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GLYCINEBETAINE APPLICATION ALLEVIATES SALINITY DAMAGE TO ANTIOXIDANT ENZYME ACTIVITY IN ALFALFA

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Abstract

We investigated whether glycinebetaine (GB) application could alleviate salinity-induced damage in alfalfa (*Medicago sativa* L.). A hydroponic culture experiment was employed, and thirty-day-old alfalfa seedlings were subjected to 0, 5, 10, and 20 mM GB and exposed to a 1% salt regime, with a nutrient solution as the control. Marked decrease was detected in the vertical shoot growth rate (VSGR), vertical root growth rate (VRGR), plant biomass, and normalized relative transpiration (NRT) due to the salinity stress, and significant increase was also detected in MDA, soluble protein content, and SOD, CAT, and POD activities. The application of GB increased VSGR, VRGR, SOD activity, and decreased the MDA, soluble protein content, and Na^+/K^+ ratio compared to the treatment without GB application. Alfalfa treated with 5 mM GB exhibited higher VSGR, VRGR, plant biomass, NRT, SOD, POD, and lower CAT activity when compared to other levels of GB application. Moreover, lower MDA and soluble protein contents were also observed in the 5 mM GB application treatment relative to the non-GB treatment. These results indicated that the GB-enhanced salinity tolerance in alfalfa was attributed to the elevation in SOD and POD activities, and the decrements in the MDA and soluble protein contents. It could be concluded that the 5 mM GB application was the proper concentration to ameliorate the damage of salt on alfalfa.

Key words: Salinity tolerance, Antioxidant enzyme, Alfalfa, Glycinebetaine, Ionic equilibrium.

Introduction

Soil salinization has aroused worldwide concern due to the limitations imposed on plant growth, productivity, and distribution (Wang *et al*., 2013). According to Researchers the area of irrigated agricultural soil subjected to salt stress is approximately 20% (Viswanathan *et al*., 2005). The reductions in growth and development were the conspicuous inhibitions of photosynthetic capacity, water imbalance and ion homeostasis disruption in plants from salinity (Hussein *et al*., 2017). Fortunately, researchers had well documented that the excessive compatible organic solutes accumulation and the reactive oxygen species (ROS) detoxification were employed to deal with osmotic and oxidative stress by plants when subjected to salinity (AbdElgawad *et al*., 2016).

Plant salt resistance results from a complex network of mechanisms that involve biochemical and physiological processes, even morphological and developmental alteration. Growth inhibition was attributed to the overproduction of ROS in mitochondria and chloroplasts (Halliwel & Gutteridge, 1985), and severe cellular damage was induced due to the oxidation of lipid, protein, and and nucleic acids by ROS overproduction (Apel & Hirt, 2004). Fortunately, the antioxidant system that included enzymatic and non-enzymatic detoxification mechanisms was developed and evolved to alleviate this detrimental effects. O_2^- was catalyzed into H_2O_2 and O_2 by superoxide dismutase (SOD), and H_2O_2 was also further detoxified by catalase (CAT), peroxidase (POD) to H_2O and O_2 (Chen *et al.*, 2018). Therefore, efficient antioxidant system was utilized to cope with salt stress by various plants, and this correlation between antioxidant system and salt resistance was well documented (Kanwal *et al*., 2013).

Glycinebetaine (GB) is found in chloroplasts of certain halotolerant plants, was also detected in various plants when exposed to salinity regimes (Rontein *et al*., 2002; Hossain & Fujita, 2010). Previous studies demonstrated that GB, as an osmoregulator, maintain the membranes integrity richly to alleviate the detrimental effects by salinity, and this regulation mechanism was attributed to the structure stabilization, antioxidant enzymes guidance, and protein complexes generation equilibrium (Sakamoto & Murata, 2002). Saneoka *et al*., (1995) indicated that enhanced GB synthesis was vital to improve salt stress tolerance in maize. However, even GB could be naturally synthesized/ accumulated in various plants, the quantity was not sufficient to against the osmotic stress, especially under severe environmental stress (Sakamoto & Murata, 2002). GB application could improve salt tolerance, and was illustrated in various crops, e.g., lettuce (Shams *et al*., 2016), cotton (Lv *et al*., 2007), and rice (Sobahan *et al*., 2016). There were many literature reported that GB was an effective ameliorating agent, and pure GB was synthetised and utilized in plants against salt stress. However, a negative effect was also observed on the growth of tomato by exogenous GB (Heuer, 2003). Therefore, the response of plants to exogenous GB is dependent according to different plant species.

Alfalfa (*Medicago sativa* L.), with the characteristics of high production, excellent quality, especially with high protein content, is utilized worldwide as primary preferred legume forage crop (Forni *et al*., 2017). Previous researches were focused on antioxidant mechanisms induced by environmental stress in alfalfa, such as salt (Forni *et al*., 2017), drought (Tina *et al*., 2017), and heavy metals (Zhou *et al*., 2008). To date, systematic analyses of the effects on antioxidant modulation by exogenous GB application in alfalfa exposed to salt regime are scanty.

In this study, we investigated the ability of alfalfa to withstand NaCl damage by exogenous GB application, and, if present, the physiological mechanism for this alleviation of NaCl damage was established.

Material and Methods

Plant materials and growth conditions: Disposable plastic cups (upper diameter was 6.8 cm, and lower diameter was 4.8 cm, and the height was 7.4 cm) were used as the containers, and twenty seeds of alfalfa 'Magnum Salt' were sown in each cup filled with pre-treated sand (<1 mm, washing with 1 mol L^{-1} HCl). In order to drain excess water and maintain good soil aeration of each cup, holes of 5 mm diameter were drilled in the bottom. For germination, all cups were placed randomly and kept in growth chamber (the constant temperature for day/night was 25°C/20°C, the photo-period was 16 h, the relative humidity was 50±10%, and the quantum flux density was 300 μmol photons $m^{-2}s^{-1}$ at plant height). After germination, 1/2 Hoagland nutrient solution (Hoagland & Arnon, 1950) was irrigated every two days. After 30 days of cultivation, all seedlings were removed from the cups to rinse the roots thoroughly, and transplanted into Erlenmeyer flasks (250-mL) filled with 1/2 Hoagland's nutrient solution (added with CaO_2 to provide oxygen). Preservative film and silicone rubber were employed to seal the flasks, and the idea was to inhibit the evaporation losses of water and chemicals. Then, all flasks were wrapped with aluminium foil to avoid the algal growth. After 4 days of adaptation, the water loss was determined by weighting the plant-flask system individually after every 24 h (Yu *et al*., 2010). Then, similar transpiration rates were employed to divide all plant-flask systems into five groups for salt and GB treatments. Alfalfa seedlings were subjected to certain GB and salt levels. The five treatments were as follows: CK: control treatment with only nutrient solution, T1: 1% NaCl, T2: 5 mM GB+1% NaCl, T3: 10 mM GB+1% NaCl, and T4: 20 mM GB+1% NaCl. GB and NaCl were weighted and dissolved in 1/2 Hoagland's nutrient solution for treatments. Each treatment was performed in triplicate, and all cups were randomly arranged.

Measurements

Plant growth: Vertical shoot (or root) growth rates(VSGR/VRGR) were calculated as the *D*-value in average turf canopy height (or root length) that was divided by days, and the *D*-value was the difference between the finial and initial treatment in height/length and determined by using a ruler (Huang & Liu, 2009). After 4 days' cultivation after GB/NaCl treatment, plants were removed from the flasks, and the fresh weights of shoots and roots were determined separately.

Plant transpiration was measured according to the water loss of the plant-flask systems described by Yu *et al*., (2007). Under salt-stressed conditions, in order to identify the GB application effects on plants with different initial transpiration levels, the following equation was used:

$$NRT(C,t)(\%) = \frac{(1/n)\sum_{i=1}^{n} Ti(C,t)/Ti(C,0)}{(1/m)\sum_{j=1}^{m} Tj(C,t)/Tj(C,0)} \times 100$$

where *C*-solution concentration (mM); t-time period (days); T-absolute transpiration of the plants; i-replicate 1, 2, …, n; j-control 1, 2, …, m. The NRT of the control was always set at 100%.

Antioxidant enzymes, MDA, and soluble protein content: Fully expanded leaves were excised and weighted, 0.3 g sample was placed into pre-chilled mortar, and then homogenized by pre-chilled pestle with 4 mL 50-mM phosphate buffer (pre-treated with an ice bath, pH 7.0) for the determination of SOD, CAT, POD, MDA, and soluble protein content. The homogenate was transferred into 5-mL Eppendorf centrifuge tubes, and then centrifuged using refrigerated centrifuge (Eppendorf 5427 R, Germany) at 4°C for 15min (Centrifuge speed at 15,000 g). Subsequently, supernatant was collected, and the MDA content was determined following the procedure described by Heath & Packer (1968), and the soluble protein content content was determined in accordance with the protocol described by Bradford (1976). The activities of SOD, CAT, and POD were determined by the methods of Fu & Huang (2001), Chance & Maehly (1955) and Polle *et al*., (1994), respectively.

Na^+/K^+ ratio assay: Fresh leaf were oven-dried to constant weight at 80°C, and then ground into powder used mortar. 1.0 g sub-samples were weighted and digested with H_2SO_4 and H_2O_2 at 370°C until the solution was clear, and the Na and K contents were determined as following Mostofa *et al*., (2015), using a Flame Photometer and the Na^+/K^+ ratio was calculated.

GB content assay: To determine the GB content, approximately 0.15 g of dry tissue material were ground into a fine powder carefully, and then 20 mL deionised water was added and shaken at 25°C for 16 h. Then, 1 mL of 2 N H_2SO_4, 400 μL of KI_3 solution were added successively, and the samples were kept at 4°C for 24 h. Finally, the samples were centrifugated using refrigerated centrifuge at 0°C for 15min (Centrifuge speed at 15,000 g). The supernatant was collected carefully, and the

periodide crystals in 5 mL of 1,2-dichloroethane and kept at room temperature for 2 h. The absorbance was determined using spectrophotometer at 365 nm with ultraviolet-2600 (Lokhande & Nikam, 2010).

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Treatment means were separated using Fisher's least significant difference (LSD) test at a significance level of 0.05.

Results

Plants exposed to salt regimes exhibited a lower VSGR and VRGR relative to the control (Table 1). However, 5 mM GB application was significantly ameliorated the decrements of the VSGR and VRGR, while 10 mM and 20 mM GB application showed no marked effects on VSGR and VRGR relative to the treatment without GB application when exposed to salt regime. Salt stress reduced plant biomass by 13.04% when compared with the control. Interestingly, 5 mM GB application significantly improved the plant biomass relative to the treatment without GB application under salinity conditions. However, no significant alleviation effects were observed on plant biomass by the 10 mM and 20 mM GB treatments relative to the treatment without GB application when exposed to the salt regime. Meanwhile, lower plant biomass was detected in the 10 mM and 20 mM GB application treatments under salt stress when compared with the control.

Salt stress reduced the NRT significantly in alfalfa (Fig. 1). 5 mM GB application significantly enhanced the NRT relative to the treatment with non-GB application when exposed to salinity regimes. Notably, GB application increased the NRT significantly more than non-GB treatments regardless of the GB level when exposed to salt regimes at 1 day. As the treatment-period increased, higher GB application level had a negative effect on the NRT when exposed to the salt regime.

Plants treated with salt exhibited higher MDA contents than the control, with a 64.22% increment (Fig. 2). GB application alleviated the increment of the MDA contents regardless of the GB level compared to the treatment without GB application when exposed to salinity regime. No marked difference in MDA content was observed among GB application treatments under salt stress. Similar MDA contents were determined between the control and GB application treatment under salt stress.

A higher soluble protein content was detected in salt stress treatments (M1) than the control, and the increment was 29.27% (Fig. 3). GB application alleviated the increment in the soluble protein content compared to the non-GB treatment when exposed to the salt regime, and the decrements were 14.40% to 21.98%. Plants receiving 5 mM and 10 mM GB exhibited higher soluble protein contents than the control. However, similar soluble protein contents were detected in the treatment with 20 mM GB application and CK.

Salt treatment induced remarkable increment in SOD and POD activities relative to CK (Table 2). 5 mM GB application significantly enhanced SOD and POD activity when exposed to salinity regime. Plants with GB application that exposed to salinity regime exhibited higher SOD activity than those without GB treatment regardless of the application level, and the 5 mM GB application treatment had the highest SOD activity. Similarly, the highest POD activity was also investigated in the 5 mM GB application treatment under salt stress, and no marked differences in POD activity were found between M1 and M3 or M4. Plants exposed to the salt regime exhibited higher CAT activity as compared to the control. 5 mM and 10 mM GB application alleviated this increment in CAT activity significantly compared to the treatment without GB application when exposed to salt regime, and the decrements were 50.89% and 39.36%, respectively. However, exogenous application 20 mM GB exhibited no significant effect on the activity of CAT when exposed to salt regime.

Higher Na^+/K^+ ratios were observed in salt stress treatments (M1, M2, M3, M4) than the non-salt treatment (CK) (Fig. 4). GB application induced a significant decrease in the Na^+/K^+ ratio relative to the plants without GB application exposed to salt regimes. However, no remarkable differences were detected among treatments with GB application.

Endogenous GB in both shoots and roots were induced by salt stress regardless of the GB application level relative to CK (Table 3). Meanwhile, a dramatic increase of GB contents in alfalfa plants were observed when subjected to exogenous GB application under salt stress, and the effect was dose dependent. Roots accumulated more GB than shoots when plants were subjected salt stress, and similar GB contents were accumulated in shoots and roots in the 5 mM and 20 mM GB application treatments.

Table 1. Effect of glycinebetaine (GB) on the vertical shoot growth rate (VSGR), vertical root growth rate (VRGR), and biomass of alfalfa under salt-stressed or non-stressed conditions.

Treatment	VSGR (cm d^{-1})	VRGR (cm d^{-1})	Biomass (g)
CK	0.68a	0.44a	5.65a
M1	0.42c	0.23c	4.91bc
M2	0.59b	0.36b	5.27ab
M3	0.45c	0.21c	4.53c
M4	0.45c	0.23c	3.27d

Data are expressed as the means of three replicates (n=3). Means in a column followed by a different lower-case letter for each measurement are significant by Fisher's protected least significant difference test at $p = 0.05$

Table 2. Effect of glycinebetaine (GB) on the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase isozyme (POD) of alfalfa under salt-stressed or non-stressed conditions.

Treatment	SOD (U min^{-1} mg^{-1} protein)	CAT (U min^{-1} mg^{-1} protein)	POD (U min^{-1} mg^{-1} protein)
CK	9.88d	2.65bc c	340.46c
M1	12.15c	3.85a	1222.41b
M2	18.37a	1.89c	1629.25a
M3	17.46ab	2.34bc	1313.49b
M4	15.87b	3.30ab	1374.21b

Data are expressed as the means of three replicates (n = 3). Means in a column followed by a different lower-case letter for each measurement are significant by Fisher's protected least significant difference test at p = 0.05

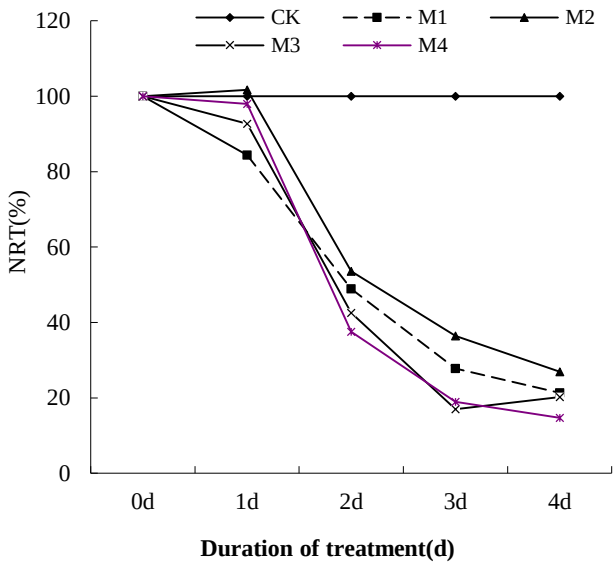

Fig. 1. Normalized relative transpiration (NRT) of alfalfa subjected to different levels of glycinebetaine (GB) applied to salt-stressed or non-stressed plants.

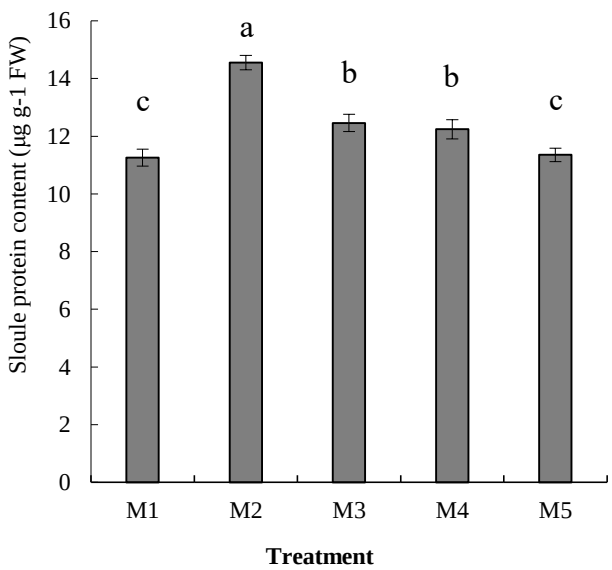

Fig. 3. Effects of glycinebetaine (GB) on the soluble protein content of alfalfa under salt-stressed or non-stressed conditions. Vertical bars on the top indicate SD, and bars with the same letter indicate no significant difference at p=0.05.

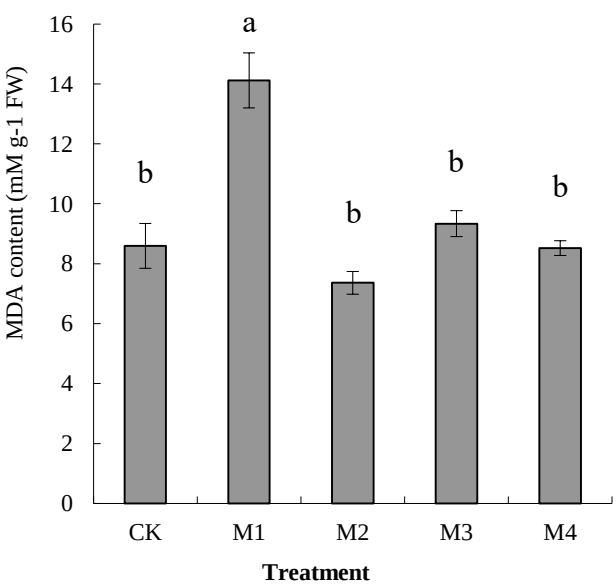

Fig. 2. Effects of glycinebetaine (GB) on the MDA content of alfalfa under salt-stressed or non-stressed conditions. Vertical bars on the top indicate SD, and bars with the same letter indicate no significant difference at p=0.05.

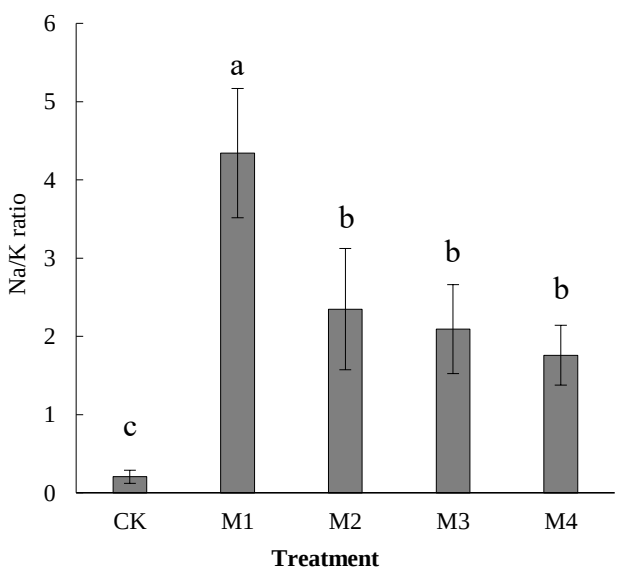

Fig. 4. Effects of glycinebetaine (GB) on the Na^+/K^+ ratio of alfalfa under salt-stressed or non-stressed conditions. Vertical bars on the top indicate SD, and bars with the different letter indicate significant difference at p=0.05.

Table 3. Effect of exogenous glycinebetaine (GB) on the GB content of shoots and roots of alfalfa under salt-stressed or non-stressed conditions.

Treatment	Shoots (mg·g^{-1} DW)	Roots (mg·g^{-1} DW)
CK	2.26Ae	2.86Ae
M1	6.32Bd	8.16Ad
M2	13.58Ac	19.10Ac
M3	18.62Bb	26.64Ab
M4	25.76Aa	31.80Aa

Data are expressed as the means of three replicates (n = 3). Means in a column followed by a different lower-case letter for each measurement are significant; means in a row followed the same upper-case letters for each tissue are not significant by Fisher's protected least significant difference test at $p = 0.05$

Discussion

Previous studies indicated that endogenous GB was accumulated to ameliorate the detrimental damage induced by adversity stress in various plants, eg. perennial ryegrass (Hu *et al*., 2012), lettuce (Yildirim *et al*., 2015), wheat (Raza *et al*., 2007), and tomato (Wutipraditkul *et al*., 2015). Our research has clearly illustrated that alfalfa accumulated GB both in shoots and roots when exposed to salt stress. The results of our research also illustrated that 5 mM GB application effectively alleviated the damage induced by salinity. Similar alleviation effects by exogenous GB application were reported on the grown development of plants when exposed to heavy metal (Ali *et al*., 2015), drought (Osman, 2015), and heat (Sorwong & Sakhonwasee, 2015) regimes.

Plant growth has been illustrated to be sensitive to salinity (Soylemez *et al*., 2017). In the present research, both the VSGR and VRGR were inhibited under salt stress regardless of GB application (Table 1). Plant roots, as the first organ to sense salinity, the growth was more inhibited than shoots due to the direct suppression of the root meristem activity that modulated elongation and hair formation (Liu *et al*., 2015). 5 mM GB treated plants had higher biomass and increased VSGR and VRGR relative to the treatment without GB application when exposed to salt regime. No positive effect of this amelioration was observed with the additional application of GB. Therefore, the proper concentration to alleviate this detrimental damage caused by salinity on the growth of alfalfa was 5 mM. However, higher GB (20 mM) application level exhibited no significant effects on the VSGR and VRGR and even decreased plant biomass compared to the treatment without GB application when exposed to salt regime due to the phytotoxicity (Mickelbart *et al*., 2006).

Plant transpiration, a reliable index of the toxic effects of salt, was measured quickly and coupled with photosynthesis (Hu *et al*., 2012). Plants treated with salt performed a lower NRT compared to CK, and this phenomenon was attributed to the inhibition of water flow, and less water was flowed from roots to shoots, even induced the toxic effects on plant growth. Fortunately, the application of 5 mM GB could alleviate this negative effect on the NRT under salt stress. This phenomenon was attributed to the increment in hydraulic conductivity induced by GB. Nevertheless, higher level of exogenous GB application (20 mM) had negative effects on the NRT suggesting phytotoxicity by GB on alfalfa.

Previous reports indicated that high concentrations of Na^+ or high Na/K ratio posed a series of osmotic and metabolic problems to plants, such as reduced photosynthesis and protein synthesis (Weissenhorn *et al*., 1995). In the present study, it was found that GB application could ameliorate salt stress based on a lower Na^+/K^+ ratio. Similar result was reported by Giri *et al*., (2007) indicating that higher K accumulation by plants exposed to salt regimes might help to maintain a high Na^+/K^+ ratio in order to prevent the disruption of various enzymatic process.

Lipid peroxidation in cellular membranes was recognized as the result of oxidative deterioration of unsaturated lipids (Health & Packer, 1968). In some cases, lower MDA and soluble protein contents could be correlated with stress tolerance (Diego *et al*., 2003). Higher accumulation of MDA and soluble protein contents in plants under salt stress conditions was observed in the present study. Plants treated with GB performed a significant decrements in MDA and soluble protein contents compared to the treatment without GB application when exposed to salinity regimes, suggesting the positive effects of GB on the stability of cell membrane maintenance. The activities of SOD and POD in leaves were increased in salinity regimes due to the activation of plant defence mechanisms (Wang *et al*., 2016). 5 mM GB application was enhanced the SOD and POD activities when exposed to salinity, suggesting that plants modulated their antioxidant enzymes to detoxify H_2O_2 (Xu *et al*., 2015). These results concurred with the findings by Yang *et al.*, (2012), who illustrated that antioxidative defence of kentucky bluegrass and creeping bentgrass under salt stress were improved by GB application. Lower CAT activity was detected in the 5 mM and 10 mM GB application treatments than in the non-GB treatment when exposed to the salinity regime. However, higher GB application level (20 mM) showed no significant effect on CAT activity relative to the treatment without GB application when exposed to salinity. These findings illustrated that the lower GB (5 mM and 10 mM) application could ameliorate detrimental effects to antioxidant enzymes, and a higher GB (20 mM) application may have negative effects on this alleviation due to phytotoxicity.

Conclusion

In summary, this study demonstrated that the GB application with proper concentrations was effective in alleviating the damage effects to alfalfa exposed to salt stress. The beneficial effects of GB were in virtue of the strong protection to peroxidation-linked membrane from detrimental damage, cleaning up free radicals, and the beneficial influence on the ionic balance of the cytoplasm.

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