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Research Article

Role of exogenous glycinebetaine and humic acid in mitigating drought stress-induced adverse effects in *Malus robusta* seedlings

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Abstract: Glycinebetaine (GB) and humic acid (HA) are 2 commercial growth regulators that are being used worldwide to enhance the tolerance of most crops, including fruit trees, to various stresses, including drought stress (DS). *Malus robusta* Rehder is considered as one of the most important rootstocks for apple in China, but its growth and productivity is believed to be adversely affected by DS. The effects of different concentrations and combinations of GB (0, 100, and 200 mg L^{-1}) and HA (0, 500, 1000, and 1500 mg L^{-1}) on *M. robusta* seedling growth, photosynthesis characteristics, osmolyte accumulation, and antioxidant responses were evaluated under DS and non-DS conditions. GB and HA applied exogenously to drought-stressed *M. robusta* seedlings increased total dry matter, net photosynthetic rate, free proline content, endogenous glycinebetaine content, soluble sugar content, and potassium content as well as the activities of superoxide dismutase, peroxidase, and catalase. However, GB and HA decreased stomatal conductance and malondialdehyde content. The above-mentioned responses were greater for combined application of GB and HA as compared to application of only GB or HA. The best responses for most of the above parameters were with the application of 100 mg L^{-1} GB and 1500 mg L^{-1} HA. These results suggest that application of GB and HA could mitigate the deleterious effects of DS on *Malus* seedlings and offer an efficient, economical, and simple means to enhance DS tolerance of the apple rootstock.

Key words: Glycinebetaine, humic acid, photosynthesis, osmolytes, antioxidant responses, drought stress, *Malus*

1. Introduction

Drought stress (DS) is one of the premier abiotic stresses that adversely affect growth and production of various crops (Ashraf et al., 2010), including fruit crops such as apple (*Malus domestica* L.). *Malus robusta* Rehder is the most important commercial rootstock for apple production in China (Bai et al., 2009; Wu et al., 2011). Unfortunately, apple trees with *M. robusta* rootstock are sensitive to DS, as is evident from the study of Han (2011), who reported reduced tree growth and fruit yield in some parts of northern China. Previous studies showed that *M. robusta* (Lebed.) Roem from a high rainfall region was more sensitive to drought than *Malus sieversii* from a lower rainfall region (Han, 2011; Wu et al., 2011). Thus, to improve drought tolerance of *M. robusta* rootstock, different plant growth regulators such as glycinebetaine (GB) and humic acid (HA) could be useful (Ashraf and Foolad, 2007; Cimrin et al., 2010).

One of the most common effects of DS on plants is osmotic stress, which leads to the reduced chemical activity of water and loss of cell turgor, thereby causing a decrease in leaf relative water content (RWC). Therefore, RWC is a potential criterion of plant water status (Taiz and Zeiger, 2002). DS-induced osmotic stress also causes the production of a variety of reactive oxygen species (ROS), e.g., superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxide, and singlet oxygen (Raza et al., 2007; Ashraf, 2009; Baloğlu et al., 2012; Sekmen Esen et al., 2012). The ROS interact with a variety of organic molecules, due to which various key pathways responsible for growth and development are perturbed (Ashraf, 2009). However, plants exposed to stressful environments have evolved various strategies to counteract ROS and reduce malondialdehyde (MDA) content in plant cells (Ashraf, 2010). For example, most higher plants overproduce key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) to counteract the ROS produced under DS (Ashraf, 2009, 2010; Wu et al., 2011; Baloğlu et al., 2012; Sekmen Esen et al., 2012). Furthermore, osmotic adjustment is also considered vital for mitigating the inhibitory effects

of DS on plants (Ashraf, 2010). This phenomenon may occur due to accumulation of inorganic ions such as K^+ and intracellular compatible solutes such as free proline (FP), GB, and soluble sugars (SSs) (Taiz and Zeiger, 2002; Zhang et al., 2009; Baysal Furtana et al., 2013).

GB, one of the most effective osmoprotectants, accumulates in certain plant species under DS. However, application of exogenous GB to plants that lack the inherent ability to synthesise GB in adequate amounts may provide an effective means of overcoming the adverse effects of DS on crop production (Ashraf and Foolad, 2007). The effectiveness of seed treatment or foliar application of GB in alleviating DS has been reported in different crops such as maize (*Zea mays* L.), tobacco (*Nicotiana tabacum* L.), soybean [*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.) (Ashraf and Foolad, 2007) and in some *Malus* Mill species such as *Malus domestica* Borkh 'Red Fuji', *Malus hupehensis* (Pamp.) Rehder, and *Malus baccata* (L.) Borkh (Jie, 2006).

HA is believed to play a significant role in improving plant tolerance to DS (Cimrin et al., 2010). However, the mechanism underlying HA-promoted plant growth is not completely understood. HA has been reported to show various biochemical effects such as increases in cell membrane permeability, potassium and phosphate uptake, photosynthesis and respiration rates, synthesis of proteins and hormones, and root cell elongation (Böhme and Thi, 1997; Nardi et al., 2002; Cimrin et al., 2010; Saruhan et al., 2011). Some previous studies have shown that HA can be used as a growth regulator to regulate hormone levels, improve plant growth, and enhance stress tolerance (Cimrin et al., 2010).

Most of the above-mentioned studies demonstrating the beneficial effects of GB and HA on crop growth have used the application of either GB or HA to plants grown under DS. We could not find a single report from the literature wherein both GB and HA were used in combination to assess their mitigating role in drought-stressed plants. Therefore, the present study was designed to investigate the efficacy of GB and HA applications to *M. robusta* seedlings in mitigating the negative effects of DS on growth, photosynthetic characteristics, osmolyte accumulation, and antioxidant responses.

2. Materials and methods

2.1. Plant material and trial location
Experiments were carried out under controlled conditions in growth chambers/glasshouses at the College of Horticulture of the Northwest A&F University, Yangling (34°20′N, 108°24′E), China. The seeds of *M. robusta* were supplied by the Horticulture College of the same university.

2.2. Plant growth and experimental design
Before using the seed for experimentation, it was stratified by placing it in moist sand in a refrigerator at 5 °C for 60–

90 days. The prestratified seeds were sown in a nursery box containing a mixture of soil, sand, and organic matter (5:3:2, v/v/v) with a layer of gravel at the bottom for drainage. The experiment was conducted in a glasshouse with full natural sunlight, day and night ambient temperatures of 28–33 and 20–25 °C, and relative humidity of 40%–70%. At the 8-leaf stage (60 days old), 3 seedlings were transplanted to plastic pots (30 cm in height and 28 cm in internal diameter) containing quartz sand and moistened with distilled water. The sand in each pot was first leached with 1 M HCl, flushed with distilled water, and then sterilised in an oven at 180 °C for 30 h. The seedlings were irrigated with deionised water for the first 2 days, with half-strength Hoagland solution (Hoagland and Arnon, 1938) for 10 days, and subsequently with full-strength nutrient solution up to harvesting. When the seedlings attained the 12-leaf stage, they were thinned to one per pot and then subjected to the desired treatments.

The main treatments included 2 water regimes for 30 days: 1) well-watered, i.e. application of full-strength nutrient solution without polyethylene glycol (PEG)-6000, which served as the non-DS control; and 2) DS, full-strength nutrient solution containing 10% (w/v) PEG-6000 to achieve –0.15 MPa osmotic potential (ψs) (Zhang et al., 2009). Subtreatments were: 1) 3 levels [0 (GB 0), 100 (GB 1), and 200 (GB 2) mg L^{-1}] of exogenous GB through rooting medium (T) (GB produced by Shiying Chemical Plant, Changping, Beijing, China); and 2) sub-subtreatments: 4 levels [0 (HA 0), 500 (HA 1), 1000 (HA 2), and 1500 (HA 3) mg L^{-1}] of exogenous HA through root growing medium (HA derived from lignitic coal using 0.1 M NaOH, produced by Yangling Lvdu Bioecology Technology Co., Ltd., Yangling, China) (Hai and Mir, 1998). The seedlings were irrigated with full-strength Hoagland solution containing the above amendments per treatment. The pH of the nutrient solutions was maintained at 6.20 ± 0.05 by adding 1.0 M HCl or NaOH. All treatment pots were arranged in a randomised complete block design of 4 replications.

The experiment was conducted twice under the same environmental conditions to ensure repeatability of the data. Data presented are means of 4 replicates of the 2 experiments (n = 8).

2.3. Total dry matter and leaf RWC observations
Total dry matter (TDM) was determined after 30 days from the start of drought and growth regulator treatments. The whole plants were harvested from all pots and placed in an oven at 105 °C for 15 min, and then dried to a constant weight at 75 °C to determine TDM.

Before harvesting the third or fourth leaf from the top of a plant, the plant was used for all in situ measurements. Leaves were sampled between 1030 and 1100 hours and immediately stored in ice packs contained in an ice chest and brought to the laboratory. They were then washed with distilled water, and the excess water was removed.

The leaf RWC was estimated using the following equation, following Gao (2000):

RWC (%) = [(W – DW) / (TW – DW)] × 100,

where W = sample fresh weight, TW = sample turgid weight, and DW = sample dry weight.

2.4. Measurement of photosynthesis parameters

A portable photosynthesis system (LI-6400; LI-COR Inc., Lincoln, NE, USA) was used to measure gas exchange parameters using the second fully developed leaf from the top between 0900 and 1100 hours, before harvest. The data were recorded for net photosynthetic rate (P_n, μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}) under a light intensity (PAR) of 1200 μmol m^{-2} s^{-1} and ambient CO_2 concentration of 360 μmol mol^{-1}. The leaf temperature was 25.5 ± 2 °C, and the relative humidity in the leaf chamber was 45% throughout the measurement period.

2.5. Osmolyte content measurement

For FP content (FPC) determination, each leaf sample was extracted using 5 mL of 3% sulphosalicylic acid and reacted with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent in a water bath at 100 °C for 30 min. After cooling, 6 mL of toluene was added, and then the mixture was poured into a separating funnel. After thorough mixing, the chromophore-containing toluene was separated and absorbance was read at 520 nm in a UV-visible spectrophotometer against a toluene blank. The concentration of proline was estimated from a standard curve prepared using varying levels of proline (Bates et al., 1973).

Leaf endogenous GB content (EGBC) was measured following the protocol described by Greive and Grattan (1983) with some modifications. Finely powdered plant material (0.5 g) was mechanically shaken with 20 mL of deionised water for 48 h at 25 °C. The samples were then filtered and the filtrate was stored in a freezer until analysis. The frozen filtrate was thawed and diluted at 1:1 with 2 N sulphuric acid and then cooled in ice water for 1 h. Cold potassium iodide–iodine reagent (0.2 mL) was added to the mixture and gently vortexed. The samples were stored at 0–4 °C for 16 h and centrifuged at 10,000 × g for 15 min at 0 °C. The supernatant was treated in 9 mL of 1,2-dichloroethane (reagent grade), and absorbance was measured at 365 nm with a UV-visible spectrophotometer.

Leaf soluble sugar content (SSC) was estimated using anthrone reagent (Zhang et al., 2009). An aliquot of 0.05 mL of the sample was made up to 1 mL and then treated with 4 mL of anthrone regent. The mixture was heated in a water bath for 8 min. After cooling, optical density of the mixture was read at 630 nm. Concentration of SSs was appraised using a standard curve prepared using varying levels of glucose.

Leaf K^+ content (KC) was determined by flame photometry after wet ashing in 18 mol L^{-1} of sulphuric acid solution following the method of Bao (2000).

The relative contribution of a soluble nitride to osmotic adjustment was indirectly reflected by comparing one solute mole concentration with another base in DW under DS, due to the better negative correlation between mole concentration of a solute and its osmotic potential (ψs) following the van't Hoff equation: $\psi s = -CiRT$, where C = mole concentration of a solute; i = solute's ionisation constant, approximately equal to 1 in these experiments due to the main zwitterionic characters of 3 organic solutes (inner-salt); R = gas constant; and T = temperature (K) (Taiz and Zeiger, 2002).

2.6. Extraction and measurement of activities of antioxidant enzymes

The fresh leaf material was homogenised in 4 mL of ice-cold 50 mM phosphate buffer (pH 7.8) containing 1% PVP (v/v) and a little quartz sand with a prechilled pestle and mortar. The homogenates were transferred to centrifuge tubes and centrifuged (10,000 × g for 20 min at 4 °C), and the supernatants were used for the following antioxidant enzymes assays.

The activity of SOD was appraised by recording a decrease in absorbance at 560 nm of superoxide-nitroblue tetrazolium complex by the enzyme. One unit of SOD was considered as the amount of enzyme required to inhibit tetrazolium (NBT) reduction by 50% (Dhindsa et al., 1981). POD activity was assayed following the method of Putter (1974) by treating the mixture with guaiacol at 470 nm, and 1 unit of enzyme activity was taken as the rate of guaiacol oxidised in 3 min. CAT activity was assayed following the method of Dhindsa et al. (1981) by determining the residual H_2O_2 by the Tris-HCl reagent. Absorbance of the enzyme mixture was read immediately every 4 min at 240 nm and 1 unit of enzyme determined the amount necessary to decompose 1 μmol of H_2O_2 per minute at 25 °C. The activities of all antioxidant enzymes were expressed as U mg^{-1} protein. Protein content was determined following the method of Bradford (1976), using bovine serum albumin as a standard.

For the measurement of MDA content, leaves were extracted with 10% trichloroacetic acid, and absorbance of the samples was read at 450, 532, and 600 nm with 0.6% thiobarbituric acid, as described by Heath and Packer (1968).

2.7. Data statistical analysis

Data for all variables were subjected to analysis of variance (ANOVA) using the SAS software package (SAS Institute Inc., 1996). Standard errors (SEs) of all means were calculated. The significance of the treatment effects was determined using the F-test, and least significant differences (LSDs) were calculated at P < 0.05. Duncan's multiple range test was used for comparing the mean values.

3. Results and discussion

Analysis of variance of the data showed that the effects of DS, GB, and HA were significant in all parameters measured in *Malus robusta* seedlings (Table). The magnitude of F values for most parameters was in the order of DS > HA > GB; for EGBC, the order was DS > GB > HA. Two-way and 3-way interactions were also significant for most of the measured parameters, except GB × HA and W × GB × HA for P_n, FPC, and CAT activity. This study showed that choosing the optimal application rate of GB and/or HA is important for overcoming the adverse effects of DS on plant growth, photosynthesis, osmolyte accumulation, and antioxidant responses (Table).

3.1. Seedling growth, water status, and photosynthesis

DS is known to perturb water relations and inhibit growth of plants. TDM and leaf relative RWC are potential criteria for growth and water status of plants that reflect the regulation of metabolic activities in tissues (Zhang et al., 2009). P_n and g_s are important gas exchange parameters that can be effectively used to evaluate plant photosynthetic responses under DS (Zhang et al., 2011). DS can cause disruption in the homeostasis of plant water status, leading to decreased RWC, rate of photosynthesis,

and, ultimately, plant growth (Pinheiro and Chaves, 2010). Plants can respond to DS by limiting leaf expansion and closing stomatal pores. This, in turn, limits water loss through transpiration, thereby enabling plants to sustain their growth under DS (Pinheiro and Chaves, 2010). The results of this study confirmed that TDM, RWC, P_n, and g_s of *Malus robusta* seedlings were considerably affected by DS, followed by HA and GB (Figures 1 and 2; Table).

GB is an effective osmoprotectant that plays a significant role in plant survival under stress cues (Ashraf and Foolad, 2007; Chen and Murata, 2008). HA is also known as a stimulator of plant growth under environmental stresses (Aydin et al., 2012). For example, barley seeds treated with GB showed increased dry matter production under DS (Zhang and Li, 2004). Rate of photosynthesis and yields were also significantly improved in wheat by exogenous application of GB under DS (Díaz-Zorita et al., 2001). Furthermore, humic substances have been shown to cause antistress effects in plants exposed to DS (Kulikova et al., 2005). HA can promote plant growth by enhancing the uptake of nutrients and photosynthesis and by reducing water loss by decreasing g_s (Nardi et al., 2002; Cimrin et al., 2010; Saruhan et al., 2011).

Table. Analysis of variance (ANOVA; mean squares) of the data for growth, photosynthesis characteristics, some osmolytes, and antioxidant responses of GB- and HA-treated *Malus robusta* Rehder seedlings grown under well-watered (control) and drought stress (DS) conditions.

Source		Water regime (W)	Exogenous GB	Exogenous HA	W × GB	W × HA	GB × HA	W × GB × HA
Growth, water status, and photosynthetic characteristics	TDM	26,547.3***	377.2***	2625.5***	289.3 ***	178.3***	3.1*	2.4*
	RWC	2610.4***	120.0***	234.9***	130.3***	207.1***	3.0*	5.0***
	P_n	6301.1***	145.6***	655.9***	126.3***	92.8***	2.1	1.1
	g_s	15,364.5***	116.5***	694.1***	116.5***	520.6***	3.9**	3.9**
Osmolyte contents	FPC	74,922.7***	304.5***	1056.4***	309.3***	1023.2***	1.68	1.55
	EGBC	63,333.6***	6561.2***	339.7***	3534.4***	302.5***	15.2***	15.1***
	SSC	12,781.9***	111.9***	1768.2***	111.9***	261.0***	7.2***	7.2***
	KC	7068.4***	143.0***	1689.9***	155.6***	367.3***	5.3***	4.2**
Antioxidant responses	SOD	51,351.5***	560.5***	726.6***	560.5***	808.9***	8.1***	8.1***
	POD	21,969.1***	666.5***	1782.6***	666.5***	1278.5***	7.4***	7.4***
	CAT	16,593.2***	334.5***	954.9***	334.3***	884.4**	2.2	2.2
	MDA	23,841.9***	76.11***	616.93***	76.18***	341.99***	3.2*	3.3**

*P = 0.05, **P = 0.01, ***P = 0.001.

Abbreviations: CAT, catalase; EGBC, endogenous glycinebetaine content; FPC, free proline content; g_s, stomatal conductance; KC, K⁺ content; MDA, malondialdehyde (MDA); P_n, net photosynthetic rate; POD, peroxidase; RWC, relative water content; SOD, superoxide dismutase; SSC, soluble sugar content; TDM, total dry matter.
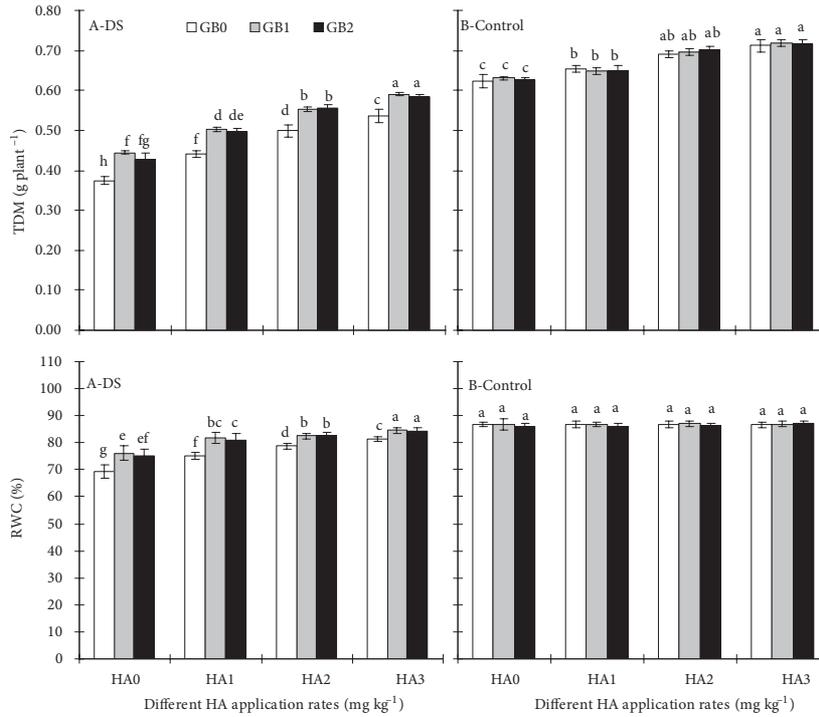
Figure 1. Effects of exogenous GB, HA, and their interactions on total dry matter (TDM) and relative water content (RWC) of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. GB 0, GB 1, and GB 2 represent 0, 50, and 100 mg kg^{-1} concentrations of GB, respectively. HA 0, HA 1, HA 2, and HA 3 represent 0, 500, 1000, and 1500 mg kg^{-1} HA, respectively.
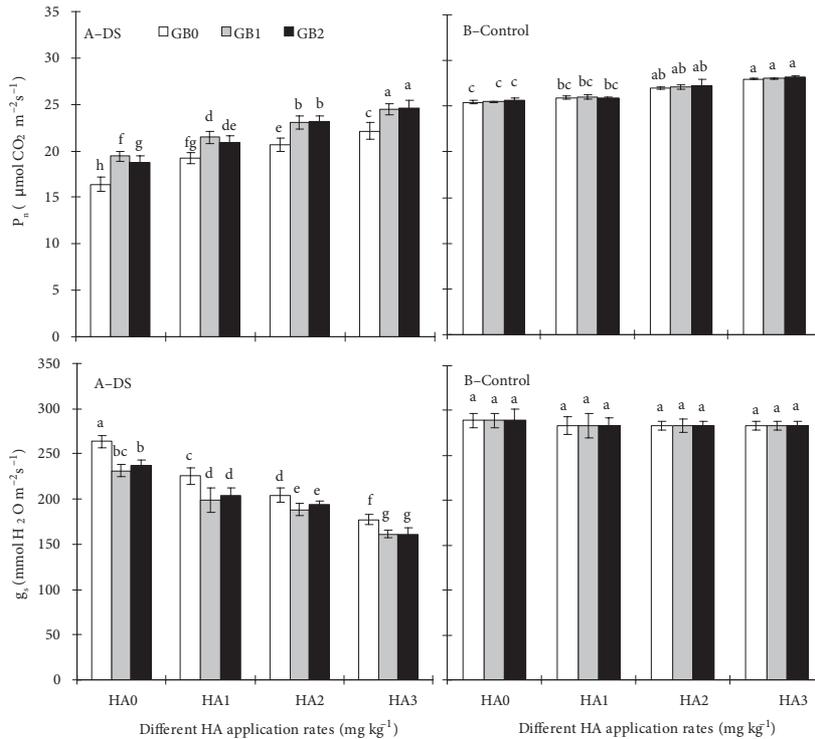
Figure 2. Effects of exogenous GB, HA, and their interactions on net photosynthesis rate (P_n) and stomatal conductance (g_s) of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. Details of culture media are given in the legend of Figure 1.

In our study, inhibition in seedling growth, RWC, and photosynthesis was significantly alleviated by exogenous application of GB and HA to *Malus robusta* seedlings under DS (Figures 1 and 2). Greater TDM, RWC, and P_n and lower g_s of seedlings resulted from GB 1 (100 mg L^{-1}) and GB 2 (200 mg L^{-1}) applications as compared to treatments with no GB. The TDM, RWC, and P_n increased consistently, while g_s decreased with increasing HA doses at a given GB dose under DS. However, the increase in TDM and P_n was greater than under the control. The seedlings receiving GB 1 and HA 3 treatment showed peak values of TDM, RWC, and P_n and the lowest g_s. There were no significant differences in growth and photosynthesis responses between HA 1 + GB 1 or GB 2 and HA 2 treatment, or GB 1 or GB 2 and HA 1 treatment. This study demonstrated a significant enhancement in TDM, RWC, and P_n and a decrease in g_s as a result of root-applied GB and HA on *Malus robusta* seedlings subjected to DS. Humic acid increased TDM and P_n in non-DS seedlings, but GB had no effect (Figure 1). These results suggest that root application of GB and HA treatments can mitigate the deleterious effects of DS on *Malus* seedlings in terms of growth, water status, and photosynthesis.

3.2. Osmolyte accumulation

A variety of low-molecular-weight organic solutes including FP, GB, and SS and inorganic ions such as K$^+$ accumulate in plants grown under environmental stresses including water deficit conditions (Baysal Furtana et al., 2013). This is an adaptive mechanism to overcome the negative effects of DS (Ashraf and Foolad, 2007; Zhang et al., 2009), because these osmolytes play an important role in stabilising membranes and/or macromolecular structures (Ashraf and Foolad, 2007). Exogenous single GB or HA application has been reported to significantly affect osmolyte accumulation in many crops under DS (Zhang and Li, 2004; Cimrin et al., 2010; Saruhan et al., 2011). Foliar spray of GB onto plants under DS significantly increased leaf FP in wheat, endogenous GB in soybean, and SS in apricot (*Prunus armeniaca* L.) (Zhang and Li, 2004; Ashraf and Foolad, 2007). In maize, Zhang et al. (2009) showed that foliar application of GB increased the FP, endogenous GB, SS, and K$^+$ contents of drought-stressed plants. This study confirmed that GB and HA applied through root growing medium significantly increase levels of FP, endogenous GB, SS, and K$^+$ in seedling leaves under DS (Figure 2). HA shows promising effects on nutrient uptake by plants. Most importantly, it is effectively involved in the transport of essential macronutrients such as N, P, and K, which are vital for the synthesis of FP, GB, and SS (Böhme and Thi, 1997). Foliar-applied GB contributes positively to stress tolerance by improving the process of osmotic adjustment and protecting some key enzymes such as delta-1-pyrroline-5-carboxylate synthetase and

betaine aldehyde dehydrogenase (Ashraf and Foolad, 2007), which enhance ATPase activity in plant cells. The enhanced ATPase activity is believed to be useful for active absorption of inorganic nutrients such as K (Zhang and Li, 2004).

In the present study, the contents of measured osmolytes, i.e. FPC, EGBC, SSC, and KC, were greater in *M. robusta* Rehder plants under DS than those under no DS (Figures 3 and 4). Exogenously applied GB and HA significantly increased FPC, EGBC, SSC, and KC values in seedling leaves under DS, which were associated with increased tolerance to DS (Figures 3 and 4). The concentrations of all osmolytes increased with increasing HA levels across all GB levels used. In contrast, increases in osmolyte levels with GB application occurred only at the first dose. The peak values of FPC, SSC, and KC were obtained in seedlings receiving GB 1 and HA 3, while the peak value in EGBC was found with the GB 2 and HA 3 treatments. In non-DS seedlings, exogenous GB had no effect on the levels of most osmolytes, except EGBC, at all HA levels, whereas exogenous HA caused continuous and consistent increases in SSC and KC only when HA was applied as HA 2 (1000 mg L^{-1}) under all GB levels. On the basis of relative concentrations of these 4 osmolytes, in each treatment decreases were in the following order: K > SS > SP > GB. As a result, the relative contribution of these osmolytes to osmotic adjustment was of the same order as that of their contents (Figures 3 and 4). Thus, these results clearly show that osmotic adjustment is a main modulation mechanism of exogenous GB and HA in mitigating the deleterious effects of DS on the seedlings of *Malus* plants.

3.3. Antioxidative enzyme activities and lipid peroxidation

The antioxidative defence system is vital for limiting oxidative damage to plants under DS by scavenging excessive ROS (Ashraf, 2009; Baloğlu et al., 2012; Sekmen Esen et al., 2012). Enhanced production of antioxidants is known to counteract lipid peroxidation and maintain macromolecular structure and function under DS (Raza et al., 2007). The increased activities of antioxidative enzymes induced by moderate DS can protect cell membranes, proteins, and metabolic machinery, which would preserve the subcellular structure from damage as a result of cell dehydration (Ashraf, 2009; Baloğlu et al., 2012; Sekmen Esen et al., 2012).

The optimal level of GB may effectively protect plant cells from environmental stresses indirectly through its beneficial effects on some key antioxidative enzymes such as SOD, CAT, and POD and membrane integrity (Ashraf and Foolad, 2007; Ashraf, 2010) (Figures 5 and 6). Zhang et al. (2009) reported increased growth of maize plants under DS with exogenous application of GB (Zhang et al., 2009) (Figure 1). However, in barley plants external GB
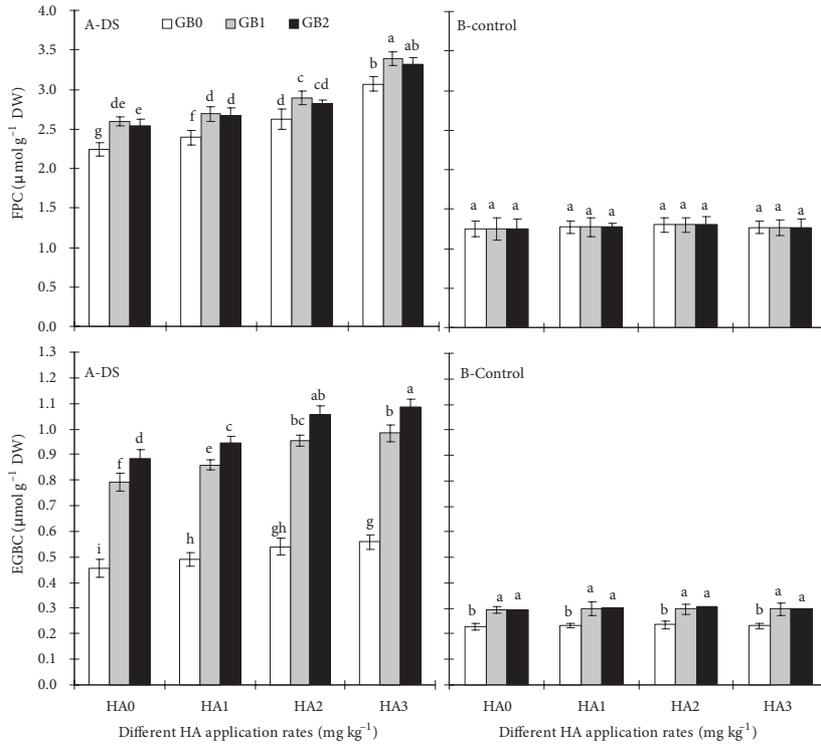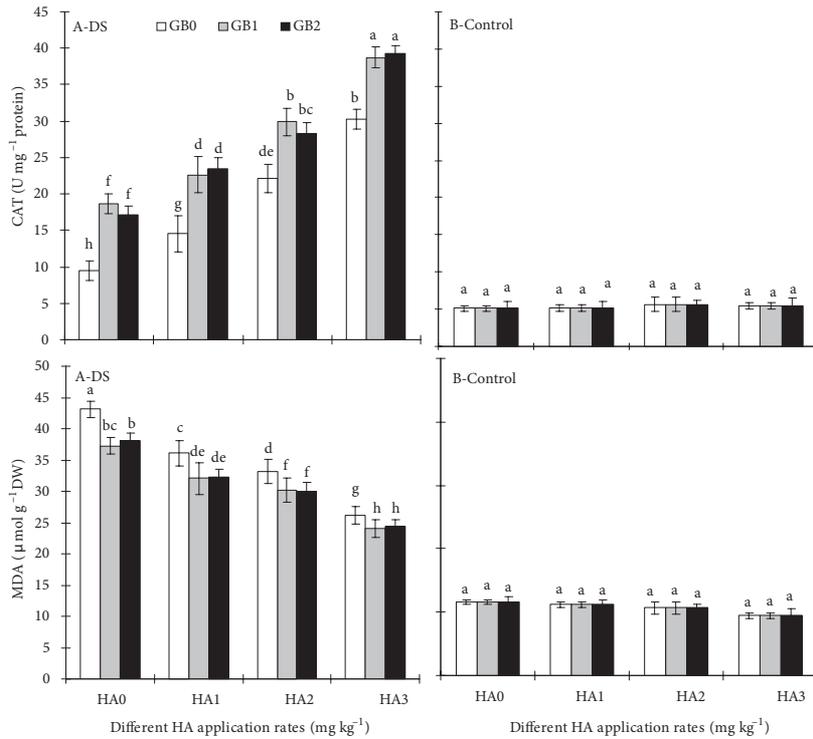
Figure 3. Effects of exogenous GB, HA, and their interactions on free proline content (FPC) and endogenous glycinebetaine content (EGBC) of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. Details of culture media are given in the legend of Figure 1.
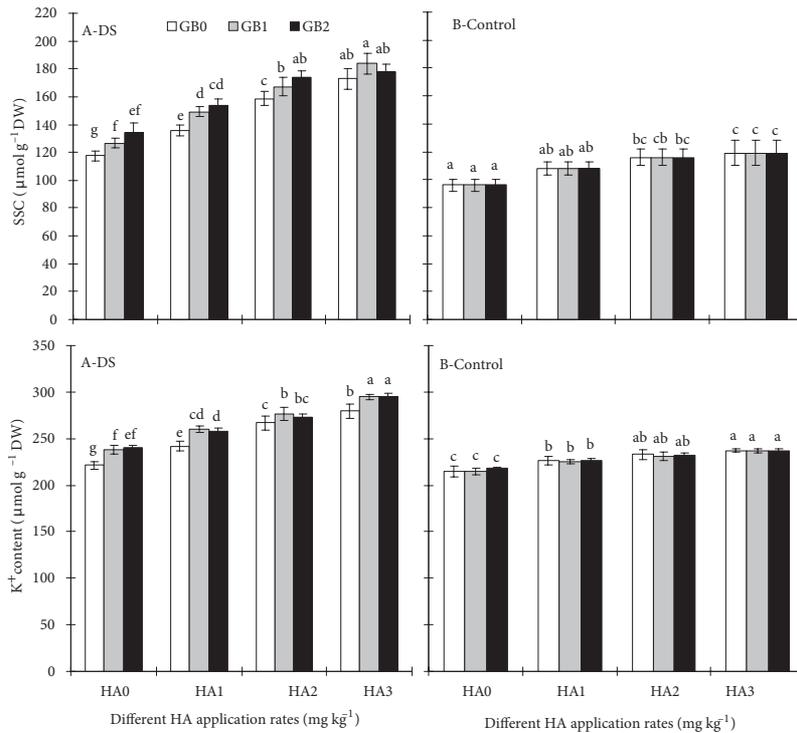
Figure 4. Effects of exogenous GB, HA, and their interactions on soluble sugar content (SSC) and K^{+} content of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. Details of culture media are given in the legend of Figure 1.
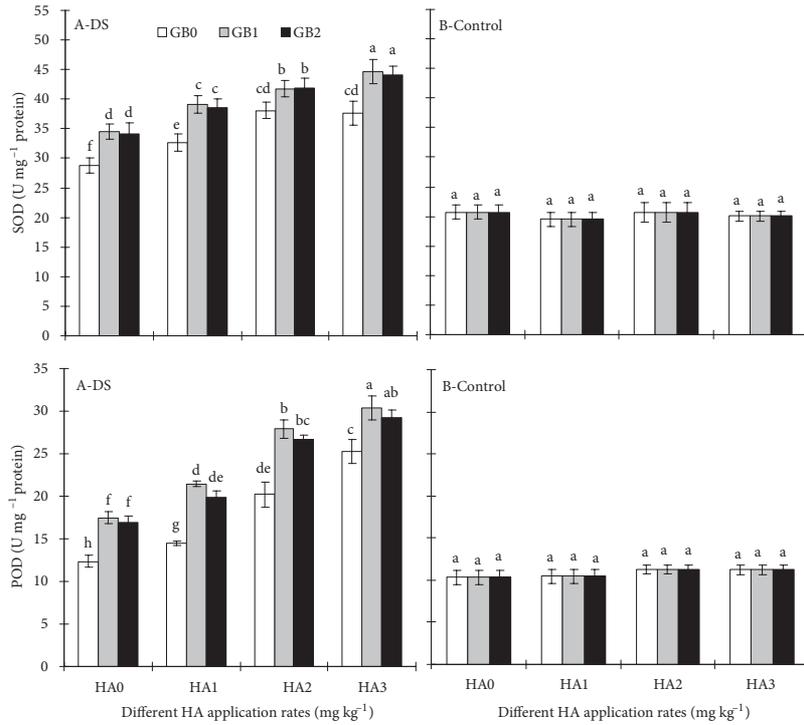
Figure 5. Effects of exogenous GB, HA, and their interactions on superoxide dismutase (SOD) and peroxidase (POD) of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. Details of culture media are given in the legend of Figure 1.

Figure 6. Effects of exogenous GB, HA, and their interactions on catalase (CAT) and malondialdehyde (MDA) of *Malus robusta* seedlings under both well-watered (control) and drought stress (DS) conditions. Mean ± SE (n = 8). Means with different letters within each parameter and water regime treatment indicate a significant difference at P < 0.05. Details of culture media are given in the legend of Figure 1.

(1 mM) caused an opposite response to osmotic stress (PEG 4000 at –0.86 MPa) by decreasing the activities of SOD and POD, as well as RWC, while increasing MDA content in leaves (Zhang and Li, 2004). It seems that the effectiveness of GB may vary with plant species, levels, and time of application, as well as environmental conditions to which plants are subjected (Ashraf and Foolad, 2007). In the current study, exogenously applied GB and HA significantly increased the activities of SOD, POD, and CAT and decreased MDA content with increases in HA levels across all external GB levels. The highest SOD, CAT, and POD activities and lowest MDA content were obtained from GB 1/GB 2 and HA 3 treatments. Exogenous GB and/ or HA had no effect on all above parameters measured for seedlings under no DS (Figures 5 and 6). GB can maintain stabilisation and integrity of cell membranes (Zhang and Li, 2004). HA has beneficial effects on growth and improves the yield and quality of many crops. Some studies suggest that HA can effectively control hormone levels, improve plant growth, and improve stress tolerance (Böhme and Thi, 1997; Nardi et al., 2002; Cimrin et al., 2010; Saruhan et al., 2011) through various biochemical effects, either at cell wall or membrane level or in the cytoplasm, including increased cell membrane permeability, enhanced protein synthesis and plant hormones, and root cell elongation (Cimrin et al., 2010; Li et al., 2011; Saruhan et al., 2011). These results show that antioxidative response is another regulation mechanism of exogenous GB and HA for mitigating the negative effects of DS on *M. robusta* plants,

but with a different mode of action in *M. robusta* seedlings.

The results presented here indicate that root-applied GB and HA can mitigate the deleterious effects of DS by increasing TDM, P_n, FPC, EGBC, SSC, and KC as well as the activities of SOD, POD, and CAT, while decreasing g_s and MDA content. In general, the response was greater when both GB and HA were applied in combination as compared to individual application. Most of the parameters measured showed improvement with increasing levels of HA across all GB levels, and until the first dose of GB at all HA levels. The best response was with the application of 100 mg L^{-1} (GB 1) and 1500 mg L^{-1} (HA 3). This study demonstrated that combined application of GB and HA has the potential to mitigate the adverse effects of DS on *Malus* plants; the mode of action was based on the levels used. Further studies are required to determine the efficiency of these amendments for mitigating DS in field conditions.

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