



## Evaluation of the Role of Cobalt Nutrition in the Oxidative Machinery of Drought-Stressed Rice (*Oryza sativa* L.) Plants at the Reproductive Stage



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**T**HIS STUDY evaluated the impact of cobalt (Co) nutrition at the optimum concentration on growth and oxidative stress in two Egyptian rice varieties (Sakha 104 and Giza 178) under drought stress. The stress was applied at different irrigation intervals (six and eight days), and irrigation after four days was set as control through the reproductive stage. The roots and shoots parameters (lengths, and fresh and dry weights), oxidative stress biomarkers; membrane injury, lipid peroxidation, proline accumulation, protein content, total phenols, and flavonoids contents, along with the activities of catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO) enzymes, were used to evaluate the strength of reactive oxygen species (ROS) detoxification in both rice varieties. Water deficit and oxidative stress affected Giza 178 less than Sakha 104. Stressed rice plants of both varieties treated with Co at the optimum concentration reduced the hurtful impact of drought, particularly in Giza 178, by enhancing root and shoot growth and biomass and the efficiency of the oxidative defense system. This was obvious in a substantial decrease in membrane injury and lipid peroxidation, while a significant increase was apparent in proline and protein content, total phenols, and total flavonoids. Additionally, the dynamic activities of antioxidant enzymes were substantially higher in Giza 178 than Sakha 104, when compared to untreated plants. The findings could be helpful in research projects aimed at developing anti-drought stress techniques for rice plants.

**Keywords:** Antioxidant enzymes, Cobalt, Drought, Lipid peroxidation, Proline, *Oryza sativa* L.

### Introduction

Climate change has significant implications for agricultural productivity in semi-arid and arid regions. Abiotic stressors, including drought, dramatically impact crops by altering the physiological functions of plants (Heshmati et al., 2021). Environmental changes and subsequent drought stress cause oxidative stress, resulting from excessive activated atmospheric oxygen, referred to as reactive oxygen species (ROS) (Aldaby et al., 2021; Heshmati et al., 2021). ROS damages molecular and cellular components due to the oxidation of biomolecules (lipids, carbohydrates, proteins, enzymes, DNA), killing plants (Shah et al., 2019; Bhuyan et al., 2020). Drought impairs cell growth (Swain et al.,

2014), biomass production (Farooq et al., 2010), photosynthesis, induced ROS overproduction, and oxidative stress in numerous plant species, such as *Z. mays* (Anjum et al., 2017), *T. aestivum* (Ahmad et al., 2019), and *O. sativa* (Cheng et al., 2020). Rice (*O. sativa* L.) is a significant grain, accounting for more than a quarter of cereal consumption worldwide, with a global output of 738.2 million tons (Khan et al., 2020). Drought stress is an essential hindrance to rice productivity since rice is a paddy field crop that requires more water for continuous growth (Khan et al., 2020).

ROS exacerbate the effects of drought stress in plants by changing cell membrane properties and causing oxidative damage to chlorophylls, lipids, proteins, and nucleic acids, causing them to

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remain inactive (Mittler, 2002). Oxidative stress damages cell membranes and other organelles when disrupting the equilibrium between ROS generation and the plant antioxidant defense mechanism. Malondialdehyde (MDA) formation is the initial consequence of membrane lipid peroxidation, occurring faster in the presence of ROS (Babaei et al., 2021). Plants generally cope with oxidative stress through an endogenous defense system that includes various enzymatic antioxidants (catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO)) and non-enzymatic antioxidants (total phenols and total flavonoids) (Kaur et al., 2019; Loutfy et al., 2020). The antioxidant defense system and ROS buildup maintain a steady-state equilibrium in plant cells (Hasanuzzaman et al., 2012). Establishing an ideal ROS level in the cell enables proper redox biology reactions and the regulation of a range of essential processes in plants, including growth and development (Mittler, 2017; Hafez & Fouad, 2020). Other osmotic stress-relieving processes in plant cells involve enhancing the synthesis and assembly of osmolytes, including proline, glycine betaine, and polyamines (Babaei et al., 2021). Drought-stressed plants have a decreased protein content, considering the increased activity of protein-degrading enzymes and accumulation of free amino acids, including proline (Babaei et al., 2021).

Plants engage their antioxidant defense mechanism to counteract the negative consequences of oxidative stress. However, antioxidant defense capability differs between plant varieties and genotypes, and between stress type (salinity, drought, heat, and heavy metals) and duration (Hasanuzzaman et al., 2020). Consequently, decreasing ROS accumulation or increasing antioxidant activity in rice organs is the most appropriate approach for rice drought tolerance improvement. Furthermore, agricultural areas worldwide are already suffering a rapid decline in irrigation water availability (Cai et al., 2013). Thus, it is vital to implement strategies to safeguard crops against extreme weather conditions, including drought. One of the vital methods of dealing with this challenge is breeding drought-tolerant crops. Unfortunately, this strategy is time-consuming and expensive (Liu et al., 2022). Accordingly, mineral nutrition utilization emerged as one of the most promising methods for alleviating the negative impact of drought on plant growth (Hassan et al., 2020).

Numerous heavy metals control and monitor plant development, and physiological and biochemical activities. Metals, including copper (Cu), cobalt (Co), manganese (Mn), chromium (Cr), and zinc (Zn), are required for plant metabolism and fundamentally impact plant development and production when present in sufficient quantities. Co is a helpful element for plants (Akeel & Jahan, 2020). Low concentrations of Co are beneficial for plant growth, whereas high concentrations are not. According to Akeel & Jahan (2020), Co plays important role in synthesizing vitamin B12 and nitrogen fixation in legumes. The ability of Co to counteract abiotic stresses, like salinity, drought, and cadmium, is documented (Gad et al., 2018; Akeel & Jahan, 2020). Foliar application of Co increased tomato growth and fruit yield (Gad et al., 2017), controlled the homeostasis of ions in cucumbers (Gad et al., 2018), and altered phytohormones in maize (Gad & El-Metwally, 2015) in response to salt stress. However, little is known about the function of Co in maintaining redox homeostasis when exposed to drought stress. Considering the beneficial effects of Co in plants at appropriate concentrations, the objective of this study was to demonstrate the effectiveness of Co nutrition at the optimum concentration by soaking grain before sowing in the soil to optimize the performance of the antioxidant defense system under different drought stress intensities in two Egyptian rice varieties.

## **Materials and Methods**

### *Materials and growth conditions*

This study was carried out as a pot experiment at the Faculty of Agriculture (Mansoura University, Egypt) throughout the growing season of 2019 (May to August) under open field conditions. The temperature was around  $30^{\circ}\text{C} \pm 5^{\circ}\text{C}$ , and the relative humidity was around 65%–73% during the experiment. Silt soil was used, and a soil sample was taken from the upper 0–30cm of the arable layer. Table 1 lists the soil characteristics. Cobalt sulfate ( $\text{CoSO}_4$ ) was purchased from Central Drug House (P) Ltd (Post Box No. 7138 New Delhi-110002). Plant materials used in the experiment were the two Egyptian varieties of Sakha104 (drought-sensitive) and Giza 178 (drought-tolerant), purchased from the Rice Research and Training Center (Sakha, Kafr El-Sheik, Egypt). The features of the two rice varieties were adopted according to Rice Research and Training Center data from 2011.

TABLE 1. Physical and chemical properties of the soil used

Soil characteristics	pH	EC (dS/m)	O. C (%)	T.N (%)	C/N ratio	T.S.S (%)	W.H.C (%)		
	8.17	0.52	0.71	0.26	2.73	0.52	48.5		
Soluble ions (mg/100 g dry mass soil)									
	SO <sub>4</sub> <sup>2-</sup>	CaCO <sub>3</sub>	HCO <sub>3</sub> <sup>-</sup>	CO <sub>3</sub> <sup>2-</sup>	Cl <sup>-</sup>	K <sup>+</sup>	Na <sup>+</sup>	Ca <sup>2+</sup>	P
	0.38	3.47%	0.45	Not detected	0.57	0.38	0.73	0.321	1.18

W.H.C, water holding capacity; EC, electrical conductivity; O.C, organic carbon; T.N, total nitrogen; T.S.S, total soluble solids; SO<sub>4</sub><sup>2-</sup>, sulfate; CaCO<sub>3</sub>, calcium carbonate; HCO<sub>3</sub><sup>-</sup>, bicarbonate; CO<sub>3</sub><sup>2-</sup>, carbonate; Cl<sup>-</sup>, chloride; K<sup>+</sup>, potassium; Na<sup>+</sup>, sodium; Ca<sup>2+</sup>, calcium; P, phosphorus.

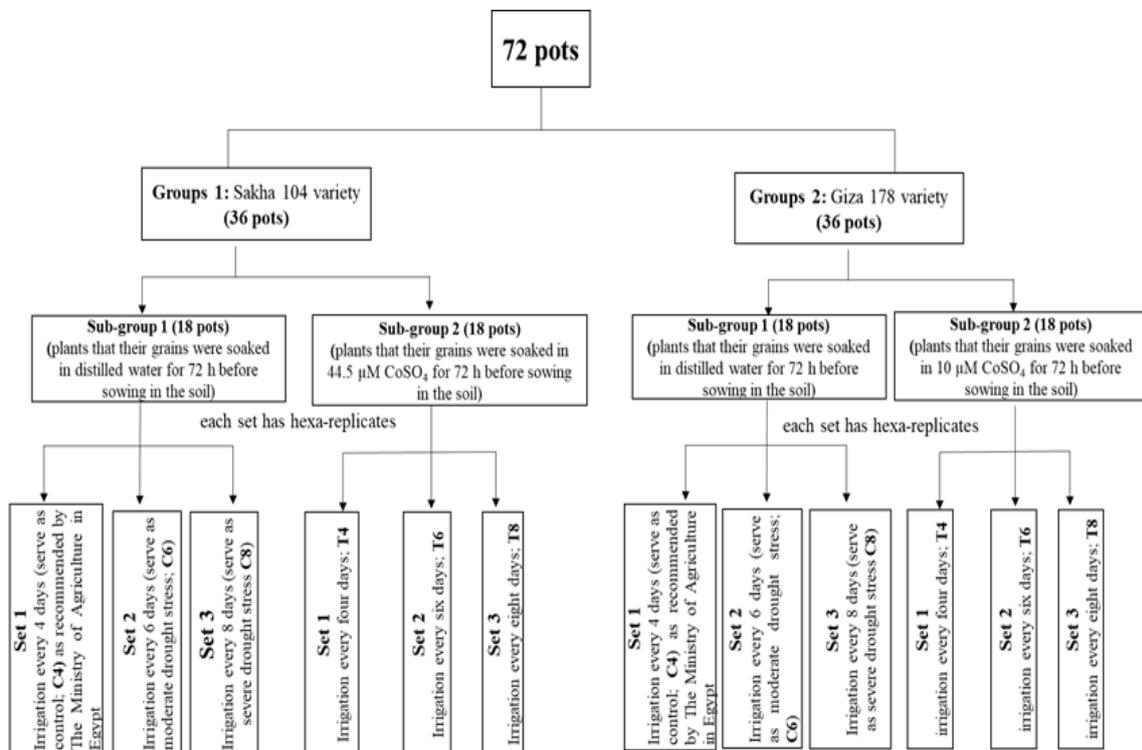
### Grain soaking

Two uniformly sized lots of homogenous rice grains of Sakha 104 and Giza 178 were surface sterilized via immersion in 4.0% sodium hypochlorite solution for 20min. The sterilized grains were washed with distilled water. One group from each variety was soaked in distilled water (control), and the second group was soaked in CoSO<sub>4</sub> (44.5µM for Sakha 104 and 10µM for Giza 178) in plastic boxes and incubated in the dark at 27°C± 2°C for 72h. The two concentrations of CoSO<sub>4</sub> were used based on previous preliminary germination experiments on both varieties in the Laboratory of Plant Physiology, Botany Department, Faculty of Science (Mansoura University, Egypt), and were applied as optimum concentration for each variety.

### Pot culture experiments and drought application

At the end of the soaking period, 20–25 grains were sown in each plastic pot with 10kg of soil. These pots were divided into two groups (10 pots each) representing the two rice varieties. Each group was divided into two subgroups. The first included five pots representing the grains soaked in distilled water for 72h, and the second includes five pots representing the grains soaked in the optimum concentration of CoSO<sub>4</sub> for 72h. The pots were irrigated daily with tap water (5L/pot) until day 28.

After day 28, an identical lot of plants from each variety was taken and transplanted in plastic pots (10 plants/pot) containing 7kg of soil. Seventy-two pots in a completely randomized complete block design with six replicates were divided into the following scheme:



Thinning occurred a week from transplantation leaving five homogenous plants per pot for experimentation. Application of drought stress occurred two weeks from the date of transplantation to avoid any shock. According to instructions of The Ministry of Agriculture (Egypt), recommended doses of fertilizers (N–P<sub>2</sub>O<sub>5</sub>–K<sub>2</sub>O), besides insect and weed control, were applied at the appropriate times throughout the experiment. At day 92 (representing the reproductive stage), plants from every treatment of both varieties were harvested, and the root and shoot lengths, root/shoot (R/S) ratio, root and shoot fresh and dry weights, and different metabolic and enzymatic parameters were measured. The illustrated data in tables and figures are the mean values of ten readings for roots and shoots parameters and three readings for each chemical analysis from each treatment.

#### *Estimation of total protein*

The soluble protein concentration was determined in the leaves at wavelength 595nm using a spectrophotometer (JENWAY designed and Manufactured in the UK by Bibby Scientific Ltd, Stone Staffs, UK, ST150SA-Model 7315) and the Brilliant Blue G-250 reagent (Bradford, 1976).

#### *Estimation of proline content*

Leaf proline levels were calorimetrically determined by the reaction with ninhydrin as described by Bates et al. (1973). The developed color was extracted in 4 ml of toluene and calorimetrically measured at 520 nm. The proline content was calculated as  $\mu\text{g g}^{-1}$  of the fresh weight.

#### *Estimation of cell membrane injury*

According to Shi et al. (2006), electrolyte leakage was evaluated by cutting freshly harvested rice leaves into thin discs, placing them in a test tube, and washing them three times with 20mL of distilled water to remove the electrolytes produced during leaf cutting. The tubes were filled with 30mL of distilled water and stood in the dark for 24h at room temperature. Electrical conductivity (EC1) was measured with an EC meter (CM-21P; DKK-TOA Corporation, Tokyo, Japan) at the end of the incubation period. The tubes were heated at 95°C in boiling water for 20min before cooling to room temperature. The electrical conductivity (EC2) of the finished product was determined.

$EC = (EC1/EC2) \times 100$  computed membrane leakage (EC).

#### *Determination of lipid peroxidation*

Lipid peroxidation in fresh leaves of rice plants was determined by estimating the content of MDA as the method adopted by Hajlaoui et al. (2009). The MDA levels were estimated using a 155mM  $\text{cm}^{-1}$  extinction coefficient and displayed as  $\mu\text{M MDA g}^{-1}$  of the fresh weight.

#### *Estimation of antioxidant compounds*

The extraction method was performed according to Singleton & Rossi (1965) and Kosem et al. (2007). One g of air-dried rice tissue was extracted in 10mL of 50% methanol for a week at 37°C and then filtered. The total phenolics content in extracts was determined using a procedure published by Turkmen et al. (2005) squash, green beans, peas, leek, broccoli and spinach. Total phenolics content of fresh vegetables ranged from 183.2 to 1344.7mg/100g (as gallic acid equivalent. An ultraviolet (UV)–spectrophotometer (JENWAY designed and Manufactured in the UK by Bibby Scientific Ltd, Stone Staffs, UK, ST150SA-Model 7315) was used to detect the absorbance at 765nm. The results were represented in mg of gallic acid equivalent to  $\text{g}^{-1}$  dry weight. The total flavonoid content was determined according to Marinova et al. (2005) dogwood berries (432.0mg GAE/100g) and Atanassova et al. (2011), and the absorbance of the reaction mixture was measured at 510nm. The flavonoid concentration was calculated via a standard curve of quercetin in the range 0–120 $\mu\text{g/mL}$ .

#### *Estimation of antioxidant enzymes activity*

About 0.5 g of fresh rice leaf material of each treatment was homogenized at 0–4°C in 3mL of 50mM Tris buffer (pH 7.8), including 1mM ethylene diamine tetra acetic acid disodium salt (EDTA-Na<sub>2</sub>) and 7.5% polyvinyl pyrrolidone, for enzyme assays in rice plants. The homogenates were centrifuged (12,000rpm, 20min, and 4°C), and the total soluble enzyme activities in the supernatant were determined via spectrophotometer (Hafez et al., 2014). All measurements were performed at 25°C with a spectrophotometer model UV-160A (Shimadzu, Japan).

According to Aebi (1984), the activity of catalase (CAT, EC 1.11.1.6) was determined.

The UV absorption of hydrogen peroxide ( $H_2O_2$ ) at 240nm decreases as  $H_2O_2$  is decomposed by CAT. This drop helps calculate enzyme activity. The reaction mixture contained 2mL 0.1M Naphosphate buffer (pH 6.5), 100 $\mu$ L hydrogen peroxide, and 50 $\mu$ L leaf extract supernatant for a final volume of 2.15mL. After mixing the solution, the absorbance change was measured using quartz at 240 nm for three minutes.

The activity of peroxidase (POX, EC 1.11.1.7) was measured using the method of Hammerschmidt et al. (1982). The reaction mixture consisted of 2.9mL of a 100mm sodium phosphate buffer (pH 6.0) containing 0.25% (v/v) guaiacol (2-methoxy phenol) and 100mm  $H_2O_2$ . Changes in absorbance at 470nm were detected every 30 seconds for three minutes. Enzyme activity was expressed as an increase in absorbance minute<sup>-1</sup> g<sup>-1</sup> of fresh weight.

The activity of polyphenol oxidase (PPO, EC 1.14.18.1) was evaluated in a mixture of 1.5mL of 0.1M sodium phosphate buffer (pH 6.5) and 0.1mL of enzyme extracts. About 0.2 ml of 0.01M catechol was added to initiate the reaction. The absorbance change was measured at 495 nm and expressed as a change in absorbance minute<sup>-1</sup> g<sup>-1</sup> of fresh tissue (Hammerschmidt et al., 1982).

#### *Statistical analysis*

Experimental data were statistically analyzed using the one-way analysis of variance with the post hoc Duncan test. The P value  $\leq 0.05$  was statistically significant, and the analysis was performed using the statistical package for social science (version 22) for windows.

## **Results**

#### *Root and shoot parameters*

Data in Table 2 showed that root length and the R/S ratio were significantly increased in stressed plants compared to the control in both varieties. Sakha 104 had the largest increase. Results also showed that shoot length, fresh and dry weights of roots, and shoots were significantly decreased in both varieties under drought stress. The decrease was much more pronounced in Giza 178. Co application, either alone or combined with drought stress, significantly increased all previous parameters compared to untreated plants in both varieties in a comparable pattern (Table 2).

#### *Changes in protein content*

The protein content decreased as the drought level increased in both varieties (Fig. 1A). However, the decrease was more pronounced with severe drought stress in Sakha 104. The protein content reduced under treatment C6 by -14.12% and -10.64%, while the protein content under treatment C8 reduced by -20.67% and -19.07% compared with treatment C4 in Sakha 104 and Giza 178, respectively. The combination of both the control and drought with Co recorded a significant increase in protein content of both varieties, with the percentage enhancement being more apparent in Giza 178. Treatment T4 enhanced the protein content by 10.42% and 12.71%, and treatment T6 enhanced the protein content by 6.73% and 7.15%. The protein content was enhanced by 4.34 % and 6.49% under treatment T8 in Sakha 104 and Giza 178, respectively, compared to treatments C4, C6, and C8.

#### *Changes in proline content*

Proline content in both varieties increased exponentially as drought stress increased, compared to the control (Fig. 1B). Giza 178 pronounced higher level of proline under severe drought stress. Under treatment C6, proline content increased by 19.01% and 20.89%, while proline content increased by 29.20% and 35.05% under treatment C8 in Sakha 104 and Giza 178, respectively, compared to treatment C4. However, a significant increase was observed in both varieties with the Co combination compared to untreated plants. The increase was more apparent with severe drought stress in Giza 178. Proline content under treatment T4 increased by 5.68% and 9.90%, and it increased under treatment T6 by 21.30% and 22.79%. Proline content under treatment T8 increased by 32.37% and 37.65% in Sakha 104 and Giza 178, respectively, compared to treatments C4, C6, and C8.

#### *Changes in cell membrane integrity and lipid peroxidation*

Data in Fig. 2 indicated a progressive percentage increase in electrolyte leakage and MDA content in the treated varieties as drought stress increased. Sakha 104 exhibited the highest percentage of membrane injury and MDA content with severe drought stress. Electrolyte leakage increased by 30.63% and 26.30% in Sakha 104 and Giza 178 under treatment C6,

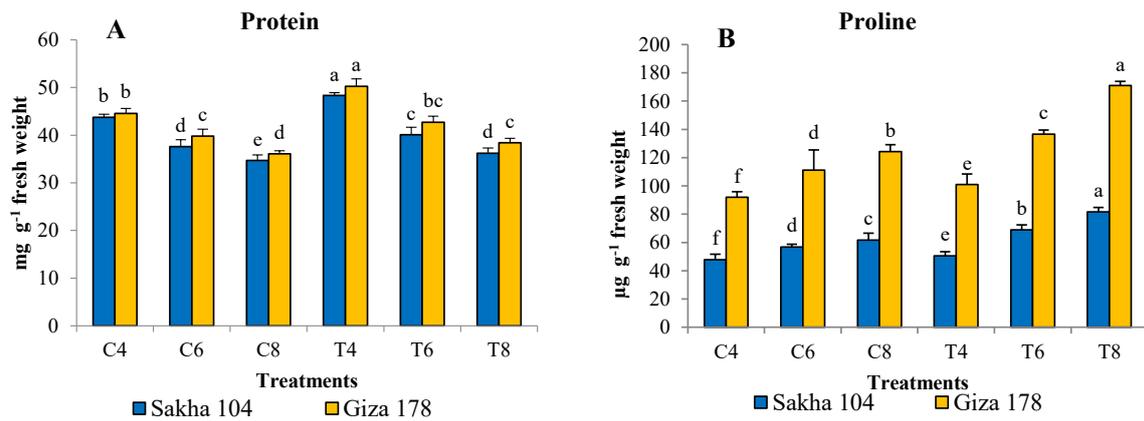
while electrolyte leakage increased by 35.98% and 34.09% under treatment C8 in Sakha 104 and Giza 178, respectively, compared to treatment C4 (Fig. 2A). Conversely, MDA content increased by 5.20% and 2.79% under treatment C6 in Sakha 104 and Giza 178, while MDA content increased by 6.94% and 5.35% under treatment C8 in Sakha 104 and Giza 178, respectively, compared to treatment C4 (Fig. 2B). Co treatments recorded a significant reduction in electrolyte leakage and MDA content below untreated plants in both varieties. Electrolyte leakage reduced by -19.62% and -20.88%, and

MDA content reduced by -8.51% and -9.07% under treatment T4 in Sakha 104 and Giza 178, respectively. Electrolyte leakage reduced by -16.34% and -19.19%, and MDA content reduced by -8.08% and -8.60% under treatment T6 in Sakha 104 and Giza 178, respectively. Treatment T8 reduced electrolyte leakage by -1.39% and -2.10% and reduced MDA content by -6.18% and -7.28% in Sakha 104 and Giza 178, respectively, compared to treatments C4, C6, and C8.

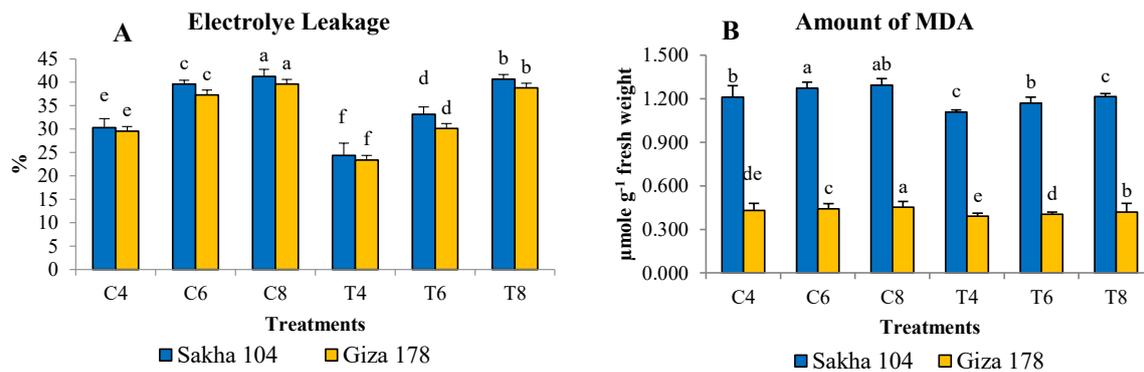
**TABLE 2.** Effects of drought stress either alone or in combination with  $\text{CoSO}_4$  on root and shoot parameters of Sakha 104 and Giza 178 plants at the reproductive stage. Data are the means of ten replicates  $\pm$  standard error

Variety	Treatments	Root length (cm)	Shoot length (cm)	R/S ratio	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)
Sakha 104	C4	26.259 <sup>d</sup> $\pm 0.276$	68.108 <sup>c</sup> $\pm 0.177$	0.386 <sup>e</sup> $\pm 0.224$	28.154 <sup>b</sup> $\pm 0.282$	47.524 <sup>b</sup> $\pm 0.263$	5.616 <sup>a</sup> $\pm 0.042$	12.419 <sup>a</sup> $\pm 0.067$
	C6	28.240 <sup>c</sup> $\pm 0.309$	64.752 <sup>c</sup> $\pm 0.168$	0.436 <sup>e</sup> $\pm 0.234$	25.317 <sup>c</sup> $\pm 0.349$	43.609 <sup>c</sup> $\pm 0.325$	5.463 <sup>a</sup> $\pm 0.021$	12.213 <sup>ab</sup> $\pm 0.106$
	C8	30.552 <sup>b</sup> $\pm 0.217$	60.438 <sup>f</sup> $\pm 0.335$	0.506 <sup>a</sup> $\pm 0.254$	23.489 <sup>d</sup> $\pm 0.398$	40.578 <sup>d</sup> $\pm 0.319$	5.398 <sup>a</sup> $\pm 0.059$	12.301 <sup>ab</sup> $\pm 0.064$
	T4	28.783 <sup>c</sup> $\pm 0.205$	75.341 <sup>a</sup> $\pm 0.243$	0.382 <sup>f</sup> $\pm 0.223$	31.629 <sup>a</sup> $\pm 0.342$	52.272 <sup>a</sup> $\pm 0.244$	5.621 <sup>a</sup> $\pm 0.051$	12.504 <sup>a</sup> $\pm 0.048$
	T6	30.512 <sup>b</sup> $\pm 0.330$	70.594 <sup>b</sup> $\pm 0.315$	0.432 <sup>d</sup> $\pm 0.311$	28.005 <sup>b</sup> $\pm 0.289$	47.701 <sup>b</sup> $\pm 0.369$	5.571 <sup>a</sup> $\pm 0.117$	12.351 <sup>a</sup> $\pm 0.087$
	T8	32.539 <sup>a</sup> $\pm 0.257$	66.419 <sup>d</sup> $\pm 0.220$	0.490 <sup>b</sup> $\pm 0.241$	25.158 <sup>c</sup> $\pm 0.337$	41.682 <sup>d</sup> $\pm 0.296$	5.373 <sup>a</sup> $\pm 0.052$	12.010 <sup>b</sup> $\pm 0.073$
Giza 178	C4	22.860 <sup>c</sup> $\pm 0.204$	63.500 <sup>b</sup> $\pm 0.264$	0.360 <sup>c</sup> $\pm 0.211$	26.141 <sup>b</sup> $\pm 0.672$	48.205 <sup>b</sup> $\pm 0.672$	4.290 <sup>b</sup> $\pm 0.051$	10.940 <sup>b</sup> $\pm 0.177$
	C6	24.500 <sup>b</sup> $\pm 0.264$	59.300 <sup>c</sup> $\pm 1.566$	0.413 <sup>c</sup> $\pm 0.262$	23.327 <sup>c</sup> $\pm 0.200$	44.259 <sup>c</sup> $\pm 0.200$	4.116 <sup>c</sup> $\pm 0.023$	10.718 <sup>b</sup> $\pm 0.204$
	C8	26.000 <sup>a</sup> $\pm 0.264$	54.960 <sup>d</sup> $\pm 0.243$	0.473 <sup>a</sup> $\pm 0.242$	21.505 <sup>d</sup> $\pm 0.187$	40.549 <sup>d</sup> $\pm 0.187$	4.007 <sup>d</sup> $\pm 0.125$	10.565 <sup>b</sup> $\pm 0.021$
	T4	24.480 <sup>b</sup> $\pm 0.385$	70.000 <sup>a</sup> $\pm 0.264$	0.350 <sup>f</sup> $\pm 0.275$	29.636 <sup>a</sup> $\pm 0.224$	55.882 <sup>a</sup> $\pm 0.224$	4.440 <sup>a</sup> $\pm 0.123$	11.578 <sup>a</sup> $\pm 0.094$
	T6	25.840 <sup>a</sup> $\pm 0.367$	66.212 <sup>b</sup> $\pm 0.192$	0.390 <sup>d</sup> $\pm 0.265$	26.007 <sup>b</sup> $\pm 0.179$	48.609 <sup>b</sup> $\pm 0.179$	4.281 <sup>b</sup> $\pm 0.111$	10.838 <sup>b</sup> $\pm 0.083$
	T8	26.780 <sup>a</sup> $\pm 0.242$	64.400 <sup>b</sup> $\pm 0.321$	0.416 <sup>b</sup> $\pm 0.251$	23.160 <sup>c</sup> $\pm 0.257$	44.434 <sup>c</sup> $\pm 0.257$	4.128 <sup>c</sup> $\pm 0.147$	10.560 <sup>b</sup> $\pm 0.104$

Mean values with the same superscripts are not significantly different at  $P \leq 0.05$ .



**Fig. 1.** Effects of drought stress either alone or in combination with  $\text{CoSO}_4$  on protein and proline contents of Sakha 104 and Giza 178 rice plants at the reproductive stage [Vertical bars represent the standard error ( $\pm$ SE). Different bar letters show significant differences among treatments separately] [C4: Control, T4: Control+  $\text{CoSO}_4$ , C6: plants irrigated every 6 days, T6: plants irrigated every 6 days +  $\text{CoSO}_4$ , C8: plants irrigated every 8 days, T8: plants irrigated every 8 days +  $\text{CoSO}_4$ ]



**Fig. 2.** The effects of drought stress either alone or in combination with  $\text{CoSO}_4$  on electrolyte leakage and MDA amount of Sakha 104 and Giza 178 rice plants at the reproductive stage [Vertical bars represent the standard error ( $\pm$ SE). Different bar letters show significant differences among treatments separately] [Legends are the same as in Fig. 1]

### Antioxidant system

#### The changes in antioxidant compounds

The total phenols and flavonoids content increased as the level of drought stress increased compared to the control in both varieties (Fig. 3). The total phenols content increased by 6.07% and 7.08%, and the total flavonoids content increased by 4.54% and 6.57% under treatment C6. The total phenols content increased by 9.92% and 11.43%, and the total flavonoids content increased by 6.73% and 7.95% under treatment C8 in Sakha 104 and Giza 178, respectively, compared to treatment C4. However, Co treatments appeared to induce additional increases in the total phenols and

flavonoids contents in both varieties compared to untreated plants. The percentage increase was most pronounced in treatment T8 in Giza 178. The total phenols content increased by 7.72% and 8.17%, and the total flavonoids content increased by 2.62% and 3.52% under treatment T4 in Sakha 104 and Giza 178, respectively. The total phenols content increased by 10.52% and 13.46%, and the total flavonoids content increased by 6.12% and 8.92% under treatment T6 in Sakha 104 and Giza 178, respectively. The total phenols content increased by 12.18% and 14.84%, and the total flavonoids content increased by 9.13% and 10.20% under treatment T8 in Sakha 104 and Giza 178, respectively,

compared to treatments C4, C6, and C8 (Fig. 3).

#### *Changes in antioxidant enzymes*

Figure 4 shows that the activity values of CAT, POX, and PPO in leaves of Sakha 104 and Giza 178 had varied increases corresponding to increased drought stress compared to the controls. CAT activity increased by 30.26% and 34.88%, PPO activity increased by 50% and 54.55%, and POX activity increased by 66.67% and 76.47% under treatment C6 in Sakha 104 and Giza 178, respectively. CAT activity increased by 57.24% and 63.95%, PPO activity increased by 87.50% and 90.91%, and POX activity increased by 66.67% and 76.47% under treatment C8 in Sakha 104 and Giza 178, respectively, compared to treatment C4. Conversely, the Co treatments appeared to induce additional comparable increases in all enzyme activities in both varieties. The magnitude of increases is generally higher with severe drought stress, particularly in Giza 178 compared to Sakha 104. CAT activity increased by 9.21% and 11.63%, PPO activity increased by 37.50% and 45.45%, and POX activity increased by 80% and 82.35% under treatment T4 in Sakha 104 and Giza 178, respectively. CAT activity increased by 35.35% and 38.31%, PPO activity increased by 66.67% and 72.41%, and POX activity increased by 84.0% and 85.33% under treatment T6 in Sakha 104 and Giza 178, respectively. CAT activity increased by 49.79% and 56.68%, PPO activity increased by 73.33% and 80.95%, and POX activity increased by 89.29% and 96.97% under treatment T8 in Sakha 104 and Giza 178, respectively, compared to treatments C4, C6, and C8 (Fig. 4).

#### *Principal component analysis, Pearson correlations, and heatmap of the Co-treated and Co-non-treated rice plants grown under drought and non-drought conditions*

All the 16 investigated variables, including growth attributes (root length, shoot length, R/S ratio, root fresh weight, shoot fresh weight, root dry weight, shoot dry weight), the content of protein, proline, electrolyte leakage (EL), MDA, antioxidant compounds, and antioxidant enzymes were subjected to a PCA (Fig. 5A and 5B). The first principal component (PC1) accounted for the larger proportion of variance (73.13%) for Sakha 104 and (68.84%) for Giza 178, whereas PC2 captured smaller portion of variance (19.27%) for Sakha 104 and (24.12%) for Giza 178 (Fig. 5A

and 5B). Referring to both varieties, shoot length, root and shoot fresh weights, root dry weight, shoot dry weight (in Giza 178 only), and protein content were mostly associated with Co-treated plants under control and moderate drought stress (T4 and T6), whereas root length, proline, total phenols, total flavonoids, CAT, POX, and PPO were linked with Co-treated plants grown under severe drought stress (T8) (Fig. 5A and 5B). On the other hand, R/S ratio, EL, and MDA content were associated with Co-untreated plants grown under moderate and severe drought stress (C6 and C8). No association was detected with Co-untreated plants grown under control conditions (C4), except shoot dry weight in Sakha 104 (Fig. 5A). Heatmap correlation analysis of both varieties revealed a variable negative correlation between (root length and R/S ratio) and the other growth parameters (shoot length, root and shoot fresh weights, roots and shoots dry weights), protein content, and MDA content (in Sakha 104 only). While strong positive correlations were observed between (root length and R/S ratio) and antioxidant biomarkers (proline, MDA (Giza 178 only), EL, total phenols, total flavonoids, CAT, POX, PPO) (Fig. 5C and 5D).

## **Discussion**

### *Root and shoot parameters*

The extreme typical symptom of drought stress is suppression of plant growth (Sairam & Srivastava, 2001). Our study revealed that the progress in drought stress considerably increased the root length and R/S ratio while dramatically lowering shoot length and fresh and dry weights of roots and shoots in both varieties. This is consistent with Comas et al. (2013), who observed that plants require long roots with a high density to maintain output in the face of water constraints, especially when the water is deeper. Additionally, elevated values of the R/S ratios under elevated drought stress levels are attributed to the prevention of biomass accumulation in the shoots. This observation was reported by Liu et al. (2004) in wheat, and Xu et al. (2015) in rice under drought stress. The difference in growth in the two varieties were explained by variations in phenotypes. Since Sakha 104 is drought-sensitive, necessitating adaptation and exploration of deeper soil for water and nutrients, Giza 178 is more drought-tolerant that requires less modification (Abdelraheem et al., 2019).

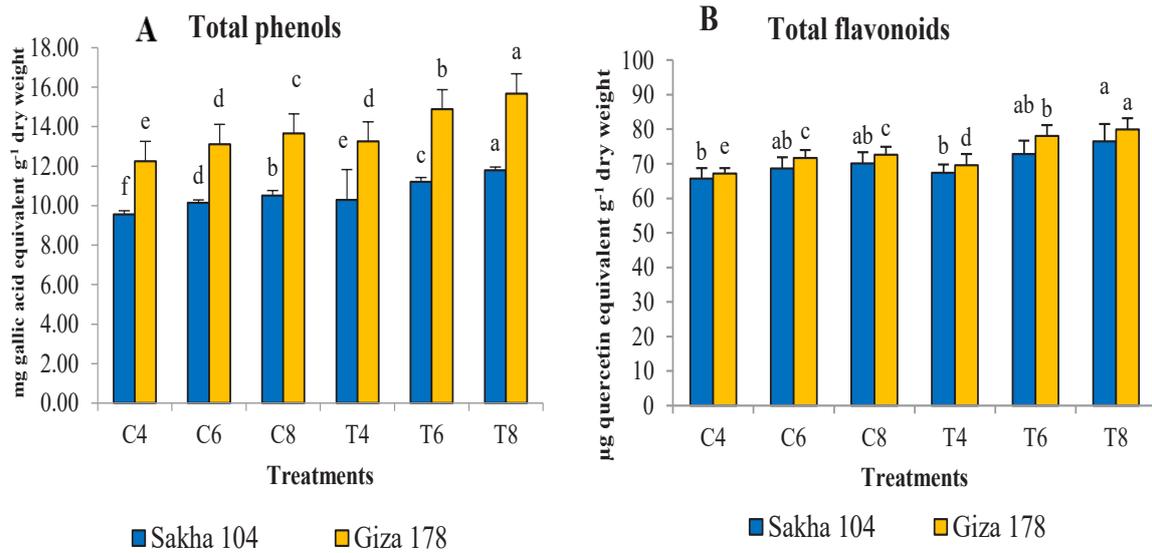


Fig. 3. Effects of drought stress either alone or in combination with  $\text{CoSO}_4$  on total phenols and total flavonoids of Sakha 104 and Giza 178 rice plants at the reproductive stage [Vertical bars represent the standard error ( $\pm$ SE). Different bar letters show significant differences among treatments separately. (Legends are the same as in Fig. 1)]

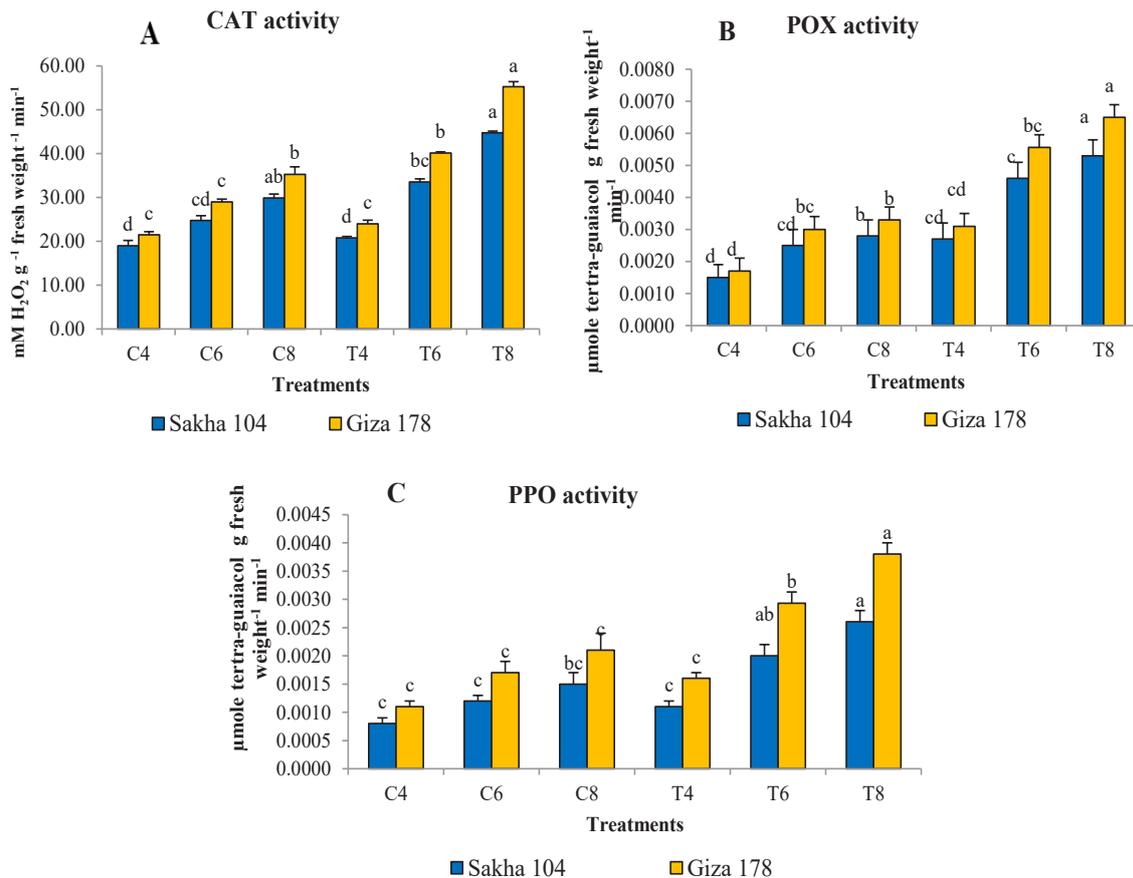
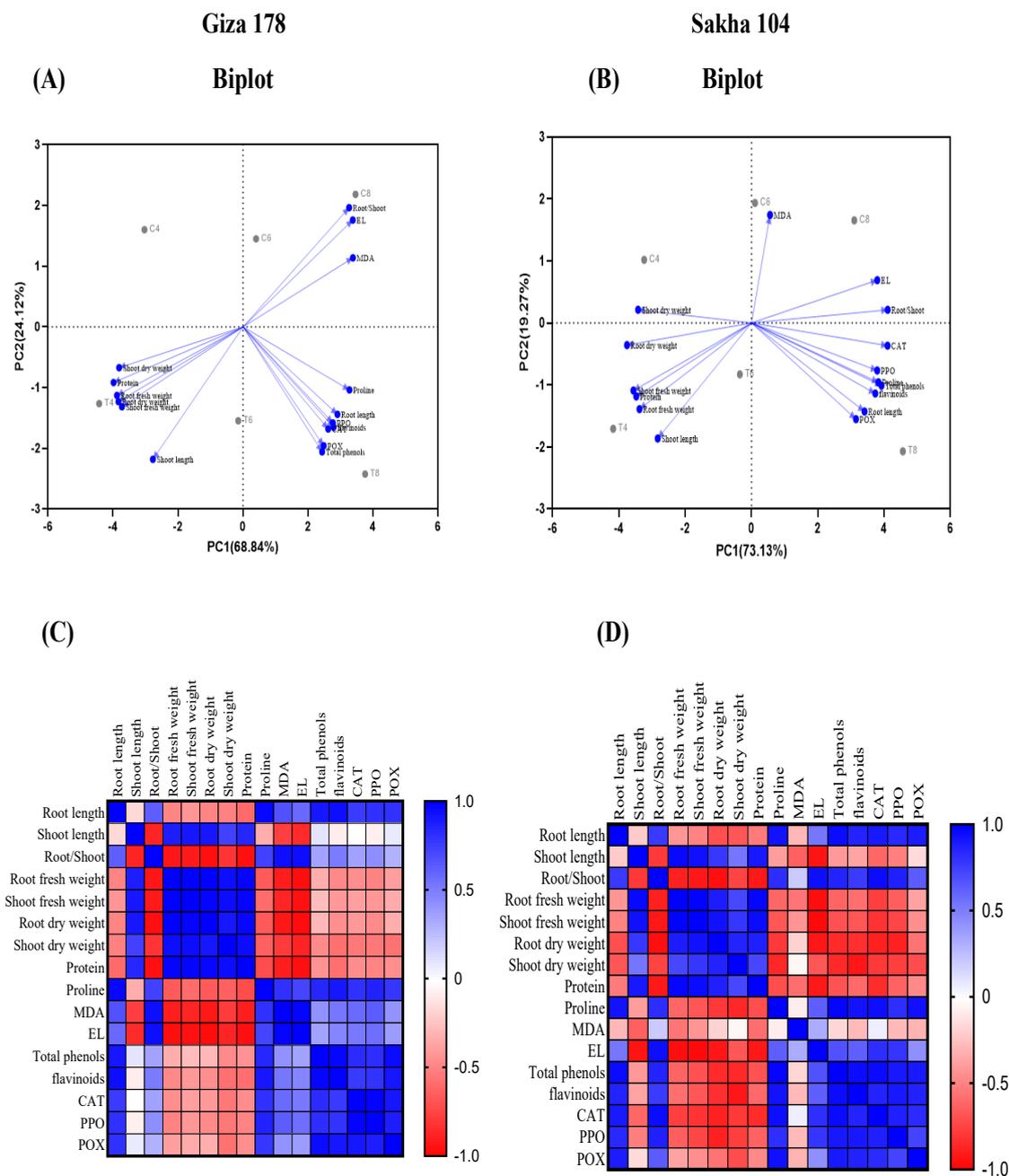


Fig. 4. Effects of drought stress either alone or in combination with  $\text{CoSO}_4$  on the activities of CAT, POX, and PPO enzymes of Sakha 104 and Giza 178 rice plants at the reproductive stage [Vertical bars represent the standard error ( $\pm$ SE). Different bar letters show significant differences among treatments separately. (Legends are the same as in Fig. 1)]



**Fig. 5. (A and B) Principal component analysis (PCA) and (C and D) Heatmap of Pearson correlation coefficient of growth parameters and drought stress biomarkers under the effects of drought stress either alone or in combination with CoSO<sub>4</sub> in Giza 178 and Sakha 104 varieties [Positive and negative correlations are shown in blue and red colors, respectively, according to the color scale. (Legends are the same as in Fig. 1)]**

The positive effect of Co on root length, shoot length, R/S ratio, and biomass may be due to an enhanced effect on both water channel proteins, known as aquaporins. Aquaporins adjust root hydraulics in response to various stimuli, including drought stress. Also, abscisic acid modulates hydraulic conductivity and promotes root cell elongation, aiding the plant's recovery

from water scarcity and increasing biomass production (Kudoyarova et al., 2011). In general, plants show multiple response mechanisms to drought stress, like the production of specific proteins, and high levels of metabolites besides gene expression to increase growth and productivity (Anjum et al. 2016). The production of ROS increases in stress conditions, resulting

in damaged plant cells (Ullah et al., 2017). Plants have numerous mechanisms to adapt to stress conditions, including the accumulation of solutes (like sugar and proline), enzyme activation (POX, superoxide dismutase (SOD), ascorbate (ASC) and CAT, and non-enzymatic (reduced glutathione (GSH)) substances (Anjum et al. 2016). In this study, the enhancement differences in growth under Co application in both varieties resulted from the accumulation and production variation of protein, proline and antioxidant compounds, and antioxidant enzymes in both varieties (Figs. 1, 2, 3, and 4), depending on drought tolerance traits in each variety.

#### *Protein content*

Drought generated differences in the production of soluble protein, which substantially decreased as the used levels of drought stress increased in both varieties compared with the controls, as shown in this study's results. These results align with Kumar et al. (2020), who reported that leaf protein levels reduced dramatically under multi-stage drought conditions, with the highest drop occurring during the reproductive stage, followed by the vegetative stage, while the least reduction occurred during the seedling stage. Martinez-Acedo et al. (2012) mentioned that ROS, produced in the various cells due to dehydration response and altered oxidation-reduction processes, lead to protein degradation. This protein content disparity is an essential aspect of a plant's stress reaction and adaptation to changing environmental conditions (Hieng et al., 2004). Furthermore, the adverse effect of drought stress was more severe in Sakha 104 than Giza 178, particularly at the high drought stress level (C8). These data demonstrate that pretreating Sakha 104 and Giza 178 rice grains with Co improved protein content under normal and stressed conditions, reflecting research completed by Gad et al. (2011) in tomatoes and Gad (2012) in *Arachis hypogaea*. Mounika et al. (2010) found that Co increased amino acid and protein content in *Z. maize*. Thus, the total protein content's increase in response to Co application may result from its integral role in binding amino acids to build specific proteins.

#### *Proline content*

According to Nxele et al. (2017), proline buildup in stressed plants is a major defense mechanism to maintain osmotic pressure in cells. The capacity to accumulate proline under stress conditions correlates with stress tolerance in several

plant species. In this study, Giza 178 maintained higher leaf proline concentrations under the used drought stress levels, particularly at the severe level, indicating that this variety is more drought-tolerant and showing less deterioration in growth attributes than the sensitive variety of Sakha 104 in its respective control treatments. This reflects Anjum et al. (2011), who reported that proline accumulation under stress in numerous plant species correlated with stress tolerance, and its concentration increased to higher values in stress-tolerant plants (Giza 178) than stress-sensitive ones (Sakha 104). However, Co nutrition induced a significant progressive increase in proline content in rice plants of both varieties compared with untreated plants, particularly in Giza 178 rather than Sakha 104. This demonstrates that Co improved drought tolerance in stressed rice plants of both varieties to varying degrees (depending on drought tolerance characteristics) via increasing proline accumulation, which was linked to oxidative damage suppression (Fig. 1B).

#### *Cell membrane injury and lipid peroxidation*

When ROS exceeds a certain threshold, lipid peroxidation increases in both cells and organelles membranes, affecting normal cellular activity. Lipid peroxidation aggravates oxidative stress through the production of lipid-derived radicals that can react with and damage proteins and DNA (Sharma et al., 2012). Additionally, Bajji et al. (2002) the major part of electrolytes was removed within 15 min. Varying the stress conditions influenced both the percent and the kinetics of electrolyte leakage during rehydration. Electrolyte leakage exhibited a characteristic pattern reflecting the condition of cellular membranes (repair and hardening found that maintenance of membrane integrity in leaf segments under osmotic stress correlates with the drought tolerance in durum wheat based on growth of whole plants. This is consistent with our findings, which showed a progressive significant increase in electrolyte leakage and MDA content in both varieties as the level of drought stress increase, particularly in Sakha 104. Khaleghi et al. (2019) found that the general increase in membrane lipids peroxidation is proportional to drought stress intensity of and may derive from the spontaneous reactions of ROS with organic molecules contained in the membrane. However, a reduction in membrane fluidity and/or an increase in membrane leakage may have increased lipid peroxidation together. From our results, MDA

content in Giza 178 was consistently lower than Sakha 104, which confirmed more drought tolerance compared to Sakha 104, as tolerance to a water deficit is correlated with the capacity to scavenge and detoxify ROS (Liu et al., 2011).

The membrane stability and lowering MDA content of stressed Giza 178 plants under Co nutrition led to lower electrolyte leakage and significant lipid peroxidation from its tissue, especially under moderate drought stress level, compared with Sakha 104. This agrees with Li et al. (2005), who reported that the cell membrane injury, lipid peroxidation, and degradation in chlorophyll content that was induced under osmotic stress via polyethylene glycol 4000, which were mitigated owing to the presence of Co ions in testing solution in potato seedlings leaves. The depression in the concentration of MDA in stressed rice plants of both varieties treated with Co might confirm its antioxidant property (Marschner, 2011). As a result, Co may be one of the critical micronutrients during plant drought stress. It significantly improved processes related to maintaining plant redox homeostasis. For instance, protecting cell membranes and repairing lipid content, as well as enhancing the performance of the antioxidant defense system (Figs. 1, 2, 3, and 4). Conversely, phenols (flavonoids in particular) stabilize membranes under drought stress by decreasing their fluidity, which in turn limits the diffusion of free radicals and reduces peroxidation of membrane lipids. The stabilization of membranes is due to the ability of phenolics (especially flavonoids) to bind to some integral membrane proteins and phospholipids (Michalak, 2006). In this study, the effects of phenolic compounds on membrane integrity and lipid peroxidation under drought stress were more pronounced in Co treatments, compared to untreated plants (Fig. 3).

#### *Antioxidant system*

Phenolic compounds have antioxidant characteristics due to their chemical composition, allowing them to chelate transition metal ions, neutralize ROS, inhibit lipid peroxidation by encapsulating the lipid alkoxyl radical, and reduce plasma membrane permeability (Younis et al., 2018). Thus, increased content of these compounds is useful for plants under oxidative stress (Younis et al., 2018). This study showed that drought stress significantly increased the total phenols and flavonoids of both rice varieties compared to the

controls, corroborating the phenomena of acquired drought resistance significantly obvious in Giza 178 compared to Sakha 104. However, Trejo-Tapia et al. (2001) highlighted the participation of Co in the synthesis and deposition of secondary metabolites (like saponin steroids), as well as betalains. Increased diversity of various secondary metabolites in plants protects against stress (Bhat et al., 2020). The additional significant increases in total phenols and flavonoids in response to treatments of stressed plants of both varieties with Co, support the positive action of Co to venerate the antioxidant properties of these compounds.

A variety of antioxidant enzymes' (CAT, POX, and PPO) activities constrain the ROS concentration, and their increased activities in response to drought stress has been widely employed as an indicator of drought resistance (Apel & Hirt, 2004). In this study, the activity of the CAT enzyme showed a progressive increase at a moderate level of drought stress, while a significant increase was apparent at the severe level. The higher CAT activity in Giza 178 suggests that the H<sub>2</sub>O<sub>2</sub> scavenging mechanism is more operative compared to Sakha 104. Osipova et al. (2011) observed the changes in CAT activities in wheat genotypes subjected to water deficits and linked its greater activity to increased drought tolerance. Boaretto et al. (2014) demonstrated the differences in CAT activity in sugarcane varieties with contrasting responses to drought. The integrated CAT action in the more tolerant variety (Giza 178) was an essential system for drought stress tolerance.

Moreover, POX enzyme activity increased considerably in both the leaves of both varieties in response to drought stress (Liu et al., 2007). The resistance of rice to the consequences of water stress may be attributed to improved antioxidant enzymes activity, which lowers MDA generation (Zain et al., 2014). This suggests that drought tolerance improved with increased POX and CAT activity, followed by high proline production (Fig. 1B). Moreover, the POX enzyme is important in preventing oxidative damage and maintaining cell membrane integrity by lowering H<sub>2</sub>O<sub>2</sub> concentration and removing MDA.

PPO activity progressively increased in both varieties with elevated drought stress. Compared to the various enzymatic activities under drought stress, the significant up-regulation in SOD

and CAT and the decline of PPO activities were observed in most rice varieties (Swapna & Shylaraj, 2017). POX and PPO activity was higher in Giza 178 than Sakha 104, consistent with the findings of Selote & Khanna-Chopra (2010), who revealed that drought treatment increased POX activity in tolerant wheat leaves compared to susceptible wheat leaves. Varied POX and PPO activity is attributed to genotype differences. Chakrabarti et al. (2006) found that metals play an integral role in the life processes of living organisms. In both varieties of this study, Co nutrition under the used drought stress levels enhanced the performance of non-enzymatic and enzymatic antioxidants in scavenging ROS production under drought stress, particularly in Giza 178. This is because Co mitigates the adverse effects of oxidative stress by inhibiting the synthesis of ethylene (Roychoudhury & Chakraborty, 2022), which is typically synthesized under stress conditions, increasing oxidative stress and the production of photoassimilates (Pilon-Smits et al., 2009). Additionally, Hu et al. (2021) revealed that Co application at appropriate concentrations activates antioxidative enzymes, reducing ROS-caused damage.

### **Conclusion**

The current study revealed how the growth of Giza 178 and Sakha 104 declined at different degrees under different strengths of drought stress due to excessive ROS accumulation. Drought-induced stress was effectively mitigated by exogenous application of Co, which reduced oxidative stress damage. This was proven by strengthening the antioxidant defense system and decreasing the production of ROS, which was demonstrated by increasing CAT, POX, and PPO enzymes activity, and phenol and flavonoid compounds production. Also, Co improved protein and proline levels, and reduced membrane damage and lipid peroxidation. This suggests that soaking rice grains in Co at the optimum concentration before sowing in soil could be used as a promising approach to mitigate drought stress' damaging effects in rice plants. Further research on the molecular and genetic levels is necessary to obtain more data on the ameliorative mechanisms associated with Co nutrition in stressed rice plants.

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### تقييم دور التغذية بالكوبالت على الآلية المؤكسده لنباتات الأرز (*Oryza sativa* L.) المجهد تحت تأثير الجفاف أثناء مرحلة الإنجاب

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قامت هذه الدراسة بتقييم تأثير التغذية بالكوبالت عند التركيز الأمثل على النمو والإجهاد التأكسدي في صنفين من الأرز المصري (سحا 104 وجيزه 178) تحت تأثير إجهاد الجفاف. تم تطبيق الإجهاد علي فترات ري مختلفة (6 و8 أيام) وكذلك الري كل 4 أيام كعينه ضابطه في مرحلة التكاثر. تم استخدام مقاييس الجذور و السيقان (الاطوال، الأوزان الطازجة والجافة)، المؤشرات الحيوية للإجهاد التأكسدي متمثله في معدل تسرب الالكترونوليتات من الخلايا، المسار فوق التاكسدي للدهون، تراكم البرولين، محتوى البروتين، محتوى الفينولات والفلافونويدات، بالإضافة إلى أنشطة إنزيمات الكاتاليز CAT والبيروكسيداز POX والبولي فينول اكسيداز PPO لتقييم قدرة نظام الدفاع التاكسدي في ازالة سمية أنواع الأكسجين التفاعلية الناتجة في كلا الصنفين. لوحظ أن الصنف جيزة 178 كان أقل تأثراً بنقص المياه والإجهاد التأكسدي من صنف سحا 104. كما لوحظ ان نباتات الأرز المجهدة لكلا الصنفين والتي عولجت بالكوبالت بالتركيز الأمثل، قد قللت التأثير الضار لإجهاد الجفاف، خاصة في صنف جيزة 178 من خلال تعزيز النمو والكتلة الحيوية للجذور والسيقان وزيادة فعالية نظام الدفاع التأكسدي. وقد تجلي ذلك من خلال الانخفاض الملحوظ في معدل تسرب الالكترونوليتات والمسار فوق التاكسدي للدهون، بينما كانت هناك زيادة معنوية في محتوى البرولين والبروتين والمركبات الفينولية والفلافونويدات. بالإضافة إلى ان الأنشطة الديناميكية للأنزيمات المضادة للأكسدة كانت أعلى بكثير في صنف جيزة 178 منها في سحا 104، مقارنة بالنباتات غير المعالجة. قد تكون نتائج هذه الدراسة مفيدة في المشاريع البحثية التي تهدف إلى تطوير تقنيات الإجهاد لمكافحة الجفاف في نباتات الأرز.