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Transgenerational Seed Exposure to Elevated CO_2 Involves Stress Memory Regulation at Metabolic Levels to Confer Drought Resistance in Wheat

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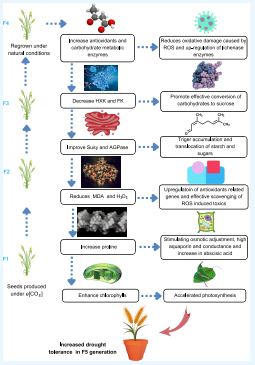
ABSTRACT: Drought is the worst environmental stress constraint that inflicts heavy losses to global food production, such as wheat. The metabolic responses of seeds produced overtransgenerational exposure to $e[CO_2]$ to recover drought's effects on wheat are still unexplored. Seeds were produced constantly for four generations (F1 to F4) under ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations, and then further regrown under natural CO_2 conditions to investigate their effects on the stress memory metabolic processes liable for increasing drought resistance in the next generation (F5). At the anthesis stage, plants were subjected to normal (100% FC, field capacity) and drought stress (60% FC) conditions. Under drought stress, plants of transgenerational $e[CO_2]$ exposed seeds showed markedly increased superoxide dismutase (16%), catalase (24%), peroxidase (9%), total antioxidants (14%), and proline (35%) levels that helped the plants to sustain normal growth through scavenging of hydrogen peroxide (11%) and malondialdehyde (26%). The carbohydrate metabolic enzymes such as aldolase (36%), phosphoglucomutase (12%), UDP-glucose pyrophosphorylase (25%), vacuolar invertase (33%), glucose-6-phosphate-dehydrogenase (68%), and cell wall invertase (17%) were decreased significantly; however, transgenerational seeds produced under $e[CO_2]$ showed a considerable increase in their activities in drought-stressed wheat plants. Moreover, transgenerational $e[CO_2]$ exposed seeds under drought stress caused a marked increase in leaf Ψ_w (15%), chlorophyll *a* (19%), chlorophyll *b* (8%), carotenoids (12%), grain spike (16%), hundred grain weight (19%), and grain yield (10%). Hence, transgenerational seeds exposed to $e[CO_2]$ upregulate the drought recovery metabolic processes to improve the grain yield of wheat under drought stress conditions.



1. INTRODUCTION

As a staple food, wheat (*Triticum aestivum* L.) is the world's most demanded cereal crop that provides protein, carbohydrates, vitamins, and vital nutrients to human diet.[1] The worldwide climate variability has drastically reduced wheat production and raised serious concerns of food security. Hence, the world's need of wheat grains for the global population has become a huge challenge for future climate change scenarios.[2,3] The climate change induced by increased greenhouse gases, in particular, carbon dioxide (CO_2) concentrations, causes extreme temperature fluctuations, warming climate, changing rainfall pattern, and water balance that extremely affects the ecological processes and crop yield.[4−6] The severity of drought spells as the main consequence of climate variability has been considered the most critical factor posing severe threats to agricultural production.[7,8]

Drought prevalence in plants instigated by limited water supply causes severe reduction in leaf surface expansion by wilting and curling of leaves, membrane and chlorophyll degradation, and disruption of enzyme functions.[9] More specifically, several cellular and metabolic processes such as stomatal conductance, RuBisCO enzyme activity, photosynthetic apparatus, photosynthetic CO_2 fixation and assimilation, nutrient uptake, and plant water status are negatively affected due to drought stress, consequently reducing the plant development seriously.[10,11] The drought stress in plants also disturbs the effective translocation of assimilates from source to sink that, further, limits the normal development of grains.[12] Under mild to severe drought, overgeneration of reactive oxygen species (ROS) such as H_2O_2, O_2^-, and OH^- due to imbalance between biochemical and photochemical functions predominantly causes peroxidation to nucleic acids, proteins, lipids, and cellular structures, thereby leading to electrolyte leakage and inhibiting photosynthetic efficiency in plants.[13] The drought exposure is also conducive to reduce the activity

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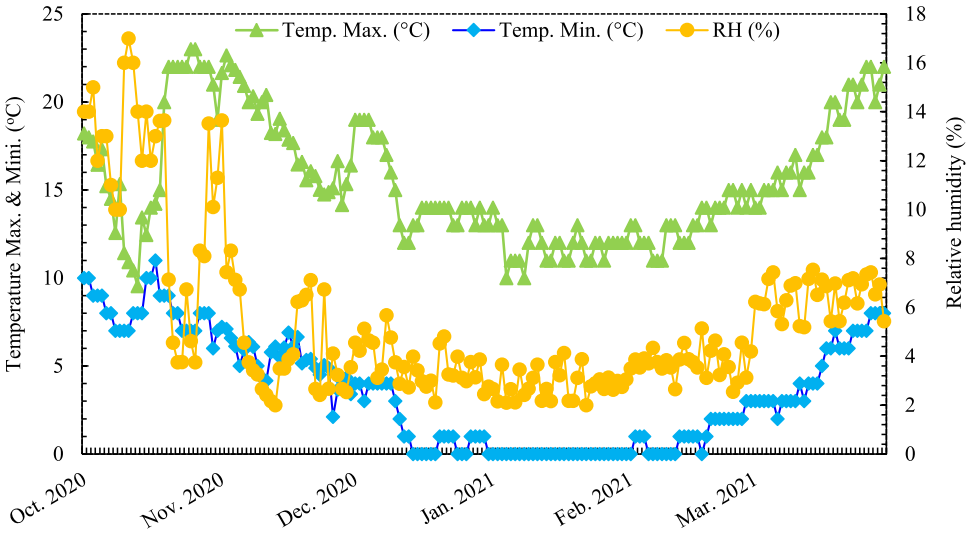

Figure 1. Meteorological conditions of the experimental site during the growth period of wheat.

of certain enzymes such as invertases,[14] ADP-glucose pyrophosphorylase,[15] and sucrose synthase,[16] thus disrupting metabolic and physiological mechanisms with a subsequent decrease in optimum growth and development.

The rapidly elevated CO_2 ($e[CO_2]$) concentration in the atmosphere is the extreme effect of climate change and is expected to increase globally from 420 to 1300 ppm.[17] As predicted, the varied responses of plants to $e[CO_2]$ depend on the CO_2 exposure that the maternal or offspring plant experiences. It is well known that plants exposed to $e[CO_2]$ for two or three generations show a higher growth response than those grown for one generation in an $e[CO_2]$ environment.[18,19] The positive effect of $e[CO_2]$ of promoting the net photosynthetic efficiency, which subsequently increased the crop yield and quality, have been shown in numerous studies.[20,21] A prominent increase in leaf area expansion with $e[CO_2]$ under drought stress is associated with enhanced CO_2 assimilation and improved water use efficiency by reducing the stomatal density and transpiration rate, therefore leading to higher crop growth and productivity.[22,23] Moreover, $e[CO_2]$ could possibly decrease the oxidative damage to plants by reducing the ROS levels via maintenance of a higher antioxidant potential, which successively helps the plants to survive in drought environments.[24] An $e[CO_2]$ environment also assists the plants in synthesizing carbohydrates and starch contents through upregulation of the antioxidant metabolism, hence contributing to a higher grain weight and yield under drought conditions.[3]

Multiple studies have focused on the positive responses of plants to $e[CO_2]$ for an increased potential of drought resistance within a single generation or over multiple generations; however, the potential metabolic processes involved in stress memory regulation for drought recovery in wheat seeds produced overtransgenerational exposure to $e[CO_2]$ are not well described. In this study, wheat seeds that were produced continuously for four generations under ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations were grown under natural CO_2 conditions to explore the effects of transgenerational exposure to $e[CO_2]$ on stress memory regulation in plants. However, we hypothesize that wheat seeds produced from overgenerational exposure to $e[CO_2]$ memorize the stress memory to increase the drought resistance in the next generation of plants through upregulation of metabolic processes when regrown under natural CO_2 environments.

2. MATERIALS AND METHODS

2.1. Seed Material and Site. Four generation seeds of wheat crop (*T. aestivum* L. "var. Lianmai 6") were obtained from plants continuously grown under climate-controlled conditions of $a[CO_2]$ (400 μmol L^{-1}) and $e[CO_2]$ (800 μmol L^{-1}) concentrations during 2014 to 2017. The seeds of all four generations (F1 to F4) were grown for four growing seasons, i.e., F1 generation from February 15 to July 15, 2014; F2 generation from September 15 to January 31, 2015; F3 generation from October 01 to February 20, 2016; and F4 generation from November 20 to April 25, 2017.[1] The study was conducted at the Research Area of University of Poonch Rawalakot, Azad Jammu and Kashmir, Pakistan (N 25.61°, E 55.94°).

2.2. Cultivation Conditions. F4 generation seeds were initially tested for their viability. The randomly selected uniform, mature, and physically pure seeds were disinfected with NaOCl solution (10%, sodium hypochlorite) for 5 min, rinsed thoroughly with distilled water to remove the traces of NaOCl and then air-dried up to their original moisture content. Six seeds were sown at the top soil (3 cm depth) in earthen pots of 4 L with 21 cm diameter, 20.5 cm height, and one central drainage hole. The pots were filled with 3.5 kg of air-dry, ground, and screened (2 mm mesh) soil mixture comprising loam soil and peat material (3:1, w/w). The textural class and physio-chemical characteristics of the experimental soil were determined by following standard procedures.[25,26] The soil was characterized as follows: sand 40%, silt 40%, clay 20%, pH 6.5, organic matter 3.12%, electrical conductivity 0.47 mS cm^{-1}, nitrogen 0.14%, calcium 10.66 mg kg^{-1}, sodium 107.31 mg kg^{-1}, chloride 0.74 mg kg^{-1}, phosphorus 78.21 mg kg^{-1}, and potassium 0.05 mg kg^{-1}. The nutrient demands for NPK fertilizers were determined using the optimum rates of urea (120 kg ha^{-1}), diammonium phosphate (60 kg ha^{-1}), and potassium sulfate (50 kg ha^{-1}) corresponding to 210, 105, and 88 mg pot^{-1}, respectively. All P, K, and 1/third N were mixed with the top soil layer (0−15 cm) during filling of pots, whereas the remaining 2/third N

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was top-dressed in two series with each 1/third at 5 leaf (Zadoks code 15) and flag leaf ligule visible (Zadoks code 39) stages.[27] All plants were kept in a wire house equipped with a portable rain-protected plastic sheet under natural conditions for 153 days during the growth period of 2020−21 (October 23rd to March 24th). The climatic conditions of the experimental site such as average air temperatures (min and max) and relative humidity during the cultivation period are described in Figure 1. Water was applied to the plants daily to retain the soil mix at optimum moisture content (i.e., 100% FC, field capacity). At the emergence of the fourth leaf (Zadoks code 14), 3 plants were left in each pot by thinning. The pots were rotated randomly within each block for every 1 week. Weeds were removed manually in each pot when required.

2.3. Experimental Setup. F4 generation seeds collected from continuously grown plants separately in $a[CO_2]$ and $e[CO_2]$ concentrations for four generations were raised under natural CO_2 conditions (without $e[CO_2]$ exposure). At the anthesis stage (Zadoks code 61), plants were subjected to drought by keeping the soil moisture content at 60% FC level, whereas the 100% FC level in the soil mix served as well-watered treatment (normal). All plants received normal watering at the end of the drought stress (i.e., after 28 days of drought imposed). In total, this study had 4 treatments based on drought stress and CO_2 environments (two drought levels × two transgenerational CO_2-treated seeds). Each treatment was repeated 3 times and arranged randomly under a CRD factorial design. Leaf samples were taken from each treatment (pot) after termination of drought stress (28 days after the drought was imposed) for physiological and enzymatic analysis. The experiment ended at physiological maturity.

2.4. Assay for Antioxidative Enzymes. The fresh leaf sample (1 g) was homogenized in 50 mM potassium phosphate buffer (pH 7.0), 1 mM dithiothreitol (DTT), and 0.1 mM EDTA and centrifuged for 20 min at 13,000 rpm.[28]

Catalase (CAT) activity was measured in an assay solution (3 mL) containing 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2, and 0.1 mL of enzyme extract. The decrease in absorbance for every 20 s was recorded at 240 nm and absorbance change of 0.01 units min^{-1} was defined as one unit CAT activity.[29]

Peroxidase (POD) activity was assessed from the reaction mixture containing 0.1 mL of enzyme extract, 50 mM phosphate buffer (pH 7.0), 40 mM H_2O_2, and 20 mM guaiacol. Absorbance was recorded at 470 nm and 1 unit POD activity was defined as an absorbance change of 0.01 unit min^{-1}.[30]

The superoxide dismutase (SOD) activity of the reaction mixture (3 mL) comprising 13 mM methionine, 2 μM riboflavin, and 75 μM p-nitroblue tetrazolium chloride (NBT) was measured spectrophotometrically by illuminating under 500 μmol m^{-2} s^{-1} PPFD.[31]

Total antioxidants were determined using trolox equivalent antioxidant capacity.[32]

2.5. Carbohydrate Metabolic Enzyme Determination. Leaf material (500 mg) was extracted in a buffer (1 mL) comprising Tris−HCl (40 mM, pH 7.6), PMSF (0.1 mM), EDTA (1 mM), $MgCl_2$ (3 mM), β-mercaptoethanol (14 mM), benzamidine (1 mM), and NADP (24 μM) with a semi-high-throughput analytical platform.[33] An aliquot (25 μL) of the extract supplemented with the individual enzyme mixture (i.e., total reaction volume of 160 μL) was incubated in a 96-well plate reader for 40 min at 30 °C in ultraviolet (UV)-transmissive flat-bottom 96-well plates.

The enzyme activities of fructose-1,6-bisphosphate aldolase (Ald), phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGPase), vacuolar invertase (vacInv), glucose-6-phosphate-dehydrogenase (G_6PDH), cell wall invertase (cwInv), hexokinase (HXK), fructokinase (FK), sucrose synthase (SuSy), and ADP-glucose pyrophosphorylase (AGPase) were determined.[33]

2.6. Detection of H_2O_2 and MDA. Fresh leaf material (0.5 g) was ground in 0.1% trichloroacetic acid solution (5 mL). The extract was homogenized at 12,000 rpm for 15 min, and an aliquot of the supernatant (0.5 mL) was mixed with 1 M potassium iodide (1 mL) and 10 mM potassium phosphate buffer (0.5 mL, pH 7.0). The absorbance of the mixture was noted at 390 nm using a spectrophotometer (Hitachi-120, Japan) to determine the hydrogen peroxide (H_2O_2) contents.[34]

For determining malondialdehyde (MDA) contents in fresh leaves, the weighed sample (0.5 g) was homogenized in trichloroacetic acid solution (0.1%) and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant (2 mL) was thoroughly mixed with 0.6% thiobarbituric acid (2 mL) solution prepared in 10% trichloroacetic acid. After incubation at 95 °C for 30 min, the mixture was centrifuged at 10,000 rpm for 10 min and used to record the absorbance at 532 and 600 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to measure the MDA contents.[35]

2.7. Estimation of Proline and Leaf Ψ_w. The homogenized fresh leaf material (0.5 g) with a 3% aqueous solution of sulfosalicylic acid (10 mL) was filtered to 2 mL and incubated at 100 °C for 1 h after mixing with a glacial acetic acid and acid ninhydrin solution (2 mL). After terminating the reaction in the ice bath, toluene (4 mL) was used to extract the reaction mixture, which formed a chromophore. A continuous air stream was then passed through the reaction mixture to isolate the aqueous phase from the chromophore containing toluene. The isolated colored phase was allowed to stand at room temperature for 2−3 min and used to measure its absorbance spectrophotometrically at 520 nm.[36]

$$\mu\text{mol proline g}^{-1}\text{ FW} = [(\mu\text{g proline mL}^{-1} \times \text{mL toluene})/115.5]/[(\text{g sample})/5]$$

Leaf water potential (Ψ_w) was estimated using a Scholander type pressure chamber (Model 1000, PMS New York).

2.8. Measurement of Chlorophyll Content. Fresh leaf material of 0.5 g was ground in 80% acetone solution and allowed to extract overnight at 4 °C. The mixture was adjusted to 5 mL using acetone solution and further centrifuged at 10,000 rpm for 5 min. The filtered supernatant was then used to record the absorbance at 645, 647, and 663 nm using a spectrophotometer (Hitachi-120, Japan) for estimating the chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b), and carotenoid (Car) contents.[37]

$$Chl_a\ (\text{mg g}^{-1}) = (12.7A^{663} - 2.69A^{645}) \times V/1000 \times W$$

$$Chl_b\ (\text{mg g}^{-1}) = (22.9A^{645} - 4.68A^{663}) \times V/1000 \times W$$

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$$\text{Car (mg g}^{-1}) = 1000A^{470} - (1.82\ \text{Chl. } a) - (85.02\ \text{Chl. } b)/198$$

2.9. Determination of Yield and Yield Attributes. At physiological maturity (Zadoks code 90), crop plants from each pot were harvested to record the data of the number of grains per spike, hundred grain weight, and grain yield following standard procedures.

2.10. Statistical Analysis. The linear model analysis of variance (ANOVA) technique for two-factor analysis was employed using Statistix 8.1 to test the significance of water, CO_2-treated seeds, and their interaction treatments on the measured traits. The treatments' means were compared using Tukey's HSD test (p = 5%). Pearson's correlation analysis was done using the *ggcorrplot* function. A heatmap for all traits was developed using the *R*-package (Version 4.2.6 software). In order to investigate the research hypothesis, the structural equation model (SEM) was developed to analyze the structural relationships between the variables and underlying constructs. The coefficients of this model are calculated using the Maximum Likelihood Technique, accompanied by standard errors and scaled statistics, combining the elements of Factor Analysis and Multiple Regression Analysis. Additionally, we examined modification indexes and residuals to determine whether to include or exclude the variables. The fit of the model was evaluated using a variety of metrics, such as chi-square test, Goodness of Model Fit, and Standard Root Mean Square Residuals within the framework of the structural equation model. Within this framework, enzymatic and yield-contributing parameters were treated as latent variables, while grain yield was treated as an observable variable.

3. RESULTS

3.1. Antioxidative Enzyme Activities. Drought caused a remarkable increase in CAT, POD, and SOD activities, whereas transgenerational seeds produced under $e[CO_2]$ considerably increased the antioxidative capacity of CAT and TA activities, while it had no significant ($p < 0.05$) effect on the POD and SOD potential under drought stress (Table 1). A substantial increase in CAT, POD, SOD, and TA activities by 23, 37, 19, and 14% was exhibited in drought-stressed plants grown with seeds produced by overtransgenerational exposure to $a[CO_2]$. Plants of transgenerational seeds exposed to $e[CO_2]$ showed notable increase in the CAT, POD, and SOD activities by 24, 9, and 16%, respectively, when subjected to drought stress (Figure 2a−c). Overgenerational $e[CO_2]$ exposure to wheat seeds markedly increased the TA activity by 35 and 14% when raised under both normal and drought conditions, respectively (Figure 2d).

3.2. Carbohydrate Metabolic Enzymes. Transgenerational $e[CO_2]$ exposure under drought stress had significant ($p < 0.05$) effects for Ald, PGM, HXK, FK, cwInv, vacInv, and AGPase enzymes, while UGPase, SuSy, and G_6PDH enzymes showed no significant responses for their activities (Table 1). Multiple-generation $a[CO_2]$-treated seeds grown under drought conditions markedly reduced the enzymatic activities of Ald, PGM, UGPase, vacInv, and G_6PDH, whereas a notable decline by 36, 12, 25, 33, and 68%, respectively, was recorded in wheat plants as compared to normal plants. Quite the reverse, overgenerational $e[CO_2]$-exposed seeds showed improved Ald, PGM, UGPase, vacInv, and G_6PDH activities in the leaves of wheat plants by 20, 38, 16, 57, and 57%, respectively, compared to plants raised with transgenerational $a[CO_2]$-treated seeds upon subjecting to drought stress (Figure 3a−e). Similarly, drought exposure caused a profound decline in cwInv activity as the highest decrease by 17% was exhibited in plants of overgenerational $a[CO_2]$-exposed seeds. Transgenerational $e[CO_2]$-exposed seeds resulted in a further decrease in the cwInv activity of plants by 7 and 35% compared to $a[CO_2]$-grown seeds under either normal or drought conditions, respectively (Figure 3f).

Under drought conditions, HXK and FK activities were increased by 31 and 27%, respectively, in plants of overgenerational $a[CO_2]$-exposed seeds in contrast to normal plants raised with exposed seeds of $a[CO_2]$. Overgenerational exposure of $e[CO_2]$ to seeds considerably decreased the HXK and FK activities by 25 and 17%, respectively, in drought-prone plants compared to $a[CO_2]$ treatment. On the contrary, HXK activity was improved by 56% in plants grown with overgenerational $e[CO_2]$-treated seeds under normal water supply conditions (Figure 3g,h). SuSy and AGPase activities were increased by 35 and 38%, respectively, in plants of transgenerational $a[CO_2]$-exposed seeds under drought

Table 1. Significance (p-Values) for Antioxidants, Carbohydrate Metabolism, Oxidative Destruction Compounds, Proline, Ψ_w, Chlorophyll Content, and Yield-Related Traits of Wheat at 95% Confidence Interval of Mean[a]

variables	drought (D)	CO_2 treatments (CO_2)	$D \times CO_2$ interaction
CAT	<0.0001	0.0018	0.0205
POD	0.0002	0.0333	0.6312
SOD	0.0019	0.0399	0.3444
TA	1.0000	0.0001	0.0125
Ald	<0.0001	0.0680	0.0037
PGM	0.3229	0.0001	0.0070
UGPase	0.0005	0.0137	0.7443
vacInv	0.2326	<0.0001	0.0001
G6PDH	<0.0001	0.0001	0.2392
cwInv	<0.0001	0.0006	0.0121
HXK	0.0063	0.0017	<0.0001
FK	<0.0001	<0.0001	0.0246
SuSy	<0.0001	0.0047	0.7452
AGPase	0.5138	<0.0001	0.0023
H_2O_2	<0.0001	0.0115	0.9553
MDA	0.0001	0.0044	0.0196
Proline	<0.0001	<0.0001	0.0041
Ψ_w	0.0004	0.0516	0.2861
Chl. *a*	0.0001	0.0015	0.5045
Chl. *b*	<0.0001	0.0012	0.0423
Car	<0.0001	0.0004	0.0202
G/S	<0.0001	0.0028	0.5767
HGW	0.0001	0.0037	0.5989
GY	<0.0001	0.0001	0.0380

[a]p-values for D, CO_2, and $D \times CO_2$ interaction indicate statistical significance at $p < 0.05$. CAT, catalase; POD, peroxidase; SOD, superoxide dismutase, TA, total antioxidants; Ald, fructose-1,6-bisphosphate aldolase; PGM, phosphoglucomutase; UGPase, UDP-glucose pyrophosphorylase; vacInv, vacuolar invertase; G6PDH, glucose-6-phosphate-dehydrogenase; cwInv, cell wall invertase; HXK, hexokinase; FK, fructokinase; SuSy, sucrose synthase; AGPase, ADP-glucose pyrophosphorylase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; Ψw, water potential; Chl. *a*, chlorophyll *a*; Chl. *b*, chlorophyll *b*; Car, carotenoids; G/S, grains per spike; HGW, hundred grain weight; GY, grain yield.

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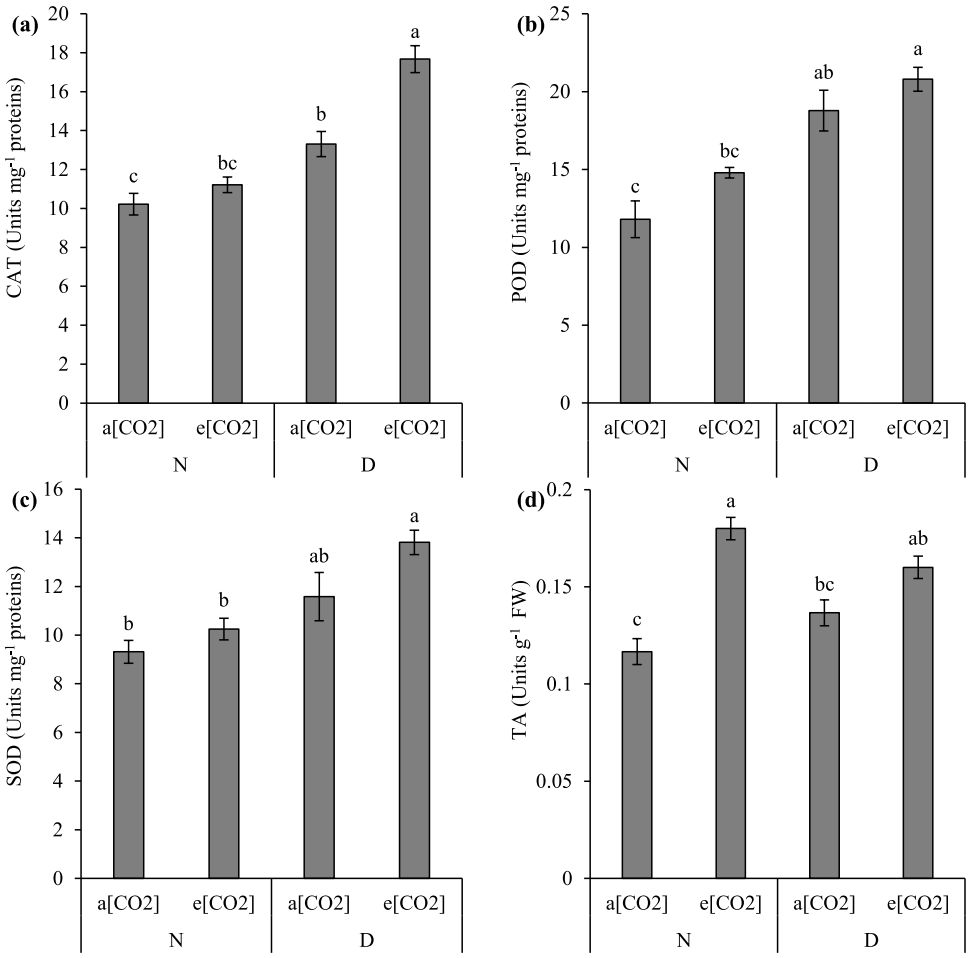

Figure 2. Response of catalase (CAT) (a), peroxidase (b), superoxide dismutase (SOD) (c), and total antioxidants (d) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test ($p < 0.05$). N, normal water supply; D, drought stress.

stress. Overgenerational seeds exposed to $e[CO_2]$ revealed a significant improvement in SuSy and AGPase activities by 15 and 54%, respectively, in plants as compared to the plants of multigenerational $a[CO_2]$-exposed seeds (Figure 3i,j).

3.3. H_2O_2 and MDA. Overgenerational $e[CO_2]$-exposed seeds under drought stress showed remarkably ($p < 0.05$) reduced MDA contents in wheat plants, while the interaction effect for H_2O_2 was not significant (Table 1). H_2O_2 content was increased significantly in plant leaves subjected to drought stress compared to normal plants. A marked increase in H_2O_2 content was found by 35% in plant leaves grown with transgenerational $a[CO_2]$-exposed seeds while treated with drought stress. Plants raised with treated seeds of transgenerational $e[CO_2]$ showed the H_2O_2 content significantly reduced by 11% compared to $a[CO_2]$-exposed seeds subjected to drought stress conditions. Similarly, the H_2O_2 content was decreased by 18% with $e[CO_2]$ treatment under normal water conditions (Figure 4a).

Likewise, MDA content was increased significantly by 34% in transgenerational $a[CO_2]$-exposed seeds under drought stress than under normal conditions. Under normal water supply conditions, overgenerational $e[CO_2]$-treated seeds showed a slight decrease in leaf MDA content by 5% than control ($a[CO_2]$-exposed seeds). A notable decline in MDA content by 26% was observed in plants grown with transgenerationally treated seeds of $e[CO_2]$ in parallel to the plants of $a[CO_2]$-exposed seeds under drought stress conditions (Figure 4b).

3.4. Proline and Leaf Ψ_w. Transgenerational exposure of $e[CO_2]$ to wheat seeds significantly ($p < 0.05$) affected the proline content under drought stress conditions; however, the effects of drought stress on leaf Ψ_w were only significant (Table 1). Proline content was increased markedly in drought-prone plants by 38% in parallel to normal plants grown with overgenerational $a[CO_2]$-induced seeds. A marked increase in proline content was found in plants raised with transgenerational $e[CO_2]$-treated seeds under either normal or drought conditions. The combination of transgenerational $e[CO_2]$-exposed seeds with drought stress improved the proline content by 35% in drought-stressed plants in comparison to the plants grown with overgenerational $a[CO_2]$-treated seeds (Figure 5a).

Exposure of wheat seeds to transgenerational $e[CO_2]$ caused a positive gain in the leaf Ψ_w of normal or drought-stressed plants. A more positive gain in leaf Ψ_w by 15% was exhibited in drought-affected plants grown with overgenerational $e[CO_2]$-treated seeds than plants of $a[CO_2]$-exposed seeds. Similarly, a positive gain in leaf Ψ_w by 5% was found in plants raised with multigenerational $e[CO_2]$-treated seeds under normal water conditions (Figure 5b).

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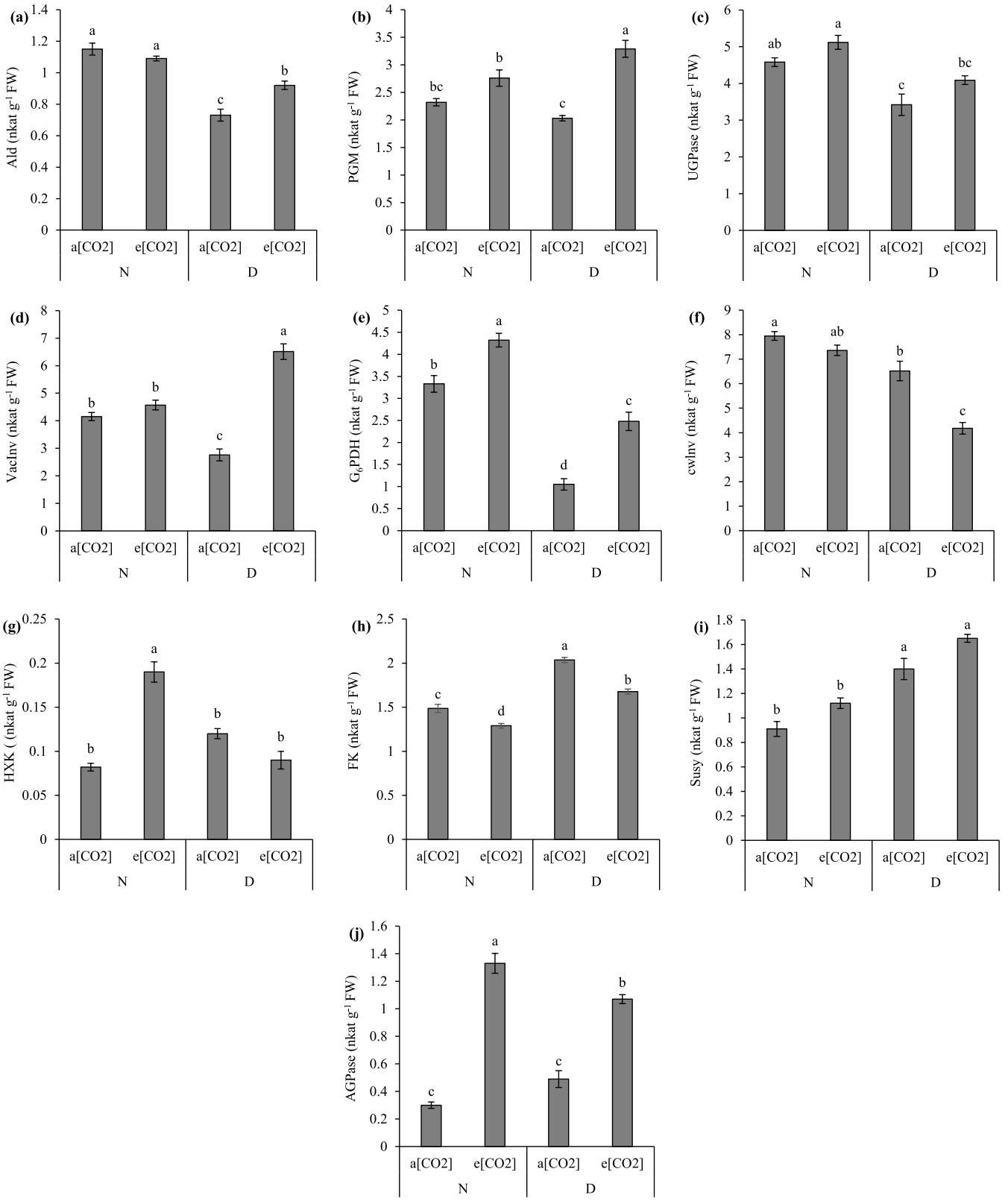

Figure 3. Response of fructose-1,6-bisphosphate aldolase (Ald) (a), phosphoglucomutase (PGM) (b), UDP-glucose pyrophosphorylase (UGPase) (c), vacuolar invertase (vacInv) (d), glucose-6-phosphate-dehydrogenase (G_6PDH) (e), cell wall invertase (cwInv) (f), hexokinase (HXK) (g), fructokinase (FK) (h), sucrose synthase (SuSy) (i), and ADP-glucose pyrophosphorylase (AGPase) (j) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test ($p < 0.05$). N, normal water supply; D, drought stress.

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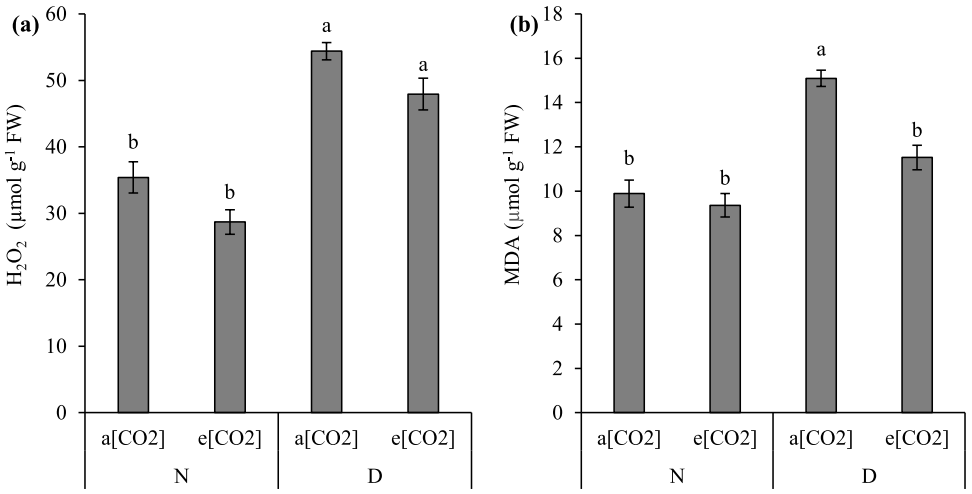

Figure 4. Response of hydrogen peroxide (H_2O_2) (a) and malondialdehyde (MDA) (b) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test (p < 0.05). N, normal water supply; D, drought stress.

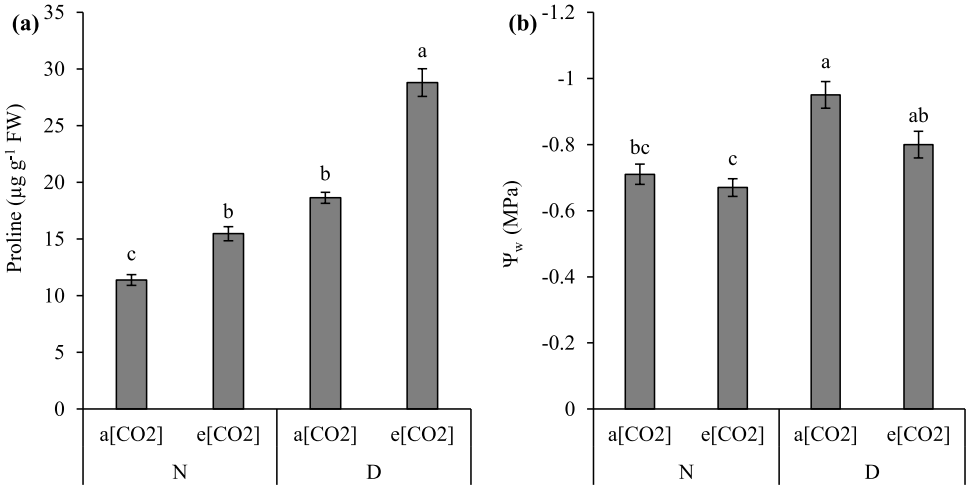

Figure 5. Response of proline (a) and water potential (Ψ_w) (b) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test (p < 0.05). N, normal water supply; D, drought stress.

3.5. Chlorophyll Content. Chlorophyll contents (Chl. *a*, Chl. *b*, and Car) were considerably (p < 0.05) affected under drought stress and transgenerational $e[CO_2]$ exposure (Table 1). Chl. *a* and Chl. *b* contents were decreased sharply in plants subjected to drought stress. A marked reduction in Chl. *a* and Chl. *b* contents by 27 and 21%, respectively, was found in drought-stressed plants over normal plants. Overgenerational treated seeds with $e[CO_2]$ showed enhanced leaf Chl. *a* and Chl. *b* contents by 19 and 8%, respectively, compared to plants of $a[CO_2]$-treated seeds subjected to drought stress conditions (Figure 6a,b). Drought also caused a prominent decline in Car content; however, a notable decline of 24% was exhibited in plants produced under transgenerational $a[CO_2]$. Plants grown with overgenerational $e[CO_2]$-exposed seeds revealed a highest increase in Car content by 12% than $a[CO_2]$-treated seeds subjected to drought conditions (Figure 6c).

3.6. Yield and Yield-Related Parameters. Exposure to drought stress resulted in a significant (p < 0.05) reduction in yield and yield-related attributes; however, transgenerational $e[CO_2]$ exposure to seeds enhanced the yield of wheat (Table 1). Plants grown with transgenerational seeds exposed to $a[CO_2]$ showed a notable decline in G/S, HGW, and GY by 29, 33, and 22, respectively, when subjected to drought conditions in contrast to normal plants. Overgenerational seeds exposed to $e[CO_2]$ markedly increased the G/S and HGW by 16 and 19%, respectively, compared to $a[CO_2]$-treated seeds under drought stress conditions. Similarly, a remarkable increase in GY by 10% was found in plants grown with transgenerational $e[CO_2]$ exposed to drought stress conditions (Figure 7a–c).

3.7. Correlation of Antioxidants, Carbohydrate Metabolic Enzymes, H_2O_2, MDA, Proline, Leaf Ψ_w, and Chlorophyll with Grain Yield and Yield-Related Traits. The association of Ald, UGPase, and G_6PDH enzymes with G/S, HGW, and GY was determined to be strong and positive, whereas HXK was positively correlated with the GY and Car of wheat subjected to transgenerational $e[CO_2]$ exposure under drought stress. Chlorophyll contents (Chl. *a*, Chl. *b*, and Car)

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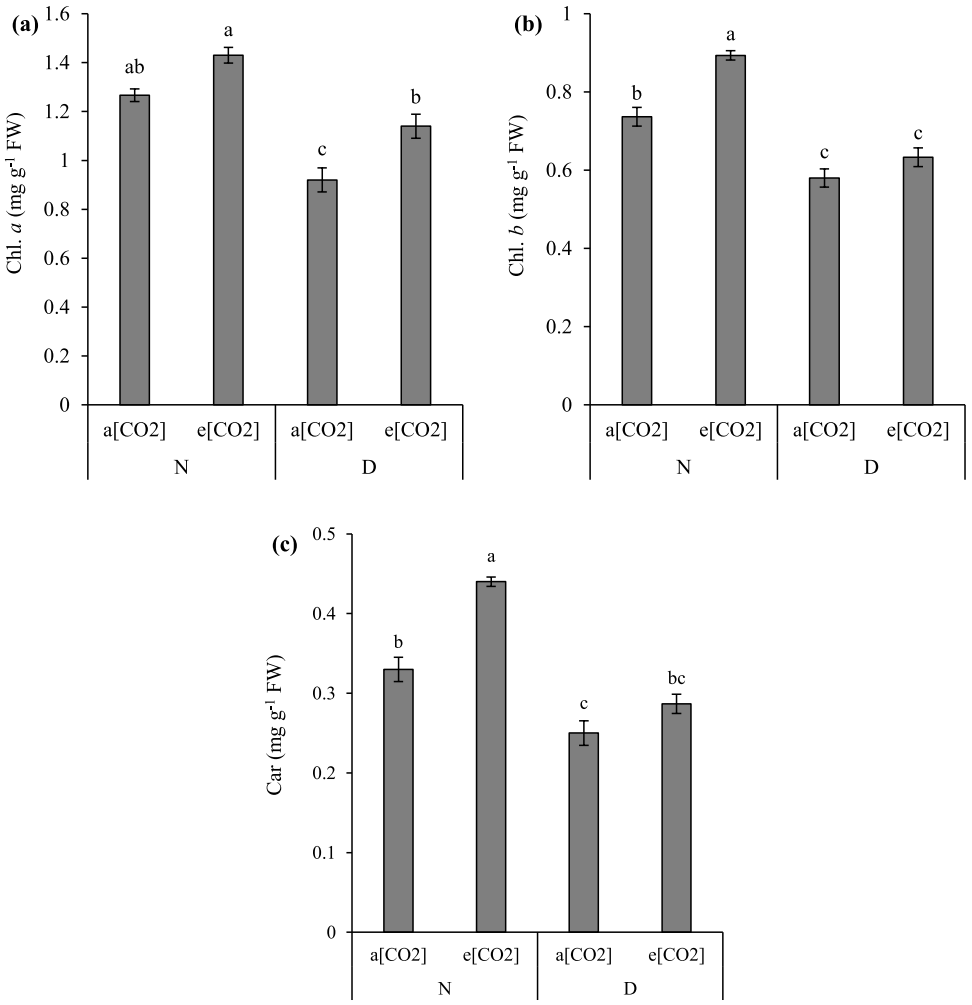

Figure 6. Response of chlorophyll *a* (Chl. *a*) (a), chlorophyll *b* (Chl. *b*) (b), and carotenoids (Car) (c) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test ($p < 0.05$). N, normal water supply; D, drought stress.

showed a positive correlation with GY, G/S, and HGW. On the contrary, H_2O_2, MDA, and leaf Ψ_w were negatively correlated with the G/S, HGW, and GY of wheat (Figure 8).

3.8. Clustered Heatmap of Antioxidants, Carbohydrate Metabolic Enzymes, H_2O_2, MDA, Proline, Leaf Ψ_w, Chlorophyll, and Grain Yield. CAT, POD, and SOD were positively clustered for D ($e[CO_2]$), whereas a strong association for TA was found in N ($e[CO_2]$). A positive and strong association for PGM, VacInv, and Susy was observed with D ($e[CO_2]$). The enzymes like UGPase, G_6PDH, HXK, and AGPase were clustered positively in N ($e[CO_2]$), while a strong and positive relationship of Ald and cwInv was depicted with N ($a[CO_2]$). H_2O_2, MDA, FK, and WP were clustered positively with D ($a[CO_2]$), whereas proline was strongly correlated with D ($e[CO_2]$). A strong positive association of Chl. *a*, Chl. *b*, Car, G/S, HGW, and GY was revealed with N ($e[CO_2]$) (Figure 9).

3.9. Structural Equation Modeling (SEM). The path diagram represents the structural relationships, including causality, covariance, and variances, between the observed and latent variables in the SEM model. As illustrated in Figure 10a, the estimated regression coefficient of GY on EA and YC is 0.02 and 0.09, respectively, with a covariance −11.77 between both variables. All of the nonenzymatic and yield-contributing traits have positive direct and indirect effects on wheat grain yield. Likewise, in Figure 10b, enzymatic and yield-contributing traits have positive relationships with grain yield with an estimated regression coefficient of GY on EA of 0.02 and on YC of 0.29, and with a covariance of −1.21 between both variables. Additionally, as shown in Figure 10c, yield-promoting traits have positive relationships with grain yield in wheat with an estimated regression coefficient of GY on GP of 4.13 and on YC of 0.13, with a covariance of −0.72 between both variables, indicating that they are directly related to each other.

4. DISCUSSION

Drought is the worst challenge of climatic change causing massive losses to crop yield globally.[38] Drought exposure is generally considered to cause disturbances in metabolic and physiological processes such as reduction of water relations, limiting of stomatal conductance and carbon assimilation, curling and wilting of leaves, inhibition of cell division, and oxidative damage to nuclei, cellular membranes and chlorophyll pigments, consequently reducing the normal growth of plants.[39,40] The gradual increase in environmental CO_2 is the

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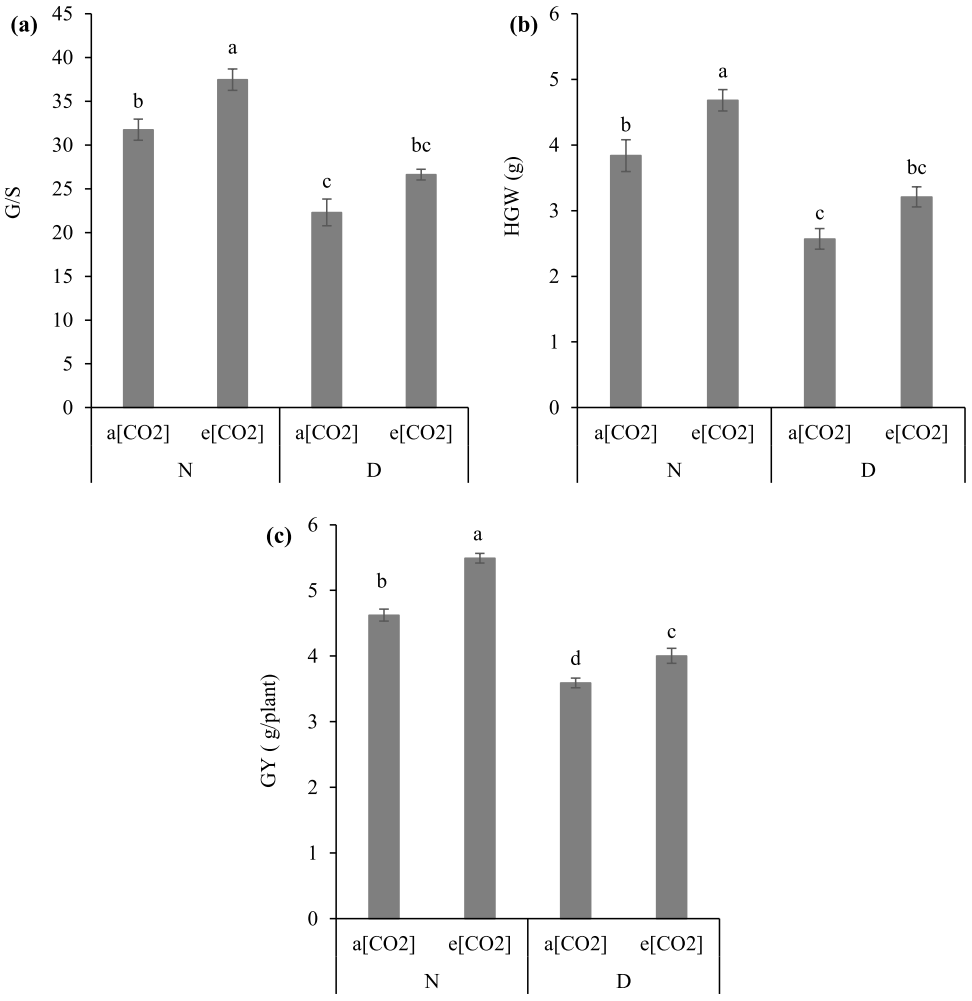

Figure 7. Response of grains per spike (G/S) (a), hundred grain weight (HGW) (b), and grain yield (GY) (c) in wheat plants grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. Means ± SE (n = 3) with different lower-case letters indicate significant differences following HSD Tukey's test ($p < 0.05$). N, normal water supply; D, drought stress.

main consequence of climate variability that interacts with drought to influence the ecological and physiological systems in plants.[6] It is evident that an increased concentration of CO_2 is involved in alleviating the drastic effects of drought via accelerating the cytoplasmic accumulation of osmolytes, protecting the photosynthetic system, and by improving the water use efficiency.[3,41,42] In the present study, the positive effects of transgenerational exposure of seeds to $e[CO_2]$ on the metabolic processes involved in stress memory regulation for inducing drought tolerance in wheat are of interest.

Drought stress is the complex and extreme effect of climate change that severely affects the growth of plants.[43] During the initial periods of drought, plants frequently adopt certain physiological adjustments like cellular accumulation of solutes, elimination of ROS through enhanced activity of antioxidative enzymes, and reduced transpiration by deposition of the waxy layer on leaf surfaces, but work for a short duration during droughts.[9] Numerous studies have suggested that $e[CO_2]$ may enhance the carbon to nitrogen ratio and leaf surface area for higher photosynthesis, and leaf water contents, thereby contributing to better crop growth and grain yield.[3,20] In the present study, transgenerational seed exposure to $e[CO_2]$ was found to largely improve the drought tolerance potential in wheat plants through the increased capacities of CAT, POD, and SOD. The increased activities of such antioxidative enzymes may effectively upregulate the defense system of plants for scavenging of ROS, causing peroxidation to membranes, proteins, and DNA, eventually enhancing the crop growth and yield under drought situations.[44] A prominent increase in CAT, POD, and SOD activities has also been described in *Selaginella tamariscina*,[45] *Helianthus annuus*,[9] *Medicago sativa*,[7] and *Ilex verticillata*,[40] suggesting that plants mostly avoid drought abnormalities by scavenging of ROS-free radicals via stimulating the antioxidant enzyme activities. As a defensive role of antioxidants, particularly, SOD is actively involved in detoxifying the O_2^- and preserving the cellular membranes from oxidative injuries, hence protecting the plants from the toxic effects of ROS instigated by drought.[47] Likewise, the upregulation of CAT was indicated in drought-stressed wheat plants raised from transgenerational seeds exposed to $e[CO_2]$, which could potentially maintain a higher net photosynthesis and grain yield via removal of H_2O_2 radicals.[3,48] Furthermore, CAT was also involved in increasing the level of ROS through its active reaction with certain hydroperoxides like methyl hydrogen peroxide, thus resulting in the enhanced potential of drought resistance in plants.[49]

As an abundant energy source for normal plant growth and physiological processes, the disturbed activities of enzymes

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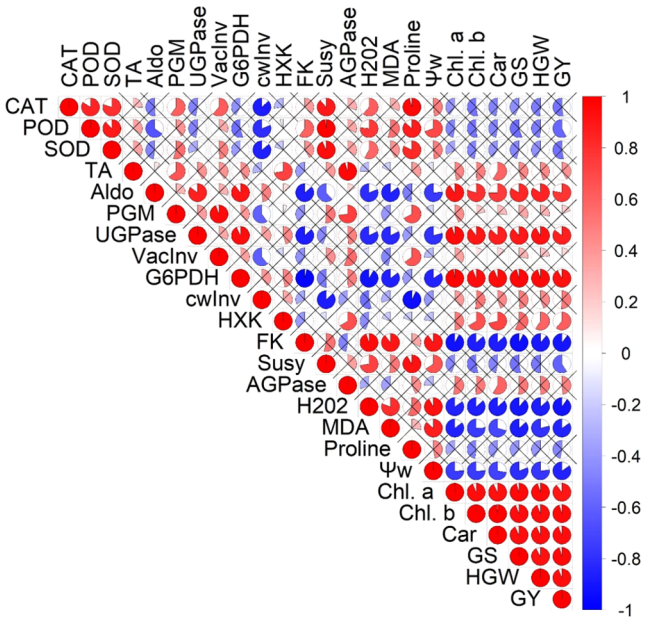

Figure 8. Correlation coefficients (r) indicating the association among antioxidants, carbohydrate metabolism, H_2O_2, MDA, proline, leaf Ψ_w, chlorophyll content, and yield-related traits of wheat following transgenerational seeds' exposure to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. The variation in color represents the significant differences ($p < 0.05$). CAT, catalase; POD, peroxidase; SOD, superoxide dismutase, TA, total antioxidants; Ald, fructose-1,6-bisphosphate aldolase; PGM, phosphoglucomutase; UGPase, UDP-glucose pyrophosphorylase; vacInv, vacuolar invertase; G_6PDH, glucose-6-phosphate-dehydrogenase; cwInv, cell wall invertase; HXK, hexokinase; FK, fructokinase; SuSy, sucrose synthase; AGPase, ADP-glucose pyrophosphorylase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; Ψ_w, water potential; Chl. a, chlorophyll a; Chl. b, chlorophyll b; Car, carotenoids; G/S, grains per spike; HGW, hundred grain weight; GY, grain yield.

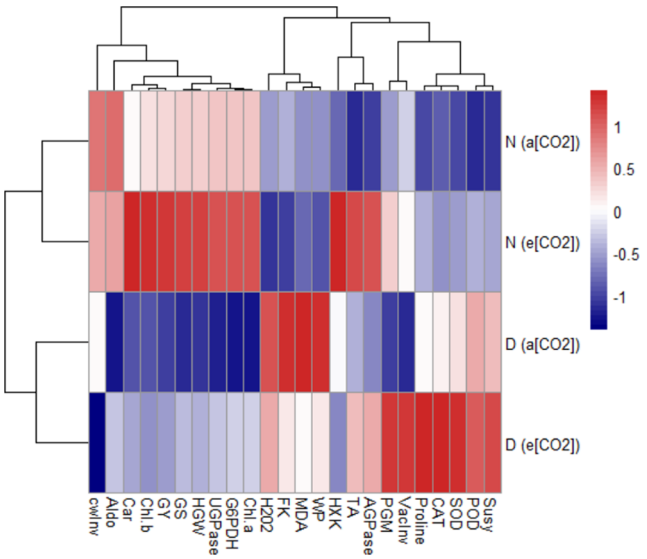

Figure 9. Clustered heatmap of antioxidants, carbohydrate metabolism, H_2O_2, MDA, proline, leaf Ψ_w, chlorophyll content, and yield-related traits of wheat following transgenerational seeds' exposure to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal (N) and drought (D) conditions. The variation in color represents the significant differences ($p < 0.05$). CAT, catalase; POD, peroxidase; SOD, superoxide dismutase, TA, total antioxidants; Ald, fructose-1,6-bisphosphate aldolase; PGM, phosphoglucomutase; UGPase, UDP-glucose pyrophosphorylase; vacInv, vacuolar invertase; G_6PDH, glucose-6-phosphate-dehydrogenase; cwInv, cell wall invertase; HXK, hexokinase; FK, fructokinase; SuSy, sucrose synthase; AGPase, ADP-glucose pyrophosphorylase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; WP, water potential; Chl. a, chlorophyll a; Chl. b, chlorophyll b; Car, carotenoids; G/S, grains per spike; HGW, hundred grain weight; GY, grain yield.

involved in carbohydrate metabolism were ascribed to drought-prone plants.[50] In the current results, drought stress caused a prominent decrease in carbohydrate metabolic enzymes such as Ald, PGM, UGPase, vacInv, G_6PDH, and cwInv, which might be associated with upregulation of proteins related to α-amylase and β-amylase enzymes. Overexpression of such enzymes is normally responsible for degradation of starch into soluble sugars to increase the cellular accumulation of osmoregulators, thus providing a large amount of energy to plants for optimal cell division and metabolism under drought stress.[51,52] A prompt increase in the activities of carbohydrate metabolic enzymes such as Ald, PGM, UGPase, vacInv, G_6PDH, and cwInv in wheat plants raised from transgenerational seeds exposed to $e[CO_2]$ is strongly related to the key role of $e[CO_2]$ in suppressing the upregulation of the lichenase enzyme responsible for degradation of carbohydrates within the plant system under drought stress.[53] It is also evident that carbohydrate metabolic enzymes like Ald, PGM, UGPase, vacInv, G_6PDH, and cwInv have essential roles in drought tolerance in plants.[54] In previous reports, Ald and PGM have been described to increase under $e[CO_2]$ concentration and to have key functions in glycolysis, starch-biosynthesis, cytoplasmic gluconeogenesis, and carbon dioxide fixation. The increase in Ald activity could enhance the carbon flow via the calvin cycle, however, stimulating the amino acids and sucrose synthesis in plants.[55,56] In present study, the increased activity of cwInv in plants of transgenerational seeds exposed to $e[CO_2]$ might be attributed to the overexpression of cwInv gene (CIN1), which helps the plants to delay senescence, regulate the source-sink balance, scavenge ROS, and supply carbohydrates through the apoplastic pathway, thereby leading to a higher grain yield under drought stress.[3] Similarly, the increased activity of the G_6PDH enzyme might be attributed to the enhanced antioxidative capacity of wheat plants, thus aggravating plant performance under drought stress.[57]

HXK, FK, SuSy, and AGPase activities were considerably increased in wheat plants during drought stress. As the more stable form of the carbohydrate, sucrose is rapidly phosphorylated with increased activity of FK enzyme in the citric acid cycle, thereby causing poor plant development due to enhanced respiration rates. Under drought stress, transgenerational seeds produced under $e[CO_2]$ revealed much less FK and HXK activities in wheat plants. The downregulation of FK activity under $e[CO_2]$ may be contributing to suppress or cause more effective conversion of carbohydrates to sucrose, which helps the plants to survive during drought stress periods.[53,58] A consistent increase in Susy and AGPase activities in wheat plants grown with overgenerational seeds exposed to $e[CO_2]$ is concomitant with the increased accumulation and translocation of starch and sugars for normal photosynthetic activities.[59]

Exposure to drought stress caused a substantial increase in the H_2O_2 and MDA contents in wheat plants. In fact, H_2O_2 is a

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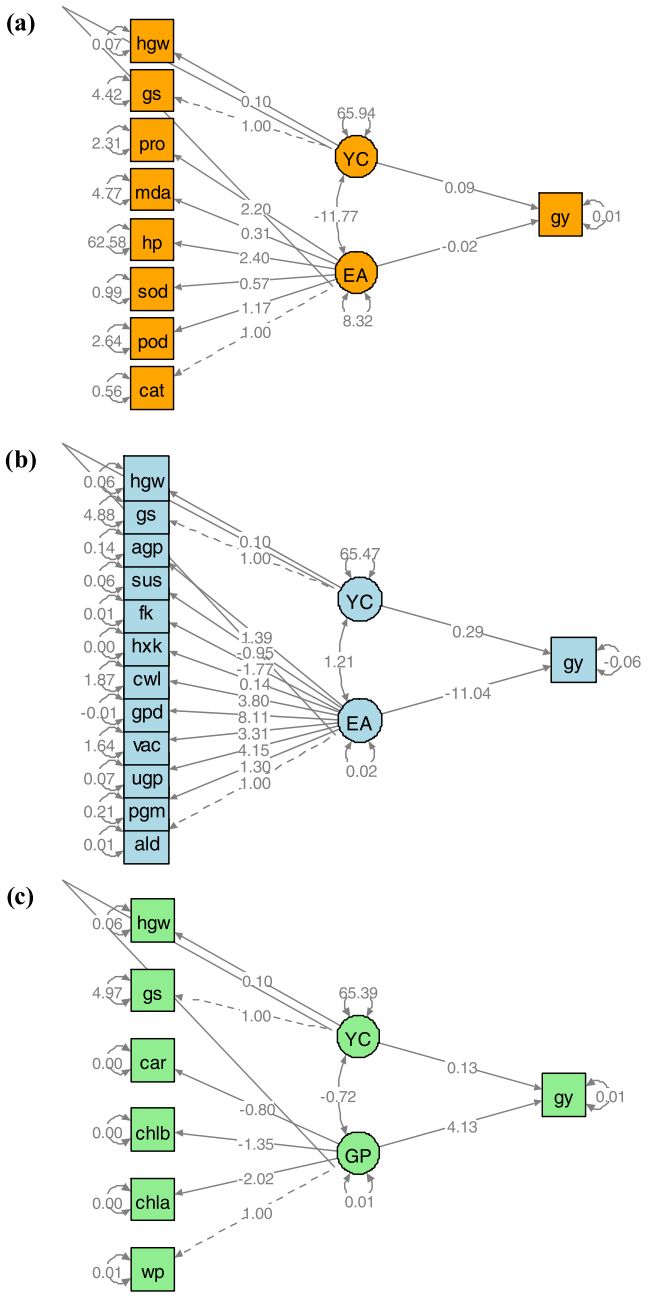

Figure 10. Structural equation model showing the direct and indirect influence of (a, b) enzymatic, nonenzymatic, and yield (c) contributing traits on wheat grain yield grown from transgenerational seeds exposed to ambient CO_2 ($a[CO_2]$, 400 μmol L^{-1}) and elevated CO_2 ($e[CO_2]$, 800 μmol L^{-1}) concentrations under normal and drought conditions. CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; Ald, fructose-1,6-bisphosphate aldolase; PGM, phosphoglucomutase; UGP, UDP-glucose pyrophosphorylase; VAC, vacuolar invertase; G_6PDH, glucose-6-phosphate-dehydrogenase; cwInv, cell wall invertase; HXK, hexokinase; FK, fructokinase; SuS, sucrose synthase; AGP, ADP-glucose pyrophosphorylase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; WP, water potential; Chl. *a*, chlorophyll *a*; Chl. *b*, chlorophyll *b*; Car, carotenoids; GS, grains per spike; HGW, hundred grain weight; GY, grain yield.

highly toxic and reactive ROS that is produced as a consequence of drought stress through conversion of O_2^- to H_2O_2. As a byproduct of H_2O_2-induced peroxidation to membranes, DNA, lipids, chloroplast and proteins, MDA compound reduces the integrity and fluidity of the membranes, thus causing a major loss to crop productivity.[48,60,61] In the current results, plants raised with transgenerational seeds produced under $e[CO_2]$ revealed a marked decline in H_2O_2 and MDA contents. The supremacy of $e[CO_2]$-exposed seeds for reducing H_2O_2 and MDA contents in wheat plants during drought stress is related to the upregulation of antioxidant-related differentially expressed genes, indicating their key functions of increasing Cu and Zn-SOD activity for effective scavenging of ROS-induced toxic compounds.[24] It was recently suggested that $e[CO_2]$-induced expression of late embryogenesis-abundant proteins could be possibly involved in protecting the membranes, nucleic acids, and enzymes from the oxidative stress of H_2O_2 and MDA via promoting the scavenging potential of antioxidant enzymes.[46,62]

As an osmolyte, proline is an effective nonenzymatic antioxidant that could potentially improve the drought tolerance in plants via scavenging of ROS, in particular, O_2^- and OH^{-8}. In the present study, proline content was increased to a significant level in drought-prone wheat plants. In conformity to our findings, proline is initially accumulated in plants as an osmoprotectant through upregulation of enzymes related to proline biosynthesis that maintain the water status of plants under drought stress.[9,63] An increase in proline content in drought-stressed wheat plants with transgenerational seeds exposed to $e[CO_2]$ indicates its positive role in stimulating the osmotic adjustment ability through cellular accumulation of proline, therefore enhancing the drought resistance in plants.[21] Drought prevalence also caused a marked decline in the leaf Ψ_w of plants. The drought exposure in wheat plants caused a more negative gain in Ψ_w, while transgenerational seeds produced under $e[CO_2]$ revealed a more positive leaf Ψ_w. The decrease in Ψ_w in drought-stressed plants was an index of the enhanced cytoplasmic accumulation of osmoprotectants such as glycine betaine, proline, sorbitol, sucrose, and fructose to cope with the drought events via maintaining the osmotic balance.[64] The reduced Ψ_w in wheat plants also coincides with the previous reports that drought-induced lipid peroxidation in plants causes damage to membrane proteins and enzymes involved in regulating the water channels and transporters for water movement.[48,65] Plants grown from transgenerational seeds exposed to $e[CO_2]$ had a higher Ψ_w, suggesting its critical involvement in maintaining the higher abundance of aquaporin proteins and hydraulic conductance, consequently improving the water status of plants.[66] It is further suggested that a stimulatory influence of $e[CO_2]$ in maintaining higher leaf water balance might be attributed to its key role in increasing the abscisic acid level that could effectively regulate the stomatal conductance for reducing the transpirational water loss in response to accelerating evaporative demands during drought.[67,68]

Chlorophyll content as an active component of photochemical reaction is damaged by drought stress.[69] In the current results, Chl. *a*, Chl. *b*, and Car contents were decreased drastically in drought-induced wheat plants. A substantial decline in chlorophyll content, i.e., Chl. *a*, Chl. *b*, and Car, is concomitant with the decomposition of chloroplast and thylakoid structures and light-harvesting pigment proteins via synthesis of oxygen-free radicals, hence resulting in reduced chlorophyll pigments.[70] Plants grown with transgenerational seeds exposed to $a[CO_2]$ had lower Chl. *a*, Chl. *b*, and Car contents than plants of $e[CO_2]$-treated seeds to drought conditions. The increase in chlorophyll contents in drought-

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stressed plants raised from transgenerational seeds exposed to $e[CO_2]$ may be accredited to the less oxidative damage to chlorophyll contents via quenching of free radicals.[71,72]

Drought instigated a considerable decrease in yield and yield attributes of wheat plants raised with transgenerational $a[CO_2]$-exposed seeds. Plants of transgenerational seeds treated with $e[CO_2]$ showed a considerably improved grain yield under drought stress. A dominant effect of overgenerational seeds treated with $e[CO_2]$ in alleviating the drought adversities in terms of higher development is related to the upregulation of metabolic processes in plants. These results further indicate the role of $e[CO_2]$ in sustaining the grain yield of wheat during drought conditions.[3] These also support the current finding that antioxidants, chlorophyll, and carbohydrate metabolic enzymes were strongly and positively associated with the grain yield and yield-related traits of wheat. It is also evident that transgenerational seeds exposed to $e[CO_2]$ maintained higher grain weights of wheat plants due to efficient utilization of carbon during photosynthesis,[42] thus leading to a higher grain yield under drought stress.

5. CONCLUSIONS

This study provides the first report describing that transgenerational seeds exposed to $e[CO_2]$ memorize stress memory via upregulation of metabolic processes to mitigate the drastic effects of drought in wheat. Our findings indicate that drought stress causes significant losses to leaf water status, carbohydrate metabolic enzymes, chlorophyll pigments, antioxidative potential, and finally, the grain yield of wheat. Nevertheless, transgenerational $e[CO_2]$-exposed seeds showed alleviated inhibitory effects of drought, which were manifested through improved leaf Ψ_w, proline, total antioxidants, and chlorophyll *a*, *b*, and carotenoid contents. The increased activities of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase in plants produced from overgenerational $e[CO_2]$-exposed seeds contributed better to eliminate the H_2O_2 and MDA contents, particularly under drought conditions. Additionally, seeds subjected to transgenerational $e[CO_2]$ exhibited a more pronounced increase in the functioning of enzymes involved in carbohydrate metabolism, consequently enhancing the wheat gain yield during drought periods.

■ ASSOCIATED CONTENT

Data Availability Statement

All data sets analyzed in current study are included in the manuscript.

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Notes

The authors declare no competing financial interest.

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