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Full Length Article

Seeds Pretreatment with Zeatins or Maize Grain-Derived Organic Biostimulant Improved Hormonal Contents, Polyamine Gene Expression, and Salinity and Drought Tolerance of Wheat

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Abstract

The strategy of seed priming has been effectively addressed with plant extracts containing biostimulants for healthy growth and productivity of stressful plants. Therefore, the effect of maize grain extract (MGE) on wheat plant performance, hormones, polyamines (PAs) gene expression, and antioxidant system was examined under combined stress compared to synthetic cytokinins (CKs). *Cis* (*c*-Z) and *trans*-zeatin-type cytokinin (*t*-Z) were the synthetic CKs, and 75 m*M* NaCl-salinity + 60% of soil water-holding capacity (SWHC) was the combined stress used in this study. *C*-Z or *t*-Z was applied as seed priming at 20 µM versus 2% MGE. Under normal conditions, *c*-Z, *t*-Z, or MGE pretreatment positively affected wheat growth, grain yield, photosynthetic efficiency, and the contents of soluble sugars, α-tocopherol, ascorbate (AsA) and glutathione (GSH). The redox state of AsA and GSH, the contents of phytohormones, PAs and metabolism enzyme activity of proline, PAs gene expression, and enzymatic activities were also improved. In contrast, malondialdehyde (MDA) and H_2O_2 contents were reduced compared to the control. Under the combined stress, negative effects were recorded on all above attributes including deleterious increases in Na^+, Cl^-, MDA, and H_2O_2 contents compared to the control. However, *c*-Z, *t*-Z, or MGE pretreatment alleviated the combined stress effects and significantly improved all above attributes including significant decreases in Na^+, Cl^-, MDA, and H_2O_2 contents compared to the stressed control. Compared to *c*-Z or *t*-Z, seed priming using 2% MGE conferred better results. Therefore, MGE is recommended to promote wheat growth and grain yield by reducing the influences of deficit saline irrigation water-induced oxidative stress. © 2020 Friends Science Publishers

Keywords: Salinity and drought; Wheat growth; Yield; Antioxidant reducing power; Defense systems; Phytohormones; Gene expression

Introduction

Throughout its life cycle, there are different types of environmental abiotic stressors that act against plant performance. Deficit irrigation water (DIw) and salinity are of these abiotic stressors that limit agricultural crop productivities, especially in drought-affected (arid and semi-arid) regions (El-Mageed *et al.* 2017; Khrueasan *et al.* 2020). As a result, low salt washing from soil due to DIw is one of the major reasons of increasing salinization of agricultural lands. In addition to DIw, poorly managed water under hot climate of such low-rainfall regions also contribute to soil salinization (Tester and Bacic 2005; Yang *et al.* 2019).

One of the expected consequences of global climate change is the increasing periods, intensity and frequency of drought, which increases DIw that will undoubtedly exacerbate this problem in the near future, at least in dry (arid and semi-arid) regions (IPCC 2014). This adverse event, which is getting worse day by day, must be confronted with further research to adopt and develop simple technological methods for farmers/producers to apply to stressed-plants to prevent loss of agricultural productions. Plants that grow in this type of adverse conditions (salinity + DIw) not only suffer from severe droughts but also from the effects of soil erosion and increased levels of Na^+, Cl^- and SO_4^- in soil (IPCC 2014).
Plant responds to salt stress in a similar way of drought stress. Osmotic mechanisms are implicated in salinity like in drought limiting water availability and thus reducing growth along with metabolic changes (Munns 2002). Salinity and DIw severely decrease plant ability to utilize water,

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disrupting water content and cell turgor. They also disrupt cell expansion, gas exchange, nutrient balance, photosynthesis, and other metabolic processes, inhibit enzyme catalysts including Rubisco enzymes, and increase specific toxic Na^+ and Cl^- ions, and finally plant death (Munns 2002; (Farooq *et al*. 2017; Hussain *et al*. 2018a).

To withstand stress, plants develop protective enzymatic and non-enzymatic antioxidant system *e.g*., catalase, superoxide dismutase, glutathione, glutathione peroxidase, ascorbate, ascorbate peroxidase, free proline, *etc*. (Hussain *et al*. 2018 Semida *et al.* 2018; Tabasssum *et al*. 2018; Rady *et al.* 2019a). In most cases, plants fail to tolerate high stress depending on their endogenous antioxidant system components. Consequently, exogenous applications (*e.g*., cytokinins; *cis*- and *trans*-zeatin, and plant extracts) should be used to raise the tolerance of plants to salt and/or drought stress (Semida and Rady 2014; Schäfer *et al.* 2015; Rady *et al.* 2019a, b). As a group of adenine-derived phytohormones, cytokinins (CKs) regulate various aspects of plant physiology. As a group of CKs, *cis*-Zeatin-type cytokinin (*c*-Z) has a lower activity than *trans*-zeatin-type cytokinin (*t*-Z) in the classical bioassays of CKs. Therefore, research using *c*-Z has been limited (Schäfer *et al*. 2015). CKs can improve plant resistance to abiotic stress (Barciszewski *et al*. 2000).

At present, natural extracts of maize grains (MGE) are used to prime seeds to enhance plant performance (growth and output) under the stress conditions of salinity (Semida and Rady 2014; Farooq *et al*. 2019; Rady *et al.* 2019c), and nutrient deficiency (Rehman *et al.* 2018). As an organic biostimulant, MGE is rich in auxins, cytokinins, gibberellins, various antioxidants and vitamins, osmoprotectants, and different macro- and micro-nutrients. Therefore, exogenous application of MGE is promoted morphological and physio-biochemical processes and induced plant tolerance to adverse stress conditions (Semida and Rady 2014; Rehman *et al.* 2018; Rady *et al.* 2019c). As far as we know, there is little work on MGE as an effective biostimulant. In addition, this is the first report that used MGE to stimulate growth of pre-treated wheat plants under combined stress (salinity + deficit irrigation). Due to that MGE is conferred seed and their emerged seedlings the power to resist the adverse effects of stress (Alzahrani and Rady 2019; Rady *et al.* 2019c), MGE is a potent strategy to solve the problem of stress hazards.

Worldwide, wheat (*Triticum aestivum* L.) is an important food cereal crop for human due to its high protein content and calories (approximately 82–85%). However, environmental stresses strongly affect wheat productivity, despite its high level of adaptation to rainy, irrigated, subtropical, and tropical regions (Rahaie *et al.* 2013; Farooq *et al*. 2014). Wheat is classified as a moderate plant in terms of salt tolerance with a threshold without loss of yield at 6 dS m^{-1} (Mass and Hoffmann 1977; Munns *et al.* 2006), but yield was reduced by 43 and 50% under 11 dS m^{-1} (Rady *et al.* 2019b) and 13 dS m^{-1} (Mass and Hoffmann 1977), respectively. In addition, there are some factors that can affect wheat plant's response to drought stress including genotype, growth stage, duration and severity of stress, and growth physiological process (Chaves *et al.* 2003), as well as differential gene expression patterns (Denby and Gehring 2005), *etc*.

Therefore, this study aimed at investigating the effects of seed priming in 2% MGE (organic biostimulant) on wheat physio-biochemical systems versus *cis*-zeatin or *trans*-zeatin as synthetic stimulants. Positive alterations were determined for pretreated plant performance, hormones, polyamines (PAs) gene expression, and antioxidant system after exposure to combined stress (75 *mM* NaCl-salinity+60% of SWHC). Moreover, connection between alterations in antioxidative system components and gene expression, and range of plant tolerance, concerning the promotions of wheat growth and yield, were also evaluated

Materials and Methods

Growing conditions, treatments, and experimental layout

Plant material and growing conditions: For preliminary and main experiments, certified wheat (*Triticum aestivum* L.) seeds (cv. Giza-168) were obtained. Both experiments were carried out in a greenhouse under the following conditions: the average temperatures were 19 ± 3 and 10 ± 2°C for day and night with average lengths of 13 and 11 h, respectively, and the average humidity was 62.0–65.1%.

Preparation for treatments: Solutions of *cis*-zeatin (*c*-Z) and *trans*-zeatin (*t*-Z) were prepared at the concentration of 20 μM for both. The level (20 μM) used of both *c*-Z (Olchemim Ltd.,) and *t*-Z (Duchefa) was obtained as a dilution from a stock solution (100 m*M*) in 0.5 *M* NaOH (Roth) as described by Großkinsky *et al.* (2013). Maize grain extract (MGE) was prepared at 2% for priming the seeds. Besides, 75 m*M* of NaCl along with half-strength nutritious solution (Hoagland and Arnon 1950) was prepared for plant irrigation at 60% of soil water-holding capacity (SWHC). The components of Hoagland's nutritive solution (pH 5.9) were $Ca(NO_3)_2{\times}4\ H_2O$, KNO_3, KH_2PO_4, $MgSO_4{\times}7\ H_2O$, H_3BO_3, $MnCl_2{\times}4\ H_2O$, $ZnSO_4{\times}7\ H_2O$, $CuSO_4{\times}5\ H_2O$, $Na_2MoO_4{\times}2\ H_2O$, and Fe^{3+}-$EDTA^+$ at the concentrations of 1.25 m*M*, 1.25 m*M*, 0.25 m*M*, 0.50 m*M*, 11.6 μM, 2.4 μM, 0.24 μM, 0.08 μM, 0.13 μM, and 22.5 μM, respectively. For the control and stress-free treatments, the nutritive solution free from NaCl salt was used for plant irrigation at 100% of SWHC. Concentrations of growth promoters (MGE, c-Z, and t-Z) and stress limits (75 m*M* NaCl and 60% SWHC) used in this study were selected based on a preliminary study (data not shown).

Treatments: A weight of 0.50 kg seeds was soaked in 1.0 L of each of the *c*-Z, *t*-Z or MGE solutions for 6 h. The seeds of the control were soaked in distilled water for 6 h as well. The seeds were then redried under shade by a forced-air until

obtaining the original seeds weight (Sundstrom *et al.* 1987). A filtered calcium hypochlorite (1%) was used to sterilize the seeds for 1 h. The seeds were then washed directly with sterilize-deionized water. Treatments are detailed as follows: (1) Control; seeds were primed in distilled water, and then irrigated throughout with pure Hoagland's nutritive solution, (2) c-Z treatment; seeds were primed in c-Z solution, and then irrigated throughout with pure Hoagland's nutritive solution, (3) t-Z treatment; seeds were primed in t-Z solution, and then irrigated throughout with pure Hoagland's nutritive solution, (4) MGE treatment; seeds were primed in MGE solution, and then irrigated throughout with pure Hoagland's nutritive solution, (5) NaCl*DI treatment; seeds were primed in distilled water, and then irrigated throughout with Hoagland's nutritive solution containing NaCl at 60% of SWHC, (6) NaCl*DI+c-Z treatment; seeds were primed in c-Z solution, and then irrigated throughout with Hoagland's nutritive solution containing NaCl at 60% of SWHC, (7) NaCl*DI+t-Z treatment; seeds were primed in t-Z solution, and then irrigated throughout with Hoagland's nutritive solution contained NaCl at 60% of SWHC, and (8) NaCl*DI+MGE treatment, seeds were primed in MGE solution, and then irrigated throughout with Hoagland's nutritive solution containing NaCl at 60% of SWHC. Experiment was laid out following completely randomized design (CRD) under greenhouse conditions. Each treatment was replicated with 20 pots by sowing 10 seeds in each pot.

Experimental management: Plastic pots (0.3 m diameter, 0.25 m depth) filled with ion-free pure sand were used to grow all seed of all treatments. The 75 m*M* NaCl-containing nutrient solution was used at the deficit irrigation level of 60% of SWHC as a combined stress. All treatments were initially irrigated with the pure half-strength nutritive solution at 100% of SWHC up to 20 DAS. The combined stressed treatments were then irrigated with 75 m*M* NaCl-containing nutritive solution at 60% of SWHC up to 60 DAP. Then, the pure nutritive solution was used at 100% of SWHC until the termination of the experiment (up to 140 DAS). Irrigation with the nutritious solution was applied 2-day intervals for all treatments up to 130 DAS, and at 140 DAS the grain yields were assessed. In all treatments of combined stress, the concentration of NaCl was maintained at 75 m*M* in the growth medium. To control the concentration of NaCl, inductively coupled plasma atomic emission spectrometry (ICP-AES, IRIS-Advan type, Thermo, USA) was used.

Plant sampling: Plant samples were taken at 60 DAS to assess growth traits, attributes of physiology and biochemistry, polyamines contents and their genes expression, phytohormones contents, and activity of antioxidant system components. At the end of the trail (140 DAS), grain yield components were assessed.

Preparation of maize grain extract (MGE): The full method outlined in Rehman *et al.* (2018) with some modifications in Alzahrani and Rady (2019) was used to obtain the extract of maize grains (MGE) using local genotype of Egyptian maize. The distilled water and ethyl alcohol (95%) were used to obtain aqueous and alcoholic extracts, respectively. Both extracts were mixed with each other, and additional distilled water was used to specify the tested MGE levels (1, 2 and 3%) that were used immediately.

Plant hormones, including zeatin-type cytokinins were detected in MGE (GC/MS; Lavrich and Hays 2007), thus they were employed in this investigation to compare with MGE. Other major ingredients that were detected in MGE are as follows: Contents of free amino acids (Dubey and Rani 1989), proline (Bates *et al.* 1973), soluble sugars (Irigoyen *et al.* 1992), ascorbate (Kampfenkel and Montagu 1995), polyamines (Flores and Galston 1982; Guo *et al.* 2014), and antioxidant activity (DPPH-radical scavenging; Lee *et al.* 2003) were assessed and are shown in Table 1.

Determination of growth and yield parameters and efficiency of photosynthesis: Sixty-day-old seedlings were selected and gently extracted from 3 randomly selected pots. A bucket filled with water was used to gently clean the seedlings from the sand particles. After determining the fresh weight (FW) of shoots, they were dried at 70°C up to obtaining a constant dry weight (DW). The grain yield components were assessed at 140 DAS (harvest stage).

At 23, 30, 37, and 44 days after applying the stress treatments, stomatal conductance (*gs*) was assessed 4 times (2h time interval from 7:00 to 17:00) using a leafy Porometer. Assessments were taken 4 times. The SPAD-502 (Minolta, Japan) chlorophyll meter was used to determine chlorophyll content using the top fully (third and fourth)-expanded leaves. The methods of Maxwell and Johnson (2000) and Clark *et al.* (2000) were utilized to assess *Fv/Fm* and performance index (PI).

Determination of the contents of K^+, Na^+, and Cl^-: The dried powdered top fully (third and fourth)-expanded leaves were utilized to determine the contents of K^+, Na^+, and Cl^- after digesting the samples. Content of Cl^- was assessed as outlined in the method of Chapman and Pratt (1961). The methods outlined in the method of Lachica *et al.* (1973) were used to determine K^+ and Na^+ contents.

Assessment of sugars, tocopherol, oxidative stress biomarkers (H_2O_2 and MDA), and proline metabolism enzyme: The methods described in Irigoyen *et al.* (1992), Konings *et al.* (1996) and Ching and Mohamed (2001), Velikova *et al.* (2000), and Heath and Packer (1968) were utilized to determine the contents of total soluble sugars, α-tocopherol (α-TOC), hydrogen peroxide (H_2O_2), and lipid peroxidation (in terms of malondialdehyde; MDA), respectively.

The content of free proline was determined as outlined in Bates *et al.* (1973). Due to the interferences between P5C and free proline during reading the absorbance of free proline, free proline values were subtracted from P5C values, which were obtained with applying a standard (*e.g.*, DL-Δ1-pyrroline-5-carboxylate acid; Miller *et al.* 2009).

The extracts were prepared as outlined in Wang *et al.* (2011) by homogenizing fresh frozen leaf (3 g) with a cold mortar and a 100 m*M* buffer (K-phosphate; pH 7.4) solution. The supernatant was used to assay the activity of enzyme or maintain on − 80°C until use. It was utilized also to determine the content of protein according to the method of Bradford (1976).

The method of Wang *et al.* (2011) was utilized to assay the activity of P5CS (EC 2.7.2.11) (Unit mg^{-1} protein) by rising the absorbance value at 340 nm due to oxidation of NADPH ($\varepsilon = 6.22 \times 10^{-6}$ M^{-1} cm^{-1}). Each 1 unit activity of P5CS was identified as a 0.001 rise of in the absorbance min^{-1}. The method of Sakuraba *et al.* (2001) was utilized to assay the activity (U mg^{-1} protein) of ProDH (EC 1.5.5.2). The reduction in the absorbance was observed at 600 nm due to the reduction of DCIP ($\varepsilon = 21.5 \times 10^{-3}$ M^{-1} cm^{-1}). Each 1 unit activity of ProDH was specified as the amount of enzyme needed to stimulate each 1 mole of DCIP reduction min^{-1}.

Determination of content and redox state of ascorbate and glutathione

The fresh top fully (third and fourth)-expanded leaves were utilized to determine the content (μmol g^{-1} FW) of ascorbate (AsA) as outlined in the method of Kampfenkel and Van Montagu (1995). The extract was added to a mixture of a 30 m*M* buffer (K-phosphate, pH 7.4), 2.5% TCA, 8.4% H_3PO_4, 0.8% bipyridyl, and 0.3% $FeCl_3$. After conducting the reaction for 30 min on 40°C, the absorbance was read at 525 nm. The content of the oxidized AsA (DHA) + AsA was determined after adding the extract to 0.5 *M* of DTT to assess the total reduction of AsA through reading the absorbance at 525 nm. The L-AsA was served as a standard. The AsA redox state was calculated [AsA redox state (%) = AsA ÷ (AsA + DHA) × 100].

The fresh top fully (third and fourth)-expanded leaves were utilized to determine the content (µmol g^{-1} FW) of the reduced GSH and the total GSH (reduced GSH + oxidized GSSG) as outlined in the method of Griffth (1980). To determine the GSH, the reaction mixture containing the extract, 0.13 *M* and 7 m*M* of buffers (Na-phosphate, pH 7.4 and 6.8, respectively), and 6 m*M* of DTNB was heated at 30°C for 10 min. The absorbance was then read at 412 nm. Total level of GSH was assessed after GSSG reduction to GSH through the addition of the extract to 130 m*M* of buffer (Na-phosphate, pH 7.4) and 1 unit GSH-reductase. The GSH and GSH+GSSG contents were calculated [GSH redox state (%) = GSH ÷ (GSH + GSSG) × 100].

Assay the activity of antioxidant enzymes

For extraction of enzymes, a weight of 200 mg of freeze-dried top fully (third and fourth)-expanded leaves was homogenized with 100 m*M* of a 2 mL buffer (K-phosphate, pH 7.0). AsA (2 m*M*) was added to 100 μM EDTA to comprise the extraction buffer to assay the activity of ascorbate peroxidase (APOX). The homogenate was filtered through a nylon cloth and centrifuged (12,000 × g, 15 min). All previous steps were practiced on 4°C. The extract was stored on −25°C till use.

Table 1: Contents of antioxidants and plant hormones detected in maize grain extract (MGE)

Parameter	Unit	Value
Antioxidants:		
Proline	(mmol g^{-1} DW)	30.4
Ascorbic acid	(µmol g^{-1} DW)	6.14
Glutathione		2.42
DPPH radical-scavenging activity	%	88.4
Phytohormones:		
Total cytokinins (CKs)	(µmol g^{-1} DW)	4.16
Trans-Zeatin (*t*-Z)		1.06
Cis-Zeatin (*c*-Z)		0.67
Salicylic acid (SA)		2.34

The assay of the activity (μM H_2O_2 min^{-1} g^{-1} protein) of both CAT (EC 1.11.1.6) and APOX (1.11.1.11) was performed applying the methods outlined in Harvir and MacHale (1987) and Nakano and Asada (1981), respectively. CAT activity assay was conducted by reading the absorbance reduction on 240 nm (because of breakdown of H_2O_2; $\varepsilon = 36$ $mole^{-1}$ cm^{-1}). APOX activity assay was carried out by reading the absorbance reduction on 290 nm (because of AsA oxidation; $\varepsilon = 2.8 \times 10^{-3}$ $mole^{-1}$ cm^{-1}). GPOX activity assay was done by using the Assay Kit (Abcam, Ref. ab102530, Cambridge, U.K.). The reduction in NADPH reading on 340 nm ($\varepsilon = 6.22$ mM^{-1} ·cm^{-1}) indicates the activity value as described (Martinez *et al.* 2018). The activity (U mg^{-1} protein) of SOD (EC 1.15.1.1) was assayed by determining its ability to inhibit NBT photochemical reduction (Beauchamp and Fridovich 1971). Each 1 U activity of SOD was determined as the enzyme amount needed to inhibit 50% of the photoreduction rate of NBT.

Determination of polyamines (PAs) contents: At 4°C, extraction of PAs was implemented by utilizing 500 mg of top fresh fully (third and fourth)-expanded leaves with 4 mL fresh 5% (v/v) $HClO_4$. The supernatant obtained after centrifugation (15,000 × g, 30 min) was utilized to detect the free PAs (*e.g.*, PUT, SPM, and SPD) using HPLC system (Flores and Galston 1982; Guo *et al.* 2014). Identification and quantification of PAs were conducted by comparing the retention times with peaks areas using the standards of PAs (observed on 254 nm using a 2487 dual UV-detector; Waters, Milford, MA, U.S.A.).

RNA isolation, cDNA synthesis, and quantitative analysis of real-time (qRT-PCR): As described by the manufacturer's protocol, 0.1 g of top fresh (third and fourth) fully-expanded wheat leaf was prepared to isolate the total RNA using TRIsure (Bioline). Digestion of RNA samples was performed using DNase I (Thermo Scientific). Then, quantification of RNA was performed using spectrophotometer apparatus, and total RNA purity and integrity were then determined using Agarose gel electrophoresis. One μg of total RNA was reverse-

transcribed to cDNA using a kit of Sensifast first cDNA synthesis (Bioline), based on the manufacturer instructions. According to NCBI, the primers (5′–3′) were designed and the wheat Genome Database was as follows: ADC (F: CAACGACTTTGTTAGCTTTGG, R: CAGGCTTGGCTTTGGTAA), ODC (F: GGCCACTTCTTCTAGGTTCA, R: ACTCGGCGTCTTATATAGCG), SAMDC (F: CGAGCTTGTGTTGCGTCAG, R: ATACATTCGCTCACACTGGCA), SPDS (F: CTGAGAGTATGTGGTTGCAT, R: CATAGTGGACAGAACCCTTG), SPMS (F: AGTAGAGAAGATTTTGTACCAGG, R: GGACATTCCCATAGGTTGAAG), DHS (F: TCACTCGGAGACATGCTGTT, R: CAGCCTTATATCTTGTACAATGTCG), and GAPDH (F: TTGCTCTGAACGACCATTTC, R: GACACCATCCACATTTATTCTTC).

The qPCR was implemented using samples of diluted cDNA in a reaction mixture (20 mL) containing 10 mL of SensiFast SYBR Lo-Rox 2X mix (Bioline) with 1.2 mL (300 n mole) of each primer. The PCR was implemented by using a STRATAGENE MxPro-3000P (Agilent Technologies) as the following: for 2 min on 95°C, denaturation was implemented, then 40 cycles for 10 s on 95°C, and 30 s on 60°C were performed. Immediately after the reaction of PCR, melt curve was analyzed. Calculation of relative expression was implemented by using the method of 2-DDCt, where the level of mRNA relative expression was normalized versus the internal standard gene (GAPDH) and was then compared with the control.

Hormonal extraction and assessment

After excluding midribs, the top fresh (third and fourth) fully-expanded leaves were frozen in liquid N and then grounded. Thereafter, extraction of phytohormones (*cis*-zeatin-type cytokinin; *c*-Z, *trans*-zeatin-type cytokinin; *t*-Z, and salicylic acid; SA) was performed and they were analyzed (Novák *et al.* 2008).

Analysis of the experimental data

The completely randomized design (CRD) was the layout of this study. ANOVA was followed to statistically analysis of all data, with Tukey's Multiple Comparison Test (SPSS 14.0; SPSS, Chicago, IL, USA) at $P \leq 0.05$.

Results

Growth and yield components and efficiency of photosynthesis

Under normal condition, seed pretreatment using 20 μM *c*-Z, 20 μM *t*-Z, or 2% MGE significantly increased shoot fresh weight, shoot dry weight, grain yield plant^{-1}, weight of 1000 grains, and efficiency of photosynthesis (*e.g.*, gs, SPAD chlorophyll content, Fv/Fm, and PI) compared to normal control (Table 2). MGE significantly exceeded *c*-Z or *t*-Z and increased the abovementioned attributes by 33.5, 42.2, 19.1, 19.6, 23.0, 23.0, 7.5, and 12.7%, respectively compared with normal control. The positive impact of both *c*-Z and *t*-Z was in parallel line. Exposing wheat plants to combined stress (75 m*M* NaCl + 60% SWHC) extremely decreased shoot fresh weight, shoot dry weight, grain yield plant^{-1}, weight of 1000 grains, gs, SPAD chlorophyll content, Fv/Fm, and PI by 56.4, 71.1, 88.7, 54.9, 61.8, 51.8, 22.5, and 49.2%, respectively compared to normal control. However, *c*-Z, *t*-Z or MGE pretreatment mitigated the combined 75 m*M* NaCl + 60% SWHC stress impacts and significantly elevated plant growth and yield component, and photosynthetic efficiency attributes compared to stressed (75 m*M* NaCl + 60% SWHC) control. Pretreatment with *c*-Z significantly exceeded *t*-Z, however, best findings were obtained by MGE pretreatment, which exceeded stressed control by 109.8 for shoot fresh weight, 207.7 for shoot dry weight, 677.4 for grain yield pot^{-1}, 84.2 for 1000-grain weight, 123.5 for gs, 81.0 for chlorophyll, 25.8 for Fv/Fm, and 83.0% for PI. All priming treatments showed more effectiveness under stress than normal condition (Table 2).

Sodium (Na^+), chlorine (Cl^-), and potassium (K^+) contents

Under normal condition, *c*-Z or *t*-Z pretreatment did not affect K^+, Na^+, and Cl^- contents, and K^+/Na^+ ratio, while K^+ content and K^+/Na^+ ratio were significantly increased by 11.7 and 12.6%, respectively by pre-applying MGE compared to normal control (Table 3). Combined 75 m*M* NaCl + 60% SWHC stress significantly increased Na^+ and Cl^- contents by 553.5 and 613.5%, respectively, while decreased K^+ content and K^+/Na^+ ratio by 51.9 and 92.7%, respectively compared to normal control. However, these results were reversed by *t*-Z pretreatment, which gave lower results than *c*-Z pretreatment, however, MGE pretreatment was the best. This best pretreatment reduced Na^+ and Cl^- contents by 66.2 and 71.3%, respectively, while increased K^+ content and K^+/Na^+ ratio by 109.7 and 527.3%, respectively compared to the stressed control. Results obtained from all seed soaking treatments were more pronounced under stress than normal condition (Table 3).

Plant hormones

Under non-stress condition, *c*-Z, *t*-Z, and salicylic acid (SA) contents were significantly increased by *c*-Z, *t*-Z, or MGE pretreatment with an exception (*t*-Z content was not affected with *c*-Z pretreatment) compared to normal control (Table 4). Generally, MGE was the best pretreatment increasing *c*-Z content by 1100%, *t*-Z by 833%, and SA content by 219% compared to normal control. Combined stress significantly increased *c*-Z, *t*-Z, and SA contents by 1411, 1188 and 258%

Table 2: Changes in growth and yield components, and photosynthesis efficiency of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Grain yield (g pot^{-1})	1000-grain weight (g)	*gs* (mmol^{-2} S^{-1})	SPAD chlorophyll	*Fv/Fm*	PI (%)
Control	18.8 ± 2.0c	4.5 ± 0.4c	46.7 ± 3.3c	22.4 ± 2.1c	178 ± 5c	39.2 ± 1.3c	0.80 ±0.04b	8.80±0.45c
c-Z	23.2 ± 2.4b	5.5 ± 0.6b	52.4 ± 4.3b	24.0 ± 2.2b	199 ± 7b	44.3 ± 1.5b	0.84±0.04ab	9.49±0.49b
t-Z	22.8 ± 2.2b	5.3 ± 0.5b	51.8 ± 4.2b	23.7 ± 2.3b	194 ± 6b	43.6 ± 1.4b	0.83±0.05ab	9.41±0.47b
MGE	25.1 ± 2.8a	6.4 ± 0.6a	55.6 ± 5.4a	26.8 ± 2.5a	219 ± 8a	48.2 ± 1.8a	0.86 ±0.05a	9.92±0.54a
NaCl*DI	8.2 ± 0.9g	1.3 ± 0.1g	5.3 ± 5.0g	10.1 ± 0.9g	68 ± 2g	18.9 ± 0.7g	0.62 ±0.02d	4.47±0.24g
NaCl*DI + *c*-Z	14.3 ± 1.5e	3.2 ± 0.4e	29.8 ± 3.2e	15.8 ± 1.7e	130 ± 3e	30.1 ± 1.0e	0.71 ±0.03c	7.48±0.36e
NaCl*DI + *t*-Z	12.4 ± 1.3f	2.8 ± 0.3f	24.6 ± 2.7f	14.6 ± 1.4f	111 ± 3f	25.5 ± 0.8f	0.69 ±0.03c	6.79±0.32f
NaCl*DI + MGE	17.2 ± 1.7d	4.0 ± 0.4d	41.2 ± 4.3d	18.6 ± 1.9d	152 ± 4d	34.2 ± 1.2d	0.78 ±0.04b	8.18±0.42d
LSD value at $P \leq 0.05$	1.2	0.2	2.8	1.1	14	2.7	0.04	0.41

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE = Maize grain extract; Combined stress = 75 m*M* NaCl + irrigation at 60% of SWHC (NaCl*DI); *c*-Z= Cis-zeatin-type cytokinin; *t*-Z= Trans-zeatin-type cytokinin; gs= Stomatal conductance; Fv/Fm= Efficiency of PSII maximal quantum; and PI= Performance index of photosynthesis

Table 3: Changes in leaf Na^+ and K^+ ion contents, and K^+/Na^+ ratio of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Parameters			
	Na^+ content (mg g^{-1} DW)	Cl^- content (mg g^{-1} DW)	K^+ content (mg g^{-1} DW)	K^+/Na^+ ratio
Control	1.42 ± 0.04e	2.08 ± 0.06e	2.14 ± 0.06b	1.51 ± 0.04b
c-Z	1.41 ± 0.04e	2.08 ± 0.06e	2.16 ± 0.07b	1.53 ± 0.04b
t-Z	1.40 ± 0.04e	2.10 ± 0.08e	2.17 ± 0.07b	1.55 ± 0.04b
MGE	1.41 ± 0.04e	2.08 ± 0.05e	2.39 ± 0.08a	1.70 ± 0.05a
NaCl*DI	9.28 ± 0.28a	14.84 ± 0.46a	1.03 ± 0.04e	0.11 ± 0.00f
NaCl*DI + *c*-Z	4.05 ± 0.12c	6.11 ± 0.19c	1.67 ± 0.05c	0.41 ± 0.02d
NaCl*DI + *t*-Z	4.74 ± 0.15b	7.20 ± 0.25b	1.34 ± 0.05d	0.28 ± 0.01e
NaCl*DI + MGE	3.14 ± 0.09d	4.26 ± 0.13d	2.16 ± 0.06b	0.69 ± 0.02c
LSD value at $P \leq 0.05$	0.69	0.98	0.29	0.12

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE= Maize grain extract; NaCl*DI= Combined (stress= 75 m*M* NaCl + irrigation at 60% of SWHC) (NaCl*DI); c-Z= Cis-zeatin-type cytokinin; and t-Z= Trans-zeatin-type cytokinin

Table 4: Changes in leaf contents of phytohormones of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Parameters		
	c-Z (ng g^{-1} DW)	*t*-Z (ng g^{-1} DW)	SA (ng g^{-1} DW)
Control	18 ± 0h	84 ± 1g	276 ± 4h
c-Z	223 ± 3f	86 ± 1g	646 ± 9g
t-Z	49 ± 1g	928 ± 11f	822 ± 10f
MGE	216 ± 3e	784 ± 10e	880 ± 10e
NaCl*DI	272 ± 3d	1082 ± 13d	988 ± 13d
NaCl*DI + *c*-Z	387 ± 4b	1428 ± 17b	1528 ± 18b
NaCl*DI + *t*-Z	324 ± 4c	1214 ± 16c	1324 ± 16c
NaCl*DI + MGE	449 ± 6a	1689 ± 25a	1789 ± 22a
LSD value at $P \leq 0.05$	30	112	49

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE= Maize grain extract; NaCl*DI= Combined (stress= 75 m*M* NaCl + irrigation at 60% of SWHC) (NaCl*DI); *c*-Z= Cis-zeatin-type cytokinin; and *t*-Z= Trans-zeatin-type cytokinin

compared to normal control. These hormonal contents were further elevated by *c*-Z pretreatment, showing better results than *t*-Z pretreatment; however, MGE pretreatment was the best, exceeding stressed control by 65.1% for *c*-Z content, 56.1% for *t*-Z content, and 81.1% for SA content. Results obtained from all seed soaking treatments were more effectiveness under stress than normal condition (Table 4).

Osmoprotectants, oxidative stress biomarkers, antioxidants and redox state

Under non-stress condition, soluble sugars and α-TOC contents, oxidative stress biomarkers (H_2O_2 and MDA contents), AsA and GSH contents and redox states were not affected by all pretreatments with minor exception (soluble sugars and α-TOC contents were significantly increased by MGE pretreatment by 16.1 and 14.6%, respectively) compared to normal control (Table 5). Combined 75 m*M* NaCl + 60% SWHC) stress treatment significantly elevated soluble sugars, α-TOC, AsA, and GSH contents, and AsA and GSH redox states by 76.3, 53.7, 58.5, 100.0, 15.7, and 60.8%, respectively. These increases were synchronized with increases in H_2O_2 and MDA contents (by 114.6 and 153.1%, respectively) compared with normal control.

Table 5: Changes in soluble sugars, α-tocopherol (α-TOC), hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), AsA, and GSH contents, and antioxidant redox state of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Parameters							
	Soluble sugars (mg g^{-1} DW)	α-TOC (μmol g^{-1} DW)	MDA (μmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)	AsA content (μmol g^{-1} FW)	AsA redox state (%)	GSH content (μmol g^{-1} FW)	GSH redox state (%)
Control	11.8 ± 0.3d	1.64 ± 0.04f	24.6 ± 0.5e	12.8 ± 0.3e	1.30 ± 0.04e	66.9 ± 0.9e	0.86 ± 0.02e	15.8 ± 0.4e
c-Z	11.6 ± 0.3d	1.61 ± 0.03f	24.2 ± 0.5e	12.4 ± 0.2e	1.31 ± 0.03e	67.0 ± 0.8e	0.88 ± 0.02e	15.7 ± 0.3e
t-Z	11.8 ± 0.3d	1.62 ± 0.04f	24.1 ± 0.4e	12.6 ± 0.3e	1.30 ± 0.03e	66.8 ± 0.8e	0.86 ± 0.02e	15.8 ± 0.3e
MGE	13.7 ± 0.4c	1.88 ± 0.05e	24.2 ± 0.5e	12.4 ± 0.3e	1.32 ± 0.04e	67.1 ± 0.9e	0.89 ± 0.02e	15.9 ± 0.4e
NaCl*DI	20.8 ± 0.6b	2.52 ± 0.07d	52.8 ± 1.2a	32.4 ± 0.8a	2.06 ± 0.05d	77.4 ± 1.2d	1.72 ± 0.05d	41.2 ± 0.9d
NaCl*DI + *c*-Z	21.2 ± 0.6b	3.08 ± 0.08b	34.1 ± 0.8c	16.8 ± 0.4c	2.62 ± 0.08b	91.3 ± 1.5b	2.22 ± 0.07b	52.6 ± 1.0b
NaCl*DI + *t*-Z	21.0 ± 0.6b	2.81 ± 0.07c	38.9 ± 1.0b	19.6 ± 0.5b	2.30 ± 0.07c	84.2 ± 1.3c	1.94 ± 0.06c	47.2 ± 0.9c
NaCl*DI + MGE	28.4 ± 0.8a	3.40 ± 0.09a	28.2 ± 0.6d	14.2 ± 0.3d	3.12 ± 0.09a	98.6 ± 1.8a	2.46 ± 0.07a	58.2 ± 1.2a
LSD value at $P \leq 0.05$	1.6	0.23	2.4	1.8	0.26	3.8	0.16	2.7

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE= Maize grain extract; NaCl*DI= Combined (stress= 75 m*M* NaCl + irrigation at 60% of SWHC) (NaCl*DI); *c*-Z= Cis-zeatin-type cytokinin; *t*-Z= Trans-zeatin-type cytokinin; α-TOC= α-Tocopherol; MDA= Malondialdehyde; H_2O_2= Hydrogen peroxide; AsA= Ascorbate; and GSH= Glutathione

However, *c*-Z, *t*-Z, or MGE pretreatment further increased soluble sugars, α-TOC, AsA, and GSH contents, and AsA and GSH redox states, while significantly reduced H_2O_2 and MDA contents compared with stressed control. Pretreatment with *c*-Z yielded better results than *t*-Z pretreatment, however, MGE pretreatment awarded best results, exceeding stressed control by 36.5% for soluble sugars content, 34.9% for α-TOC content, 51.5% for AsA content, 43.0% for GSH content, 27.4% for AsA redox state, and 41.3% for GSH redox state. In addition, this best pretreatment reduced H_2O_2 content by 46.6% and MDA content by 56.2% compared to the stressed control. Results obtained from all seed soaking treatments were more pronounced under stress than normal condition (Table 5).

Proline and proline-5-carboxylate (P5C), and enzymatic antioxidants

Under non-stress condition, proline and P5C contents, and P5CS, ProDH, SOD, CAT, APOX, and GPOX activities were not affected by *c*-Z, *t*-Z, or MGE pretreatment compared with normal control (Table 6). Combined 75 m*M* NaCl + 60% SWHC stress significantly elevated proline and P5C contents, and P5CS, SOD, APOX, and GPOX activities (by 266.7, 65.5, 135.0, 55.9, 31.8, and 32.6%, respectively), while activities of ProDH and CAT were significantly decreased (by 46.8 and 40.7%, respectively) compared with normal control. However, *c*-Z, *t*-Z, or MGE pretreatment further increased all abovementioned attributes compared with stressed control. Pretreatment with *c*-Z conferred better results than *t*-Z pretreatment, however, MGE pretreatment granted best findings, exceeding stressed control by 73.2% for proline content, 37.5% for P5C content, 36.3% for P5CS activity, 310.1% for ProDH activity, 35.1% for SOD activity, 93.0% for CAT activity, 48.8% for APOX activity, and 39.4% for GPOX activity. Results obtained from all seed soaking treatments were more effectiveness under stress than normal condition (Table 6).

Polyamines (PAs) and relative expression of PAs biosynthetic genes

Under no stress, PUT, SPD, and SPM contents, and relative expressions of PAs biosynthetic genes (*e.g.*, ADC, ODC, SPDS, SPMS, SAMDC, and DHS) were not affected by *c*-Z, *t*-Z, or MGE pretreatment compared with normal control (Table 7). Under combined 75 m*M* NaCl + 60% SWHC stress, PUT, SPD, and SPM contents were significantly increased by 74.1, 16.7, and 21.7%, respectively. In addition, the relative expressions of ADC, SPDS, SAMDC, and DHS genes were significantly increased by 120.0, 170.0, 70.0, and 90.0%, respectively, while the relative expressions of ODC and SPMS genes were not affected compared with normal control. However, *c*-Z, *t*-Z, or MGE pretreatment further increased all mentioned attributes, except for the relative expression of ODC and SPMS genes compared to stressed control. Pre-applying *c*-Z had better results than *t*-Z, however, best findings were obtained by MGE pretreatment, which exceeded stressed control by 44.6% for PUT content, 24.9% for SPD content, 28.3% for SPM content, 40.9% for ADC relative expression, 40.7% for SPDS relative expression, 52.9% for SAMDC relative expression, and 52.6% for DHS relative expression. Results obtained from all seed soaking treatments were more effectiveness under stress than normal condition (Table 7).

Discussion

Results indicated that MGE (at 2% concentration of bioactive components) is a distinctive mean as a natural plant growth biostimulant to replace costly synthesized CKs. MGE was found to be a potent catalyst for wheat growth against the combined stress under study. After seed priming, the MGE bioactive components (Table 1) may easily translocate to the seed to provide the ability to germinate rapidly and strongly and generate a strong seedling that withstand stress conditions effectively. MGE exceeded CKs (*c*-Z or *t*-Z) in mediating antioxidant

Table 6: Changes in contents of proline and P5C, and activities of P5CS, ProDH, SOD, CAT, APOX, and GPOX enzymes in leaves of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Parameters							
	Proline content	P5C content	P5CS activity	ProDH activity	SOD activity	CAT activity	APOX activity	GPOX activity
	(μmol g^{-1} DW)			(U mg^{-1} protein)		(μmol H_2O_2 min^{-1} g^{-1} protein)		
Control	2.51 ± 0.04e	0.29 ± 0.01e	24.6 ± 0.4e	44.7 ± 0.8d	3132 ± 55e	172 ± 3b	15.4 ± 0.2e	22.4 ± 0.3e
c-Z	2.54 ± 0.04e	0.29 ± 0.01e	25.0 ± 0.3e	45.0 ± 0.8d	3141 ± 56e	174 ± 3b	15.6 ± 0.2e	22.7 ± 0.3e
t-Z	2.50 ± 0.04e	0.28 ± 0.00e	24.8 ± 0.3e	44.8 ± 0.7d	3138 ± 57e	172 ± 3b	15.5 ± 0.2e	22.6 ± 0.3e
MGE	2.55 ± 0.04e	0.30 ± 0.01e	25.1 ± 0.4e	45.2 ± 0.7d	3144 ± 52e	175 ± 3b	15.6 ± 0.2e	22.8 ± 0.3e
NaCl*DI	8.20 ± 0.12d	0.48 ± 0.01d	57.8 ± 0.8d	23.8 ± 0.3e	4884 ± 79d	102 ± 2d	20.3 ± 0.3d	29.7 ± 0.4d
NaCl*DI + *c*-Z	12.3 ± 0.16b	0.59 ± 0.02b	72.6 ± 0.9b	86.4 ± 1.2b	5922 ± 92b	175 ± 3b	26.8 ± 0.4b	37.0 ± 0.5b
NaCl*DI + *t*-Z	10.2 ± 0.16c	0.54 ± 0.01c	64.2 ± 0.8c	80.2 ± 1.1c	5408 ± 86c	154 ± 3c	24.0 ± 0.4c	33.8 ± 0.5c
NaCl*DI + MGE	14.2 ± 0.18a	0.66 ± 0.02a	78.8 ± 1.1a	97.6 ± 1.4a	6596 ± 99a	196 ± 4a	30.2 ± 0.5a	41.4 ± 0.7a
LSD value at $P \leq 0.05$	0.94	0.05	3.1	4.6	321	15	2.4	2.7

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE= Maize grain extract; NaCl*DI= Combined (stress= 75 m*M* NaCl + irrigation at 60% of SWHC) (NaCl*DI); *c*-Z= Cis-zeatin-type cytokinin; *t*-Z= Trans-zeatin-type cytokinin; P5C= Pyrroline-5-carboxylate; P5CS= Pyrroline-5-carboxylate synthase; ProDH= Proline dehydrogenase: SOD= Superoxide dismutase; CAT= Catalase; APOX= Ascorbate peroxidase; and GPOX= Glutathione peroxidase

Table 7: Changes in contents of PAs (PUT, SPD, and SPM) and relative expression of PAs biosynthetic genes (by qPCR) of combined stressed-wheat plant pretreated with CKs (*c*-Z or *t*-Z) or MGE

Treatments	Parameters								
	PUT content	SPD content	SPM content	ADC	ODC	SPDS	SPMS	SAMDC	DHS
	(nmol g^{-1} DW)								
Control	11.6 ± 0.3e	68.4 ± 0.9e	60.3 ±0.8e	1.0±0.02e	1.00±0.03a	1.0±0.01e	1.00±0.02a	1.0±0.01e	1.0±0.02e
c-Z	11.3 ± 0.2e	68.6 ± 0.9e	60.9 ±0.8e	1.0±0.02e	1.00±0.02a	1.0±0.01e	1.00±0.01a	1.0±0.01e	1.0±0.02e
t-Z	11.1 ± 0.2e	68.3 ± 0.8e	60.9±0.7e	1.0±0.03e	1.00±0.03a	1.0±0.02e	1.00±0.01a	1.0±0.02e	1.0±0.01e
MGE	11.4 ± 0.2e	68.8 ± 0.9e	61.1±0.9e	1.1±0.02e	1.05±0.03a	1.1±0.02e	1.06±0.01a	1.0±0.02e	1.0±0.02e
NaCl*DI	20.2 ± 0.4d	79.8 ± 1.1d	73.4±1.1d	2.2±0.04d	1.04±0.02a	2.7±0.05d	1.06±0.02a	1.7±0.03d	1.9±0.03d
NaCl*DI + *c*-Z	25.3 ± 0.5b	92.6 ± 1.5b	86.6±1.5b	2.7±0.04b	1.03±0.02a	3.4±0.06b	1.05±0.02a	2.2±0.03b	2.4±0.04b
NaCl*DI + *t*-Z	23.1 ± 0.5c	86.4 ± 1.4c	80.4±1.2c	2.4±0.05c	1.04±0.03a	2.9±0.05c	1.05±0.02a	1.9±0.03c	2.1±0.03c
NaCl*DI + MGE	29.2 ± 0.6a	99.7 ± 1.7a	94.2±1.6a	3.1±0.05a	1.05±0.03a	3.8±0.06a	1.06±0.02a	2.6±0.04a	2.9±0.04a
LSD value at $P \leq 0.05$	1.7	5.9	5.6	0.2	NS	0.1	NS	0.1	0.2

Means sharing different letters, within a column for each trait, differ significantly from each other at $P \leq 0.05$ according to LSD test), different small letters after means ± SE indicate significant differences

CKs= Cytokinins; MGE= Maize grain extract; NaCl*DI= Combined (stress= 75 m*M* NaCl + irrigation at 60% of SWHC) (NaCl*DI); *c*-Z= Cis-zeatin-type cytokinin; *t*-Z= Trans-zeatin-type cytokinin; PAs= Polyamines; PUT= Putrescine; SPD= Spermidine; SPM= Spermine; ADC= Arginine decarboxylase; ODC= Ornithine decarboxylase; SPDS= Spermidine synthase: SPMS= Spermine synthase; SAMDC= S-Adenosyl methionine decarboxylase; DHS= Deoxyhypusine synthase; and NS= Non-significant

defenses and K^+/Na^+ transporters to boost stress tolerance in wheat plant as noted from results of this study (Tables 2–7). Under combined stress (75 m*M* NaCl + 60% SWHC), the ratio of K^+/Na^+ was decreased as a result of decreasing K^+ uptake on the expense of Na^+ and Cl^- uptake (Table 3). This result was associated with increases in lipid peroxidation (measured as malondialdehyde; MDA) and H_2O_2 contents (Table 5), which led to decrease in plant growth, disorders in the efficiency of photosynthesis (Table 2) and cellular metabolism (Tables 3–7) with a great loss in wheat yield (Table 2). Wheat growth and yield components were restricted because of metabolic processes disorders and elevated rate of respiration due to the increased requirements of energy, reducing meristem and cell expansion activities (Safi-naz and Rady 2015). To cope with these undesirable outputs due to combined stress, wheat plant developed and adopted antioxidant defense system components, including antioxidant redox state, antioxidant enzymes, PAs and their gene expression, and phytohormones (Tables 2–7). These inner antioxidant systems were supported by pretreatment with MGE and CKs to survive under long term stress and sustain plant life.

Under stress in this study, wheat plant maintained its growth as well as its later yield because it had many catalysts due to pretreatment with MGE that exceeded CKs (*c*-Z or *t*-Z) in this regard. Wheat growth and yield improvements were associated with improved photosynthetic efficiency due to MGE or CKs pretreatment (Table 2). These positive results may be due to improved nutrient uptake (Rehman *et al.* 2018; Rady *et al.* 2019c), especially K^+ that antagonized Na^+ ions, increasing K^+/Na^+ ratio and reducing Na^+ and Cl^- ion contents (Table 3). As confirmed in this study, a decrease in K^+ efflux and an increase in K^+/Na^+ ratio in stressful plants were expressed by the exogenous application of MGE (Rady *et al.* 2019c) and CKs (Shabala *et al.* 2009). The positive ionic balance obtained in the stressful wheat plants in this study indicates that pivotal mechanisms may function in the roots of stressed plants to avoid Na^+ xylem loading. Also, compartmentalization of Na^+ may increase, donating an elevation of K^+ influx to plant leaves (Assaha *et al.* 2017; Rehman *et al.* 2018). This finding leads to an elevated K^+/Na^+ ratio in cytosol as a pivotal indicator of plant tolerance to salt stress. Additionally, improvements in the contents of plant hormones (Table 4) and the activity of antioxidant defense system (Tables 5–7) by MGE or CKs pretreatment contributed to increased wheat plant tolerance to the combined (75 m*M* NaCl + 60% SWHC) stress. This

alleviated combined stress-induced oxidative stress and resulted in decreased levels of MDA and H_2O_2 in plant tissues (Table 5), helping to increase the growth and production of wheat plant (Table 2).

Further away than CKs (*c*-Z or *t*-Z), MGE alleviated the combined stress and helped the photosynthetic machinery to function effectively (Table 2) and improved cell metabolism (Rady *et al.* 2019c). This improved cell metabolism increased plant hormonal content under stress (Table 4). Different plant hormones are mediated specific plant response to stress. Based on interaction of plant with stress, CKs and/or salicylic acid (SA) and their signaling ingredients mainly regulate plant defensive reactions (Robert-Seilaniantz *et al.* 2011). CKs modulate the defensive responses of many plant species to stress through various mechanisms such as regulating defense genes and other plant hormones like SA (Jiang *et al.* 2013), which have been demonstrated to be CKs responsive (Großkinsky *et al.* 2013). This result is confirmed by the results of this study using *c*-Z or *t*-Z (Table 4). In this regard, c-Z has been discovered with physiological functions in all parts of the plant (Kudo *et al.* 2012). Current study showed that seed pretreatment with *c*-Z or *t*-Z significantly increased their contents along with SA content and improved plant tolerance to combined stress (75 m*M* NaCl-salinity + 60% SWHC) (Tables 2–7). Previous studies have shown that *t*-Z has generally higher activity than *c*-Z, and the differences between *c*-Z and *t*-Z are related to transport, degradation, and conjugation processes (Gajdosová *et al.* 2011; Kudo *et al.* 2012). In this study, these processes contributed to reversing differences between the elevated contents of *c*-Z and *t*-Z, which most likely contributed to superiority in *c*-Z performance compared to *t*-Z (Tables 2–7). These results can be attributed to that *c*-Z has higher activity under stress conditions compared to *t*-Z for transport, degradation and conjugation processes, indicating a higher stimulation of defense mechanisms against stress at least in wheat. Compared to *t*-Z, *c*-Z caused a higher tolerance to the combined stress in the wheat plant, which could be explained at least partially by the potential of the physiological role of *c*-Z to confer a higher increase in SA accumulation under such stress.

Additionally, the improved cell metabolism by MGE or CKs pretreatment activated the components of the antioxidant defense system (Tables 5–7). This result contributed to scavenging excessive ROS including H_2O_2 and prohibiting oxidation of plasma membranes, effectively lowering MDA and H_2O_2 levels under the combined stress conditions (Table 5). The physiological interplaying effects between MGE/CKs and other distinctive defensive mechanisms mediated tolerance induced by other improved attributes such as α-TOC, AsA, GSH, antioxidant redox state (Table 5). Besides, proline and its metabolism enzyme activity, antioxidative enzyme activity (Table 6), and PAs and their genes expressions (Table 7) contributed as distinctive defensive mechanisms. This might enhance the overall stress responses and improve plant tolerance to stress. The integration of various defensive mechanisms, which are regulated by MGE or CKs not only assesses the effectiveness in restricting the harmful stress effects but also affects physiological state to restrict plant integrity trade-off connected with defensive response. As a pivotal growth enhancers, therefore, MGE or CKs pretreatment significantly increased levels of antioxidants and PAs gene expression, reduced levels of ROS in conjunction with reducing lipid peroxidation (MDA) and H_2O_2, and induce plant growth and production (Tables 2–7).

Regarding the results of this study, wheat plants can survive better with application of MGE than CKs (*c*-Z or *t*-Z) in regions that suffer from stresses. These findings may be attributed to that MGE stimulated a pronounced increase in the metabolism of proline through two pathways analyses; P5CS anabolism and ProDH catabolism, conferring lowered activity of P5CS and increased activity of ProDH to balance proline content within plant tissues (Rady *et al.* 2019b). MGE also reduced effectively the accumulation of H_2O_2 and MDA, as well as membrane leakage (EL) giving useful effect in relieving the stress-caused oxidative damages (Table 5). It contributed effectively to accumulate soluble sugars and proline to provide protection for cells by keeping a balance between osmotic strength of cytosol and osmotic strength of cellular vacuole and that of external environment (Sairam *et al.* 2002). As boosted by MGE, antioxidant enzymes are special stress biochemical signals and their high activity can relieve stress-catalyzed oxidative stress. With further activation of SOD, CAT, GPOX, and APOX, low oxidative damage was found MGE-pretreated wheat plant grown under combined stress (Table 6). Pretreatment with MGE caused further increase in PUT, SPD, and SPM levels in stressed wheat plant. These excess PAs, along with other antioxidants and phytohormones (Tables 3–7), could be contributed to relieve the effects of tough combined stress due to their antioxidative roles (Rady and Hemida 2015; Ebeed *et al.* 2017) and their gene expressions (Table 7). PAs are acted as signaling molecules to stimulate the action of antioxidants against abiotic stress. The potential functions of PAs mainly focus on plant metabolism regarding the preservation against the specific stresses (Groppa and Benavides 2008; Rady and Hemida 2015). The excessive levels of endogenous PAs under the combined stress conditions were associated with up-regulation of expression of SPDS, ADC, DHS, and SAMDC, but not ODC and SPMS genes (Table 7). Similar to our results, Ebeed *et al.* (2017) have suggested that PUT is synthesized under stress through the pathway of ADC not of ODC in wheat plant. Many enzymes are implicated in the pathway from PUT to SPM and SPD, including SPDS, SPMS, and SAMDC. Findings of this study also displayed an elevation in SPM content under combined stress and up-regulation of SAMDC gene expression while SPMS was not altered. This finding indicates a pivotal role of SAMDC gene in SPM synthesis in wheat under stress (Ebeed *et al.* 2017).

In this study, SPDS was up regulated under combined stress with an increase in the endogenous SPD content, which further elevated by MGE. The elevated levels of PUT, SPD, and SPM by MGE in combined stressed-wheat plant were associated with upregulated expression levels of ADC, SPDS, SAMDC, and DHS genes, conferring wheat plants powerful antioxidative defenses to withstand the combined stress (Tables 2–7).

MGE has effectively exceeded CKs (*c*-Z or *t*-Z) in improving wheat plant growth, grain yield, physio-biochemistry, and all components of antioxidant defense system (Tables 2–7). These results may be attributed to that MGE contains high contents of proline, AsA, GSH, SA, and CKs including *c*-Z and *t*-Z. Also, it has a high antioxidant activity (88.4%) measured as DPPH radical-scavenging activity because of various antioxidants among its ingredients. The high antioxidant activity in MGE makes it possess axial mechanisms to increase plant antioxidant status and inhibit or at least reduce oxidative stress effects, including membrane lipid peroxidation (Alzahrani and Rady 2019). Thus, MGE is a vigorous natural organic growth biostimulant and an environmentally friendly strategy. It has been used in some works to protect common bean, sunflower, and wheat plants against salt, nutrient deficiency, and cadmium stress conditions (Semida and Rady 2014; Rehman *et al.* 2018; Alzahrani and Rady 2019; Rady *et al.* 2019c).

Conclusion

Beyond synthetic cytokinins, 2% MGE can be used efficiently to boost tolerance to combined (75 m*M* NaCl-salinity + 60% SWHC) stress in wheat. The high contents of antioxidants and hormonal components, including cytokinins (especially zeatins) in MGE represented mechanisms to induce combined stress tolerance in wheat. Besides, MGE has high antioxidant activity (88.4%; assessed as DPPH radical-scavenging activity) due to its possession of several antioxidants. Further studies are needed to find the exact possible mechanisms provided by MGE to improve plant performance as observed in this study.

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