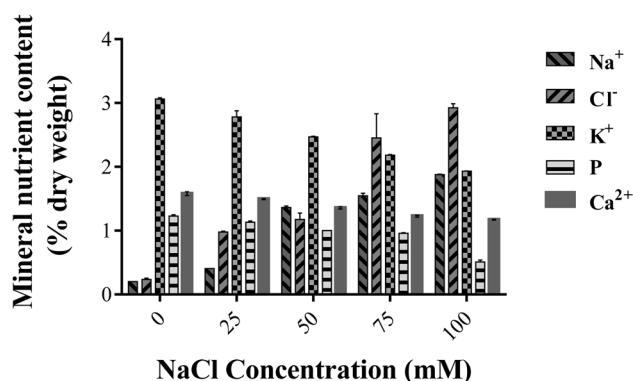


Fig. 4 Effect of different NaCl levels on the amounts of Na^+ , Cl^- , P, Ca^{2+} , and K^+ accumulation in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests

amounts of P, Ca^{2+} , and K^+ ions decrease, significantly (Fig. 4).

Measurement of the Electrolyte Leakage, Lipid Peroxidation, Other Aldehydes, and Hydrogen Peroxide Contents

Our results showed that the amounts of electrolyte leakage in 14-day-old sprouts of *L. draba* are considerably enhanced in all samples by increasing concentrations of NaCl (Fig. 5a). 1.13-, 1.71-, 1.97-, and 2.14-fold up-regulation in electrolyte leakage contents were estimated under 25, 50, 75, and 100 mM NaCl in comparison with the normal condition, respectively (Fig. 5a). Lipid peroxidation in the 14-day-old sprouts of *L. draba* plant was measured as MDA content and is shown in Fig. 5b. In this study, increasing NaCl concentrations led to the increasing MDA contents in all samples, significantly (Fig. 5b). Under different concentrations of NaCl including 25, 50, 75, and 100 mM, enhancement

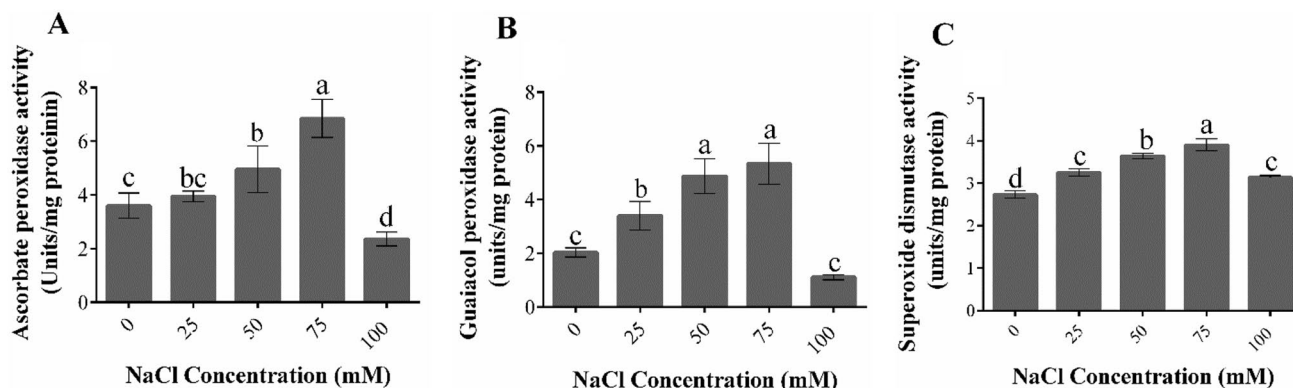


Fig. 3 Effect of different NaCl levels on the ascorbate peroxidase (a), guaiacol peroxidase (b), and superoxide dismutase (c) enzymes activities in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests

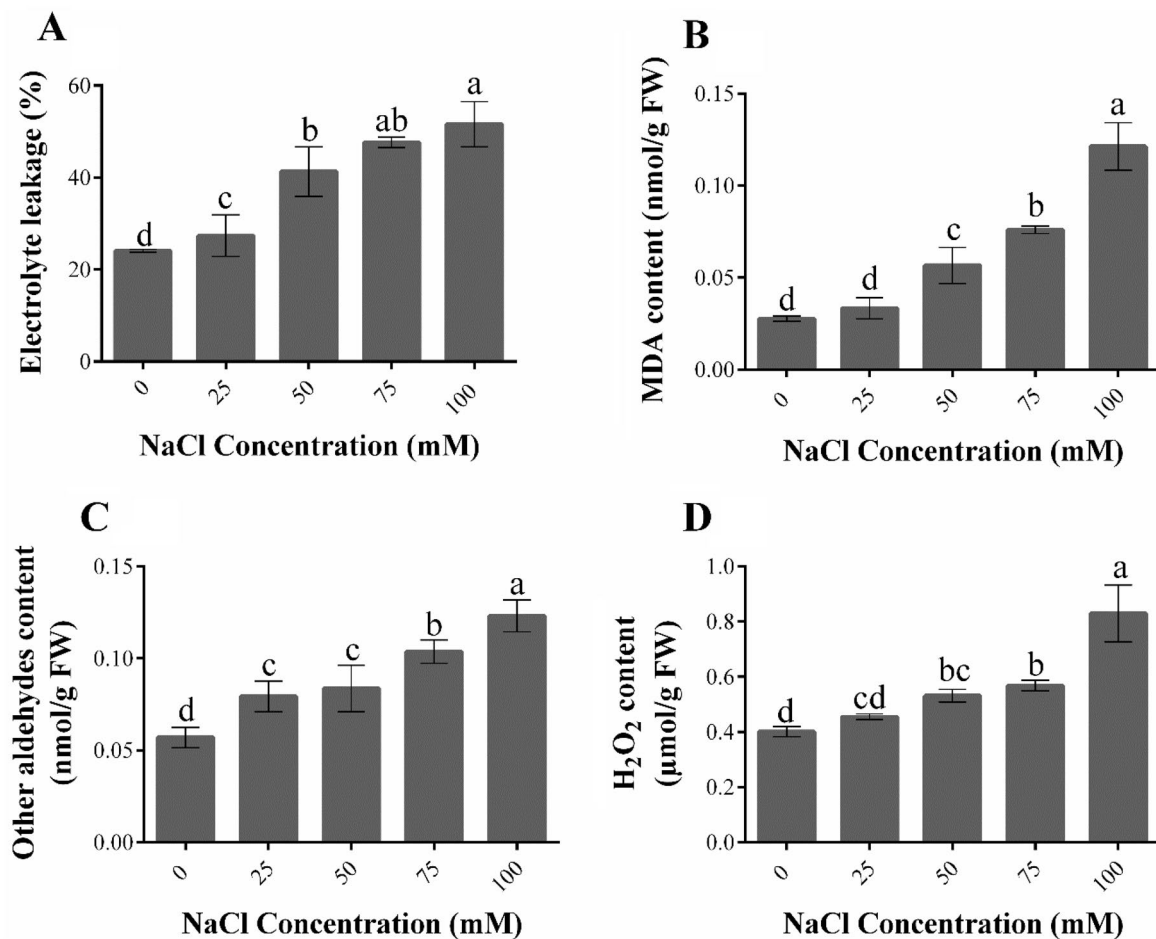


Fig. 5 Effect of different NaCl levels on the electrolyte leakage (a), lipid peroxidation (b), other aldehydes (c), and hydrogen peroxide (d) contents in 14-day-old *L. draba* sprouts. Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests

of 1.2-, 2.04-, 2.74-, and 4.38-fold was calculated for lipid peroxidation compared to normal environment in this plant, respectively (Fig. 5b). Also, 1.39-, 1.46-, 1.81-, and 2.16-fold up-regulations were observed for other aldehydes contents under 25, 50, 75, and 100 mM NaCl in comparison with 0 mM, respectively (Fig. 5c). Increasing NaCl levels increased the H₂O₂ concentration in sprouts of this plant in all samples (Fig. 5d). 1.13-, 1.32-, 1.41-, and 2.06-fold up-regulations were observed for H₂O₂ concentrations under 25, 50, 75, and 100 mM NaCl compared to normal condition, respectively (Fig. 5d).

Measurement of the Total Phenolic Content

In this study, total phenolic content is enhanced by enhancement of NaCl concentrations in all samples (Fig. 6a). Enhancement of 1.2-, 2.24-, 2.55-, and 3.52-fold was calculated for 25, 50, 75, and 100 mM NaCl relative to the normal condition, respectively (Fig. 6a). Under NaCl stress, flavonoid content increased with increasing concentrations

of sodium chloride in all samples (Fig. 6b). Also, under 100 mM NaCl concentration, the highest concentration of flavonoids was measured (Fig. 6b). Additionally, between all NaCl concentrations, a significant difference was observed in all samples between flavonoids content (Fig. 6b). Up-regulation of 1.1-, 1.13-, 1.15-, and 1.67-fold was estimated for flavonoid content under 25, 50, 75 and 100 mM NaCl in comparison with the 0 mM, respectively (Fig. 6b). Under different levels of salinity stress, with enhancing NaCl concentration, anthocyanin content increased in all samples, but a significant difference was not observed between 0 and 25 mM of NaCl (Fig. 6c). Regarding these results, anthocyanin content was improved by increasing levels of NaCl in comparison with the control condition (Fig. 6c). Enhancement of 1.08-, 1.33-, 2.04-, and 2.33-fold was calculated for anthocyanin content under 25, 50, 75, and 100 mM NaCl in comparison with the 0 mM, respectively (Fig. 6c). As shown in Fig. 6d, in a dose-dependent manner, the contents of total free amino acids increased with induction of NaCl in all samples (Fig. 6d). 1.1-, 1.22-,

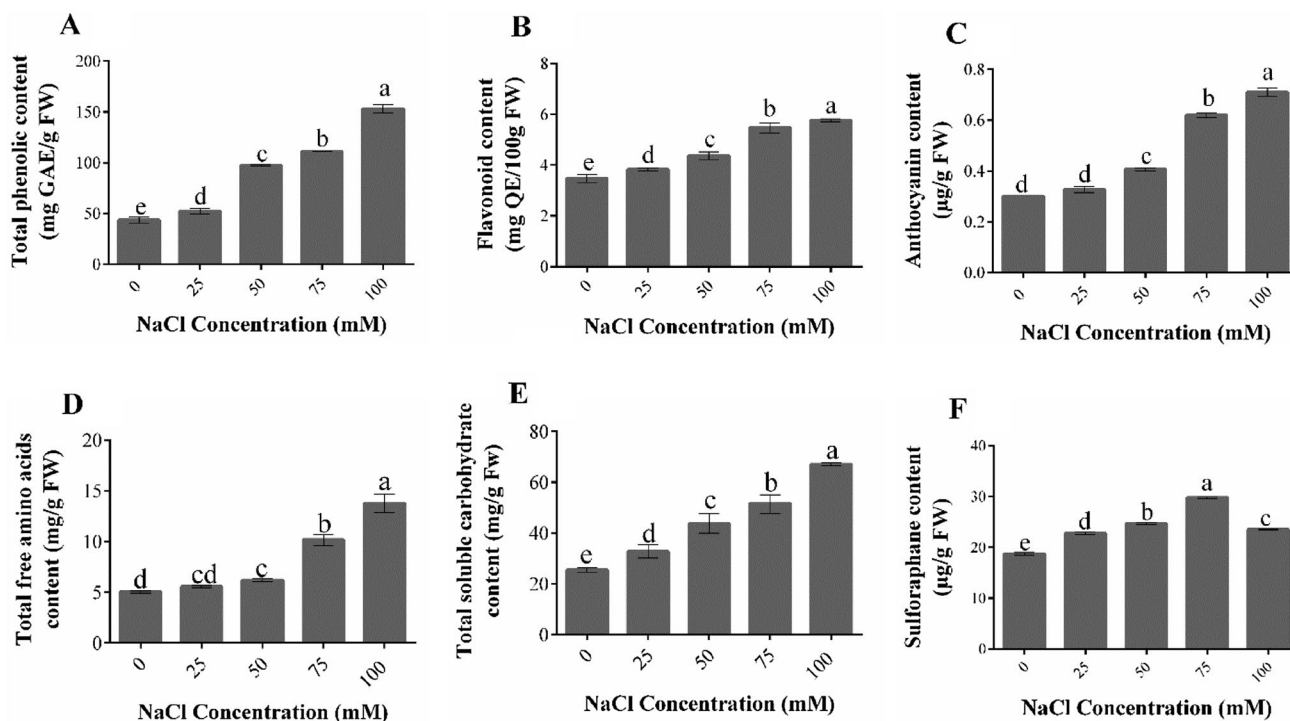


Fig. 6 Effect of different NaCl levels on the total phenolic (a), flavonoid (b), anthocyanin (c), total free amino acids (d), total soluble carbohydrate (e), and sulforaphane (f) contents in 14-day-old *L.*

draba sprouts. Bars with different letters are significantly different at $P < 0.05$, according to Duncan's multiple range tests

2.02-, and 2.74-fold up-regulations were observed for the free amino acids contents under 25, 50, 75, and 100 mM NaCl compared to 0 mM, respectively (Fig. 6d). Contents of total soluble carbohydrate in a dose-dependent manner were elevated by increasing concentrations of NaCl in all samples (Fig. 6e). Enhancement of 1.28-, 1.71-, 2.01-, and 2.62-fold was calculated for total soluble carbohydrate under 25, 50, 75, and 100 mM NaCl in comparison with 0 mM, respectively (Fig. 6e). The retention time of SFN was about 4 min after injection into the column. Variance analysis showed significant differences between NaCl treatments, and means comparison showed the following results (Fig. 6f). Under salt stress in 14-day-old *L. draba* sprouts, SFN content was increased by increasing concentrations of sodium chloride up to 75 mM and after that it was decreased (Fig. 6f). Also, in all NaCl concentrations, a significant difference was observed between SFN contents (Fig. 6f). 18.72, 22.76, 24.69, 29.8, and 23.53 $\mu\text{g g}^{-1}$ FW were computed for SFN content under 0, 25, 50, 75, and 100 mM NaCl, respectively (Fig. 6f).

Amplification, Sequences Alignment, and Expression Level of *CYP79F1* Gene

For *CYP79F1* gene amplification, cDNA library was used as a template in the presence of specific primers.

The amplified fragment (928 bp) was observed on 1% agarose gel (Fig. 7a). mRNA sequence identified as 491 nucleotides related to the codon region of this enzyme was accurately recognized and recorded in GenBank with KT626603 accession number. Amino acid sequence (168 amino acids) encoded by this sequence was also extracted and recorded with KT626603 accession number in GenBank. The recorded sequence of *L. draba CYP79F1* mRNA showed 85% and 84% identity with *CYP79F2* mRNA sequence of *Arabidopsis lyrata* and *Arabidopsis thaliana*, respectively. Also, Blast results showed that the *CYP79F1* protein sequence of *L. draba* has 83%, 84%, and 82% identity with the *CYP79F1* protein sequence of *Brassica napus*, *Brassica oleracea* and *Arabidopsis lyrata*, respectively. In this study, $2^{-\Delta\text{Ct}}$ method was applied to calculate the *CYP79F1/GAPDH* mRNA ratio. Variance analysis showed highly significant differences between NaCl treatments. Expression of *CYP79F1* gene under salinity stress conditions was studied in 5 levels, and means comparison showed the following results. Expression of *CYP79F1* gene was increased by increasing concentrations of sodium chloride up to 75 mM and decreased at higher concentration (Fig. 7b). In order to validate the PCR product (real-time PCR product), separation of amplified product was performed using 1% agarose gel (Fig. 7c).

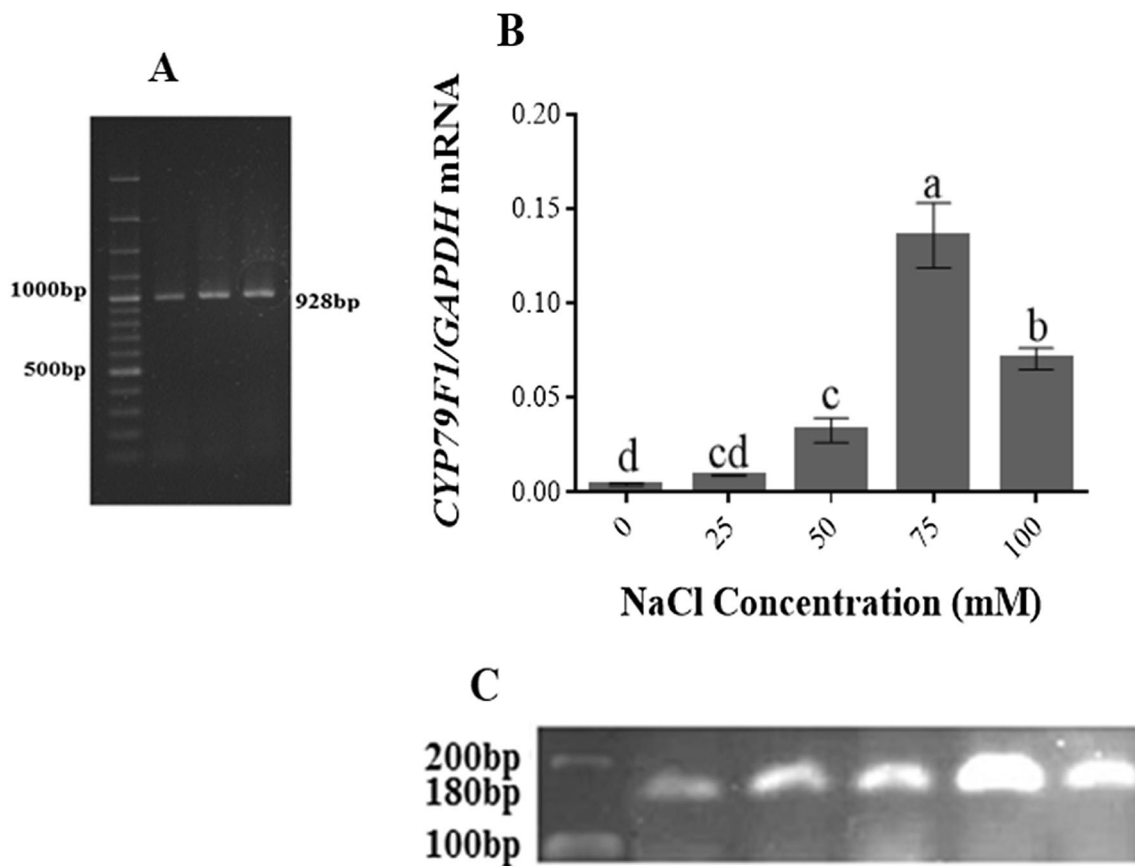


Fig. 7 *CYP79F1* amplification band (928-base pair band was observed on a 1% agarose gel after PCR amplification) (a), expression level of *CYP79F1* gene relative to the *GAPDH* as internal control (b), and real-time quantitative RT-PCR amplification fragments' specificity (amplified fragments obtained after qRT-PCR were sepa-

rated by agarose gel electrophoresis) (c) under 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

Dendrogram Analyses and Estimates of Evolutionary Divergence for *CYP79F1* mRNA

Dendrogram analyses and pairwise distances computation of *CYP79F1* mRNA were drawn by MEGA7 software. The results showed that *L. draba* with *Arabidopsis thaliana* have a common ancestor (Fig. 8). The number of base substitutions per site between sequences was shown in Table 2. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004). Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 206 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Discussion

In this study, five different *L. draba* ecotypes of Kerman province in Iran including: Lalezar, Baft, Rafsanjan, Kerman, and Zarand had various reactions to germination under 50 mM NaCl. Rafsanjan and Kerman ecotypes, which grew in warmer temperatures, had higher germination percentage, respectively. Also, germination percentage of Rafsanjan ecotype, which was selected as the experimental material of this study, decreased with increasing concentrations of NaCl. In sweet sorghum genotypes that were exposed to the different doses of salinity stress, germination percentage was declined with increasing levels of salinity (Almodares et al. 2007). One of the most important proceedings in plant

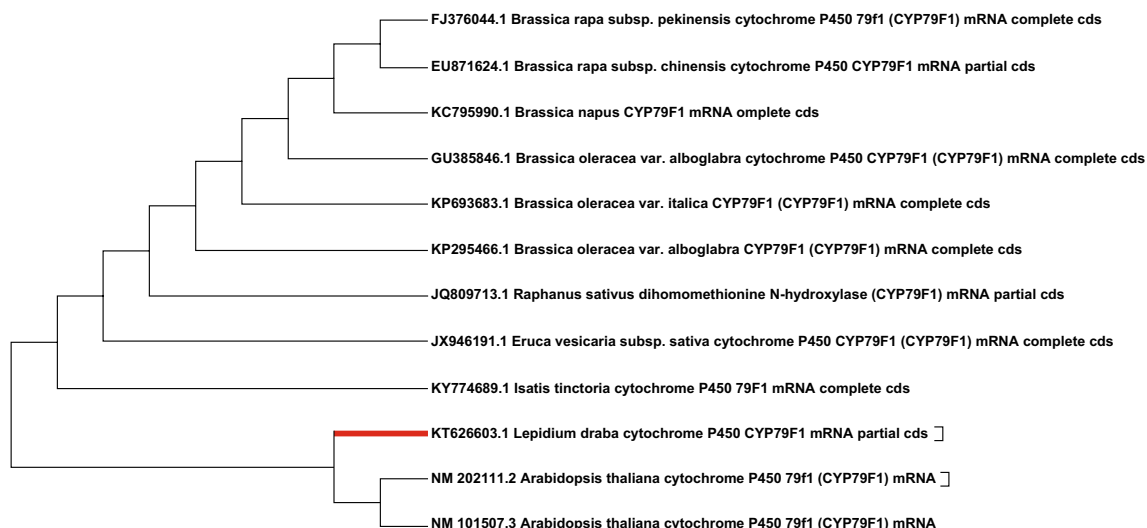


Fig. 8 Dendrogram analyses of *CYP79F1* mRNA

Table 2 Estimation of the evolutionary divergence between sequences (computing pairwise distances)

		1	2	3	4	5	6	7	8	9	10	11
1	KC795990.1											
2	GU385846.1	0.00										
3	JX946191.1	0.07	0.06									
4	FJ376044.1	0.00	0.00	0.07								
5	KP693683.1	0.00	0.00	0.06	0.00							
6	NM_202111.2	0.24	0.24	0.31	0.24	0.24						
7	NM_101507.3	0.24	0.24	0.31	0.24	0.24	0.00					
8	KP295466.1	0.00	0.00	0.06	0.00	0.00	0.24	0.24				
9	KT626603.1	0.24	0.23	0.24	0.24	0.23	0.22	0.22	0.23			
10	JQ809713.1	0.04	0.03	0.09	0.04	0.03	0.24	0.24	0.03	0.26		
11	KY774689.1	0.09	0.08	0.10	0.09	0.08	0.20	0.20	0.08	0.23	0.11	
12	EU871624.1	0.00	0.01	0.08	0.00	0.01	0.24	0.24	0.01	0.25	0.04	0.09

extension is seed germination that can be declined by abiotic stresses, such as salinity and drought. Salinity decreases germination percentage, seedling growth, and the number of seedlings. Also, it considerably increases dispersion of germination (Almansouri et al. 2001; Kaya et al. 2006). It is specified that when plants are exposed to salinity stress, seed establishment, seed germination, leaf area, and plant biomass production reduce under the destructive effects of salinity (Tanveer et al. 2018). Low temperature increases abscisic acid (ABA) content during seed development, and several studies reported seeds that grow and develop at cooler temperatures are generally more dormant and have less germination than those that grow and develop at warmer temperature, such as wheat (Biddulph et al. 2007), weedy rice (Gu et al. 2006), and *Arabidopsis* (Donohue et al. 2008) and these results are quite similar to our results.

Application of NaCl significantly reduced shoot and root lengths in *L. draba* plants when compared to the control environment. Several studies have shown that NaCl stress decreased the shoot and root lengths of various plants in comparison with the normal condition (Ahmad et al. 2019, 2017; Kaur et al. 2018; Bai et al. 2011). Plant development decreases under abiotic stresses by affecting various physiological and biochemical mechanisms such as hormonal signaling, photosynthesis, and antioxidant systems (Sharma et al. 2016). Reduction of stem and root lengths occurs either directly via the decline in the level of growth by reduction of the leaf surface and then reducing the turgor (Bouchabke et al. 2006), inhibiting cell division (Granier et al. 2000), and declining the total biomass (Labdelli et al. 2014), or indirectly by reducing the number of leaf-bearing organs (Labdelli et al. 2014).

According to the results of this study, *APX*, *GPX*, and *SOD* enzymes activity increased up to 75 mM NaCl and then decreased. Numerous researches have demonstrated that in different plants treated with NaCl, activities of *APX*, *SOD*, *CAT* (catalase), and *GR* (glutathione reductase) increased compared with those in the control plants (Ahmad et al. 2016, 2018, 2019), *SOD* and *APX* in mustard and maize (Ahmad et al. 2017; Bai et al. 2011), and *GPX*, *SOD*, and *CAT* in UCB-1 (Goharrizi et al. 2019). Also, another study has proven that salinity stress causes the expression levels of *SOD*, *CAT*, and *APX* genes to increase, significantly (Kaur et al. 2018). Also, it is proved that *GPX* activity increased in maize under salinity stress (de Azevedo Neto et al. 2006). Furthermore, many studies have reported the increased activity of antioxidant enzymes in other plants like *Morus alba* (Ahmad et al. 2014), *C. aeritimum* (Ahmad et al. 2016), and *Brassica juncea* (Ahmad et al. 2015). When plants are exposed to salinity stress, ROS accumulation increases and leads to lack of balance between ROS production and scavenging system, which results in cellular oxidative damage. Plants have evolved an effective defense system of scavenging ROS including enzymatic (*APX*, *SOD*, *CAT*, *GPX*) and non-enzymatic (carotenoids, flavonoids, anthocyanin, and so on) antioxidants defense systems to protect versus cellular detriment and retain the useful action of ROS-mediated signal transduction which operates in a normal plant (Shi et al. 2014; Gengmao et al. 2015; Wei et al. 2015). Most likely, *APX*, *GPX*, and *SOD* in this study increased in order to decrease the produced ROS under salinity stress.

In this study, the results of Na⁺, Cl⁻, P, Ca²⁺, and K⁺ ions accumulation under 0, 25, 50, 75, and 100 mM NaCl showed that Na⁺ and Cl⁻ ions increased with increasing the concentrations of NaCl, unlike with the P, Ca²⁺, and K⁺ ions that reduced. Ahmad et al. showed that in mung bean treated with 50 and 100 mM NaCl, the concentration of Na⁺ increased, while Ca²⁺ and K⁺ decreased compared with those in the control plants, significantly (Ahmad et al. 2019). A previous research has shown that in roots and leaves of pistachio rootstocks, Na⁺ and Cl⁻ increase, while K⁺ decreases, significantly (Akbari et al. 2018). Also, another research showed that P ion decreases under NaCl stress in faba bean plant (Tavakkoli et al. 2010). Furthermore, it has been shown that the Ca²⁺ concentration in the roots of two Canola (*Brassica napus* L.) was declined with NaCl application (Bahrani 2013). Previous researches have shown the distinguished importance of Na⁺ for several plant species. These researches have proved that Na⁺ acts as a reason for ion-specific detriment during salinity tension. The high amounts of Na⁺ in metabolic processes lead to competition between the Na⁺ and K⁺ ions and consequently, causes enzymes to become inactive (Amtmann and Sanders 1998; Munns and Tester 2008; Tester and Davenport 2003). Elimination of Na⁺ and/or Cl⁻ minimizes ion toxicity in plants,

but precipitates water deficit. Also, osmotic adjustment can be facilitated by absorption of Na⁺ and/or Cl⁻, but can also lead to nutritional imbalance and specific ion toxicity. Salinity caused by high levels of NaCl reduces plant growth by the accumulation of high doses of both Na⁺ and Cl⁻ ions. To defeat the harm of salt stress, plants have the ability to reduce uptake or transport Cl⁻ and Na⁺ from the roots to the leaves to maintain ion homeostasis (Li et al. 2014). In this research, possibly induced NaCl causes Na⁺ and Cl⁻ ions to increase and P, Ca²⁺, and K⁺ to decrease in 14-day-old *L. draba* sprouts. Most probably, high absorption of Na⁺ and Cl⁻ by *L. draba* plant root is the main reason for the lower level of P, Ca²⁺ and K⁺ in sprouts of this plant.

Our data showed that electrolyte leakage content significantly increased with increasing NaCl concentrations, and these data are completely similar to the previous research by (Hniličková et al. 2019). Many studies have shown that the increasing NaCl content results in increasing electrolyte leakage content in mung bean, tomato, chickpea, and mustard plants, significantly (Ahmad et al. 2016, 2017, 2018, 2019; Kaur et al. 2018). Also, Mahmoudi et al. in (2011) have proven that NaCl increases electrolyte leakage in lettuce (Mahmoudi et al. 2011). The preliminary site of ion-specific salinity damage is plasma membrane (Mansour and Salama 2004). Therefore, electrolyte leakage content can be considered as one of the most important indexes for plants' resistance (Ashraf and Ali 2008). In this study, most likely, NaCl stress causes the electrolyte leakage content to increase with increasing NaCl levels.

MDA, which is an index for lipid peroxidation, and other aldehydes contents in the sprouts of *L. draba* plant were measured. The results of this study showed that both MDA and other aldehydes contents increase under NaCl stress in 14-day-old *L. draba* sprouts up to 100 mM. Several studies have shown that in green bean, mung bean, chickpea, tomato, and mustard, MDA content increases in response to salinity stress, significantly (Yasar et al. 2008; Ahmad et al. 2016, 2017, 2018, 2019; Kaur et al. 2018). In 2019, Goharrizi et al. showed that in UCB-1, 100 mM NaCl increased the content of MDA, significantly (Goharrizi et al. 2019). Also, it is proved that when MDA content increases, the plants' growth decreases (Cavalcanti et al. 2004). Kabiri et al. showed that other aldehydes contents increase under salinity stress, significantly (Kabiri et al. 2014). ROS generation in response to salinity stress is unavoidable (Abogadallah 2010), and ROS causes membrane lipid peroxidation in plants. MDA and other aldehydes contents are generally two indexes for resistance of plants under unfavorable conditions of stresses such as salinity and drought (Abogadallah 2010). For example, Taïbi et al. have proved that salt-tolerance plants have less membrane lipid peroxidation (Taïbi et al. 2016). According to our data, it can be concluded that increasing MDA and other aldehydes contents are oxidative

damages of salinity stress to the 14-day-old *L. draba* sprouts which occur because of the enhancement in NaCl levels.

According to our results, enhancement of H_2O_2 occurs with increasing doses of NaCl in all samples. Several researchers have shown that the content of H_2O_2 increases in different plants under salinity stress, significantly (Wang et al. 2016; Ahmad et al. 2016, 2017, 2018, 2019; Kaur et al. 2018; Bai et al. 2011). NaCl can impair plant productivity in several various ways (Meloni et al. 2003). One of the detriments induced by salinity stress is the growth in reactive oxygen species (ROS) such as hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), and superoxide (O_2^-) (Yen et al. 2013). Higher plants generate ROS under stress conditions and excessive accumulation of ROS in response to salinity stress can cause oxidative stress (Yen et al. 2013; Czegeny et al. 2014) and extremely disrupt natural metabolism by membrane lipids peroxidation (Fridovich 1986; Wise and Naylor 1987), nucleic acid mutation (Fridovich 1986; Imlay and Linn 1988), and protein destruction (Davies 1987). Enhancement of H_2O_2 content in this study occurs because of the destructive effects of salinity in this plant.

In this study, enhancement of total phenolic content was observed under salinity stress. Our results showed that total phenolic content was increased by increasing NaCl levels. A previous research declared that by increasing the concentrations of salinity, the amount of total phenolic content significantly increases (Akbari et al. 2018). Also, it has been shown that salinity stress can also change the plants phenolic compounds which depend on the salt sensibility of plants (Yuan et al. 2010). The accumulation of total phenolic content under salinity stress might be related to its synthesis pathway. Phenolic compounds in the plant are generally generated by the phenylpropanoid pathway, and this pathway can be induced under salinity tension conditions (Lim et al. 2012). Under salinity stress conditions, plants induce several phytohormones, such as methyl jasmonic acid and jasmonic acid, in which they induce phenylalanine ammonia lyase. In plants, phenylalanine ammonia lyase is the main enzyme in the phenolic metabolism, resulting in the phenolic compounds accumulation (Rebey et al. 2017).

In this research, flavonoid content was evaluated under different concentrations of salinity and with increasing concentration of NaCl, flavonoid content increased in all samples. Additionally, variance analysis showed a significant difference between flavonoid content in all NaCl concentrations. The results of flavonoid content are completely similar to the previous results reported by (Gengmao et al. 2015; Ahmad et al. 2017). Gengmao et al. showed that with increasing concentration of salinity stress up to 150 mM NaCl, flavonoid content has increased in Safflower plant (Gengmao et al. 2015). Also, it was specified that under abiotic stress environments, plants increase the synthesis of flavonoids to fight against the unfavorable conditions of

stress (Sharma et al. 2019b). In the same article, Sharma et al. showed that salinity, drought, cold, UV, and heavy metal stresses are able to stimulate the phenolic biosynthetic pathways, which finally result in enhanced abiotic stress tolerance (Sharma et al. 2019b). In response to salinity stress, plants use secondary metabolites to eliminate and enervate the damaging effects of salt stress. Oxidative defense metabolism, compatible solutes, inorganic nutrients, hormonal regulation as well as secondary metabolites are used in response to salinity stress by plants (Parida and Das 2005; Duan et al. 2008; Ashraf 2009; Noreen et al. 2010; Mittler 2002). Flavonoids are one of the most important secondary metabolites which reduce destructive effects of osmotic stress such as salinity (Buer et al. 2010). Flavonoids accumulate in vacuoles, chloroplasts, and nuclei and vacuole flavonoids interfere in detoxification of hydrogen peroxide (Shoeva et al. 2016).

In this research, variance analysis showed that the effect of NaCl < 100 mM on anthocyanin content is meaningful and the comparison between means proved that with increasing concentration of NaCl, anthocyanin content increased in all treatments. Numerous researches proved our results (Piao et al. 2001; Parida and Das 2005; Hughes et al. 2010; Keutgen and Pawelzik 2007; Eraslan et al. 2008). One of the flavonoids is anthocyanin, which has a positive charge at the oxygen atom of the C-ring of basic flavonoid construction (Khoo et al. 2017). Also, Anthocyanins are a large class of water-soluble flavonoid pigments that meaningfully influence how leaves reply to abiotic stresses. Anthocyanins are involved in tolerance to stresses such as drought, salinity, heavy metal, UV-B, as well as resistance to biotic stresses like herbivores and pathogens (Landi et al. 2015). Also, anthocyanin is one of the major plant pigments of which its content presents a valuable index of the plant's physiological condition especially under osmotic stress in leaves and sprouts (Gitelson and Merzlyak 2004). Anthocyanins are involved in photoprotection of chlorophyll and in response to osmotic stresses such as salinity in plants (Gould et al. 2000; Landi et al. 2015). Also, in a review paper, Sharma et al. concluded that in plants, tolerance increasing is correlated with the multiple functions of polyphenols, commonly including capacity of several polyphenol classes to preserve the plant from visible light (anthocyanins) and excessive light such as UV (flavonoids) and/or in their ROS scavenging ability (Sharma et al. 2019b).

In this study, total free amino acids content increased with increasing concentrations of NaCl in all samples. Cusido et al. in a study have shown that in *Nicotiana rustica* L. and under salinity stress, the contents of free amino acids increased, significantly (Cusido et al. 1987). In this study, probably protein synthesis disruption is the main reason of total free amino acids increasing under salinity stress, and most likely, total free amino acids increasing occurs

for osmotic adjustment under Na^+ toxicity and low water potential.

Total soluble carbohydrate was elevated by salinity stress in 14-day-old *L. draba* sprouts. Under salinity stress in rice, content of sugar increased compared with those in the control plants, significantly (Amirjani 2011; Pattanagul and Thit isaksakul 2008). Also in chickpea plants, total soluble carbohydrate increased under salinity stress (Ahmad et al. 2016). It is proven that plants in order to decrease the osmotic stress produced under salinity stress, increase the contents of sugars along with other compatible solutes (Dubey and Singh 1999), which allow the plants to support their fundamental metabolism under stressed conditions (Hurry et al. 1995). Likely, increasing total soluble carbohydrate in this study can help *L. draba* to synthesize more protein and enhance its growth under stress conditions.

In the current study, the SFN content and expression of *CYP79F1* gene were investigated under different concentrations of NaCl. Our results showed that NaCl treatment has a significant effect on SFN content as well as the expression level of *CYP79F1* gene. SFN content and *CYP79F1* gene expression increased with enhancement of NaCl levels up to 75 mM and after that decreased. Guo et al. reported that SFN content in concentration of 100 mM NaCl in broccoli sprouts has a significant increase in comparison with the control condition which is very similar to the results of this study (Guo et al. 2013). No report is available for expression of *CYP79F1* under salinity stress, but *CYP79F1* gene that is involved in biosynthesis of aliphatic glucosinolates was induced by methyl jasmonate (Mikkelsen et al. 2003). Additionally, the same article showed that after methyl jasmonate treatment, the concentration of long-chain aliphatic glucosinolates increases (Mikkelsen et al. 2003; Borek et al. 1998).

Cytochromes are a big gene family that has the ability to metabolize insecticides and poisonous secondary metabolites (Feyereisen 1999; Mao et al. 2011). Cytochromes P450 are key players in the biosynthesis of glucosinolates (Nafisi et al. 2006). Glucosinolates have a defensive role in plants (Pedras et al. 2009) and are accumulated in the cell vacuole and released in reaction to cell injuries or pathogens attack. When glucosinolates are exposed to myrosinase, they are hydrolyzed by myrosinase and produce glucose and aglycone, and aglycone can be converted to thiocyanates, isothiocyanates, and nitriles (Rask et al. 2000). One of the most important isothiocyanates is SFN, which can be produced through glucoraphanin hydrolysis via myrosinase activity (Gu et al. 2012). Five cytochrome P450 (CYP) gene products, *CYP79F1* and *CYP79F2*, *CYP79B2* and *CYP79B3*, *CYP79A2*, have been shown to catalyze the conversion of short- and long-chain-elongated methionine substrates (Chen et al. 2003b; Hansen et al. 2001), tryptophan (Mikkelsen et al. 2000; Hull et al. 2000), and phenylalanine (Wittstock and Halkier 2000) to the corresponding

aldoximes, respectively. Also, *CYP79F2* and *CYP79F1* play distinct roles in physiological effects of the accumulation of short- and long-chain derivatives of methionine in the mutants (Hansen et al. 2001). SFN that is derived from glucoraphanin involves in many mechanisms including inhibition of phase 1 enzymes (Clarke et al. 2008), and induction of phase 2 enzymes that detoxify carcinogens (Fahey et al. 2001). Also, Devi and Thangam (2012) showed that SFN possesses anti-proliferative effects. This increase of total aliphatic glucosinolate under salt stress could be due to the osmotic adjustment under low water potential (Qasim et al. 2003) and maybe increasing levels of aliphatic glucosinolate occur as a result of the decline in the compensation of indole glucosinolate. Therefore, it may be concluded that changes in SFN content can be affected by myrosinase activity and/or glucoraphanin content by NaCl. Also, in this study, increasing *CYP79F1* gene expression, which was induced by NaCl treatment, could be a part of response to make the plant more resistant to stress.

Conclusion

The results of this study proved that salinity stress is able to reduce the germination percentage of different ecotypes, but ecotypes which grow in warmer temperatures have higher germination percentage. Also, our results showed that shoot and root lengths reduce in response to the salinity stress. Based on our results, in response to salinity stress, enzymatic antioxidant defense system including the activities of APX, GPX, and SOD and non-enzymatic antioxidant defense systems including the contents of total phenolic, total flavonoid, total anthocyanin, total free amino acids, total soluble carbohydrate, and SFN are extremely activated to reduce the destructive effects of salinity in 14-day-old *L. draba* sprouts. From the results of this study, it can be concluded that increasing Na^+ and Cl^- concentrations are the main reasons of decreasing P, Ca^{2+} , and K^+ ions in this plant. Increasing contents of some oxidative stress parameters such as electrolyte leakage, malondialdehyde, other aldehydes, and hydrogen peroxide in this study showed the destructive effects of sodium toxicity and osmotic stress in the 14-day-old *L. draba* sprouts. According to the results, SFN content increasing under salt stress may be due to the osmotic adjustment under low water potential and sodium toxicity by aliphatic glucosinolate degradation via myrosinase activity. Also, similar patterns of SFN content and *CYP79F1* expression level were observed upon salinity stress in this plant. Finally, according to the previous and present studies, it can be concluded that the amount of sodium chloride, the time of treatment and ecotype have a remarkable effect on the contents of valuable plant components.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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