

Glycine betaine affects the antioxidant system and ion accumulation and reduces salinity-induced damage in safflower seedlings

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Abstract: Safflower (*Carthamus tinctorius* L.) is an important oilseed crop, usually grown on a small scale and in salt-affected soils. Salinity stress can cause oxidative damage to plants. Upregulation of the antioxidant defense system induced by glycine betaine (GlyBet) alleviates the damaging effects of oxidative stress in plants. In the present investigation, seeds were treated with 0, 10, 30 and 60 mM of GlyBet solutions. Germination and the primary growth of the seedling were examined using sodium chloride salt (NaCl) at 0 (non-stress), 50, 100 and 150 mM concentrations. The obtained results indicate that at 50 and 100 mM NaCl, priming with 30 and 60 mM GlyBet increased root and shoot lengths compared to the control (0 mM). In addition, at all stress levels, priming with 60 mM GlyBet led to lower malondialdehyde, total soluble sugars and proline contents than in control seedlings. Priming with GlyBet increased catalase (CAT), superoxide dismutase (SOD) enzyme activities and protein content, while it reduced the activity of peroxidase (POD) under salinity stress. In addition, priming with GlyBet reduced the Na⁺/K⁺ ratio of seedlings and increased K⁺ under all salinity stress levels. Priming with 60 mM GlyBet also reduced the Na⁺ content under 150 mM NaCl. Together, these results show that 60 mM GlyBet had the most pronounced effect on tolerance to salinity stress in safflower seedling. The GlyBet-increased tolerance to salt in safflower was mainly related to increased CAT and SOD activities, and the prevention of cell membrane damage as a result of reduced lipid peroxidation and improved ion homeostasis under salinity stress condition.

Key words: enzyme activity; germination; glycine betaine; safflower; salt stress

INTRODUCTION

Salinity is one of the major environmental stresses presenting a serious threat to field crops in many parts of the world, especially arid and semiarid regions. Salinity can cause water stress, malnutrition and accumulation of excess ions to potentially toxic levels in plants. Salt tolerance can differ dramatically between species/varieties and ontogenetic stages and is usually reflected by a reduction in biomass production, yield or survival rates [1]. Salinity influences seed germination, decreases node formation, retards plant development and decreases crop yield [2]. Seed germination is the first critical and the most sensitive process in plant growth [3]. Salt-induced inhibition of seed germination could be related to specific ion toxicity or to osmotic stress. Seedling establishment is compromised in seeds exposed to salinity stress conditions [4].

Osmolytes such as glycine betaine (GlyBet), proline, sugars, polyols and amino acids are synthesized in surplus in response to stress [5]. These osmotic adjustments maintain subcellular structures and decrease oxidative damage caused by free radicals produced in response to high salinity [6]. It has been shown that the biosynthesis of GlyBet is stress-inducible [7] and the level of accumulated GlyBet is dependent on the degree of salt tolerance [8]. Also, an exogenous supply of GlyBet increases the salt tolerance of a few plants that are otherwise unable to accumulate GlyBet [9].

The main role of GlyBet in plants exposed to saline soil is considered to be maintaining osmotic regulation in cells [10]. It was suggested that GlyBet supports various transporters for normal functioning under salt stress. From this, it can be proposed that GlyBet has a protective effect in discriminating Na⁺ against K⁺ under saline states [11]. The second pos-

sible role of GlyBet is that it might enhance vacuolar efficiency in the roots of salt-stressed plants for accumulating more Na^+ , as has earlier been reported in rice [12]. Gadallah [10] reported that GlyBet improves germination and seedling growth of many crops under stressful condition. Plants treated with GlyBet also maintain higher antioxidative enzyme activities that minimize oxidative stress [13]. Hu et al. [14] showed that exogenous application of GlyBet increased catalase (CAT) and superoxide dismutase (SOD) activity and K^+ content in perennial ryegrass under salinity stress conditions. Also, they reported that the application of GlyBet reduced Na^+ , malondialdehyde (MDA) and proline content in salt-stressed plants.

Safflower is commonly known as a moderately salt-tolerant crop and is grown in arid and semi-arid zones of the world where salinity can limit the growth of many other crops [15]. Dried seeds of safflower contain 40% protein, 21% oil, 34% carbohydrates and 5% ash. It is a very high-quality seed with a high economic performance [16]. Safflower is mostly cultivated for vegetable oil, which is considered as one of the best oils for human consumption regarding its high levels (70-75%) of linoleic acid or oleic acid [17]. The objectives of the present study were to assess the effectiveness of exogenously applied GlyBet on germination, seedling growth and some biochemical and physiological characteristics of safflower under salinity stress condition.

MATERIALS AND METHODS

Growth conditions and treatments

The safflower (*Carthamus tinctorius* L.) seeds were presoaked in solutions of different concentrations (0-distilled water, 10, 30 and 60 mM) of GlyBet for 8 h at 25°C. After presoaking, the seeds were surface-dried on filter paper and then allowed to air-dry at room temperature. Three replicates of 50 seeds were placed on two layers of filter paper (Whatman No. 1) in 9-cm diameter Petri dishes. Five ml of saline solutions containing 50, 100 and 150 mM NaCl were applied in each Petri dish to impose salinity stress, while deionized water was applied for non-saline conditions. Petri dishes were incubated at $20 \pm 1^\circ\text{C}$ under a 16/8 h regime. After 6 days, the number of total germinated seeds, and length and weight of shoots

and roots were measured. Samples of seedlings (25 seedlings per sample) were frozen in liquid nitrogen and stored at -80°C until biochemical analyses were done. The germination rate was calculated according to the equation of Ramana et al. [18]:

$$\text{GR} = \text{N1/D1} + \text{N2/D2} + \dots + \text{Ni/Di}$$

where, Ni is the number of germinated seeds per day (Di).

Determination of proline

Proline was determined following the ninhydrin method described by Bates et al. [19], using L-proline as a standard. About 0.2 g of frozen seedlings were homogenized in 3% sulfosalicylic acid, and the homogenate was centrifuged at 10000 rpm. The supernatant was used for the estimation of proline content. The reaction mixture, consisting of 2 mL of acid ninhydrin and 2 mL of glacial acetic acid, was heated at 100°C for 1 h. The reaction mixture was extracted with 4 mL of toluene and absorbance was read at 520 nm.

Determination of malondialdehyde (MDA)

For determination of MDA, frozen seedlings (0.2 g) were homogenized in an aqueous solution of 5% (w:v) trichloroacetic acid, and the homogenate was centrifuged at 14000 rpm for 20 min. Aliquots of the supernatant were heated in 0.25% thiobarbituric acid. The amount of MDA was determined from the absorbance at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated by using the extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ [20].

Total soluble sugar determination

In order to determine the content of soluble sugars frozen seedlings (0.1 g) were homogenized with 3 mL of 95% ethanol. Then the extracts were centrifuged at 3000 rpm for 10 min. The supernatant was then removed, and the content of total soluble sugars was analyzed in reactions containing 2.5 mL of concentrated sulfuric acid (96%), 0.5 mL of phenol (5%) and 50 μL from the extract. The absorbance was measured at 490 nm with standard glucose solutions from 0-100

mg/L [21] and expressed in milligrams of glucose per gram of fresh matter (mg/g FW).

Determination of antioxidant enzyme activities

Frozen seedlings (0.2 g) were homogenized at 4°C in a mortar with 4 mL of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 20000 rpm for 20 min at 4°C. The supernatant was used for determination of enzyme activities and protein content. Total protein content was determined using bovine serum albumin as a standard according to the method described by Bradford [22]. Activities of CAT and POD were measured using the method of Cakmak and Marschner [23] and Pandolfini et al. [24] with some modification. The CAT reaction solution contained 50 mM potassium phosphate buffer (pH 6.8), 10 mM of H₂O₂ and protein extract. The decrease in absorbance was recorded at 240 nm for 1 min. CAT activity of the extract was expressed as unit mg⁻¹ protein min⁻¹ (1 unit = 1 mM of H₂O₂ reduction mg⁻¹ protein min⁻¹). The POD reaction mixture contained 100 µL of protein extract, 1900 µL 50 mM potassium phosphate buffer (pH 6.8), 500 µL 28mM guaiacol and 500 µL 5 mM H₂O₂. The increase in absorbance was recorded at 470 nm for 1 min (U-2000, Hitachi Instruments, Tokyo, Japan). POD activity of the extract was expressed as unit mg⁻¹ protein min⁻¹. One unit of POD was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaiacol per min. The activity of SOD was determined using the method described by Giannopolitis and Ries [25] with some modifications. The reaction solution contained 2300 µL 50 mM potassium phosphate buffer (pH 7.8), 100 µL 12 mM L-methionine, 100 µL 1 mM riboflavin, 100 µL 0.1 mM EDTA (pH 7.8), 100 µL 50 mM calcium carbonate, 100 µL 75 mM nitroblue tetrazolium (NBT) and 200 µL of the protein extract in a final volume of 3 ml. The test tubes containing the reaction mixture were irradiated under the light of white fluorescent lamps (120 W) at a distance of 40 cm for 15 min. The absorbance of the irradiated solution at 560 nm was determined with a spectrophotometer. Tubes without extract and containing potassium phosphate buffer, were either exposed or not exposed to light, and served as blanks in the reaction. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT.

Determination of Na⁺ and K⁺ ion contents

The seedlings were dried at 60°C for 48 h. One g of samples was powdered and burned in 550°C for 6 h to obtain ashes, which were subsequently digested in 10 mL of 1 N HCl. The concentration of Na⁺ and K⁺ in the digested samples was determined using a flame photometer (Model PFP7, Germany).

Statistical analysis

The experiments were performed in a completely randomized design arranged as a factorial with three replications. Mean comparison was performed using the LSD test at the 5% level of significance (P<0.05). The statistical analysis was performed using SAS software.

RESULTS

Germination characteristics

Salinity reduced the germination rate as the NaCl level increased (Fig. 1). However, both in non-stress and all of the salinity stress levels, priming with high levels of GlyBet (30 and 60 mM) resulted in an increase in germination rate in comparison the control without GlyBet. In non-stress conditions, seeds primed with 60 mM GlyBet showed a 65% germination increase as compared to the control. At the highest level of stress (150 mM NaCl), priming with 60 mM GlyBet resulted in a 23% increase in the germination rate (Fig. 1).

Salinity reduced the length of shoots and roots as the NaCl level increased. At non-stress conditions, priming with 30 and 60 mM GlyBet increased the shoot length in comparison with the control (Fig. 2A). At non-stress conditions, priming with all of concentrations of GlyBet increased the root length. In 50 and 100 mM NaCl, priming with 30 and 60 mM GlyBet concentrations increased the root length and in 150 mM NaCl, priming with all of the GlyBet concentrations also increased it compared to the control (Fig. 2B). As the NaCl concentration increased, the shoot and root dry weight decreased. At non-stress conditions, priming with all of the GlyBet amounts increased the shoot dry weight. In 50 and 100 mM NaCl, priming with 30 and 60 mM GlyBet levels increased

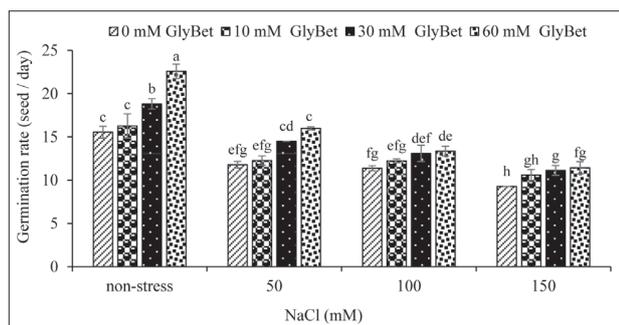


Fig 1. Effects of GlyBet pretreatment and NaCl concentrations on germination rate of safflower. Values are means \pm SE obtained from three replicates. Means followed by the same letter are not significantly different at $P < 0.05$ according to LSD test.

the shoot dry weight and in 150 mM NaCl, priming with all of GlyBet levels increased the dry weight (Fig. 2C). Priming with GlyBet at all tested concentrations increased the root dry weight compared to the control under non-stress and stress conditions (Fig. 2D).

Biochemical characteristics

As the NaCl concentrations increased, the MDA content increased as well. Under all of the stress conditions, priming with GlyBet reduced the MDA content

compared to the control, and the largest reduction was in seedlings pretreated with 60 mM GlyBet (Fig. 3A). The total soluble sugars content increased with the increase in NaCl concentrations, and proline content slightly increased with the increase in NaCl concentrations. In non-stress and salinity stress conditions, priming with GlyBet concentrations increased total soluble sugars and proline content as compared to the control (Fig. 3B, C).

Antioxidant enzyme activities

As the NaCl concentrations increased, the activity of SOD increased, whereas POD activity and protein decreased. Also, salinity slightly increased CAT activity. At all stress levels, priming with GlyBet increased CAT and SOD activity (Fig. 4A, B). Priming with 30 and 60 mM GlyBet increased CAT activity compared to the control under non-stress conditions and with 50 mM NaCl. In 100 and 150 mM NaCl, priming with all of GlyBet levels increased CAT activity (Fig. 4A). SOD activity increased at 30 and 60 mM GlyBet compared to the control under non-stress conditions and 50 and 100 mM NaCl. At 150 mM NaCl, priming with GlyBet at all tested concentrations increased SOD ac-

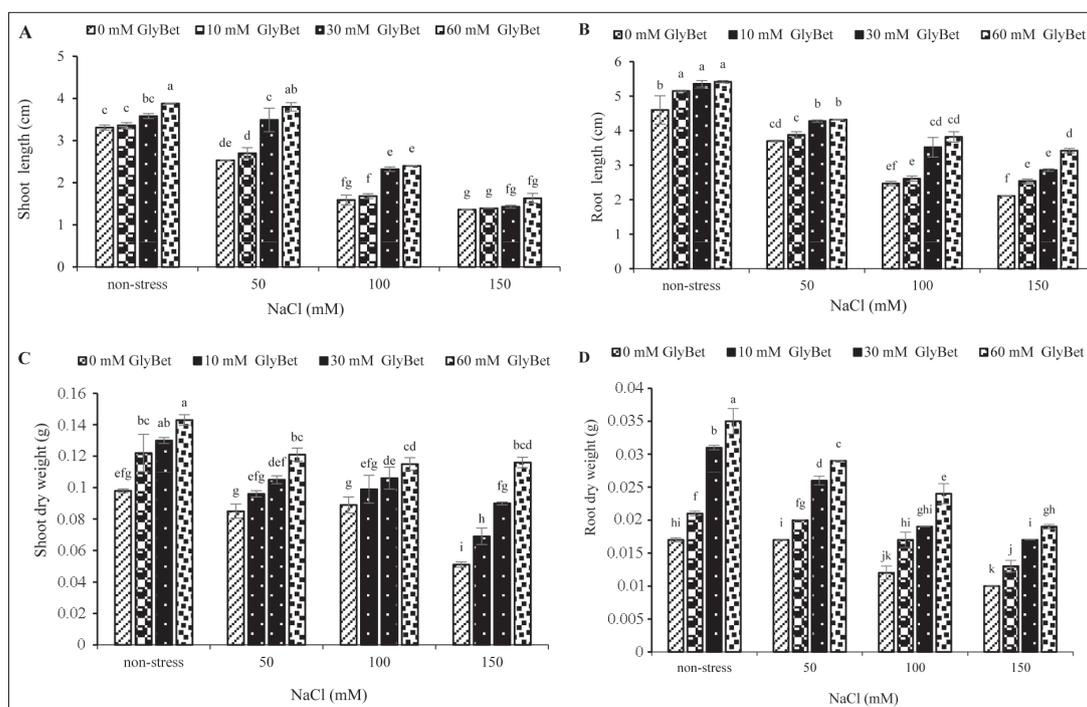


Fig 2. Effects of GlyBet pretreatment and NaCl concentrations on shoot length (A), root length (B) and shoot dry weight (C), root dry weight (D) of safflower. Values are means \pm SE obtained from three replicates. Means followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test.

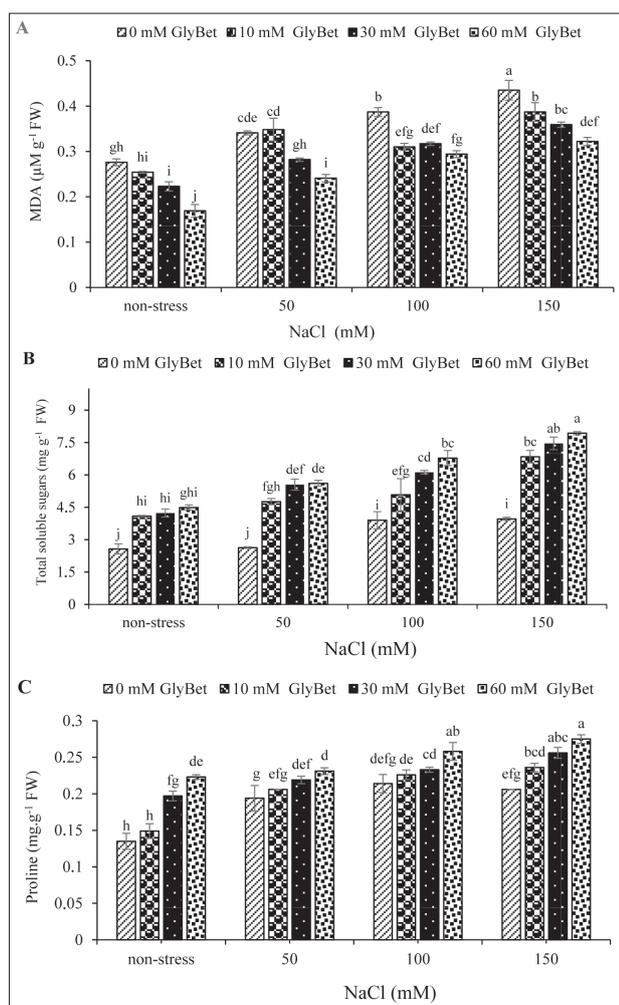


Fig. 3. Effects of GlyBet pretreatment and NaCl concentrations on MDA (A), total soluble sugars (B) and proline (C) concentrations in safflower. Values are means±SE obtained from three replicates. Means followed by the same letter are not significantly different at $P < 0.05$ according to LSD test

tivity (Fig. 4B). The highest CAT and SOD activities were observed at 150 mM NaCl and 60 mM GlyBet. The lowest CAT and SOD activities were observed at non-stress condition and in the control (Fig. 4A, B).

Under non-stress and salinity stress conditions, priming with 60 mM GlyBet decreased POD activity compared to the control (Fig. 4C). Priming with all of the GlyBet concentrations increased the protein content as compared to the control under non-stress and salinity stress conditions (Fig. 4D).

Ion content

Priming with 30 and 60 mM GlyBet reduced the content of Na^+ of seedlings under 50 and 100 mM NaCl concentrations. In 150 mM NaCl, priming with 60 mM GlyBet reduced the Na^+ content (Fig. 5A). At all stress levels, priming with GlyBet at all tested concentrations increased K^+ content and decreased the Na^+/K^+ ratio compared to the control. The highest Na^+ concentration and Na^+/K^+ ratio were obtained in the control and 150 mM NaCl (Fig. 5A, C). The highest amount of K^+ was observed in 60 mM GlyBet under non-stress conditions (Fig. 5B).

DISCUSSION

The results of the study showed that an increase in salinity reduced the germination rate and growth of safflower seedlings. Other researchers also described a reduction in the germination rate and growth seedling with increasing salinity stress levels in wheat [26,27]. Salinity stress can influence seed germination through a decrease in water absorption leading to moisture stress (osmotic effect), by ion toxicity and/or ionic imbalance, or by the accumulation of Na^+ and Cl^- ions. Also, salinity stress through inhibition of the absorption of several necessary nutrients such as K^+ causes nutritional imbalance in plants and/or accumulation of several parameters [28]. The reduction in root and shoot development may be due to the toxic effects of the NaCl used as well as by unbalanced nutrient absorption by the seedlings [29]. Our results showed that priming with high levels of GlyBet (30 and 60 mM) increased the germination rate and growth of seedlings when compared to unprimed under salinity stress. The application of exogenous GlyBet has an important role in water availability for the imbibition of germinating seeds during limited water [30]. GlyBet also increased seed germination under increased salt and control conditions by enhancing the osmotic pressure [7]. Our results are in agreement with Kausar et al. [31] who reported that the exogenous application of GlyBet also increased the fresh and dry weight of roots and shoots in maize under salinity stress.

MDA is commonly measured as the end-product of lipid peroxidation and is increased during salinity

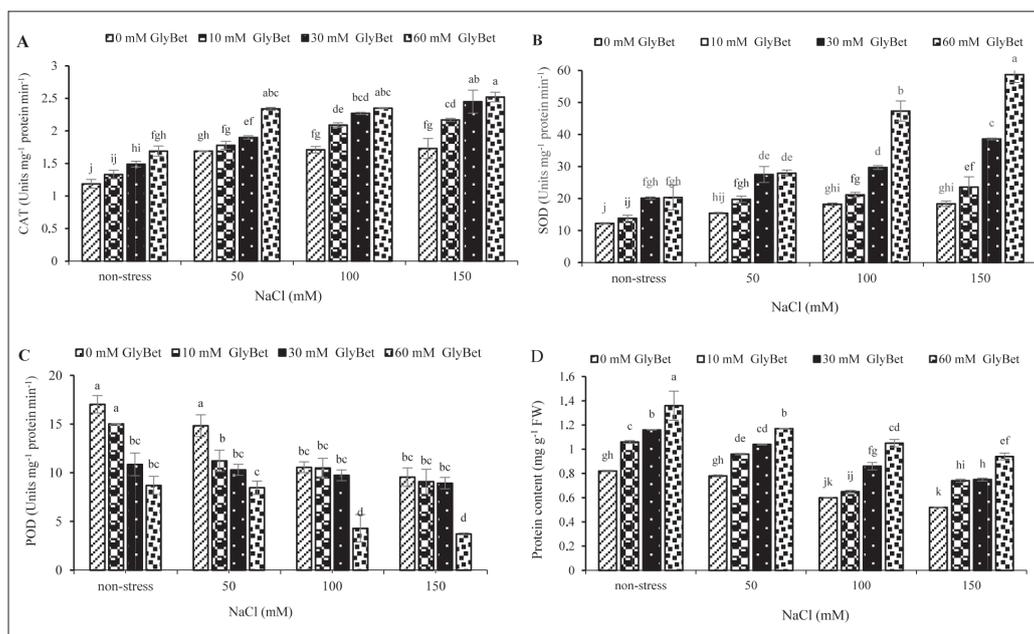


Fig 4. Effects of GlyBet pretreatment and NaCl concentrations on catalase (A), SOD (B), POD (C) and protein (D) in safflower. Values are means \pm SE obtained from three replicates. Means followed by the same letter are not significantly different at $P < 0.05$ according to the LSD test.

stress. Membrane lipid peroxidation is an indication of membrane damage and leakage under salt stress conditions [32]. In this study, as the salinity level increased, lipid peroxidation increased and the application/administration of GlyBet at high concentrations (30 and 60 mM) decreased the MDA content under salinity stress. Increased lipid peroxidation has been reported in other plants like safflower [33], wheat [34], tomato [35] and purslane [36] under salinity stress conditions. Hu et al. [14] suggested that GlyBet alleviated lipid peroxidation and facilitated the protection of membrane functions under salinity stress. This facilitation could be attributed to the GlyBet-induced antioxidant responses that support the plant from oxidative damage [5].

In this research, total soluble sugars and proline content increased with increasing salinity stress levels. The current results are in accordance with the findings of Erdal et al. [33], who showed that salinity stress increased the content of proline and soluble sugars in safflower. Soluble sugars play a significant role in the osmotic adjustment of cells during reproduction and stress conditions [37]. Proline acts as an osmoprotectant and is associated with the mechanism of salt tolerance under salinity stresses. Our results showed that priming with GlyBet led to an increase in total soluble sugars and proline content during the salinity

stress. Similarly, Demiral and Türkan [38] and Heuer [39] reported the GlyBet application increased proline content in rice and tomato under salinity stress. Also, Ibrahim and Aldesuquy [40] and Aldesuquy et al. [41] found that GlyBet led to the accumulation of more soluble sugars in sorghum and wheat under drought stress.

Reactive oxygen species (ROS) are toxic radicals and molecules that cause oxidative damage to DNA, proteins and lipids. Also, ROS function as signaling molecules that mediate various physiological processes, including programmed cell death and defense against biotic and abiotic stresses [42]. Salinity stress can stimulate ROS accumulation [43]. Plants protect themselves against oxidative stress-induced damage by their antioxidant defense systems, which include enzymatic and nonenzymatic antioxidants [44]. GlyBet protects the photosynthetic machinery by stabilizing the activity of repair proteins under high concentrations of NaCl [45]. Hoque et al. [5] reported that GlyBet at physiological concentrations provides a direct protective effect on antioxidant enzyme activities under salinity stress.

Our results suggest that salinity slightly increased CAT activity and SOD activity increased with increas-

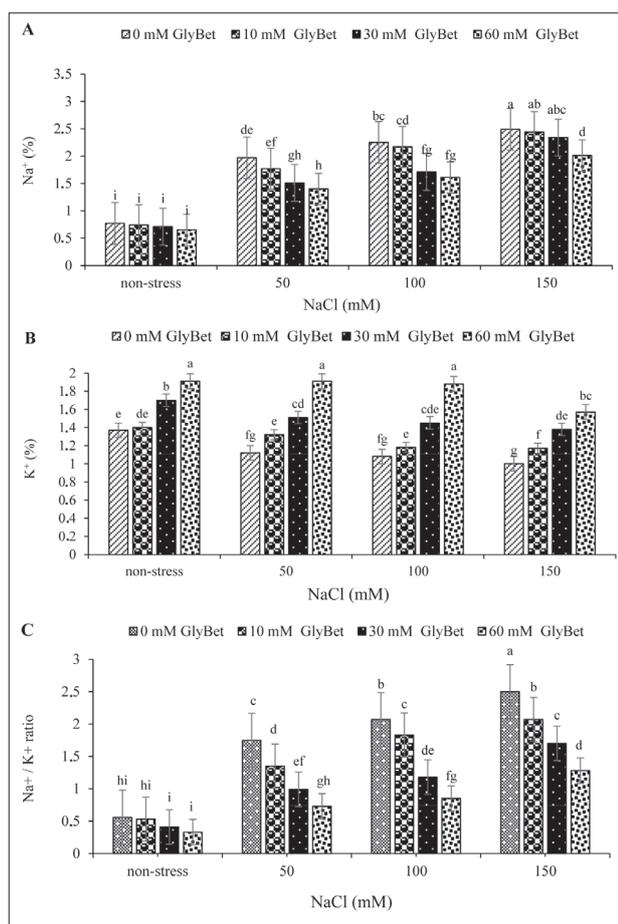


Fig 5. Effects of GlyBet pretreatment and NaCl concentrations on Na⁺ (A), K⁺ (B) and Na⁺/K⁺ ratio in safflower. Values are means±SE obtained from three replicates. Means followed by the same letter are not significantly different at P<0.05 according to LSD test.

ing NaCl concentrations. Also, GlyBet had a positive effect on the activity of the enzymes and increased their activity. With increasing salinity and GlyBet concentrations, POD activity decreased. Antioxidant enzyme activities increased in some crops such as pea [46] and wheat [47,48] under salinity stress. Kaya et al. [49] reported that salinity increased polyphenol oxidase (PPO) activity, but decreased POD and CAT activities in maize. Exogenous GlyBet alleviated the effect of salinity, most likely by scavenging free radicals and protecting antioxidant enzymes [5]. Chen and Murata [50] suggested that GlyBet overcome the deleterious effects of oxidative stress by activating or stabilizing ROS-scavenging enzymes and/or repressing the production of ROS by an unknown mechanism. Demiral and Türkan [38] have shown that exogenous

application of GlyBet enhances CAT activity in a salt-sensitive rice cultivar under high salinity stress.

In this study, priming with 60 mM GlyBet decreased Na⁺ at all concentrations of salt. With increasing NaCl concentrations and GlyBet concentrations, the K⁺ content increased and the Na⁺/K⁺ ratio decreased. Cuin and Shabala [51,52] suggested that compatible solutes, and GlyBet in particular, regulate the net fluxes of Na⁺ and K⁺ across the plasma membrane at the cellular level in response to NaCl stress. Sobahan et al. [53] reported that GlyBet may play a role in maintaining cytosolic K⁺ homeostasis by suppressing Na⁺-increased apoplastic flow to decrease Na⁺ absorption.

CONCLUSIONS

The present study revealed that priming safflower seeds with GlyBet can enhance the plant's tolerance to salt stress during the germination stage, and the higher concentration of GlyBet (60 mM) was more effective than the other concentrations. The results of this research can be a useful step toward eliminating the effects of salinity stress in the primary stages of plant growth, and the exogenous application of GlyBet appears to be a promising approach in safflower. However, considering the fact that the study was carried out in the germination stage, it is essential to assess the effects of GlyBet in the next stages of growth in the greenhouse and field.

Authors' contribution: Farnaz Alasvandyari performed the experiments and wrote the paper. Batool Mahdavi and Shahab Mahdavi Hosseini designed and organized the study and participated in writing the paper. All authors have read and approved the final manuscript.

Conflict of interest disclosure: There is no conflict of interests between the authors.

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