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Glycinebetaine-Induced Alteration in Gaseous Exchange Capacity and Osmoprotective Phenomena in Safflower (*Carthamus tinctorius* L.) under Water Deficit Conditions

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Abstract: Several inorganic and organic compounds including glycine betaine (GB) are presently being used as an exogenous application to enhance tolerance in plants to different environmental stresses. The current study assessed to what extent exogenously applied GB could improve the gaseous exchange capacity and primary and secondary metabolites in two accessions (16178 and 16180) of safflower (*Carthamus tinctorius* L.) plants under drought stress. Three-week-old plants of both safflower accessions were subjected to well-watered (control) or water-deficit conditions (60% field capacity (FC)). Three levels of GB (control, 50 mM and 100 mM) were sprayed to the foliage of the control and stressed plants after one month of drought application. After two weeks of foliar application of GB, gas exchange characteristics and other biochemical parameters were determined. The results showed that water deficiency markedly suppressed plant biomass, chlorophyll contents, photosynthesis rate (A), water use efficiency (A/E), stomatal conductance (g_s) and relative water contents (RWC) of both accessions of safflower, while it enhanced the levels of osmolytes (GB and proline), hydrogen peroxide (H_2O_2) and total phenolics. Foliar application of GB was effective in enhancing the plant biomass, chlorophyll contents, g_s, sub-stomatal CO_2 concentration (C_i), C_i/C_a ratio, osmolytes, H_2O_2, ascorbic acid (AsA), total phenolics and RWC in safflower plants under water shortage. Thus, exogenous application of GB could be used as an effective strategy to improve plant growth, photosynthetic attributes and secondary metabolites in safflower plants under water deficit conditions.

Keywords: glycine betaine; gaseous exchange; safflower; drought; metabolites

1. Introduction

All plant processes are greatly influenced by deficiency of water as water is vital for their functioning [1,2]. Water deficiency even for a short time can disturb the growth and decrease the productivity of plants by disturbing photosynthesis [3,4]. Water-deficiency-induced impairment in photosynthesis is attributed to damaged composition of thylakoid membranes in chloroplasts as the lipid contents of cell membranes are susceptible to the ROS species produced as a result of drought [3–5]. Drought stress also disturbs gaseous exchange, metabolic activities, nutrient uptake, turgor and mitosis [6,7], which may lead to reduced growth and productivity [8–10] and sometimes the death of the plant [11,12]. Plants possess defensive systems primarily as antioxidants, which can effectively reduce the drastic effects of ROS species caused by water deficiency [13–17].

Among oil-seed-producing crops, safflower (*Carthamus tinctorius*) has a capacity of producing approximately 32–40% of high-quality seed oil [18] and is a very important economic crop. Its flowers and seeds are used to extract orange-red dye used as a flavoring agent for food and dye for coloring fabrics and edible oils [19,20]. Its oil is used to cook food for patients with cardiovascular diseases as it has a high content of linoleic acid antioxidants such as vitamin E [20,21]. In view of a number of reports, it is evident that this crop, with an inherent characteristic of a deep root system, is resistant to water deficiency and thus can be grown successfully in semi-arid and arid countries of the world facing problems of water shortage [22–24].

Nowadays, a number of compounds including osmoprotectants such as proline and glycine betaine (GB) are used with exogenous application to plants to reduce the harmful effects of abiotic stresses including drought stress. GB, a quaternary ammonium substance, is an osmoprotectant that can effectively scavenge ROS in plant tissues [25] and improves the photosynthesis rate by maintaining the Rubisco ultra-structure [26]. It is present in different amounts in different plant parts including seed, stem, root and flowers [25,27]. During the early juvenile stage of the plant, it is present in very small amounts in the roots but later increases in leaves [27,28]. Different levels of GB can be observed in different plant species under different abiotic stresses [29] depending on plant species, genotype, development stage, application modes and different stress conditions [30]. GB plays an essential role to provide protection from high accumulation of ROS species in plants under water shortage [25,28] and increases the photosynthetic defensive mechanism [26,31]. Rapid change in cellular metabolism, inferior level of water potential and ABA recognition sites give rise to accumulation of GB under water stress [25,26,30]. Furthermore, exogenously applied GB enhances yield and tolerance machinery by increasing chlorophyll contents, stimulating the antioxidant defensive system, decreasing ROS and stabilizing the photosynthesis ability of photosystem II under drought stress [32]. GB is believed to contribute to better protection when the concentration of ROS increases under severe water deficiency [27,29]. GB is believed to contribute to better protection when the concentration of ROS increases under severe water deficiency, so it has been hypothesized that exogenous application of organic compounds such as GB could mitigate the adverse effects of drought stress on safflower, a potential oil-producing crop. Therefore, the aim of the present study was to examine the role of exogenously applied GB in osmoprotection and gaseous exchange in safflower (*Carthamus tinctorius* L.) under water-deficit conditions.

2. Materials and Methods

2.1. Plant Material and Growth Condition

To evaluate the influence of exogenous application of varying levels of glycine betaine (GB) on inducing water-deficiency tolerance in safflower (*Carthamus tinctorius* L.), the present study was conducted under natural environmental conditions. The experiment was arranged in a completely randomized design (CRD) with three replications of each treatment. The seeds of two accessions of safflower i.e., 16178 and 16180, were collected from the Plant Genetic Resources Institute, National Agricultural Research Centre (PGRI, NARC), Islamabad, Pakistan. The experiment continued from January through March in the research garden area of the GC University Faisalabad, Pakistan. Each pot was filled with 0.41 kg (sandy loam), soil and five seeds were sown per pot. After one week of sowing, germination started. The seedlings that emerged were thinned to maintain three almost uniform-size seedlings per pot, and three pots were used for each treatment. After 21 days of seed germination, drought stress treatments, including control (normal watering) and drought (60% field capacity) were initiated. The given water levels were checked and maintained twice a week by weighting and adjusting the moisture level of the pots. After 30 days of drought stress, three levels of glycine betaine—control (no spray), 50 mM and 100 mM—were applied as a foliar spray to stressed and non-stressed plants. After two-weeks of foliar-applied glycine betaine, plant samples were collected from each replicate, and root and shoot fresh weight were measured separately using an electrical balance after harvest. Then, the shoot and root samples were placed in an oven at 72 °C after air drying, and their dry weights were recorded. Fresh leaf samples were preserved at −20 °C for the determination of the following attributes.

2.2. Chlorophyll Contents

Following Arnon [33], chlorophyll *a* and *b* contents were determined. A proportion (0.25 g) of fresh leaf was extracted in 5 mL of 80% acetone with the help of a mortar and pestle. Then, the mixture was homogenized and kept overnight at 4 °C. The absorbance of the supernatant was recorded at 663 and 645 nm using a UV-visible spectrophotometer.

2.3. Gas Exchange Parameters

Gas exchange parameters such as rate of transpiration (E), photosynthetic rate (A), stomatal conductance (g_s), internal CO_2 concentration (Ci) and water use efficiency (A/E) were determined using an Infra Red Gas Analyzer (IRGA). The data were recorded at daytime with maximum sunshine.

2.4. Free Proline

The proline content was estimated using the procedure of Bates et al. [34]. A fresh plant leaf (0.25 g) of each sample was homogenized finely in 5 mL of (3% *w*/*v*) sulfosalicylic acid and then centrifuged. To 2.0 mL of the extract, 2 mL of ninhydrin and 2 mL of glacial acetic acid were added. This mixture was heated for 1 h at 95 °C. Toluene (4 mL) was added to the cooled sample mixture. The mixture was shaken vigorously, and two layers were formed. The absorbance of the upper layer was read at 520 nm.

2.5. Glycine Betaine (GB) Contents

The Grieve and Grattan [35] method was employed to determine glycine betaine contents. According to this method, dry leaf (0.25 g) was shaken in 10 mL toluene (0.5%) and kept at 4 °C overnight. Then, the mixture was filtered. To 1 mL of the filtrate, 1 mL of 2 N H_2SO_4 and 0.2 mL of KI_3 were added. This mixture was cooled for 90 min, and then 2.8 mL of de-ionized H_2O and 6 mL of 1, 2 dichloroethane were added to it and centrifuged. The upper layer was discarded, but the lower layer was read at 365 nm using a spectrophotometer.

2.6. Hydrogen Peroxide (H_2O_2) Concentration

The hydrogen peroxide content was estimated using the method of Velikova et al. [36]. Fresh leaf (0.5 g) was homogenized in 5 mL trichloroacetic acid (0.1%; *w*/*v*) using a mortar and pestle. After filtering the extract, an aliquot of the supernatant (500 µL) was taken in a test tube, and 1 mL of potassium iodide and 0.5 mL of phosphate buffer were added to it. The test tubes were kept at room temperature for 20 min, and absorbance was recorded at 390 nm using a spectrophotometer.

2.7. Malondialdehyde (MDA) Contents

According to the method of Cakmak and Horst [37], malondialdehyde contents were determined. TBA (thiobarbituric acid) was used for the determination of lipid peroxidation in leaf samples. A fresh leaf (0.25 g) was homogenized in 5 mL of 1% (*w*/*v*) trichloroacetic acid (TCA). The mixture was centrifuged at 15,000 rpm for 10 min. An aliquot of the supernatant (500 µL) was treated with 2 mL of 0.5% thiobarbituric acid (TBA), which was prepared in 20% TCA. After that, this mixture was boiled at 100 ℃ for 30 min and cooled in an ice bath. The optical density (OD) of the treated mixture was recorded at 600 and 532 nm using a spectrophotometer.

2.8. Total Phenolics

Total phenolic content was determined according to the method of Julkenen-Titto [38] using Folin and Ciocalteau's phenol reagent. According to this protocol, fresh leaf (0.25 g) was extracted in 80% acetone solution (5 mL). Then, the extract was filtered through a filter paper. To an aliquot (100 µL) of the supernatant, 2 mL of distilled water and one mL of Folin Phenol Ciocalteau's reagent were added and the mixture was strongly shaken. Then 5 mL of 20% sodium carbonate (Na_2CO_3) was added to the mixture and made to be a final volume of 10 mL with distilled water. The amount of total phenolics was estimated by reading the treated samples at 750 nm using a spectrophotometer.

2.9. Ascorbic Acid Content

Ascorbic acid content was measured according to the method of Mukherjee and Choudhuri [39]. A fresh leaf (0.25 g) was ground in 10 mL of 6% TCA (*w*/*v*), and the extract was filtered. To 1 mL of the filtrate contained in a test tube, 2 mL of dinitrophenyl hydrazine prepared in 9 *N* H_2SO_4 and one drop of 10% thiourea (in 70% ethanol) solution were added. The mixture was boiled for 15 min at 100 °C and cooled at room temperature. Then, 5 mL of 80% (*v*/*v*) H_2SO_4 was added to the mixture. Absorbance of the samples was recorded at 530 nm spectrophotometrically.

2.10. Relative Water Contents (RWC)

This was calculated using the method of Barrs and Weatherby [40]. Fresh leaf samples were collected from each replicate, and the fresh weight was recorded. Immediately afterward, the leaves were dipped in water for 2 h at room temperature. The leaves were then taken out of the water, extra moisture was dried with a filter paper, and turgid weight was recorded. Afterward, the leaf samples were kept in an oven for 48 h and weighed again, and the dry weight was recorded.

2.11. Statistical Analysis

Analysis of variance of data was worked out using the Cohort Statistical program. In addition, correlations were determined between plant growth and other biochemical attributes. Mean data were compared at a 0.05% probability level. Graphical presentation was conducted using Origin Pro-2017. Correlation between different variables studied in this experiment was conducted using RStudio. Similarly, the principal component analysis was also conducted to study different components in safflower using RStudio.

3. Results

3.1. Growth Attributes

A marked ($p \leq 0.001$) decrease was observed in the biomass production (fresh and dry weight of root and shoot) of both accessions (16178 and 16180) of safflower under drought stress (Table 1). However, foliar-applied glycine betaine (GB) significantly ($p \leq 0.001$) improved plant fresh and dry biomass in both accessions of safflower under control (unstressed plants) and water stress conditions (Table 1). Of both GB levels, 100 mM was more effective in enhancing the plant biomass (Figure 1).

No significant difference was observed between both accessions of safflower in terms of biomass production (Figure 1). Drought stress significantly decreased the lengths of the shoot ($p \leq 0.01$) and root ($p \leq 0.001$) in both accessions (Table 1). In contrast, exogenous application of GB was effective in improving the shoot ($p \leq 0.01$) and root ($p \leq 0.001$) lengths in both accessions (Table 1). Exogenous application of 50 mM GB was found to be as effective as 100 mM GB in promoting shoot and root lengths (Figure 1).

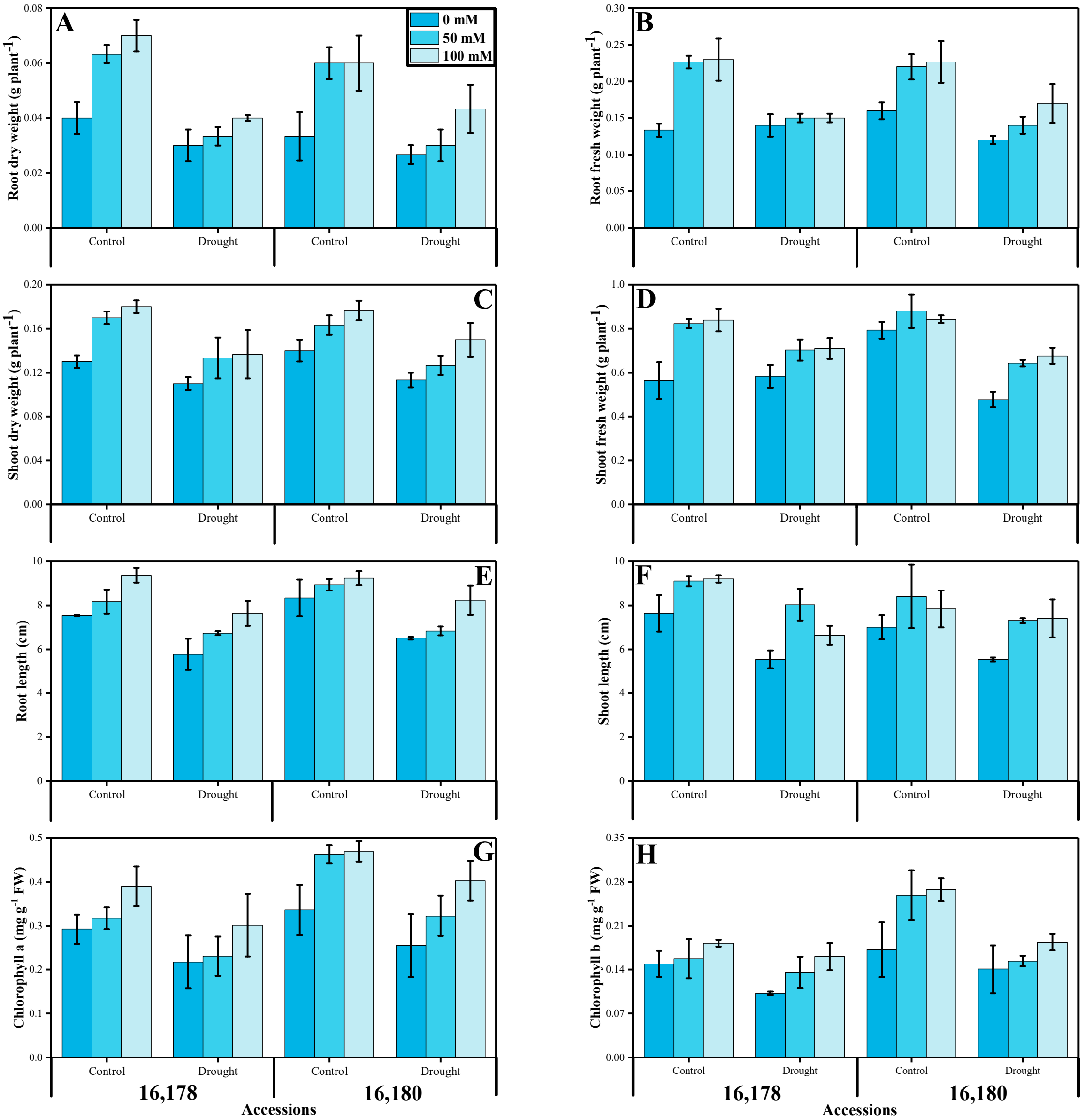

Figure 1. Effect of different concentrations of exogenous application of glycine betaine (0, 50 and 100 mM) on root dry weight (**A**), root fresh weight (**B**), shoot dry weight (**C**), shoot fresh weight (**D**), root length (**E**), shoot length (**F**), chlorophyll a (**G**) and chlorophyll b contents (**H**) of safflower (acessions) grown under control (normal watering) and drought (60% field capacity) stressed environments. Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($p < 0.05$). All the data represent the average of three replications ($n = 3$). Error bars represent standard deviation (SD) of three replicates.

Table 1. Mean squares from three-way analysis of variance of data for biomass and different biochemical processes of safflower (*Carthamus tinctoris* L.) foliarly applied glycine betaine (GB) under water deficit conditions.

Source of Variations	df	Shoot FW	Shoot DW	Root FW	Root DW	Shoot Length	Root Length
Cultivars (Cv)	1	0.0020 ns	2.5 ns	1.111 ns	1.361 ns	1.777 ns	2.054 ns
Drought stress (D)	1	0.2256 ***	0.0090 ***	0.026 ***	0.003 ***	19.067 **	24.337 ***
Treatment (T)	2	0.1035 ***	0.0043 ***	0.011 ***	0.001 ***	10.350 **	7.621 ***
Cv × D	1	0.0600 **	2.5 ns	1.777 ns	6.944 ns	1.867 ns	6.933 ns
Cv × T	2	0.0050 ns	1.583 ns	2.194 ns	2.777 ns	0.166 ns	0.217 ns
D × T	2	0.0009 ns	1.583 ns	0.003 *	3.694 ns	0.371 ns	0.174 ns
Cv × D × T	2	0.0181 ns	1.083 ns	9.361 ns	3.611 ns	0.923 ns	0.37 ns
Error	24	0.0069	3.916	8.5	1.138	1.377	0.652
		Chlorophyll *a*	**Chlorophyll *b***	**Total Chlorophyll**	**Chlorophyll *a*/*b* Ratio**	**Photosynthetic Rate (*A*)**	**Transpiration Rates (*E*)**
Cultivars (Cv)	1	0.062 **	0.0207 **	0.007 ns	0.095 ns	0.232 ns	0.011 *
Drought stress (D)	1	0.072 **	0.0239 **	0.322 ***	0.069 ns	14.83 ***	0.0096 ns
Treatment (T)	2	0.039 **	0.010 *	0.018 ns	0.181 ns	0.817 ns	0.0073 ns
Cv × D	1	3.508 ns	0.004 ns	0.219 ***	0.861 ns	1.899 *	0.0084 ns
Cv × T	2	0.004 ns	7.303 ns	0.001 ns	0.039 ns	0.033 ns	0.0019 ns
D × T	2	0.001 ns	4.515 ns	9.113 ns	0.016 ns	0.15 ns	0.027 ns
Cv × D × T	2	0.001 ns	0.002 ns	0.001 ns	0.004 ns	0.094 ns	0.001 ns
Error	24	0.0069	0.002	0.008	0.326 ns	0.329	0.0023
		Stomatal Conductance (gs)	**Sub-stomatal CO_2 Concentration (C_i)**	**C_i/C_a ratio**	**Water-Use Efficiency(A/E)**	**Proline**	**GB**
Cultivars (Cv)	1	498.77 **	3664.2 *	0.0295 *	29.71 ns	90.342 ***	5460.05 ***
Drought stress (D)	1	484 **	3098.7 ns	0.0250 ns	130.06 *	37.394 ***	415.1 ns
Treatment (T)	2	206.58 *	4054.8 *	0.0327 *	7.203 ns	7.086 **	897.8 *
Cv × D	1	113.7 ns	536.6 ns	0.0043 ns	1.5059 ns	2.393 ns	0.458 ns
Cv × T	2	35.19 ns	16.603 ns	1.34 ns	23.3928 ns	0.034 ns	930.7 *
D × T	2	8.5833 ns	46.92 ns	3.7875 ns	5.4902 ns	0.344 ns	1733.8 **
Cv × D × T	2	16.861 ns	408.3 ns	0.0032 ns	1.1206 ns	0.476 ns	1331.3 **
Error	24	41.75	807.6	0.0065	18.796	1.031	234.9

Table 1. *Cont.*

Source of Variations	df	Shoot FW	Shoot DW	Root FW	Root DW	Shoot Length	Root Length
		MDA	**AsA**	**H_2O_2**	**Total Phenolics**	**RWC**	
Cultivars (Cv)	df	1.078 ns	3.187 ns	13337 ***	199.28 ***	344.0 *	
Drought stress (D)	1	3.914 ns	17.490 ns	211408.3 ***	390.72 ***	757.4 ***	
Treatment (T)	1	21.825 ns	19.689 *	44144.0 **	102.68 **	182.5 *	
Cv × D	2	17.647 ns	4.765 ns	10378.5 ns	1.52 ns	1.028 ns	
Cv × T	1	0.756 ns	2.573 ns	6929.9 ns	94.39 **	6.005 ns	
D × T	2	2.337 ns	1.891 ns	1049.7 ns	10.0 ns	14.29 ns	
Cv × D × T	2	1.040 ns	2.233 ns	44941.7 **	37.02 ns	10.39 ns	
Error	2	8.276	4.190	6175.0	11.032	51.24 ns	

ns = non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

3.2. Chlorophyll Pigments

A slightly reducing effect of drought stress was observed on chlorophyll *a* and *b* contents ($p \leq 0.01$) and total chlorophyll contents ($p \leq 0.001$), while no significant effect was observed on chlorophyll a/b ratio in both safflower accessions (Table 1). Drought-induced reduction in chlorophyll *a* and *b* contents was more prominent in accession of 16178 than in the other accession, while no prominent difference between the two accessions was observed on total chlorophyll contents (Figures 1 and 2). The foliar-applied GB enhanced the chlorophyll *a* ($p \leq 0.01$) and *b* contents ($p \leq 0.05$) being more prominent in accession 16180 (Table 1, Figure 1). While no significant effects of foliar-applied GB were observed on total chlorophyll contents and chlorophyll *a*/*b* ratio (Table 1). There was no significant difference between both levels (50 and 100 mM) of foliar-applied GB on chlorophyll *a*/*b* ratio (Figures 1 and 2).

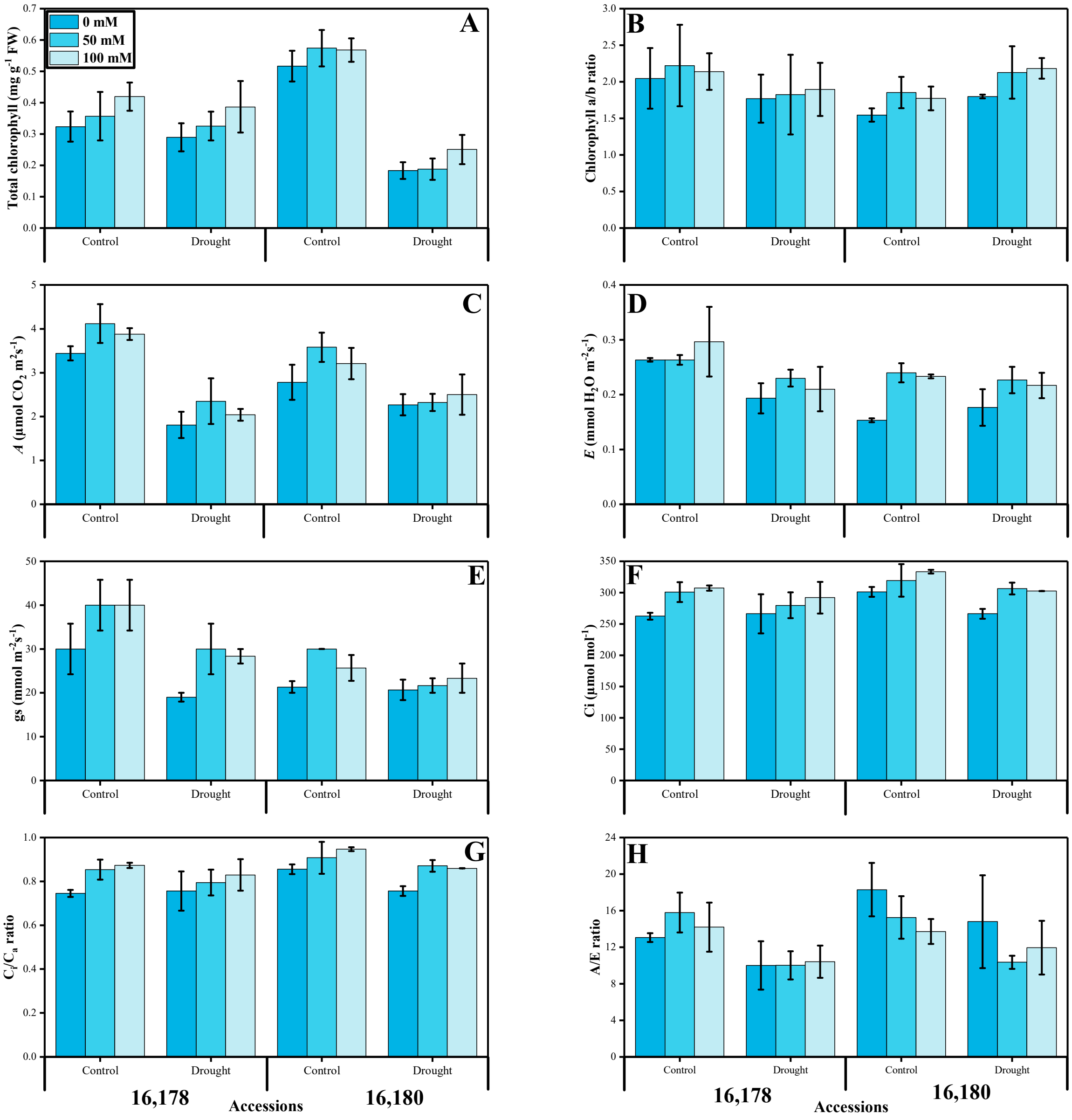

Figure 2. Effect of different concentrations of exogenous application of glycine betaine (0, 50 and 100 mM) on total chlorophyll (**A**), chlorophyll *a*/*b* (**B**), photosynthetic rate (**C**), transpiration rates (**D**), stomatal conductance (**E**), sub-stomatal CO_2 concentration (**F**), C_i/C_a ratio (**G**) and water-use efficiency (**H**) of safflower (accessions) grown under control (normal watering) and drought (60% field capacity)

stressed environment. Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($p < 0.05$). All the data represent the average of three replications ($n = 3$). Error bars represent standard deviation (SD) of three replicates.

3.3. Gas Exchange Characteristics

Drought stress significantly decreased stomatal conductance (g_s) ($p \leq 0.01$), photosynthetic rate (A) ($p \leq 0.001$) and water use efficiency (A/E) ($p \leq 0.05$), while it had no significant effect on transpiration rate (E), sub-stomatal CO_2 concentration (C_i) and C_i/C_a ratio (Table 1). Foliarly applied GB increased stomatal conductance (g_s), sub-stomatal CO_2 concentration (C_i) and C_i/C_a ratio significantly ($p \leq 0.05$), while it had no effect on photosynthetic rate, transpiration rate and water use efficiency (Table 1). In 16178 accession, the effects of drought on stomatal conductance, photosynthetic rate and GB on stomatal conductance were more prominent than those on the other accession (Figure 2). Both levels (50 and 100 mM) of GB were equally effective in affecting the gas exchange attributes (Figure 2).

3.4. Osmoprotectants

Drought stress increased proline contents significantly ($p \leq 0.001$) in both accessions of safflower, being more marked in accession 16180, while it had a non-significant effect on GB accumulation (Table 1, Figure 3). Exogenous application of GB enhanced proline ($p \leq 0.01$) and GB ($p \leq 0.05$) accumulation in both safflower accessions, but it was more prominent in accession 16180 (Figure 3, Table 1). Both levels of GB had a similar effect on intrinsic GB accumulation (Figure 3).

3.5. Lipid Peroxidation

Drought stress significantly ($p \leq 0.001$) increased the production of hydrogen peroxide (H_2O_2) in both safflower accessions, while it did not affect the MDA levels (Table 1). A significant ($p \leq 0.001$) increase was observed in stressed plants due to foliar-applied GB as compared to that in the control plants of both accessions, while no significant effect of GB application was observed on MDA levels (Table 1). A promising increase in H_2O_2 contents was observed in accession 16180 due to foliar-applied 50 mM GB (Figure 3).

3.6. Antioxidants

A considerable ($p \leq 0.001$) increase in total phenolics was observed in both safflower accessions under water shortage, being more prominent in 16178 accession, while it had a non-significant effect on AsA contents (Table 1). Exogenous application of 50 mM as well as 100 mM GB significantly improved the AsA contents ($p \leq 0.05$) and total phenolics ($p \leq 0.01$) under drought stress (Table 1). The effect of GB application on total phenolics was more prominent in accession 16178 than that in the other accession (Figure 3).

3.7. Relative Water Content

Drought stress significantly ($p \leq 0.001$) reduced the RWC of both safflower accessions. Externally applied both GB levels significantly ($p \leq 0.05$) enhanced the relative water content in both safflower accessions (Table 1; Figure 3).

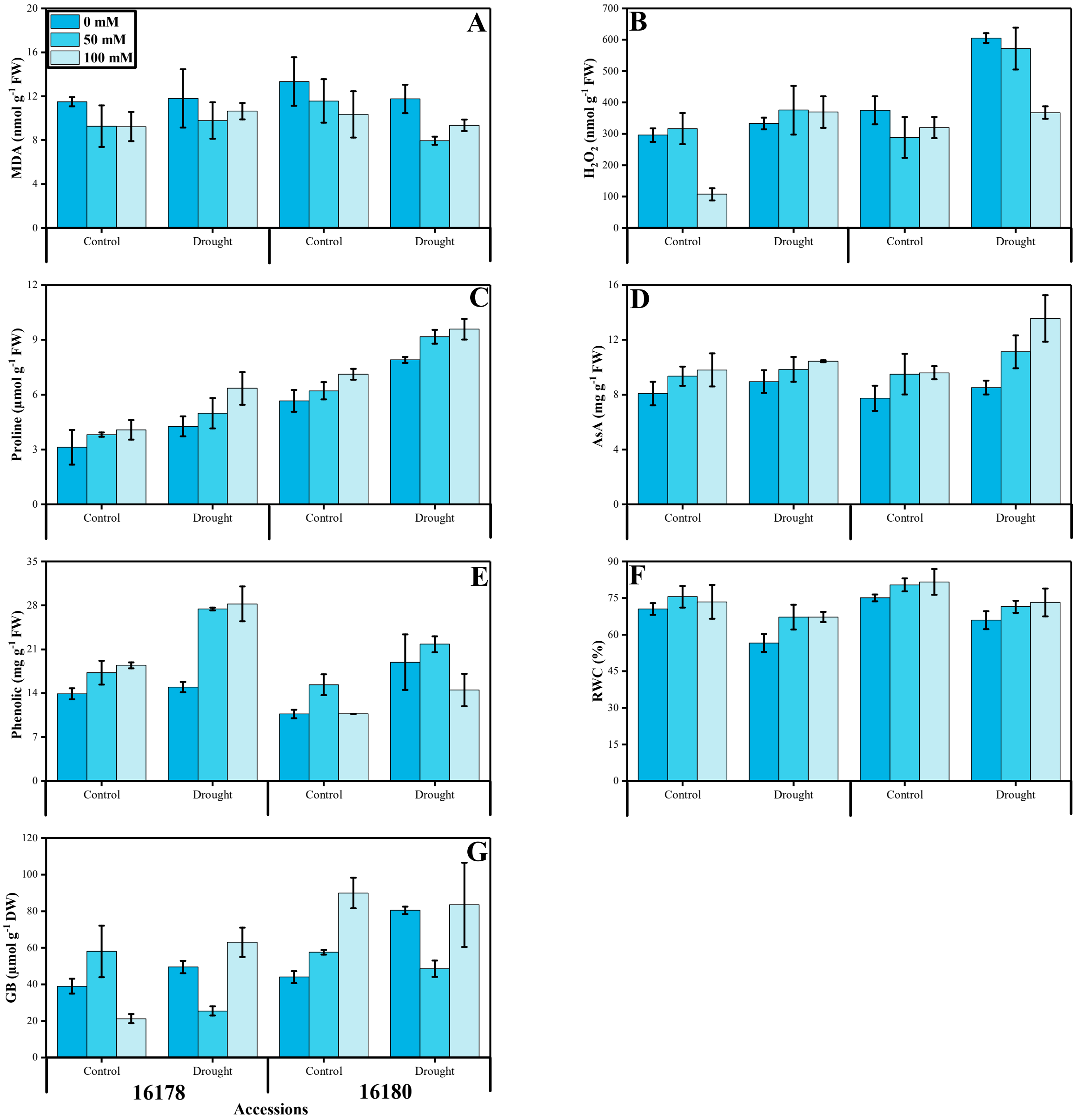

Figure 3. Effect of different concentrations of exogenous application of glycine betaine (0, 50 and 100 mM) on malondialdehyde (**A**), hydrogen peroxide (**B**), proline (**C**), ascorbic acid (**D**), total phenolics (**E**), relative water content (**F**) and glycine betaine (**G**) content of safflower (acessions) grown under control (normal watering) and drought (60% field capacity) stressed environments. Bars sharing different letter(s) for each parameter are significantly different from each other according to Duncan's multiple range test ($p < 0.05$). All the data represent the average of three replications ($n = 3$). Error bars represent standard deviation (SD) of three replicates.

3.8. Relationship

The Pearson correlation analysis was carried out to quantify the relationship between GB with growth, photosynthetic measurements, oxidative stress and antioxidants (Figure 4). The contents of GB were positively correlated with hydrogen peroxide, malondialdehyde, phenolic, proline and ascorbic acid while negatively correlated with root dry weight, root fresh weight, shoot dry weight, shoot fresh weight, root length, shoot length, chlorophyll a, chlorophyll b contents, total chlorophyll, chlorophyll a/b, photosynthetic rate, transpiration rate, stomatal conductance, sub-stomatal CO_2 concentration, C_i/C_a ratio, relative water contents and water-use efficiency. In addition, both accessions showed the same trend under the application of GB under controlled and water stress condition, so we studied only 16178. This correlation reflected the close connection between GB and growth in safflower.

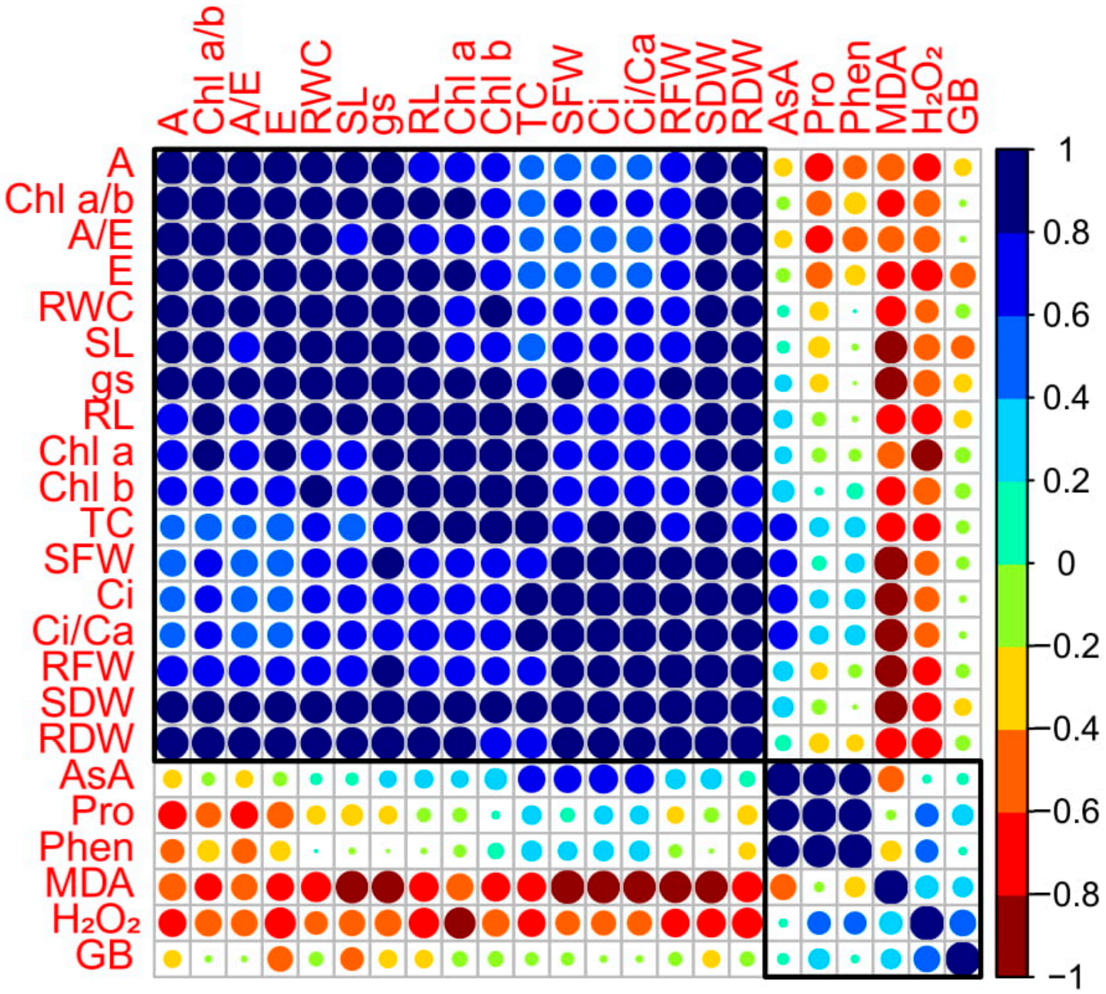

Figure 4. Correlation between GB and growth, photosynthetic measurements, oxidative stress and antioxidants in safflower. Different abbreviations used in this study are as follows: A (photosynthetic rate), Chl a/b (chlorophyll *a*/*b*), A/E (water-use efficiency), SL (shoot length), gs (stomatal conductance), RL (root length), Chl a (chlorophyll a contents), Chl b (chlorophyll b contents), TC (total chlorophyll contents), SFW (shoot fresh weight), Ci (sub-stomatal CO_2 concentration), Ci/Ca (C_i/C_a ratio), RFW (root fresh weight), SDW (shoot dry weight), RDW (root dry weight), AsA (ascorbic acid contents), Pro (proline contents), Phen (phenolic contents), MDA (malondialdehyde contents), H_2O_2 (hydrogen peroxide initiation) and GB (glysine betain contents).

3.9. Principal Component Analysis

The loading plots of principal component analysis (PCA) to evaluate the effects of various levels GB on growth, photosynthetic measurements, oxidative stress and antioxidants in safflower (16178) are presented in Figure 5. Among all the principal components, the first two components, i.e., PC1 (Dim 1) and PC2 (Dim 2), exhibited maximum contribution and accounted for 85% of the total variance in the dataset. Of these, PC1 contributed 65.9%, while PC2 contributed 19.3%. The first group of variables with which PC1 is positively correlated includes: root dry weight, root fresh weight, shoot dry weight, shoot fresh weight, root length, shoot length, chlorophyll a, chlorophyll b contents, total chlorophyll, chlorophyll *a*/*b*, photosynthetic rate, transpiration rate, stomatal conductance, sub-stomatal CO_2 concentration, C_i/C_a ratio, relative water contents and water-use efficiency. A significant negative correlation of PC1 variables was found with the variables aligned with PC2: hydrogen peroxide, malondialdehyde, phenolic, proline, ascorbic acid and GB.

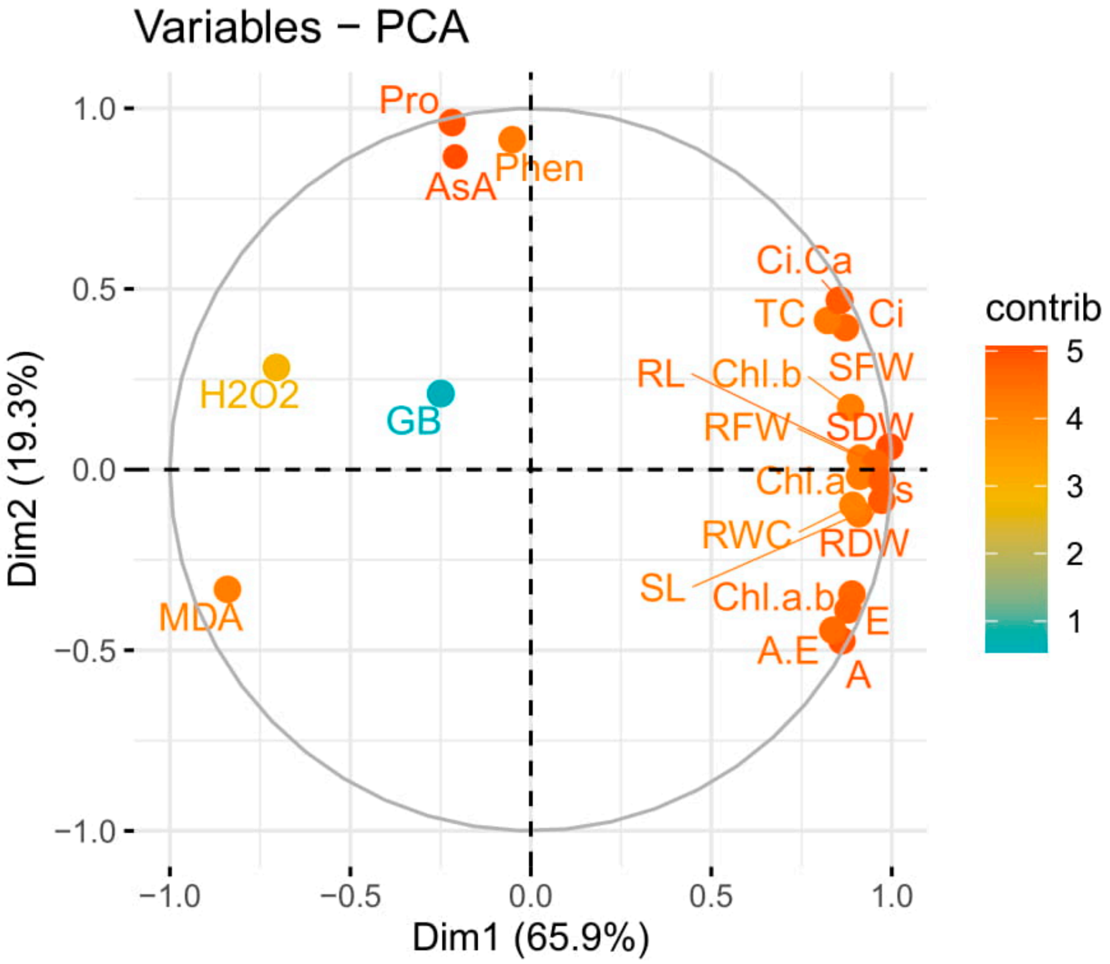

Figure 5. Loading plots of principal component analysis (PCA) on different studied attributes of safflower (16178) grown under different concentrations of exogenous application of glycine betaine (0, 50 and 100 mM) grown in control (normal watering) and drought (60% field capacity) stressed environments. Different abbreviations used in this study are as follows: A (photosynthetic rate), Chl a/b (chlorophyll *a*/*b*), A/E (water-use efficiency), SL (shoot length), gs (stomatal conductance), RL (root length), Chl a (chlorophyll a contents), Chl b (chlorophyll b contents), TC (total chlorophyll contents), SFW (shoot fresh weight), Ci (sub-stomatal CO_2 concentration), Ci/Ca (C_i/C_a ratio), RFW (root fresh weight), SDW (shoot dry weight), RDW (root dry weight), AsA (ascorbic acid contents), Pro (proline contents), Phen (phenolic contents), MDA (malondialdehyde contents), H_2O_2 (hydrogen peroxide initiation) and GB (glysine betain contents).

4. Discussion

The foremost objective of the present study was to determine whether or not exogenous application of glycine betaine (GB) at the rates of 50 and 100 mM could enhance the tolerance of safflower plants against water deficit conditions. Many studies show that water deficiency, for both long and short periods, change the physio-biochemical processes of different crops involved in seed germination, plant growth and yield production [4,18,41]. In this study, plant biomass and lengths of root and shoot of safflower plants were reduced due to drought stress. This is analogous to the findings of Aziz et al. [42] in quinoa, Reboucas et al. [43] in cowpea and Jabeen et al. [44] in spinach, in which a significant reduction in growth was observed in safflower plants due to minimal water supply. They found that obstruction in water supply could affect the rate of transpiration and water-use efficiency, cause lipid peroxidation and nutritional and hormonal imbalances. While the foliar-applied GB improved the plant biomass and shoot and root lengths of safflower plants under drought stress. Our findings are quite similar to those reported earlier in wheat [45] and corn [46], wherein the researchers had shown a significant improvement in growth due to GB application. Such GB-induced improvement in growth could be attributable to the reason that GB helps in the osmoregulation phenomenon as well as to the protection it provides to cell membranes and proteins [25,29,32].

It has been observed that water deficiency reduces the rate of photosynthesis either because of causing a decrease in the chlorophyll synthesis or damage to its molecules [47–52]. In this study, water shortage reduced the chlorophyll *a* and *b* contents and total chlorophyll contents. This is analogous to the finding in water-stressed maize [53] and sunflower [54], where a marked reduction in chlorophyll content has been observed. However, foliar-applied GB enhanced chlorophyll *a* and *b* contents of drought-stressed safflower plants. These findings are in agreement with those reported earlier in water-stressed wheat [55] and maize [46]. The GB-induced increase in chlorophyll may be due to its active role in the maintenance of photosystem II and cytoplasmic dehydration. GB can also lower photoinhibition and turgor loss and increase the stress tolerance of photosynthetic machinery [26,28].

Water deficit conditions are believed to reduce turgidity, impair nutrition balance, gas exchange and stomatal regulation [14,56–58], thereby reducing the growth and yield of plants. In our study, water deficiency reduced the A, A/E and g_s. In our study, water deficiency reduced the photosynthesis rate (A), water use efficiency (A/E) and stomatal conductance (g_s). Our results are similar to those in finger millet [59], wheat [60], barley [61] and cowpea [62]. Exogenously applied GB enhanced the stomatal conductance (g_s), sub-stomatal CO_2 concentration (C_i) and C_i/C_a ratio of safflower plants under drought stress. Similar results were observed in maize [63,64] and sunflower [65]. While critically examining the role of GB in gas exchange characteristics, Ma et al. [46] reported that GB can increase the photosynthetic rate in droughted plants by improving stomatal conductance, which in turn enhances CO_2 diffusion.

Under abiotic stresses, particularly drought stress, GB and proline accumulate in plants to a varying extent, which improves the physiological limitations by detoxifying the ROS [66,67]. In our study, drought stress increased proline and GB contents in safflower plants. A number of studies have shown that drought stress can significantly increase the proline level in different plants such as bell pepper [68] and wheat [69]. However, exogenously applied GB enhanced proline and GB accumulation in safflower plants in the present study. Foliar application of GB also increased the proline contents in corn [46] and GB in sunflower [65]. Furthermore, Raza et al. [45] also observed that exogenously applied GB enhanced proline and GB contents in wheat plants under water-deficit conditions. It has been reported that exogenously applied GB reduces damage to biomolecules and protects plants by enhancing the levels of endogenous solutes like GB and proline, resulting in alleviation of the adverse effects of drought stress [27,29].

It has been reported that hydrogen peroxide (H_2O_2), being an important ROS, helps in signal transduction, particularly under abiotic stresses [3,70]. In the current study, drought stress increased the production of hydrogen peroxide (H_2O_2) in safflower plants, which is similar to the findings observed in barley [71] and quinoa [42]. However, exogenously applied GB decreased H_2O_2 contents in safflower plants under drought stress. Our results are similar to those of Farooq et al. [4] in wheat. They observed a decrease in H_2O_2 by exogenous application of GB. It suggests that GB might have some role in the maintenance of ROS or lipid peroxidation [26,64].

It has been widely reported that enzymatic and non-enzymatic antioxidant defense systems help plants to protect against oxidative stress caused by different abiotic stresses [42,72]. AsA scavenges the H_2O_2 and other ROS species [4], and it is effective in protecting plants against oxidative damage caused by water shortage [1,22]. In the current study, drought stress did not affect the AsA contents in safflower plants, which is contrary to the findings of Secenji et al. [73] in wheat plants. Nevertheless, exogenous application of GB improved the AsA contents in safflower plants under drought stress, but in contrast, the reverse was observed in tobacco by Ma et al. [74]. Against oxidative stress, accumulation of total phenolics is vital for maintaining the oxidative stability of fatty acids in different plant species [3,18,54]. In the current study, drought stress increased the accumulation of total phenolics in safflower plants, which is in contrast to that reported in maize [64] and rapeseed [1]. Exogenous application of GB improved total phenolics in safflower plants under drought stress. Likewise, GB was effective in enhancing the levels of total phenolics in maize plants [29,30,63]. It has been reported that the increase in total phenolics by exogenously applied GB could be due to the fact that GB acts as a signaling substance in plants during drought stress [25,27,32,74].

5. Conclusions

Overall, water deficiency markedly suppressed the plant biomass (fresh and dry weight of root and shoot), lengths of root and shoot, chlorophyll *a* and *b* contents, total chlorophyll, photosynthesis rate (A), water use efficiency (A/E), stomatal conductance and RWC in both accessions of safflower, while it enhanced proline and GB contents, hydrogen peroxide and total phenolics. Drought stress did not affect the chlorophyll *a*/*b* ratio, E, stomatal conductance, sub-stomatal CO_2 concentration and C_i/C_a ratio, GB, AsA and MDA contents. Foliar applied GB was effective in enhancing the plant biomass (fresh and dry weight of root and shoot), lengths of root and shoot, chlorophyll *a* and *b* contents, stomatal conductance (g_s), sub-stomatal CO_2 concentration (C_i) and C_i/C_a ratio, proline, GB, H_2O_2, AsA, total phenolics and RWC in safflower plants under water shortage. However, no considerable change was observed in chlorophyll *a*/*b* ratio, transpiration rate, photosynthesis rate, water use efficiency and MDA contents due to GB application on both accessions of safflower under drought stress. Therefore, foliar spray of GB could be effective in improving the osmoprotective defense system of both safflower accessions under stress conditions.

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