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DECODING DROUGHT RESILIENCE IN CHINESE ANNUAL RYEGRASS (*Lolium multiflorum* Lam.): INTEGRATED PHYSIO-BIOCHEMICAL PROFILING REVEALS BOLT AS A SUPERIOR DROUGHT-TOLERANT CULTIVAR

X. Du, P. Deng, Y. Luo, T. Sangzhu and Man-Jun Yang

Science & Technology Park, Xizang Key Laboratory of Veterinary Drug, Faculty of Animal Science and Technology, Xizang Vocational Technical College, Lasa, Xizang, 850030, People's Republic of China.
Corresponding Author's e-mail: manjunyang@126.com

ABSTRACT

Annual ryegrass (*Lolium multiflorum* Lam.) cultivars, including Angus No.1, Abundant, Bolt, Jumbo, Tetragold, Barwoltra, and Diamond T, are widely cultivated in China for forage production. However, drought stress severely limits their yield and quality. This study investigated the drought tolerance mechanisms of these cultivars under 14-day extreme drought conditions. Physio-biochemical parameters, including chlorophyll content, osmoprotectants (saccharides, free amino acids, ascorbic acid), antioxidants (glutathione, polyphenols), oxidative damage markers (malondialdehyde (MDA), reactive oxygen species (ROS), superoxide anion), and structural components (cellulose, hemicellulose, lignin), were analyzed. This research found that Bolt exhibited superior drought tolerance, maintaining higher chlorophyll A (1.93 ± 0.035 mg/g), total chlorophyll (3.17 ± 0.065 mg/g), ascorbic acid (1885.9 ± 117.8 µMol/g), and glutathione (234.2 ± 25.69 µg/g) compared to other cultivars. Additionally, Bolt showed enhanced hemicellulose accumulation (241.3 ± 3.21 mg/g) and minimal reduction in cellulose. Despite elevated MDA (13.93 ± 1.1 nMol/g) and ROS (119,000 ± 4,000 relative fluorescence units (RFU) /g), Bolt's robust antioxidant system and structural adaptations likely mitigated oxidative damage. Re-watering experiments confirmed Bolt's resilience, underscoring its suitability for cultivation in arid areas. These results provide critical insights into selecting drought-tolerant ryegrass varieties for sustainable forage production in China.

Keywords: *Lolium multiflorum*, Drought stress, Antioxidants, Osmoprotectants, Cell wall components.

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INTRODUCTION

Drought stress is a critical abiotic factor limiting agricultural productivity worldwide, with escalating impacts due to climate change (Cohen *et al.*, 2021). In China, annual ryegrass (*Lolium multiflorum* Lam.) is a key forage crop, valued for its high digestibility and adaptability to marginal lands (Xiong *et al.*, 2021). However, frequent droughts have severely constrained its yield and nutritional quality, threatening livestock production systems (Fernando *et al.*, 2019). Annual ryegrass is often rotated with staple crops to avoid competition for arable land (Baldinger *et al.*, 2011), its sensitivity to water deficit necessitates the identification of drought-tolerant cultivars for sustainable cultivation.

Drought tolerance in plants involves multifaceted physiological and biochemical adaptations, including osmotic adjustment via sugars and amino acids, antioxidant defense systems (e.g., glutathione and ascorbic acid), and structural modifications such as lignin deposition (Hussain *et al.*, 2018; Ferioun *et al.*, 2023). For instance, in alfalfa (*Medicago sativa*), enhanced antioxidative capacity and reduced lipid peroxidation are pivotal for drought resilience (Zhang *et al.*, 2019). Similarly, cotton (*Gossypium* spp.) modulates cellulose synthase activity to prioritize osmoprotectant synthesis over cell wall biosynthesis under stress (Singh *et al.*, 2016). Despite these advances, the drought response mechanisms of annual ryegrass, particularly commercial cultivars in China, remain underexplored. Existing studies on *L. multiflorum* primarily focus on transcriptomic or proteomic profiles under short-term stress (Perlikowski *et al.*, 2016; Pan *et al.*, 2017), leaving a gap in understanding the integrated physio-biochemical responses under prolonged drought.

In China, seven annual ryegrass cultivars of Angus No.1, Abundant, Bolt, Jumbo, Tetragold, Barwoltra, and Diamond T, are widely cultivated, yet their comparative drought tolerance is poorly characterized. Previous research on drought stress in crops such as rice (*Oryza sativa* L.) (Sahebi *et al.*, 2018) and maize (*Zea mays* L.) (Singh *et al.*, 2023) highlights the importance of antioxidant systems and cell wall remodeling; however, analogous studies on forage

grasses like ryegrass are scarce (Sustek-Sánchez *et al.*, 2023). Furthermore, while lignin accumulation under drought is known to reduce forage digestibility (Lee *et al.*, 2012), its role in ryegrass stress tolerance remains unclear. Addressing these gaps is critical for breeding programs aimed at improving drought resilience without compromising nutritional value.

Against these drought damages, these annual ryegrass varieties of Angus No.1, Abundant, Bolt, Jumbo, Tetragold, Barwoltra and Diamond T should behave differently. This study evaluates the physio-biochemical responses of seven Chinese annual ryegrass cultivars under 14-day extreme drought stress. We hypothesize that varietal differences in (1) antioxidant capacity (glutathione, ascorbic acid), (2) osmoprotectant retention (saccharides, amino acids), and (3) structural component dynamics (cellulose, hemicellulose, lignin) determine drought tolerance. By integrating these parameters with post-rehydration recovery assays, we aim to identify cultivars with optimal drought adaptability for cultivation in arid regions of China.

MATERIALS AND METHODS

Detection kits and chemical reagents: The content assay kits of malondialdehyde (MDA), glutathione (GSH), glutathione disulfide (GSSG), total saccharide, glucose, sucrose, cellulose, hemicellulose, lignin, free amino acid and ascorbic acid were employed by Beijing Solarbio Science & Technology Company Ltd. (Beijing, China). The ROS and superoxide anion probes (catalog: S0033M and S0063) were obtained from Beyotime Institute of Biotechnology Company Ltd. (Shanghai, China). Total polyphenol assay reagent (catalog: A500467-0100) was purchased from Sangon Biotech Company Ltd. (Shanghai, China). Chemical reagents were obtained by Shanghai Aladdin Bio-Chem Technology Company Ltd. (Shanghai, China). Using Wahaha purified water as 18.9 MΩ deionized water, it's purchased from the Hangzhou Wahaha Group Ltd. (Hangzhou, China).These Solarbio kit instructions of BC0025, BC1175, BC1185, BC2715, BC2505, BC2465, BC4285, BC4445, BC4205, BC1575 and BC4635 could be downloaded from this manufacturer's website (http://www.solarbio.net/index.php, accessed on 26 May 2024). The manuals of S0033M and S0063 were obtained from their website (https://www.beyotime.com/index.htm, accessed on 26 May 2024).

Plant Materials: Annual ryegrass of *L. multiflorum* Lam. cv. Angus No.1, Abundant, Bolt, Jumbo, Tetragold, Barwoltra and Diamond T are commercial cultivars, purchased from BarenbrugAgriseeds (Bailv (Tianjin) International Grass Industry Co., Ltd., Tianjin, China), and their purity and germination rate are 90% and 98%. As described by Ling *et al.* (2020), these ryegrass varieties were cultivated by hydroponics. In detail, they were hydroponic culture and grown in vermiculite with 1/2 Hoagland's nutrient solution (Catalog: HB8870-1, Qingdao High-tech Industrial Park Haibo Biotechnology Co., Ltd., Qingdao, China). Outdoor cropping was carried out at a experimental field in Eryuan County, Yunnan Province (29°56′10.4″ N, 94°47′2.65″ E). The experiment was conducted at an average temperature of 25 ºC (± 5 ºC) and luminous intensity of 10,000 lux (± 5,000 lux).Before germinating, 2 g ryegrass seeds were weighed, and soaked in 1‰ $KMnO_4$ solution for 15 min to disinfect, and then washed with sterile distilled water. After germination at 25 ± 5 ºC, hydroponic culture (prepared with distilled water) was conducted in 1/2 Hoagland nutrient solution. 150 g vermiculite was used to fix roots of ryegrass. Here, the vermiculite had to absorb this nutrient solution, and let the nutrient solution over the vermiculite. The container is a plastic basin with a strainer, which diameter and depth is 170 mm and 73 mm respectively.

Experiment design and sample preparation: The planting conditions were light intensity of 10,000 lux ± 5,000 lux for 12h, darkness for 12h, day-night growth temperature of 25 ± 5 ºC, and kept air relative humidity of 60% ± 10%. After germination, these annual ryegrass varieties were grown for 14 days. According to Shen *et al.* (2022) and Wang *et al.* (2022b), then, the experimental groups were subjected to natural drought stress after the Hoagland nutrient solution was removed and non-irrigated till 14 days. Under an evaporation of 27.18 ± 3.4 g/day, the relative water content of these ryegrasses decreased significantly, from 93.13% to 31.07%. Using the hydroponic method, the initial water content of vermiculite employed in cultivation was maintained at 100%, wherein vermiculite solely functioned as a medium for securing ryegrass roots. Upon the initiation of stress conditions, characterized by the cessation of water flow and a 14-day drought treatment, the water content of the vermiculite was decreased to 10.3% ± 2.1%.Meanwhile, the 1/2 Hoagland nutrient solution hydroponics group was used as a control, and 3 biological duplicates were set for each group. To detect the drought tolerance of these varieties, after 14 days drought stress, they were immediately irrigated with enough 1/2 Hoagland nutrient solution to help them recover, while recovery was observed and recorded 2 days later (Shen *et al.*, 2022; Wang *et al.*, 2022b). The moisture content of the grass samples was used to calculate the net matter weight of these ryegrasses. Then the above-ground parts of these annual ryegrass varieties were collected and stored at -80 ºC until analysis.These ryegrass samples were ground with a mortar and pestle in liquid nitrogen, and 1g sample homogenate with 10 mL PBS (pH7.4, 50 mM) were ground fully setting at 4 ºC,

and then centrifuged at 12,000 rpm at 4 ºC for 10 min to remove insoluble precipitation. Then, it's determined for these physio-chemical indicators of MDA, ROS, superoxide anion, GSH, GSSG, total saccharide, glucose, sucrose, free AA, AsA and total polyphenol.

Quantification of MDA, ROS and superoxide anion production: As described by Zhang *et al.* (2019), MDA was used a commercial kit for plant. Referring to its manual, here 100 µL sample extraction was added with 300 µL testing buffer and 100 µL reagent buffer III, mixed and incubated in an 100 ºC water bath for 60 min. After cooling, this mixture was centrifuged at 12,000 rpm for 10 min at 25 ºC. Then, 200 µL supernatant was added into a 96-well to measure their absorption value at 450 nm, 532 nm and 600 nm respectively, using a microplate spectrophotometer (Epoch2, BioTek Instruments Inc., USA).

According to Zhang *et al.* (2019), ROS was detected by fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and then these samples were incubated with 10 mM DCFH-DA probe at 37 ºC for 30 min in the dark. The extraction was analyzed by a microplate reader (Varioskan LUX, Thermo Scientific, United States) at an excitation and emission wavelength of 495 and 525 nm, respectively. Each extract was measured in duplicate.

As mentioned by Devillard *et al.* (2008) and the manual of dihydroethidium (DHE) probe, superoxide anion was detected by fluorescent probe DHE, and then these samples were incubated with 10 µM DHE probe at 37 ºC for 30 min in the dark. The extraction was analyzed by a microplate reader at an excitation and emission wavelength of 495 and 525 nm, respectively. Here, DHE probe is generally prepared into an initial concentration of 10 mM by dimethyl sulfoxide, which is diluted to the final concentration of 10 µM during detecting. There were 3 biological replicates in each experimental group, which extract was measured in triplicates.

Determination of GSH and GSSG: According to the manual of GSH and GSSG kits, we detected the absorption value at 412 nm in 15 min after termination using a microplate spectrophotometer (Zhang *et al.*, 2022). Then, the initial and final absorption values were recorded according to these kits instructions and calculated the difference between these two values. Thus, the content of GSH and GSSG can be calculated by the formula of these kits instructions. There were 3 biological replicates in each experimental group, which extraction was measured in triplicates.

Detection of sacchrides, total free AA and AsA content: These extracted supernatants were transferred to a 15 mL EP tube, that's covered tightly (to prevent water loss) and placed to boiling water bath for extraction for 15 min. After cooling, these samples were centrifuged at 10,000 rpm for 10 min at 4 ºC, and the supernatant was taken to be measured by biochemical kits. Then, the absorption values were recorded according to these kits instructions and calculated the difference between these blank, standard and measuring samples.Thus, the content of total sacchride, glucose and sucrose can be calculated by the standard curve formula of these kits instructions, at 540, 505, and 480 nm respectively (Ahmed *et al.*, 2022). Here, referring to Alexou (2013), the content of total free AA and AsA could be calculated by the formula of these kit manuals, at 570 and 525 nm respectively. There were 3 biological replicates in each experimental group, which extract was measured in triplicates.

Determination of total polyphenol content: Using the Folin-Ciocalteu colorimetric method (Andre *et al.*, 2009), all the ryegrass extraction were diluted with purified water to obtain readings within the standard curve of gallic acid ($y = 0.1554x–0.0074$, $r = 0.9994$). Here, this standard curve was established by plotting the absorbance value (x-axis) against the gallic acid concentration (y-axis, mg/ml). Briefly, 125 µL of diluted ryegrass extraction was mixed with 0.5 mL of purified water in a 15 mL EP tube followed by the addition of 125 µL of Folin-Ciocalteu reagent. The samples were mixed well and then allowed to stand for 6 min before 1.25 mL of a 7% sodium carbonate ($NaCO_3$) aqueous solution was added. Water was added to adjust the final volume to 3 mL. Samples were allowed to stand for 90 min at room temperature before the absorbance was measured at 760 nm versus the blank using a microplate reader. Absorbance reads were compared with those of standards prepared similarly with known gallic acid concentrations, which were shown as mean with SD (gallic acid equivalents mg/g ryegrass, SD for 9 replications). There were 3 biological replicates in each experimental group, which extract was measured in triplicates.

Detection of chlorophyll content: As described by Pérez-Patricio *et al.* (2018) and Ciganda *et al.* (2009), the overground parts of fresh annual ryegrass were collected and dehydrated. In a mortar and pestle, 1 g sample was placed and macerated. The whole process was executed in a place protected from light. From the macerate, 8 mL of 99% acetone was mixed with 4 mL ethanol (2:1, v/v), placed in 50 mL EP tubes, and mixed for 1 min stirring, ensuring complete contact of the plant material. These were then left to stand for 30 min in the freezer in the dark, and centrifuged at 2,000 rpm for 10 min. Then 5 mL of acetone/ethanol (2:1, v/v) was added and stirred for 1 min. The extraction process should be careful to avoid light. Absorbance readings were evaluated at 663 nm and 645 nm. The control was acetone/ethanol (2:1, v/v). The obtained absorbance was substituted in the formulas to evaluate the photosynthetic capacity, at 663 nm and 645 nm, respectively. Here, using the acetone/ethanol mixture, spectrophotometer was adjusted

to zero. There were 3 biological replicates in each experimental group, which was carried out in triplicates.

Detection of cellulose, hemicellulose and lignin content: According to Balsamo *et al.* (2015) and the instructions of the cellulose assay kit at 620 nm, an extraction method was performed in two steps as follows. Firstly, extraction of cell wall material (CWM) : 300 mg (denoted as W1) sample, added 1mL extract solution, should be rapidly homogenized at room temperature, and then transferred into a water bath at 90 ºC for 20 min. After cooling, the mixture was centrifuged at 6,000 g for 10 min at 25 ºC, and their supernatant should be discarded. The precipitation was washed twice with 1.5 mL extraction solution I and acetone respectively (vortex oscillating for about 2 min, centrifuging at 6,000 g for 10 min at 25 ºC, discarding the supernatant). Thus the thick cell wall was precipitated. 1 mL extraction solution II (in order to remove starch) was added and soaked for 15 hours, and centrifuged at 6,000 g for 10 min at 25 ºC. After centrifuging, this supernatant was discarded. The precipitates were dried to obtain CWM and weighed as W2. Secondly, extraction of cellulose: 5 mg dried W2 (denoted as W3), was added with 0.5 mL distilled water to fully homogenize. Then all the mixture was transferred into a new EP tube, and added with distilled water upto the volume of 0.5 mL in an ice water bath. After that, this CWM mixture was slowly added by 0.75 mL sulfuric acid (H_2SO_4), and slowly mixed in an ice bath for 30 min. This reactant mixture was centrifuged at 8, 000 g for 10 min at 4 ºC, and then their supernatant was taken into a new EP tube and diluted 20 times with distilled water to be measured. Furthermore, diluting the 10 mg/mL standard solution with distilled water to 0.125, 0.0625, 0.03125, 0.015625, 0.0078, 0.0039 mg/mL standard solution to draw the standard curve, the content X was calculated via this standard curve. Then the cellulose contents were evaluated by a formula mentioned in kit manual (Cellulose (mg/g) = 22.52*X/W3).

The calculating methods as described in the instructions of these kits for the detection of hemicellulose and lignin, 50 mg and 3 mg samples were taken to extract respectively. Then, the testing reaction was carried out according to the procedure in the instructions, and then 200 µL was absorbed for analysis, at 540 and 280 nm respectively (Balsamo *et al.*, 2015). There were 3 biological replicates in each experimental group, which extract was measured in triplicates.

Data analysis: WPS Office for Windows (Beijing Kingsoft Office Software, Beijing, China) was used to data manipulations and transformations. Using GraphPad Prism (version 7.0, La Jolla, CA, USA), these histograms were employed to analyze the differentiation. All data were analyzed using two-way ANOVA with drought stress and cultivar as fixed factors to assess main and interaction effects, followed by Tukey's HSD post hoc tests for pairwise comparisons (p<0.05). Normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) assumptions were validated, and data were structured with 12 replicates per treatment group after outlier removal ($p < 0.05$). The model included cultivar, drought, and their interaction, with effect sizes (partial η^2) calculated to quantify variance contributions. Analyses were conducted in R (v4.3.1) using *aov*, *car*, and *effectsize* packages, and results are reported as mean ± SD with significance denoted by asterisks.This approach ensured robust identification of cultivar-specific drought responses while accounting for potential interactions between genetic and environmental factors.

RESULTS

Photosynthesis dynamic response: Chlorophyll content (Chl A, Chl B, and total Chl) varied significantly among cultivars and drought treatments. Under control conditions, as shown in Figure 1 A, Bolt exhibited the highest Chl A (3.15 ± 0.02 mg/g) and total chlorophyll (4.52 ± 0.03 mg/g), while Barwoltra showed elevated Chl B (1.37 ± 0.02 mg/g). Drought stress reduced Chl A and total chlorophyll in all cultivars (p< 0.05), with the largest reduction observed in Angus No.1 (ΔChl A = 2.32 mg/g, ΔTotal Chl = 2.64 mg/g) and the smallest in Bolt (ΔTotal Chl = 1.35 mg/g). Conversely, Chl B increased in Tetragold (Δ = 69%) and Jumbo (Δ = 16%) under drought but decreased by 58% in Barwoltra (Figure 1 B, C).Two-way ANOVA revealed significant main effects of cultivar and drought with significant cultivar × drought interactions. Post hoc analysis confirmed Bolt and Barwoltra maintained higher chlorophyll levels than other cultivars. These results demonstrate cultivar-specific drought resilience, highlighting Bolt as a promising candidate for drought-prone environments.

All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

Osmotic pressure dynamics: Drought stress and cultivar significantly influenced ascorbic acid (AsA) and free amino acid (FAA) metabolism. Under control conditions, as shown in Figure 2 A, Diamond T exhibited the highest AsA (4143.3 ± 288.7 nMol/g FW), while Tetragold showed the highest FAA (15.8 ± 1.6 µMol/g FW). Drought stress reduced AsA by 52–86% ($p < 0.05$) and FAA by 62–80% ($p < 0.05$) across cultivars. Bolt retained the highest AsA under drought (1885.9 ± 117.8 nMol/g), whereas Barwoltra maintained relatively stable FAA levels (Δ = 4.1 µMol/g). Notably, Bolt demonstrated partial drought tolerance with a minimal AsA reduction (Δ = 48%), contrasting with Angus No.1's severe decline (Δ = 85%). As shown in Figure 2 B, FAA in Tetragold decreased sharply (Δ = 79%), while Diamond T showed moderate reduction (Δ = 39%). Two-

way ANOVA revealed significant main effects of cultivar (AsA: $F(6,154) = 98.4$, $p < 0.001$, $\eta^2 = 0.79$; FAA: $F = 45.2$, $\eta^2 = 0.60$) and drought (AsA: $F = 1532.7$, $\eta^2 = 0.91$; FAA: $F = 867.3$, $\eta^2 = 0.83$), with significant cultivar × drought interactions (AsA: $F = 72.6$, $\eta^2 = 0.74$; FAA: $F = 28.9$, $\eta^2 = 0.49$). Post hoc analysis confirmed Diamond T and Barwoltra had higher AsA than other cultivars ($p < 0.05$), while Tetragold and Bolt exhibited distinct FAA accumulation patterns ($p < 0.05$). These findings highlight Bolt and Barwoltra as drought-tolerant candidates due to their biochemical stability under stress.

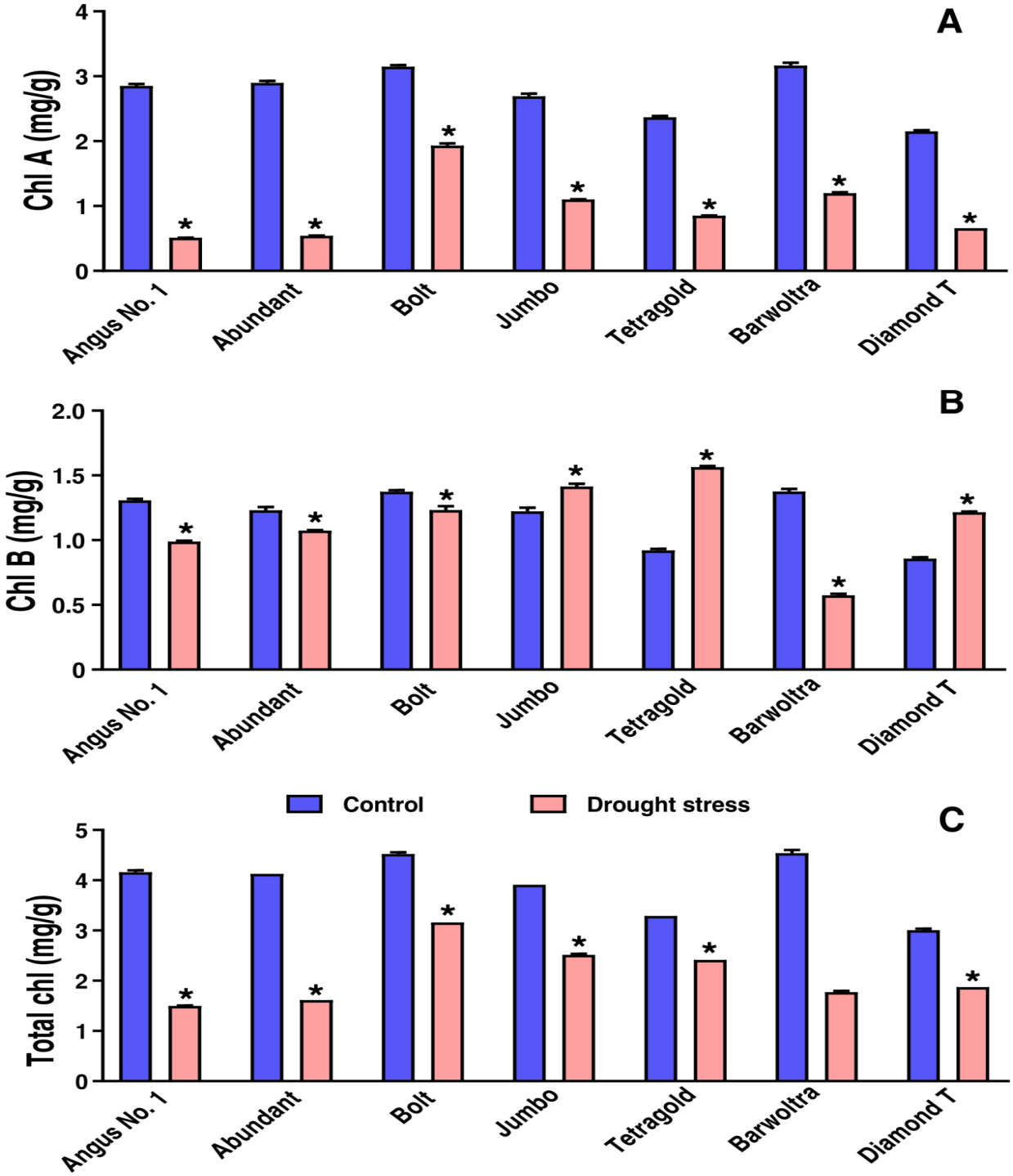

Figure 1. Effect of drought stress on chlorophyll contents of different ryegrass cultivars (A) Chlorophyll A (Chl A), (B) Chlorophyll B (Chl B), and (C) Total chlorophyll (total Chl).

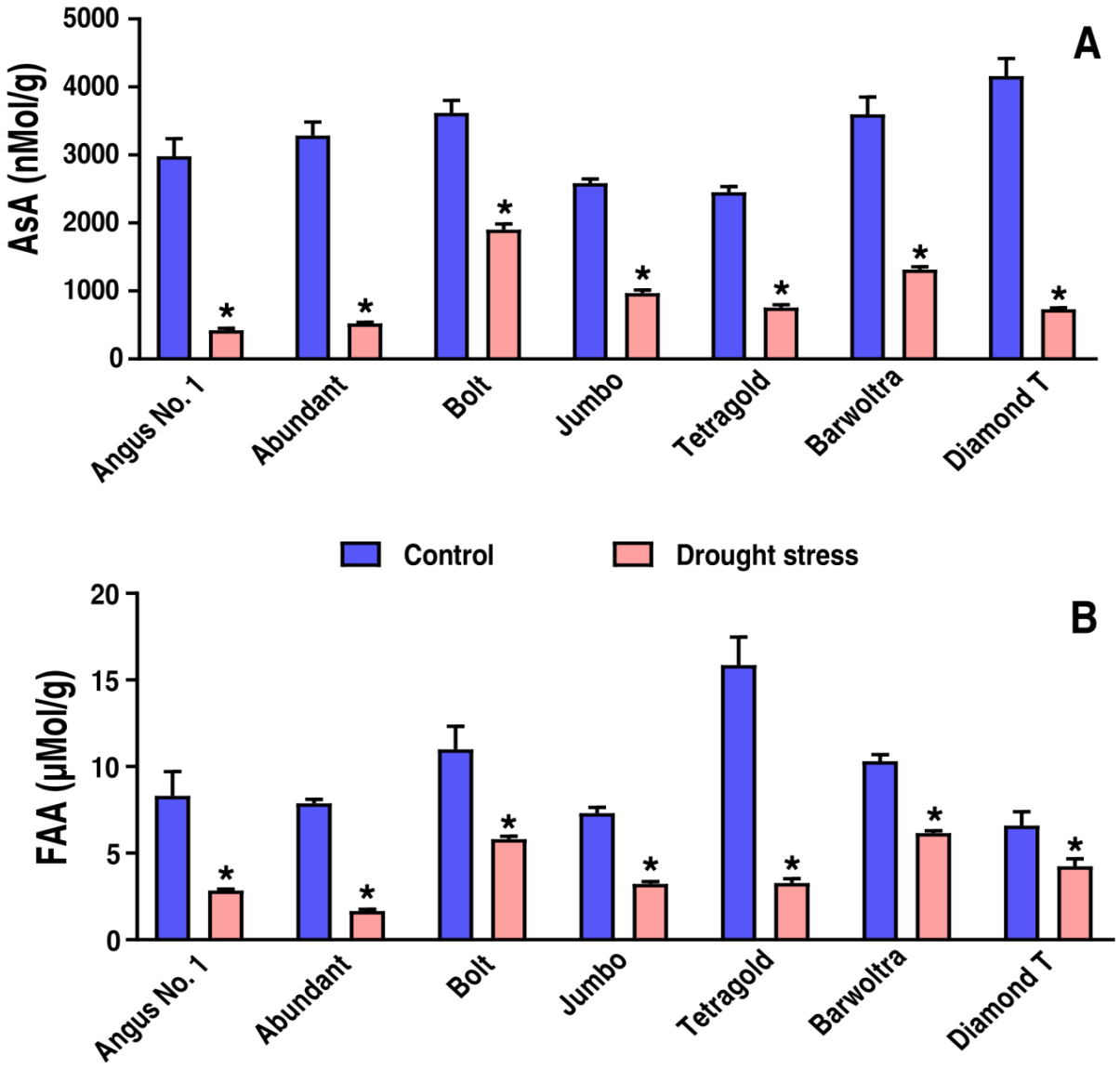

Figure 2. Ascorbic acid (AsA) and free amino acid (FAA) response to drought stress. (A) AsA content and (B) Total free AA content in seven cultivars after 14 days of drought.

All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

Carbon metabolism dynamics: Drought stress and cultivar significantly influenced glucose, sucrose, and total sugar metabolism (Figure 3). Under control conditions, as shown in Figure 3 A, Jumbo exhibited the highest total saccharide content (194.0 ± 1.8 mg/g), while Bolt showed minimal glucose reduction under drought (Δ = 6.0 µMol/g) (Figure 3 A, C). Drought stress reduced glucose by 48–89% ($p < 0.001$), sucrose by 85–95% ($p < 0.001$) (Figure 3 B), and total saccharide by 73–93% ($p < 0.001$) across cultivars. Notably, Bolt retained 91% of its glucose content under drought (62.9 ± 3.5 µMol/g vs. 68.9 ± 4.2 µMol/g in controls), contrasting with Angus No.1, which lost 81% (12.0 ± 0.3 µMol/g vs. 62.4 ± 4.3 µMol/g). As shown in Figure 3 B, Sucrose levels in Diamond T decreased by 92% (0.83 ± 0.05 mg/g vs. 10.8 ± 0.4 mg/g), while Bolt maintained 77% retention (4.3 ± 0.1 mg/g vs. 19.1 ± 3.2 mg/g). As shown in Figure 3 C, total saccharide in Bolt decreased by only 35% (81.0 ± 0.4 mg/g vs. 124.2 ± 1.1 mg/g), compared to Jumbo's 76% reduction (47.2 ± 0.9 mg/g vs. 194.0 ± 1.8 mg/g).Two-way ANOVA revealed significant main effects of cultivar (Glucose: $F(6,154) = 68.2$, $p < 0.001$, $\eta^2 = 0.73$; Sucrose: $F = 54.1$, $\eta^2 = 0.68$; Total sugar: $F = 89.5$, $\eta^2 = 0.82$) and drought (Glucose: $F = 985.4$, $\eta^2 = 0.87$; Sucrose: $F = 1120.6$, $\eta^2 = 0.89$; Total sugar: $F = 1450.3$, $\eta^2 = 0.92$), with significant cultivar × drought interactions (Glucose: $F = 45.7$, $\eta^2 = 0.64$; Sucrose: $F = 38.9$, $\eta^2 = 0.58$; Total sugar: $F = 62.3$, $\eta^2 = 0.71$). Post

hoc analysis confirmed Jumbo and Barwoltra had higher total sugar than other cultivars under control (p <0.05), while Bolt's glucose content showed no significant decline under drought. Thus, these results highlight Bolt's exceptional drought tolerance, suggesting its utility in water-limited environments.

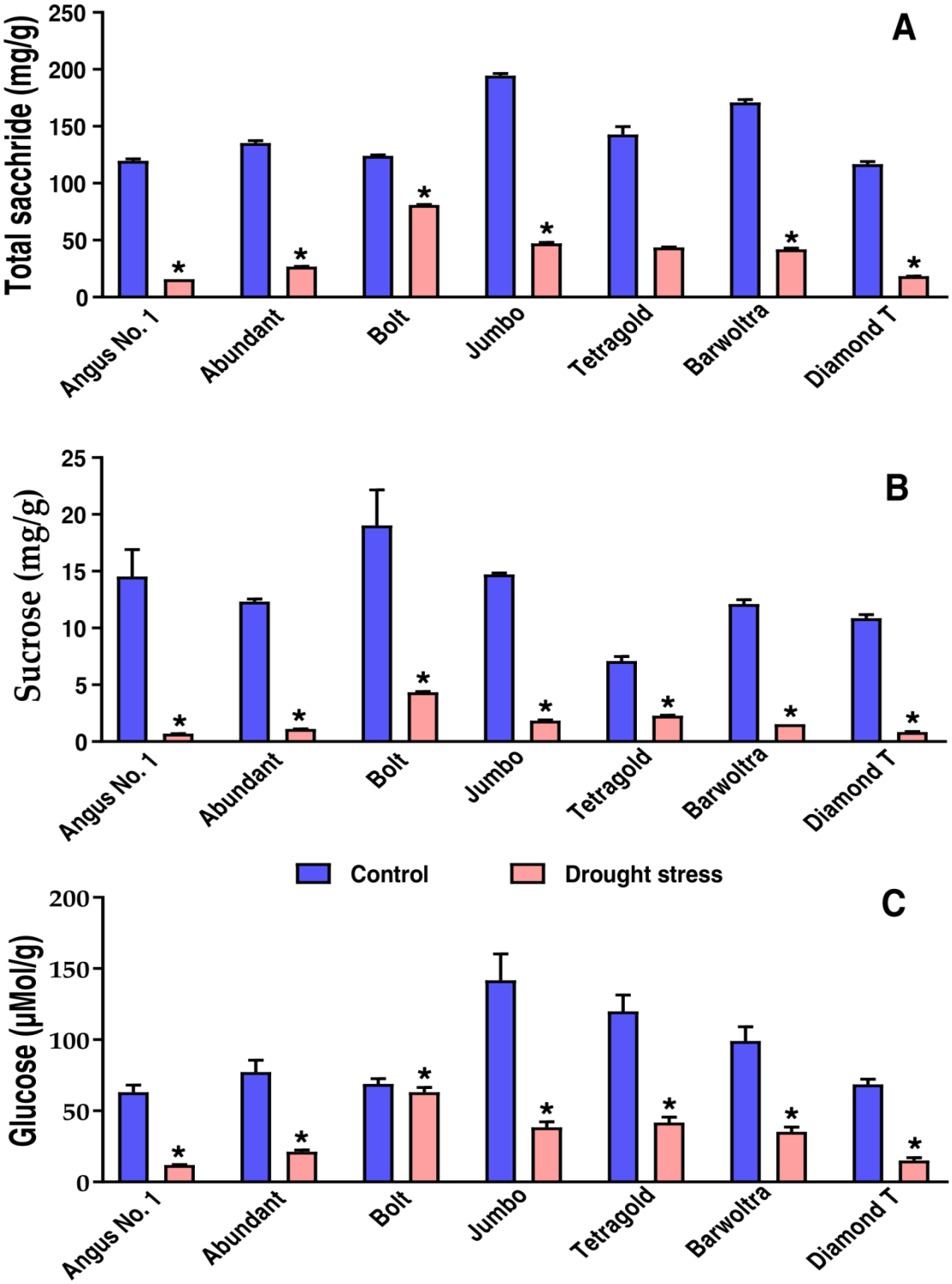

Figure 3. Carbohydrate metabolism under prolonged drought stress. (A) Total saccharides, (B) Sucrose, and (C) Glucose content in drought-stressed cultivars. All analyses were assessed using the Shapiro-Wilk test and Levene's test (* p > 0.05), confirming the assumptions required for the application of Tukey's HSD test.

Kinetic antioxidant capacity of polyphenols: Drought stress and cultivar significantly influenced total polyphenol content (Figure 4). Under control conditions, as shown in Figure 4, Bolt exhibited the highest polyphenol levels (11.1 ± 0.9 mg/g), while Diamond T showed the lowest (8.3 ± 0.6 mg/g). Drought stress reduced polyphenol content by 76–94% across all cultivars ($p < 0.001$). Bolt retained the highest polyphenol levels under drought (5.3 ± 0.2 mg/g), whereas Angus No.1 experienced the largest reduction (Δ = 9.6 mg/g). Notably, Bolt's polyphenol content decreased by only 52% compared to Tetragold's 74% reduction. Barwoltra maintained moderate stability (Δ = 8.3 mg/g), contrasting with Diamond T's sharp decline (Δ = 6.8 mg/g). Two-way ANOVA revealed significant main effects of cultivar ($F(6,154) = 38.7$, $p < 0.001$, $\eta^2 = 0.65$) and drought ($F(1,154) = 890.2$, $p < 0.001$, $\eta^2 = 0.86$), with a significant cultivar × drought interaction ($F(6,154) = 24.3$, $p < 0.001$, $\eta^2 = 0.53$). Post hoc analysis confirmed that Bolt and Barwoltra had significantly higher polyphenol content than other cultivars under both control and drought conditions ($p < 0.05$). All cultivars exhibited significant drought-induced reductions in polyphenol content ($p < 0.05$). These results highlight Bolt's resilience in preserving polyphenols under water-limited conditions, underscoring its potential as a drought-tolerant cultivar.

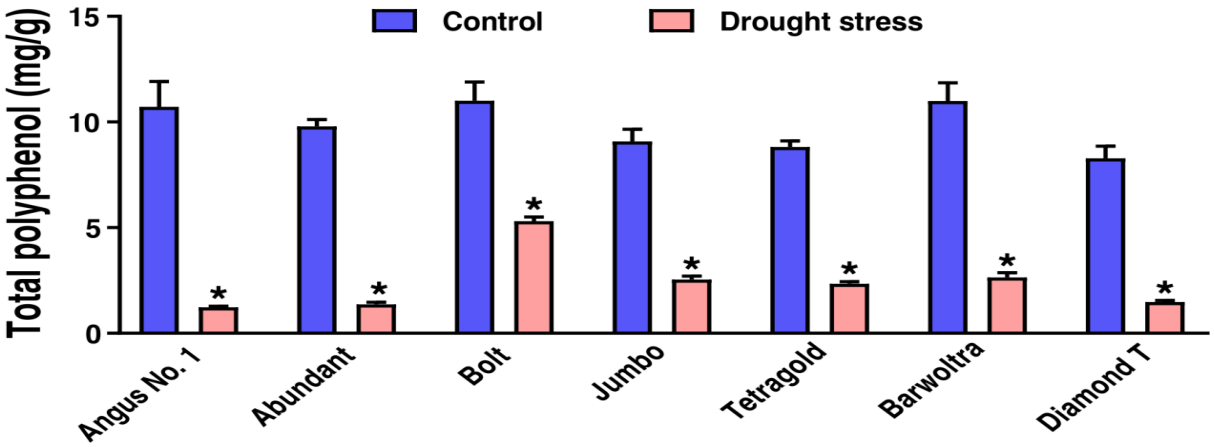

Figure 4. Total polyphenol accumulation in annual ryegrass cultivars after 14-day drought.
All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

Dynamics of cell structure synthesis: The effects of drought stress and cultivar on cell wall biosynthesis components (hemicellulose, cellulose, and lignin) were investigated using a two-way ANOVA. Under control conditions, as shown in Figure 5 A, Cellulose levels remained stable in Bolt (27.5 ± 0.7 mg/g control vs. 27.8 ± 0.6 mg/g drought), whereas Diamond T experienced a significant decline from 36.4 ± 2.1 mg/g to 27.4 ± 0.8 mg/g($p < 0.05$). As shown in Figure 5 B, Angus No.1 exhibited a hemicellulose content of 167.2 ± 1.3 mg/g, which significantly increased to 173.6 ± 1.5 mg/g under drought stress ($p < 0.05$). Conversely, Tetragold showed a reduction in hemicellulose from 160.5 ± 2.0 mg/g (control) to 118.2 ± 1.8 mg/g under drought($p< 0.05$). Bolt displayed the highest hemicellulose content under drought (241.3 ± 3.2 mg/g), representing a 52.2 mg/g increase compared to control conditions. As shown in Figure 5 C, Lignin accumulation increased universally under drought stress, with Tetragold showing the largest rise (142.4 ± 32.8 mg/g control vs. 504.9 ± 15.7 mg/g drought, Δ = +362.5 mg/g,($p < 0.05$)).Statistical analysis revealed significant effects of cultivar ($F(6, 154) = 42.1$, $p < 0.001$, $\eta^2 = 0.68$), drought stress ($F(1, 154) = 125.6$, $p < 0.001$, $\eta^2 = 0.75$), and their interaction ($F(6, 154) = 28.4$, $p < 0.001$, $\eta^2 = 0.56$) on hemicellulose. For cellulose, cultivar ($F(6, 154) = 35.7$, $p < 0.001$, $\eta^2 = 0.62$) and drought ($F(1, 154) = 15.2$, $p < 0.001$, $\eta^2 = 0.30$) effects were significant, with a notable interaction ($F(6, 154) = 12.9$, $p < 0.001$, $\eta^2 = 0.38$). Lignin accumulation was strongly influenced by cultivar ($F(6, 154) = 89.3$, $p < 0.001$, $\eta^2 = 0.83$), drought ($F(1, 154) = 543.8$, $p < 0.001$, $\eta^2 = 0.92$), and interaction effects ($F(6, 154) = 64.7$, $p < 0.001$, $\eta^2 = 0.77$).Post hoc analysis highlighted that Bolt and Barwoltra consistently exhibited higher hemicellulose and cellulose levels compared to other cultivars ($p < 0.05$), while Abundant and Tetragold displayed distinct lignin accumulation patterns. Drought universally induced lignin increases across all cultivars ($p < 0.05$), with responses varying in magnitude. For instance, Diamond T showed a 428% rise in lignin under drought, whereas Bolt exhibited a moderate 14% increase. Thereby, drought stress significantly altered cell wall biosynthesis in a cultivar-specific manner.Bolt demonstrated resilience through enhanced hemicellulose

synthesis and stable cellulose levels, while lignin accumulation emerged as a universal stress response. These findings underscore Bolt as a promising candidate for further research on drought tolerance mechanisms.

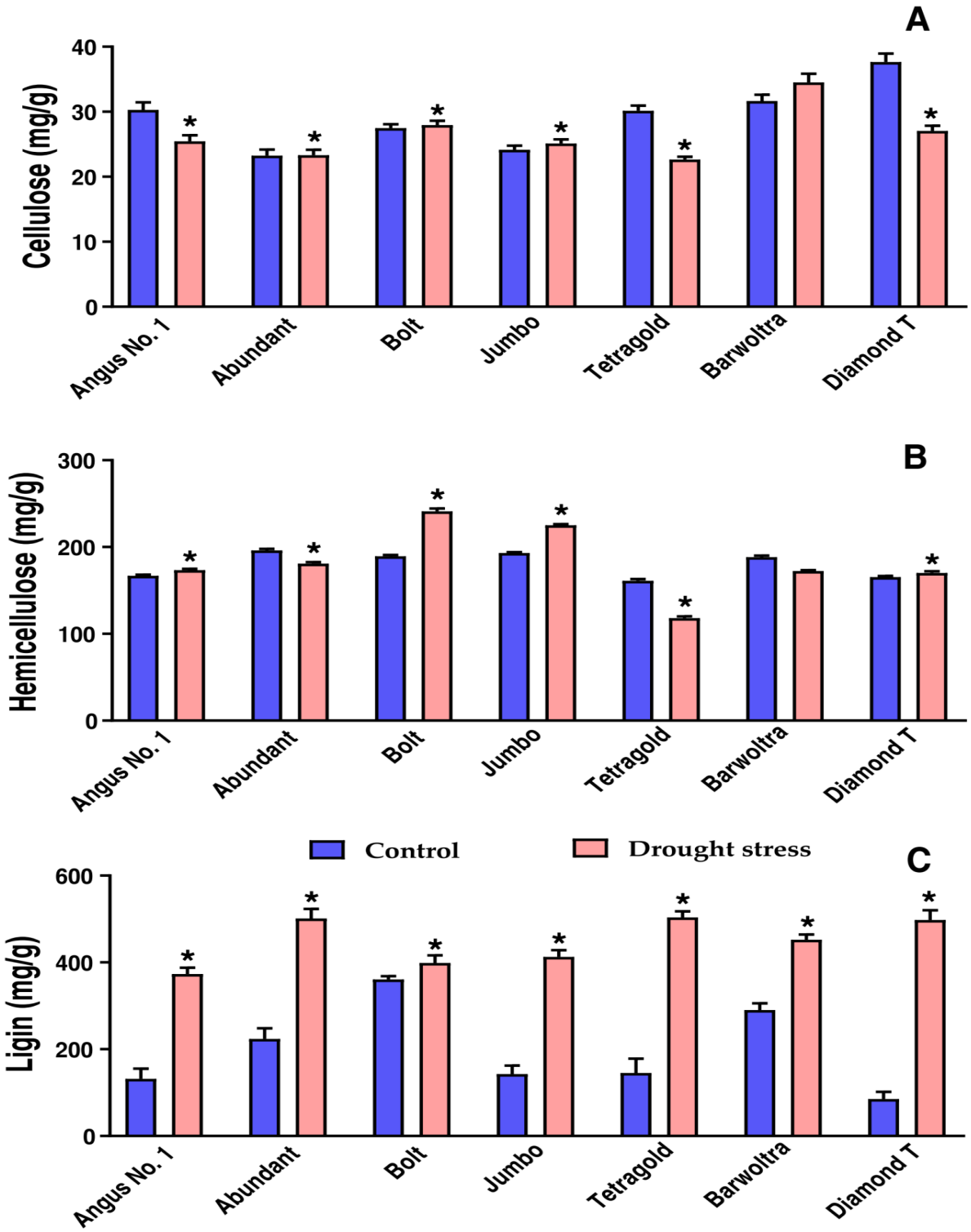

Figure 5. Cell wall component responses to drought stress. (A) Cellulose, (B) Hemicellulose, and (C) Lignin content in seven cultivars.

All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

Dynamic response of oxidative damage: The effects of drought stress and cultivar on oxidative stress markers (malondialdehyde, MDA; reactive oxygen species, ROS; and superoxide anion detected by DHE fluorescence) were analyzed using two-way ANOVA. Under control conditions, as shown in Figure 6 A, Angus No.1 exhibited MDA levels of 1.1 ± 0.4 nMol/g, which significantly increased to 3.6 ± 0.1 nMol/g under drought stress ($p < 0.05$). Similarly, as shown in Figure 6 B, ROS levels in Angus No.1 rose from 55,300 ± 2,100 RFU/g (control) to 106,000 ± 3,000 RFU/g ($p< 0.05$), while DHE fluorescence increased from 1,450 ± 300 RFU/g to 3,500 ± 200 RFU/g ($p < 0.05$). Bolt displayed the most severe oxidative damage under drought, with MDA escalating from 2.5 ± 0.8 nMol/g (control) to 13.9 ± 1.1 nMol/g ($p < 0.05$), ROS increasing from 81,800 ± 2,000 RFU/g to 119,000 ± 4,000 RFU/g ($p < 0.05$), and DHE fluorescence rising from 1,700 ± 200 RFU/g to 3,200 ± 700 RFU/g ($p < 0.05$). Barwoltra showed the highest drought-induced DHE fluorescence (4,100 ± 400 RFU/g), representing a 156% increase compared to control conditions (Figure 6 C). Statistical analysis revealed significant effects of cultivar ($F(6, 154) = 78.3$, $p < 0.001$, $\eta^2 = 0.82$), drought stress ($F(1, 154) = 1,120.5$, $p < 0.001$, $\eta^2 = 0.94$), and their interaction ($F(6, 154) = 54.8$, $p < 0.001$, $\eta^2 = 0.72$) on MDA. For ROS, cultivar ($F(6, 154) = 45.2$, $p < 0.001$, $\eta^2 = 0.68$), drought ($F(1, 154) = 890.3$, $p < 0.001$, $\eta^2 = 0.89$), and interaction ($F(6, 154) = 32.7$, $p < 0.001$, $\eta^2 = 0.58$) effects were significant. DHE fluorescence was similarly influenced by cultivar ($F(6, 154) = 62.1$, $p < 0.001$, $\eta^2 = 0.75$), drought ($F(1, 154) = 754.6$, $p < 0.001$, $\eta^2 = 0.86$), and interaction ($F(6, 154) = 48.9$, $p < 0.001$, $\eta^2 = 0.69$). Post hoc analysis indicated that Bolt and Barwoltra exhibited higher MDA and ROS levels under drought compared to other cultivars ($p < 0.05$), while Diamond T showed lower ROS accumulation relative to Jumbo and Tetragold ($p < 0.05$). Drought universally elevated oxidative markers across all cultivars ($p < 0.05$), with MDA increasing by 227–1,350%, ROS by 45–130%, and DHE by 90–250%. Notably, Bolt experienced the largest MDA increase (Δ = +11.4 nMol/g), whereas Tetragold showed moderate MDA accumulation (Δ = +6.2 nMol/g). Summatively, drought stress induced severe oxidative damage across cultivars, with MDA, ROS, and DHE levels significantly elevated. Barwoltra and Bolt were the most sensitive cultivars, while Diamond T demonstrated partial resilience. These findings highlight the importance of selecting oxidative stress-tolerant cultivars in drought-prone environments.

Dynamic response of glutathione metabolism: The effects of drought stress and cultivar on glutathione metabolism (reduced glutathione, GSH; oxidized glutathione, GSSG) were analyzed using two-way ANOVA. Under control conditions, as shown in Figure 7 A, Bolt exhibited the highest GSH content (521.6 ± 50.1 µg/g), which decreased significantly to 234.2 ± 25.6 µg/g under drought stress ($p < 0.05$). In contrast, Angus No.1 showed a drastic GSH reduction from 363.5 ± 18.2 µg/g (control) to 48.2 ± 2.1 µg/g ($p < 0.05$), representing an 87% loss. GSSG levels universally increased under drought stress, with Tetragold displaying the largest rise (70.3 ± 10.5 µg/g control vs. 443.6 ± 15.2 µg/g drought, Δ = +373.3 µg/g, ($p < 0.05$). As shown in Figure 7 B, Diamond T exhibited moderate GSSG accumulation (126.3 ± 5.8 µg/g control vs. 422.1 ± 10.4 µg/g drought, Δ = +295.8 µg/g, ($p < 0.05$). Statistical analysis revealed significant effects of cultivar ($F(6, 154) = 68.4$, $p < 0.001$, $\eta^2 = 0.75$), drought stress ($F(1, 154) = 980.5$, $p< 0.001$, $\eta^2 = 0.92$), and their interaction ($F(6, 154) = 45.7$, $p < 0.001$, $\eta^2 = 0.63$) on GSH. For GSSG, cultivar ($F(6, 154) = 54.2$, $p < 0.001$, $\eta^2 = 0.67$), drought ($F(1, 154) = 845.3$, $p < 0.001$, $\eta^2 = 0.89$), and interaction ($F(6, 154) = 32.1$, $p < 0.001$, $\eta^2 = 0.55$) effects were significant.Post hoc analysis indicated that Bolt retained 45% of its GSH under drought compared to other cultivars ($p < 0.05$), while Tetragold and Abundant showed distinct GSSG accumulation patterns ($p < 0.05$). Drought universally reduced GSH by 67–93% and elevated GSSG by 380–610% across all cultivars ($p < 0.05$). Notably, Bolt maintained the highest residual GSH levels (234.2 µg/g), whereas Tetragold exhibited the most pronounced GSSG surge (530% increase). Therefore, drought stress severely disrupted glutathione homeostasis, depleting GSH and elevating GSSG. Bolt demonstrated partial resilience through higher GSH retention, suggesting its potential as a drought-tolerant cultivar for mitigating oxidative damage.

Re-watered treatment: As depicted in Figure 8A, the comprehensive evaluation of physio-biochemical indices was presented on a radial scale ranging from 0 to 10 points. It was observed that Bolt demonstrated leadership in chlorophyll retention, osmotic regulation, and carbohydrate metabolism, exhibiting the strongest overall drought tolerance. Tetragold showcased remarkable cell wall strengthening capability. However, its photosynthetic and metabolic performance was relatively weak, making it suitable for scenarios requiring mechanical resistance. Jumbo exhibited moderate sugar metabolism, rendering it appropriate for mildly arid regions. Barwoltra displayed robust osmotic regulation but suffered from significant oxidative damage, posing a high risk. Angus No.1, Abundant, and Diamond T all exhibited low index values and were therefore not recommended for cultivation in arid environments. As shown in Figure 8 B, In addition to these physio-biochemical terms, in order to further explore the drought tolerance of these ryegrass varieties of Angus No.1, Abundant, Bolt, Jumbo, Tetragold, Barwoltra and Diamond T, they were re-irrigated to test the resilience of

each cultivar after 14 days of severe drought stress. These three varieties of Bolt and Barwoltra have some resilience, and these results suggest that they are potentially drought-tolerant varieties.

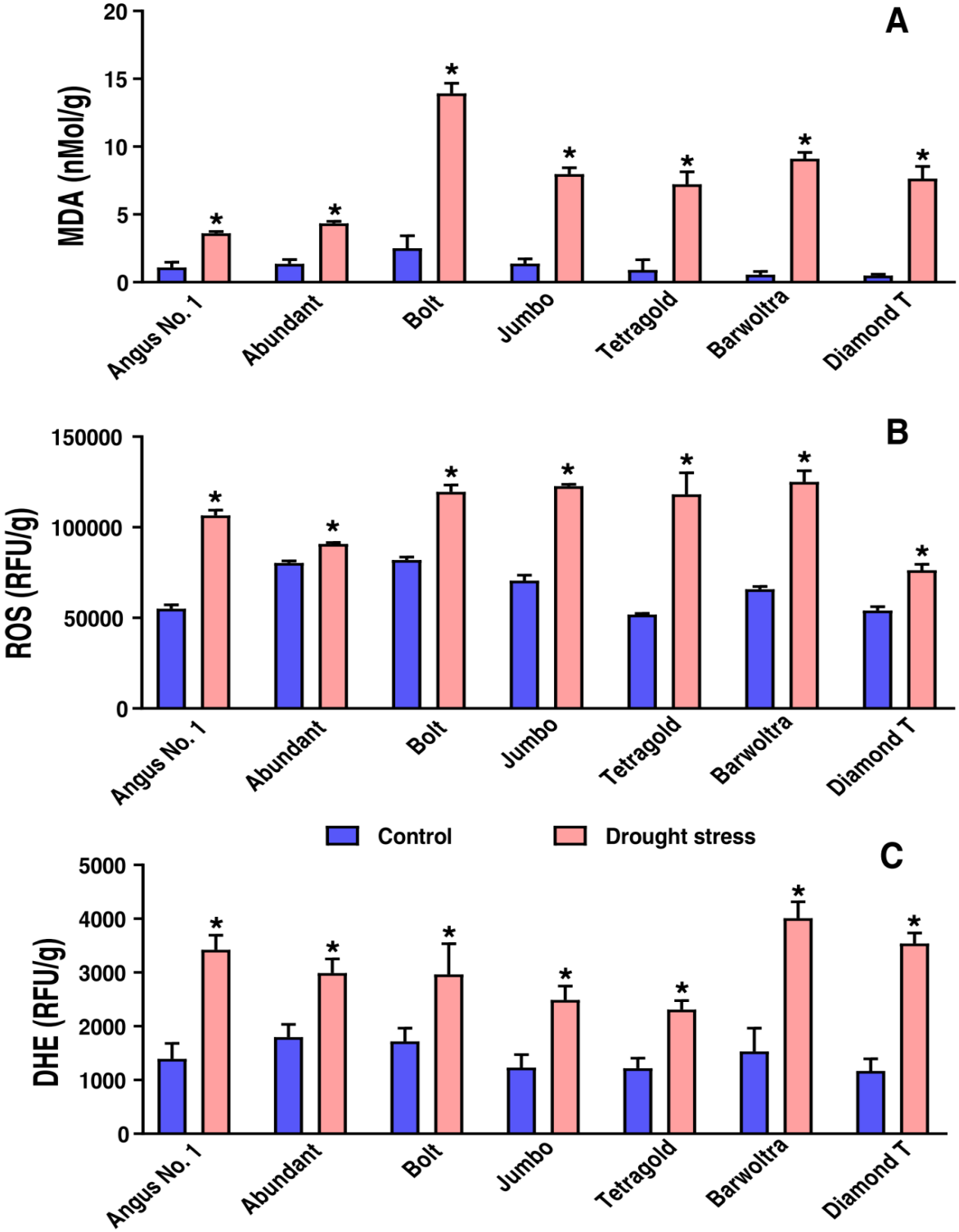

Figure 6. Oxidative damage markers under drought stress. (A) Malondialdehyde (MDA), (B) Reactive oxygen species (ROS), and (C) Superoxide anion levels.

All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

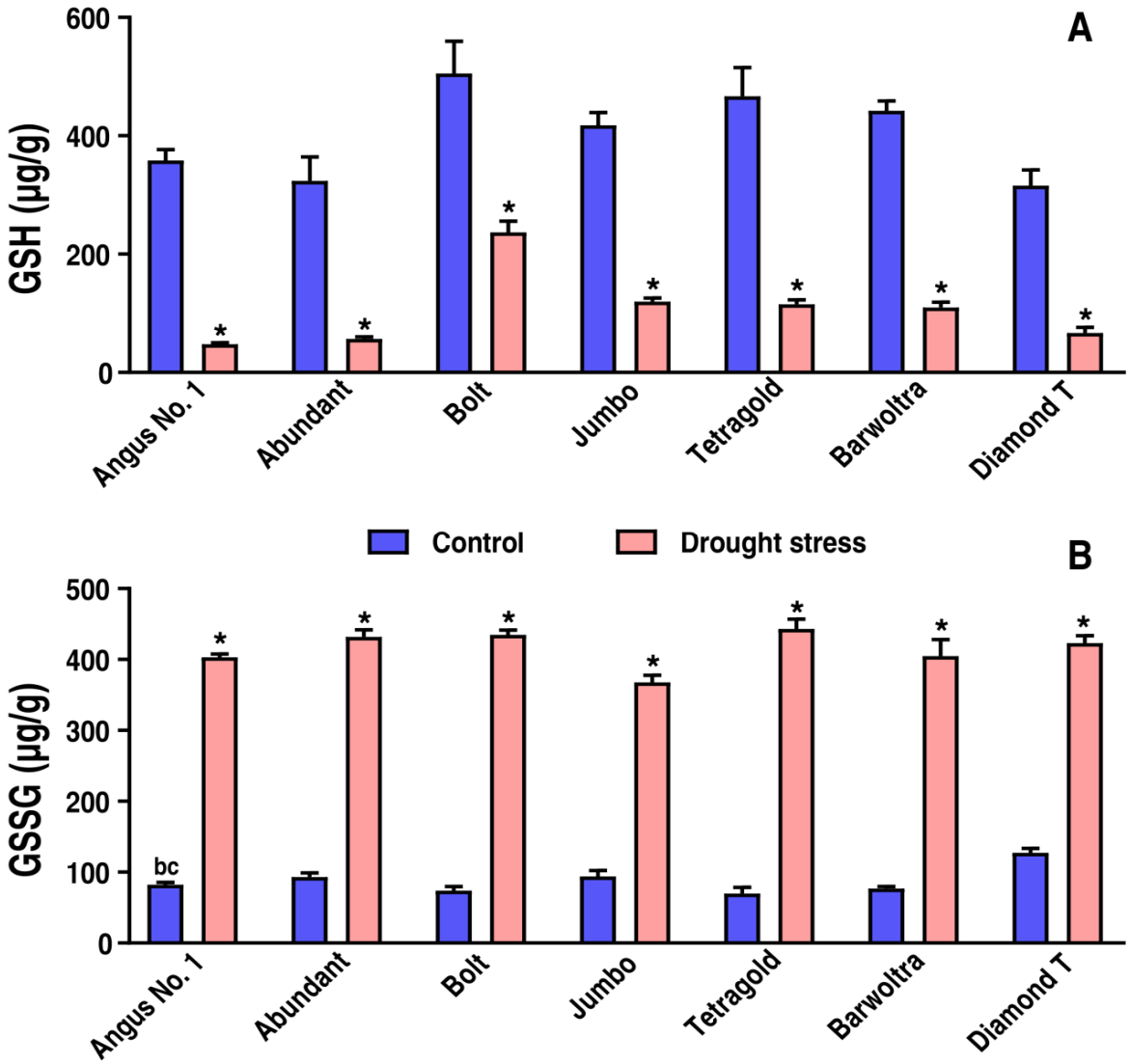

Figure 7. Glutathione metabolism dynamics in drought-stressed cultivars. (A) Reduced glutathione (GSH) and (B) Oxidized glutathione (GSSG) content. All analyses were assessed using the Shapiro-Wilk test and Levene's test (* $p > 0.05$), confirming the assumptions required for the application of Tukey's HSD test.

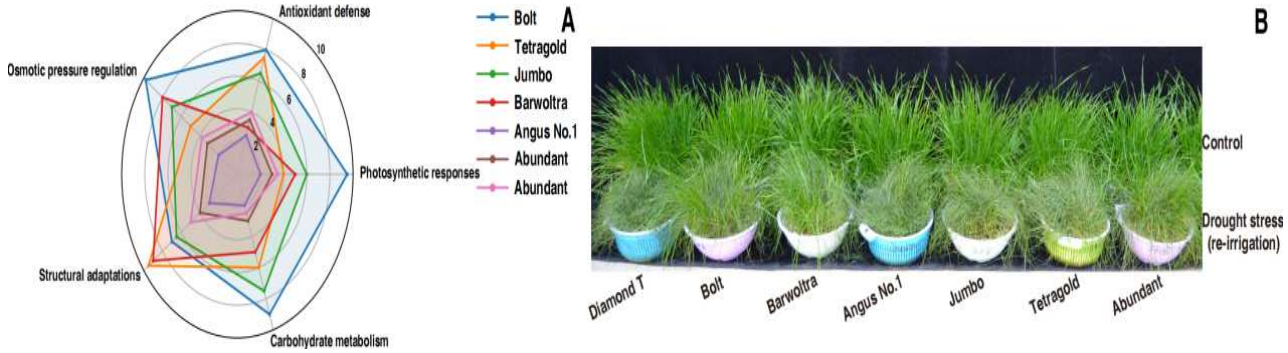

Figure 8. Recovery potential after re-watering. (A) The above physiological and biochemical indices were visualized using a radar map, and a comprehensive evaluation was conducted based on photosynthetic responses, carbohydrate metabolism, antioxidant defense, structural adaptations, and osmotic pressure regulation. (B) Resilience was evaluated 2 days after re-watering. Partial recovery was observed in Bolt and Barwoltra.

DISCUSSION

In the term of photosynthetic responses, drought stress significantly impacts the photosynthetic capacity of annual ryegrass. Chlorophyll, a crucial component in photosynthesis, showed diverse responses among the tested varieties. Unlike the up-regulated chlorophyll in chickpea reported by Ucak and Arslan (2023), chlorophyll A and total chlorophyll in these ryegrass varieties significantly decreased after 14 days of severe drought stress. However, the chlorophyll B content of Jumbo, Tetragold, and Diamond T increased. This indicates that these three varieties might have more complex mechanisms to cope with drought stress in terms of blue-violet light photosynthesis. Among all varieties, Bolt showed the smallest decline in photosynthesis. This suggests that Bolt has a good potential for drought resistance. Chlorophyll content can be used as an important indicator of drought tolerance in annual ryegrass, and Bolt's relatively stable chlorophyll levels under drought stress contribute to its better photosynthetic performance and potential for withstanding water deficits (Ucakand and Arslan, 2023).

Carbohydrate metabolism in annual ryegrass is greatly affected by drought stress. Perlikowski *et al.* (2016) found that saccharides accumulated and photosynthesis enzyme activity was improved in annual ryegrass after 10 days of drought stress. However, in this study, after 14-day severe drought stress, glucose, sucrose, and total saccharides were significantly reduced. Under mild stress, the up-regulated expression of starch/sucrose metabolism genes can increase the drought tolerance of rice cultivars (Kaur *et al.*, 2023). In our study, the varieties with slower-declining levels of these carbohydrates during severe drought stress, such as Bolt, showed relatively higher tolerance. Notably, among the tested varieties, Bolt had the highest content of free amino acids and saccharides after experiencing severe drought stress. These substances provided sufficient osmotic pressure buffering for a certain number of Bolt plants to regain vitality after re-watering irrigation. This indicates that maintaining a certain level of carbohydrate metabolism is crucial for ryegrass to resist drought stress. The decline in carbohydrate content might be related to the overall metabolic adjustment of the plant under stress to prioritize survival over growth-related processes (Perlikowski *et al.*, 2016; Kaur *et al.*, 2023).

Drought stress leads to the accumulation of harmful substances such as malondialdehyde (MDA), reactive oxygen species (ROS), and superoxide anion in annual ryegrass, which cause oxidative damage to the plasma membrane and affect plant growth and productivity (Razi and Muneer, 2021; Zou *et al.*, 2021; Ferioun *et al.*, 2023). In this study, all tested ryegrass varieties showed significant accumulations of these harmful substances. Among them, the accumulation of MDA and ROS in Bolt was significantly higher than that in other varieties, reaching the highest level among the seven tested varieties. Generally, under moderate water deficit stress, polyphenols and antioxidant capacity would increase (Farfan-Vignolo and Asard, 2012; Calone *et al.*, 2023). However, extensive drought stress in this research damaged the antioxidant capacity of the ryegrass varieties. Although the antioxidant content in these varieties, such as AsA and GSH, was reduced, they still had partial recovery ability as long as a certain amount was maintained. Remarkably, Bolt had the strongest antioxidant system after stress. It had the highest polyphenol content, ascorbic acid content, and reduced GSH content among the varieties, all of which possess strong antioxidant activities. These antioxidants can counteract the oxidative damage caused by MDA and ROS. The ratio of APX (ascorbate peroxidase) and SOD (superoxide dismutase) is a critical factor governing the drought-resistant potential of different finger millet varieties (Bhatt *et al.*, 2011). Among the annual ryegrass varieties, Bolt's relatively stronger antioxidant capacity contributed to its drought tolerance. Even though it had significant accumulations of MDA, ROS, and superoxide anion under severe drought stress, it achieved drought tolerance by slowing down the reduction of its antioxidant capacity. This shows that the antioxidant defense system plays a crucial role in the drought tolerance of annual ryegrass, and maintaining a certain balance of antioxidant substances is essential for plants to resist oxidative damage (Farfan-Vignolo and Asard, 2012; Bhatt *et al.*, 2011; Tiwari *et al.*, 2021). However, it should be noted that this study failed to reveal the molecular mechanism between the dynamic change trend of Bolt's physiological and biochemical indicators and drought-resistant genes at the genetic level. Therefore, we will conduct further research in this regard.

Drought stress impacts the structural composition of annual ryegrass, including cellulose, hemicellulose, and lignin. Balsamo *et al.* (2015) found that the synthesis of monosaccharides changed significantly under drought stress, leading to plant structure alteration. In this study, the cellulose content of Abundant, Bolt, and Jumbo showed no significant changes, while Barwoltra had an increase. For hemicellulose, an interesting phenomenon was observed in Bolt. Instead of decreasing, its hemicellulose content increased after drought stress. Since hemicellulose is an important component of the cell wall, under the osmotic stress caused by drought, the increased hemicellulose enhanced the cell mechanical strength of Bolt to a certain extent, reducing the physical damage caused by stress (Le Gall *et al.*, 2015). All ryegrass varieties showed an increase in lignin content after 14-day severe water deficit treatment. Under severe drought stress, lignification usually occurs, which reduces the growth and digestibility of forage grass (Lee *et al.*, 2012). These

changes in structural components are part of the plant's adaptation to drought stress. The increase in hemicellulose and lignin in some varieties might help strengthen the cell wall, providing mechanical support and reducing water loss. However, the excessive increase in lignin also has a negative impact on forage quality. The different responses of structural components among varieties suggest that they have different strategies for adapting to drought stress at the structural level (Balsamo *et al.*, 2015; Lee *et al.*, 2012). It is also important to note that the relationship between these structural changes in Bolt and its drought-resistant genes remains unclear, and further research is needed to explore this aspect.

Osmotic pressure regulation is an important mechanism for plants to cope with drought stress. In this study, osmoregulatory substances such as AsA and total free amino acids showed a significant decline in all tested ryegrass varieties under drought stress. Among them, Bolt and Tetragold had the smallest reduction. The content of total saccharides, sucrose, and glucose also changed under drought stress. Although these substances generally decreased, Bolt still maintained relatively high levels of these carbohydrates. As mentioned above, the high content of free amino acids and saccharides in Bolt after drought stress is crucial for its osmotic pressure regulation. These substances help maintain the osmotic balance inside and outside the cells, preventing excessive water loss. Varieties with better osmoregulation ability, like Bolt, can better adapt to drought stress. This is consistent with the view that metabolites involved in carbohydrates and amino acids can enhance the drought resistance of ryegrass (Pan *et al.*, 2017). Osmotic pressure regulation is an important aspect of the drought-tolerance mechanism of annual ryegrass, and maintaining the stability of osmoregulatory substances is beneficial for plants to survive under drought conditions (Pan *et al.*, 2017). However, the molecular mechanism underlying how Bolt maintains its osmoregulatory ability at the gene level remains to be studied, and we plan to carry out relevant research in the future.

The re-watering treatment results provide important information about the recovery potential of different ryegrass varieties after drought stress. Shen *et al.* (2022) mentioned that the levels of sugar and sugar precursors might change significantly in response to water deficit in tea plants, and metabolic recovery was only partial. Similar results were found in this study, where AsA, AA, glucose, sucrose, total saccharides, polyphenol, and glutathione steeply declined, and even after re-watering, only partial metabolic activity of Abundant, Bolt, and Barwoltra was restored. Wang *et al.* (2022b) found that the physiological responses of wheat varieties were reversible when re-watered after moderate drought. In this research, Bolt and Barwoltra showed some resilience after re-watering. The high levels of free amino acids, saccharides, and the relatively stable antioxidant substances in Bolt before re-watering provided a solid foundation for its recovery. These substances not only helped maintain the osmotic balance but also reduced oxidative damage during the re-watering process. However, the physio-biochemical indicators alone cannot fully explore the recovery potential of these varieties. Combining these indicators with re-watering verification can better screen for drought-resistant varieties. The recovery potential after re-watering is an important factor to evaluate the drought tolerance of annual ryegrass, and varieties with better recovery ability can quickly resume growth and metabolism after drought stress, which is of great significance for forage production in arid regions (Shen *et al.*, 2022; Wang *et al.*, 2022b). Future research should focus on understanding the molecular mechanisms behind the recovery process of Bolt and other varieties at the gene level to further clarify their drought - tolerance mechanisms.

Conclusion: Results revealed that Bolt exhibited superior drought tolerance through optimized chlorophyll retention, osmoregulation, and carbohydrate metabolism. Tetragold demonstrated exceptional cell wall reinforcement but compromised photosynthetic efficiency. However, the Jumbo maintained moderate carbohydrate metabolism, Barwoltra showed paradoxical traits‑strong osmotic adjustment coupled with severe oxidative damage. Therefore, prioritizing Bolt as the elite cultivar, with Tetragold serving as a niche candidate for mechanical stress environments is recommended in drought prone environments. Field validation should focus on: (1) Decoding the molecular basis of Bolt's oxidative resilience-antioxidant enzyme-related gene expression networks; (2) Quantifying the agronomic relevance of Tetragold's lignification patterns through multi-location trials.

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Author's Contribution Statements: XD, PD, YL, TS, and MJY executed the research, collected data and prepared the manuscript, XD and MJY conceived the idea, supervised the research, contributed to statistical analysis and edited the manuscript.

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