

Study of the Interactive Effects of Calcium and Abscisic Acid on Drought Stressed *Triticum aestivum* Seedlings

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IN THEIR natural habitats, plants are continuously subjected to many severe environmental challenges including drought. In this study, the effects of drought on *Triticum aestivum* seedlings were assessed, and the alleviating roles and interactive effects of calcium (Ca²⁺) and abscisic acid (ABA) to drought stress were studied. The results showed that pretreating wheat seedlings with calcium chloride (CaCl₂) or ABA led to alleviating most of the negative effects of 20% polyethylene glycol (PEG, as a drought imposing agent). Ca²⁺ improved the growth criteria, photosynthetic activity, but ABA had no significant effects on growth parameters. Ca²⁺ or ABA pretreatments have alleviated the toxic effects of the drought-induced oxidative stress which was marked with the reduction of MDA content. Ca²⁺ and/or ABA reduced the PEG-induced catalase activity, and ascorbic acid content. The use of lanthanum chloride (LaCl₃) as a calcium channel blocker has confirmed the role of Ca²⁺ in ameliorating the drought effects on plant. This study revealed that the application of Ca²⁺ has increased the endogenous ABA level of the treated seedlings to a high extent exceeding that of PEG treatment itself suggesting that ABA requires Ca²⁺ ions for its induction, and ABA is working downstream from Ca²⁺ in the relevant signaling pathways activated by drought stress. The alleviating effects of Ca²⁺ was prolonged to the yield stage. Overall, our results suggest that Ca²⁺ (10 mM), and with a less extend ABA (0.1 mM), represent signaling agents that can partially alleviate drought stress in plants growing in dry environments.

Keywords: Drought, Polyethylene glycol, Calcium signaling, Abscisic acid, Lanthanum chloride, Antioxidants.

Introduction

Plants are subjected to several environmental stresses that harmfully affect growth, metabolism, and yield. Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields (Anjum et al., 2011). Drought stress is a limiting factor at the initial phase of plant growth and establishment (Shao et al., 2008). Cell growth is considered as one of the most drought-sensitive processes due to the reduction in turgor pressure. It was reported that drought causes impaired mitosis, cell elongation and expansion resulting in reduced growth and yield traits (Hussein et al., 2008).

During events of suboptimal conditions, such as drought, different pathways can be affected differently which may make cellular homeostasis become disrupted. This process is usually

accompanied by the formation of reactive oxygen species (ROS) (Mittler, 2002). Plants are well adapted for minimizing the damage that could be induced by the ROS under natural growth conditions. However, ROS toxicity emerges when their production exceeds the quenching capacity of the protective systems due to environmental adverse conditions (Foyer & Noctor, 2003). The toxic levels of ROS can cause damage to DNA, proteins, lipids, chlorophyll and almost every other organic constituents of the living cell (Jiang & Zhang, 2001). Various plant species have an efficient enzymatic antioxidant system such as catalase, ascorbate peroxidase and glutathione reductase. In addition, plants have non-enzymatic antioxidants such as ascorbic acid (ASA) and glutathione (GSH). These antioxidant defense systems control the cascades of uncontrolled oxidation processes and protect plant cells from oxidative damage by scavenging ROS (Sewelam et al., 2016).

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DOI: 10.21608/ejbo.2018.978.1085

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Plants produce several signaling molecules, including calcium and abscisic acid, to orchestrate various stress responses and to cope with the negative effects of drought and other environmental stresses. As a second messenger in a wide range of signaling pathways, calcium connects the perception of different stimuli and stresses to their downstream cellular responses (Sanders et al., 2002 and Gilroy et al., 2016). Exogenous Ca^{2+} can enhance plant drought resistance and protect the structure of cellular plasma membranes (Chengbin et al., 2013). It maintains normal photosynthesis and regulates the metabolism of plant hormones and other important chemicals (Miqyass et al., 2007 and Gilroy et al., 2016). In addition, as a second messenger, cellular Ca^{2+} also transmits drought signals, thus regulating the physiological responses induced by drought stress (Tuberosa et al., 2007).

Various plant hormones, including ABA, have a broad effect on developmental processes and stress responses in plants (Fujita et al. 2006 and Wasternack, 2007). The complex regulatory and interaction networks occurring between hormone-signaling pathways allows the plant to activate appropriate responses to different types of stimuli (McSteen & Zhao, 2008 and Bari & Jones, 2009). It was reported that endogenous ABA concentrations increase under drought stress due to induction of ABA biosynthesis genes (Taji et al., 2001). The increase in ABA level reprograms the expression patterns of many genes to regulate water relations through adjustment of cellular osmotic pressure. Furthermore, the increase in ABA level is a central player controlling downstream responses essential for adaptation to different stresses, especially drought (Davies et al., 2002 and Giuliani et al., 2005). These responses include changes in stomatal conductance (closure of stomata), osmolyte accumulation, gene expression, a reduced leaf canopy, deeper root growth and changes in root system architecture (Davies et al., 2002; Giuliani et al., 2005; Lee et al., 2013 and An et al., 2016).

To impose water stress in plants, several methods ranging from water regimes, withdrawal of water from plants, to the use of chemicals such as polyethylene glycol (PEG), mannitol, etc., have been employed. It has been reasonably well established that polyethylene glycol-induced water stress mimics that caused by withdrawal of water from plants (Ravi et al., 2011). PEG

of high molecular weights have been long used to stimulate drought stress in plants as non-penetrating osmotic agents lowering the water potential in a way similar to soil drying (Larher et al., 1993). In addition, it has been reported that PEG induces significant water stress in plants without having any toxic effects (Emmerich & Hardegee, 1990). In the current study, PEG treatment was applied as a drought stress agent. To evaluate the roles of calcium and ABA in alleviating the effects of drought stress, wheat seedling were pretreated with calcium and/or ABA before treating with PEG. The present results suggested an important role for calcium in minimizing the negative effects of drought stress on wheat seedlings and in improving the overall growth. Also, our data revealed that calcium is working upstream from ABA during the signaling pathways involved in response to drought stress.

Materials and Methods

Plant material and growth conditions

The experiments in this study were carried out on wheat (*Triticum aestivum* L.) grains cultivar Gmiza 9. The grains were obtained from the Agricultural Research Center, Egypt. Before sowing, the grains were selected to be uniform in size and shape, sterilized in commercial clorox (1:1) for 5 min, and then washed with distilled water three times. For seedling stage, wheat grains were sown in plastic pots (10 cm diameter and 5 cm depth) filled with sand (about 100 g per pot). The sand has been washed with HCl/H₂O (1:1), and then washed three times with distilled water before sowing. Ten grains were sown per pot, irrigated with distilled water and left to grow for eight days in growth chamber under controlled conditions (12/12 day/night at 25°C/15°C ± 2°C), left to germinate and grow for eight days before the treatment. For yield experiment, 20 grains were sown in each plastic pots (40 cm diameter and 45 cm depth), filled with 20 kg clay-sandy soil (2:1 w/w) and left to grow at the greenhouse and irrigated with tap water once weekly during the experimental period.

Treatments

Based on a preliminary experiment; the sub-lethal concentration of PEG 6000 was selected to be 20%, and the proper concentration of calcium was 10 mM. While, the applied concentrations of ABA and LaCl₃ were selected based on experiments from the literature to be 0.1 mM for ABA (Tracy et al., 1993) and 5 mM for

LaCl₃ (Francisco & Juan, 2014). Eight different treatment combinations (three replicates each) were applied as represented in Fig. 1. For yield

measurements, wheat grains were grown under the same treatments as described above (after excluding the ABA treatments), and left to grow till the yield stage.

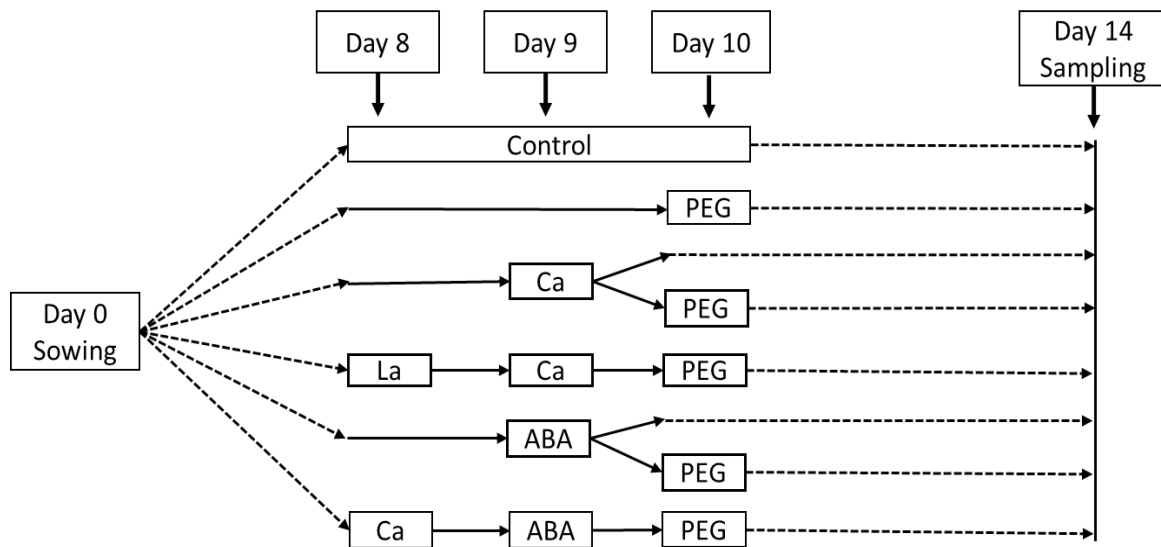


Fig. 1. A schematic diagram explaining the treatments and sampling procedures.

Plant analyses

For all measurements at the seedling stage, samples were collected four days after the PEG treatment. Yield analysis was carried out after ripening (about 120 days).

Growth criteria

Wheat seedlings were harvested and separated into shoots and roots, and weighed directly for fresh weights (FW). To determine the dry weights (DW), plant parts were dried at 60°C until constant weight was reached.

Measurement of photosynthetic efficiency

Photosynthetic efficiency (F_v/F_m) of wheat leaves was measured with a portable pulse amplitude modulation (PAM) fluorimeter (PerkinElmer, UK). The measurement of photosynthetic efficiency, on the adaxial leaf surface, was carried out as described by Gonçalves & Santos (2005). The leaves were dark-adapted for 30 min, then they were initially exposed to the weak modulated measuring beam ($< 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) to estimate the initial fluorescence (F_0) when the PS II reaction centers are opening (oxidized). Thereafter, the leaf was exposed to an 800 ms saturation pulse of high-intensity ($> 10000 \mu\text{mol m}^{-2} \text{per s}$ white light), to produce a transient closure (reduction) of the PS II reaction centers, at this point the maximum fluorescence (F_m) was

achieved. Three replicates of each treatment were measured. The fluorescence values of the initial fluorescence (F_0), the maximal fluorescence (F_m) and the maximum photochemical efficiency of PSII (F_v/F_m) were recorded from the fluorimeter. The variable fluorescence was calculated as follow ($F_v = F_m - F_0$).

Determination of lipid peroxidation level

Lipid peroxidation was measured by determining the amount of malondialdehyde (MDA), as a product of peroxidation of the unsaturated fatty acid; linolenic acid (18:3). MDA concentration was estimated by the method of Heath & Packer (1968). A sample of 0.5 g fresh leaves was extracted in 10 ml 5% (w/v) trichloroacetic acid. The homogenate was then centrifuged at 4000 rpm for 10 min. The supernatant (2 ml) was mixed with 2 ml of 0.67% (w/v) thiobarbaturic acid, and incubated at 95°C in a water bath for 20 min then cooled immediately. Absorbance was read at 532 nm and 600 nm. MDA concentration ($\mu\text{M/g f.wt.}$) was calculated using the extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

Estimation of catalase activity

A sample of 0.5 g fresh plant material was frozen, then homogenized in 8 ml of 50 mM cold phosphate buffer of pH 7.00 (Beauchamp & Fridovich, 1971). After centrifuging the homogenates for 20 min at 4000 rpm, the

supernatant was used as a raw extract for enzymatic assay. For measuring catalase activity, a sample of 3 ml of reaction mixture containing 0.1 M sodium phosphate buffer of pH 7, 2 mM H_2O_2 content and 0.1 ml enzyme extract, was prepared for the assay (Kato & Shimizu, 1987). The decrease in H_2O_2 was followed as a decline in the absorbance at 240 nm and the activity was calculated using the extinction coefficient ($40 \text{ Mm}^{-1} \text{ cm}^{-1}$ at 240 nm), and expressed in units of μM of the substrate converted per min/gram fresh weight.

Estimation of ascorbic acid content

The non-enzymatic antioxidant, ascorbic acid, was estimated according to Oser (1979). A known weight (0.1 g) of leaf tissue was homogenized in 5 ml of 5% (w/v) sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min. The reaction mixture for ascorbate consisted of 2 ml 2% Na-molybdate, 2 ml 0.15 N H_2SO_4 , 1 ml 1.5 mM Na_2HPO_4 and 1 ml tissue extract. The reaction mixture was incubated at 60°C in a water bath for 40 min, cooled and centrifuged at 3000 rpm for 10 min, and then absorbance was measured at 660 nm. Ascorbic acid content was calculated as mg/g d.wt. using a prepared calibration curve by ascorbic acid.

Estimation of endogenous ABA levels

Endogenous ABA level was estimated using Gas-Liquid Chromatography according to Shindy & Smith (1975).

Yield analyses

After ripening, about 120 days from sowing, yield criteria were measured, including number of grains per plant and the weight of 1000 grains.

Statistical analysis

All experimental determinations were replicated. The obtained data represented the mean values. Data obtained were analyzed statistically (Steel & Torrie, 1980) to determine the degree of significance between treatments. ANOVA (One-way analysis of variance) method was applied for all data and the LSD (least significant difference) at 0.05 was used to compare the means.

Results

Growth criteria

Drought stress has deleterious effects on growth of different plant species. To study the damaging effects of PEG (as a drought initiating substance) on the growth of wheat seedlings and

to assess the alleviating roles of Ca^{2+} and ABA, some of the growth criteria were measured including the fresh and dry weights of shoots and roots. The results indicated that PEG treatment led to a significant decrease in shoot fresh and dry weights where the percentages of the decrease were 15.7% and 26.7%, respectively, compared with the control. On contrary, PEG significantly increased root fresh and dry weights and the percentages of the increase were 40% and 16.7%, respectively relative to the control. The treatment with Ca^{2+} led to a significant increase in fresh weight of shoot and root relative to the control, but it induced a highly significant decrease in shoot dry weights, and had no significant effect on root dry weight (Fig. 2).

The combined treatment of Ca^{2+} and PEG had no significant effect on shoot fresh weight, but it significantly increased the shoot dry weight when compared with the PEG treatment. At the meantime, there was a highly significant decrease in root fresh and dry weights under Ca^{2+} and PEG combined treatment relative to the PEG treatment. Treating wheat seedlings with a combination of La^{3+} , Ca^{2+} and PEG resulted in a highly significant decrease in shoot fresh and dry weights, while it significantly increased the fresh and dry weights of root when compared with the combined treatment of Ca^{2+} and PEG (Fig. 2).

The treatments of ABA (singly, combined with PEG, or with Ca^{2+} and PEG) led to a significant decrease in both fresh and dry weights of shoot relative to the control. In addition, these treatments decreased root fresh weight compared with the control with the exception of ABA single treatment which had no significant effect on root fresh weight. The ABA single treatment induced a highly significant increase in root dry weight relative to the control, but the other two treatments (ABA and PEG, and Ca^{2+} with ABA and PEG) did not induce any significant effect (Fig. 2).

Photosynthetic efficiency (Fv/Fm)

Photosynthesis is one of the most sensitive physiological processes to drought stress. The changes in photosynthetic efficiency (Fv/Fm) was measured. As shown in Fig. 3, PEG led to a highly significant decrease in Fv/Fm value compared with the control. Meanwhile, the treatments of Ca^{2+} , and the combination of Ca^{2+} and PEG resulted in a highly significant increases in Fv/Fm value relative to the PEG treatment.

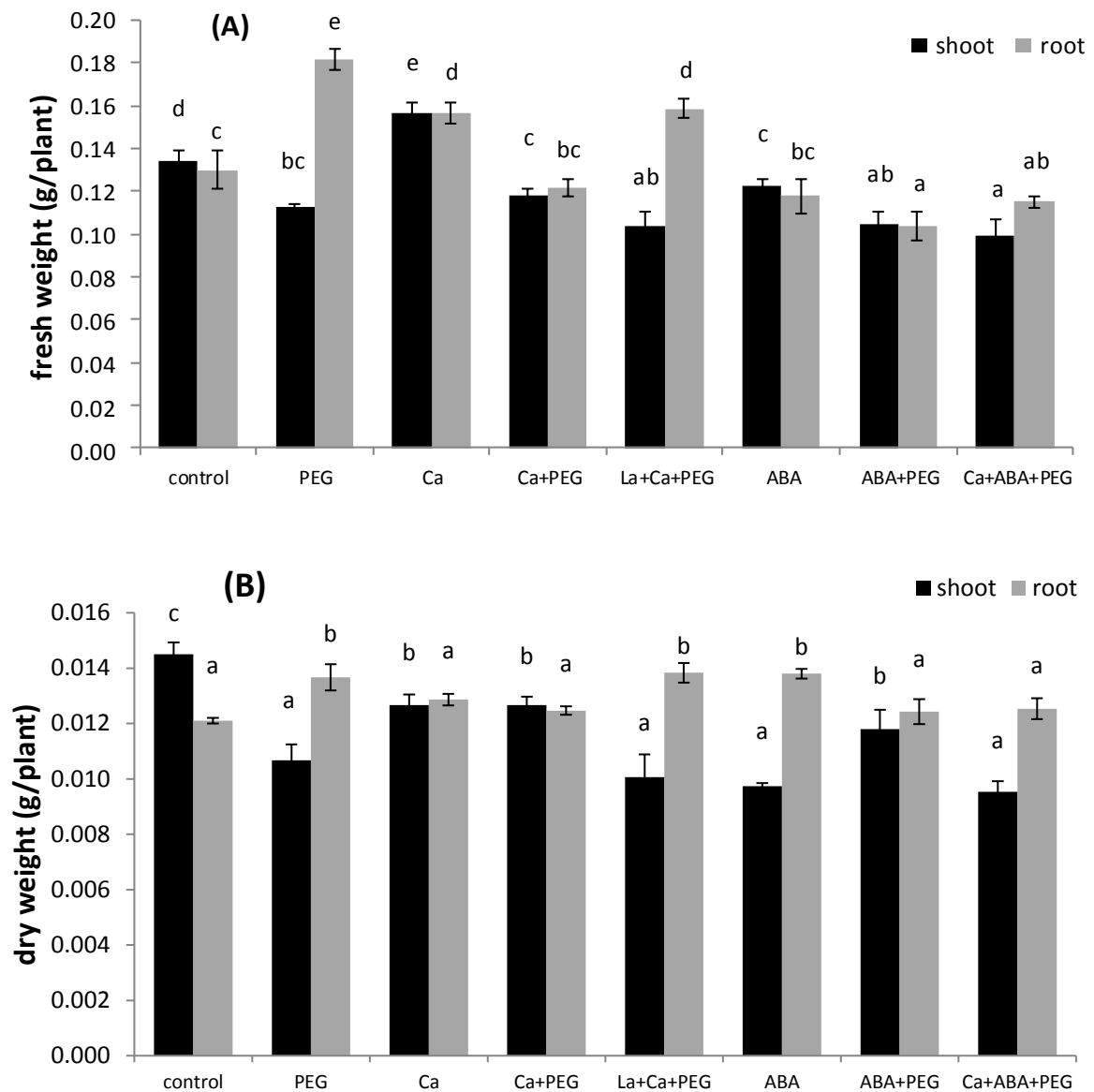


Fig. 2. The changes in shoot and root fresh and dry weights of 20% PEG-treated 14-day old *T. aestivum* seedlings with or without a pretreatment of LaCl_3 (5 mM), CaCl_2 (10 mM) and/or ABA (0.1 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
Shoot f.wt.	0.02	41.4	**
Root f.wt.	0.01	58.8	**
Shoot d.wt.	0.001	29.1	**
Root d.wt.	0.001	14.6	***

** Highly significant

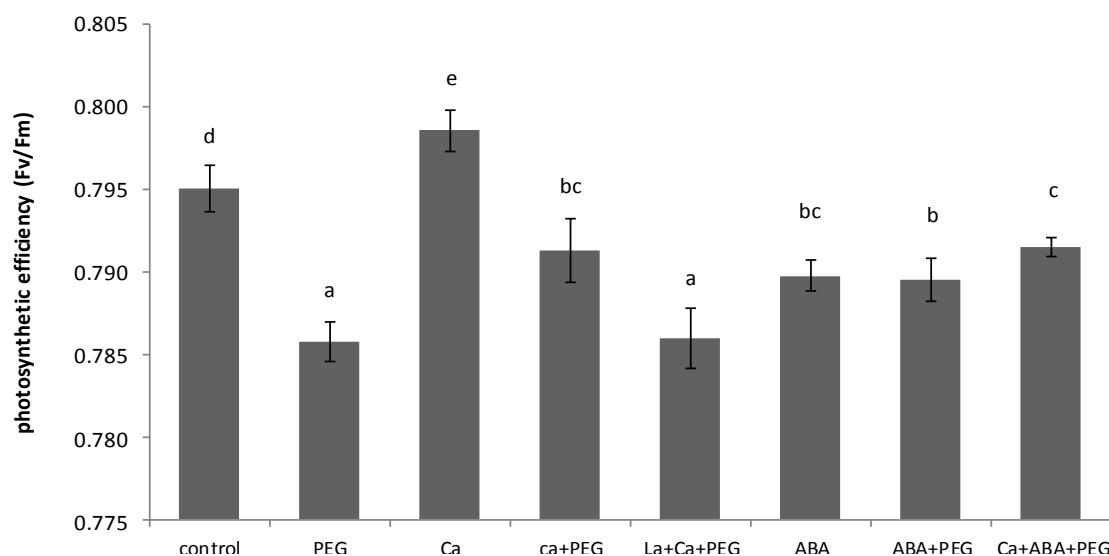


Fig. 3. The changes in photosynthetic efficiency (Fv/Fm) of 20% PEG-treated 14-day old *T. aestivum* seedlings with or without a pretreatment of LaCl_3 (5mM), CaCl_2 (10 mM) and/or ABA (0.1 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
Photosynthetic efficiency	0.002	59.88	**

** Highly significant.

Interestingly, the combined treatment of La^{3+} , Ca^{2+} and PEG didn't induce any significant effect on the Fv/Fm value compared with the PEG treatment, but led to a highly significant decrease in the Fv/Fm value when compared with the combined treatment of Ca^{2+} and PEG. However, all treatments of individual ABA or combined with PEG in the presence or absence of Ca^{2+} indicated a highly significant amelioration of the harmful effects caused by PEG, where photosynthetic efficiency was increased compared with the PEG treatment but still less than the control (Fig. 3).

Oxidative stress level

Drought stress is known to cause oxidative cell damage by generating ROS. In the current study, the oxidative stress level was monitored via measuring the changes in malondialdehyde (MDA), a lipid peroxidation product. Treating wheat seedlings with PEG induced a highly significant increase in MDA content compared with the control. The magnitude of the increase

of MDA with PEG treatment reached 85.9% when compared with the control (Fig. 4).

On the other hand, treatment with ABA or Ca^{2+} resulted in a pronounced decrease in MDA content compared with the control. Moreover, addition of either ABA or Ca^{2+} or their combination, followed by supplementation of PEG led to a marked reduction in MDA content when compared with PEG treatment. However, this effect was largely abolished when the Ca^{2+} treatment was preceded by La^{3+} treatment where the combined treatment La^{3+} , Ca^{2+} and PEG led to a highly significantly increase in MDA relative to the control treatment (Fig. 4).

Antioxidants

Plant cells have evolved a surveillance antioxidant system to cope with the oxidative damage of ROS produced under various stresses. Here, we studied the changes of the antioxidant enzyme catalase and the antioxidant compound ascorbic acid under drought stress.

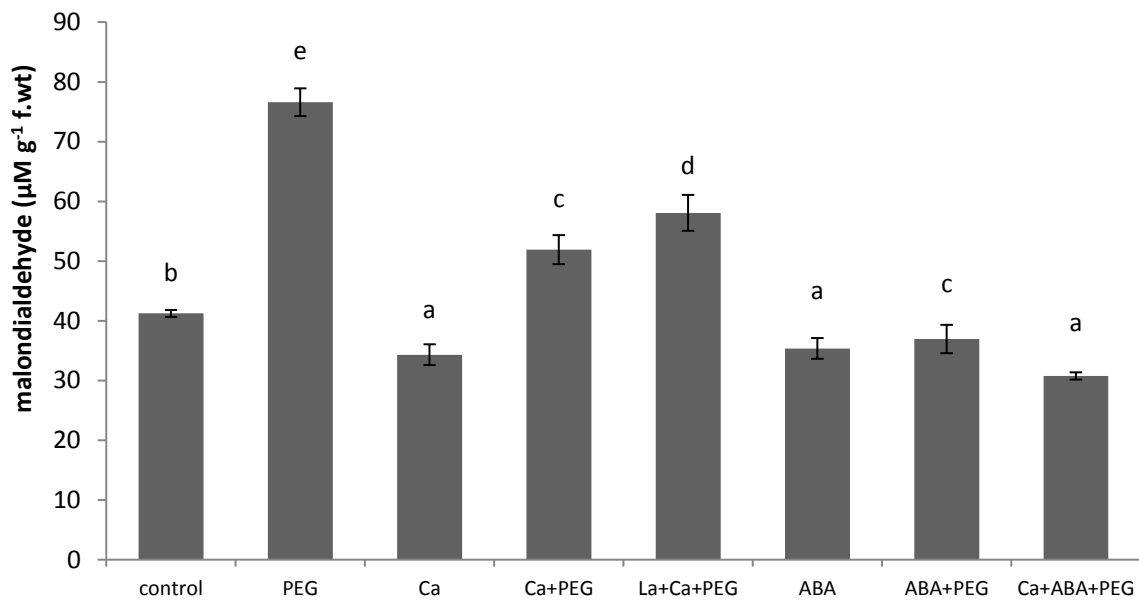


Fig. 4. The changes in malondialdehyde (MDA) content of 20% PEG-treated 14-day old *T. aestivum* seedlings with or without a pretreatment of LaCl_3 (5mM), CaCl_2 (10 mM) and/or ABA (0.1 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
Malondialdehyde content	3.86	144.27	**

** Highly significant

Catalase activity

As shown in Fig. 5, the activity of catalase was significantly increased with PEG treatment by 80%, compared with the control. The treatments of Ca^{2+} and ABA either singly or combined with PEG, as well as their combinations, led to a highly significant recovery as the catalase activity was decreased relative to the PEG treatment but still higher than that of the control. The combined treatment of La^{3+} , Ca^{2+} and PEG didn't induce any significant effect on catalase activity when compared with the PEG treatment, but its activity increased significantly compared with the combined treatment of Ca^{2+} and PEG indicating the role of La^{3+} as a calcium channel blocker.

Ascorbic acid content

As shown in Fig. 6, there was a remarkable increase in ascorbic acid content by 42.3%

under the PEG application compared with the control. It was noticeable that the combined treatments of either Ca^{2+} or ABA, or both of them, with PEG led to a significant decrease in ascorbic acid content compared with the PEG treatment indicating a recovery from the harmful effects imposed by PEG.

The ABA treatment resulted in a significant increase in the ascorbic acid content relative to the control, but the single Ca^{2+} treatment had no significant change when compared with the control. Moreover, a highly significant increase in ascorbic acid content was achieved under the combined treatments of La^{3+} , Ca^{2+} and PEG as the ascorbic acid content was increased relative to the combined treatment of Ca^{2+} and PEG, this increase indicates the role of La^{3+} in Ca^{2+} channel blocking (Fig. 6).

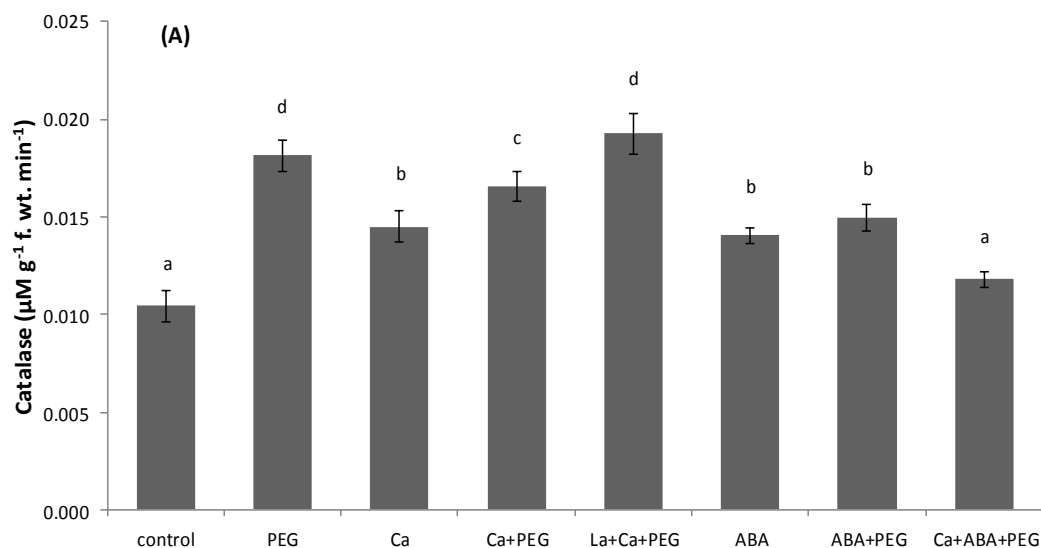


Fig. 5. The changes in catalase activity of 20% PEG-treated 14-day old *T. aestivum* seedlings with or without a pretreatment of LaCl_3 (5mM), CaCl_2 (10 mM) and/or ABA (0.1 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
Catalase activity	0.001	53.68	**

** Highly significant.

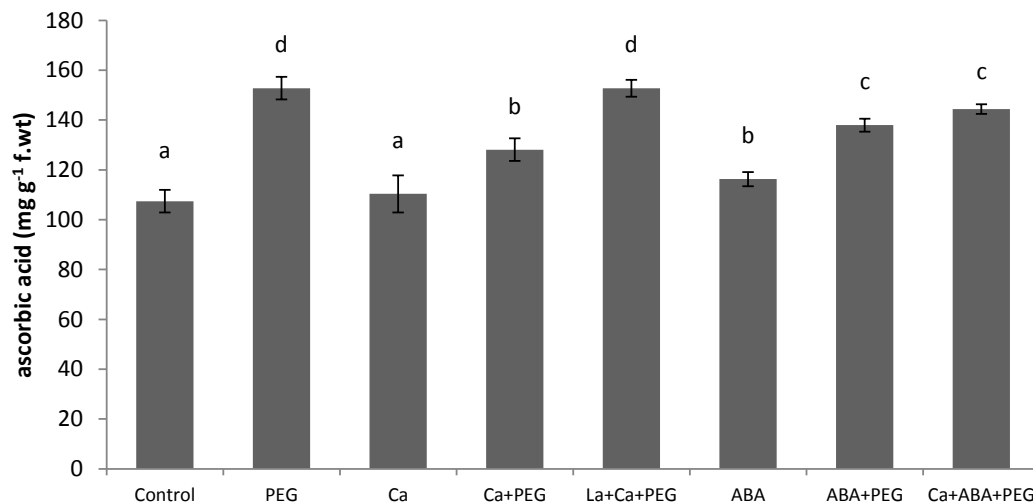


Fig. 6. The changes in ascorbic acid content of 20% PEG-treated 14-day old *T. aestivum* seedlings with or without a pretreatment of LaCl_3 (5mM), CaCl_2 (10 mM) and/or ABA (0.1 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
Ascorbic acid content	6.9	57.6	**

** Highly significant.

Endogenous ABA level

Abscisic acid is well known to play a crucial signaling role during drought stress, and its induced synthesis under drought is well documented. The results presented in Fig. 7 revealed that PEG treatment led to a highly significant increase in the endogenous ABA level, this increase reached 93% compared with the control. It was also noticeable that Ca^{2+} treatment resulted in a highly significant increase in ABA content relative to the PEG treatment, as well as to the control. The increase of ABA content under Ca^{2+} treatment compared to control and PEG was 194% and 45.7%, respectively. Additionally, the combined treatment of La^{3+} , Ca^{2+} and PEG has caused a highly

significant decrease in the endogenous ABA level relative to the control.

Yield parameters

The results presented here showed that the yield estimated parameters including the number of grains per spike and weight of 1000 grain were decreased under the PEG treatment by 24.4 % and 19.6%, respectively, compared to the control (Table 1). While, the combined treatment of Ca^{2+} and PEG led to a highly significant increase in these parameters. Noticeably, the combined treatment of La^{3+} , Ca^{2+} and PEG resulted in a highly significant decrease in both of these yield parameters when compared with the combined treatment of Ca^{2+} and PEG.

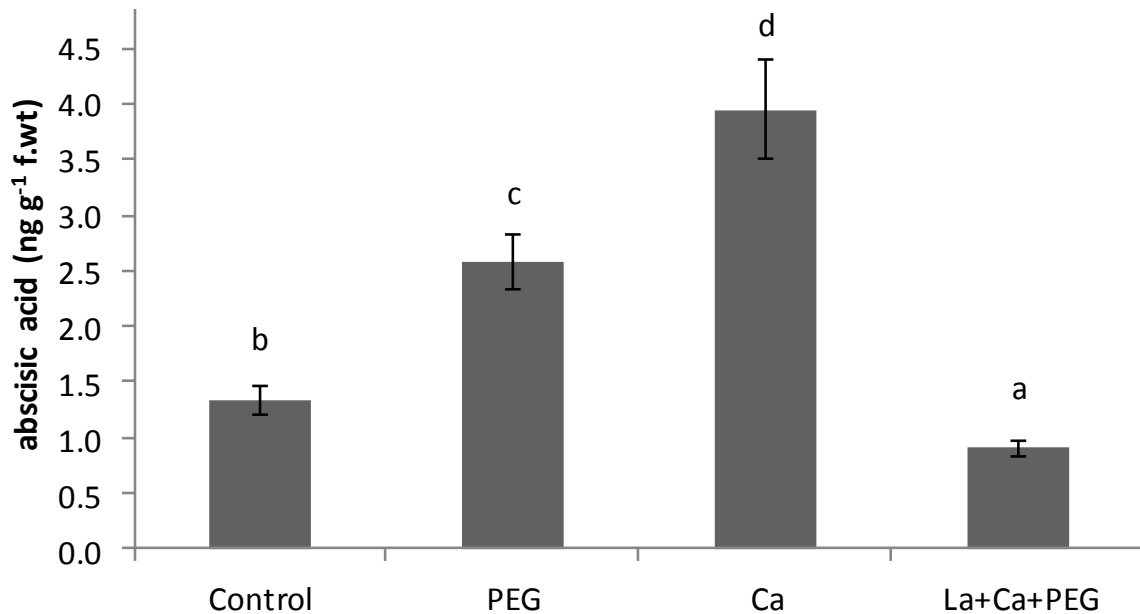


Fig. 7. The changes in the endogenous ABA level of 20% PEG-treated 14-day old *T. aestivum* seedlings under the influence of CaCl_2 (10 mM) alone or preceded by LaCl_3 (5 mM). Three biological replicates per treatment were used. Error bars represent standard deviations. Different letters indicate statistically significant ($P < 0.01$) differences.

One way ANOVA analysis ($P < 0.01$)

Parameters	LSD	F	Significance
ABA content	0.42	81.35	**

** Highly significant.

TABLE 1. The effect of 20 % PEG with or without a pretreatment of CaCl₂ (10 mM) and LaCl₃ (5 mM) on yield parameters including the number (No.) of grains per spike and weight of 1000 grains of the *T. aestivum* plants. The yield parameters were measured in mature plants (about 120-day old). Three biological replicates per treatment were used.

Treatments	No. of grains /spike	wt. of 1000 grain
Control	13.67 ± 1.15	16.30±0.27
PEG	10.33 ± 0.58	13.10±0.36
Ca	16.33 ± 1.15	20.97±1.07
Ca + PEG	12.00 ± 1.00	15.53±0.451
La + Ca + PEG	11.00 ± 1.00	13.53±0.451

One way ANOVA analysis (P < 0.01)

Parameters	LSD	F	Significance
No. of grains /spike	1.82	17.33	**
wt. of 1000 grain	1.08	84.44	**

** Highly significant.

Discussion

Calcium improves the growth and alleviates the negative effects of drought stress on growth of wheat seedlings

Drought is one of the main abiotic stresses which adversely affects crop growth and yield (Zou et al., 2015). In the present study, drought stress (applied as PEG, 20%) resulted in a significant reduction in shoot fresh and dry weights of *Triticum aestivum* seedlings (Fig. 2). These results were consistent with those reported by Riaz et al. (2010) on Turf grass, where water deficit conditions led to a significant inhibitory effect on shoot fresh and dry weights. The reduction in the monitored growth parameters may be attributed to that the reduction in soil moisture reduces the availability of nutrients to the plant and consequently reduce the growth (Razmjoo et al., 2008), in addition to the disturbance in cell physiology which may be associated with a decline in cell enlargement under water stress via low turgor pressure (Abdul, 2009 and Khayatnezhad et al., 2010).

Interestingly, results presented in this study showed that PEG (20%) induced a highly significant increase in root fresh and dry weights of wheat seedlings (Fig. 2). Similarly, it was reported that drought-stressed cotton seedlings showed a relevant increase in root length (Pace et al., 1999). The increase in root growth may be due to the capability of a prolific root system to support the accelerated plant growth during the early crop growth stage. The reason may be, also, due to the less sensitivity of root growth than that of shoot to the same low levels of water potential (Hsiao & Xu, 2000).

The present study revealed that *T. aestivum* seedlings treated with 10 mM Ca²⁺ had increased shoot fresh weight (Fig. 2). In this regard, it was reported that calcium deficiency reduce leaf growth, causing internode shortening near the apical bud, induce weak and malformed stems, as well as, necrosis (Stromme et al., 1994). Calcium was suggested to affect plant growth due to its role in mitosis and cytokinesis (Marschner, 1995). Our results reported that the lanthanum chloride (LaCl₃) has encountered the alleviating effects of Ca²⁺ on the inhibited growth of wheat seedlings caused by PEG. La³⁺ is known to reduce the magnitude of the Ca²⁺ elevation in response to drought stress caused by mannitol or salt stress treatments (Knight et al., 1996).

Data presented herein revealed that treating *T. aestivum* seedlings with 0.1 mM ABA didn't show considerable effects on growth criteria as fresh and dry weights of shoot (Fig. 2). This finding may be attributed to that the effects of ABA may be occurred at molecular level that wasn't clearly reflected in the growth criteria.

Calcium and ABA alleviate the inhibiting effects of drought stress on photosynthetic activity in wheat seedlings

Parallel to the inhibitory effects of PEG on growth, there was a highly significant decrease in the photosynthetic efficiency under PEG treatment (Fig. 3). In accordance with these findings, it was reported that drought stress led to a decrease in the Fv/Fm ratio in bean plant,

suggesting chronic photo-inhibition due to photo-oxidation of photosynthetic apparatus (Sewelam et al., 2014) and damage to the PSII oxygen-evolving complex possibly attributable to D1 protein damage under drought stress (Zlatve & Yordanov, 2004). Likewise, it was suggested that the photosynthetic efficiency has been declined in *Nothofagus* species under drought stress (Piper et al., 2007 and Luo et al., 2015). Similar effects on chlorophyll fluorescence parameters under drought stress have been observed in different species, among them *Brassica napus* (Kausar et al., 2006) and *Aegilops species* (Dulai et al., 2006).

Ca²⁺ is well documented to improve photosynthetic efficiency under drought stress. In a study carried out on *Zoysia japonica*, Chengbin et al. (2013) reported that the Fv/Fm ratio was higher in plants pretreated with 10 mM CaCl₂, and this is in agreement with our results (Fig. 3). Where Ca²⁺ treatment has increased the Fv/Fm and also alleviated the decrease in Fv/Fm caused by PEG treatment. This action of Ca²⁺ was confirmed through pretreating wheat seedlings with the calcium channel blocker LaCl₃, which has abolished the effects of Ca²⁺ (Fig. 3). These findings may be attributed to the requirement of Ca²⁺ as a co-factor in PSII for the oxygen evolving system (Miqyass et al., 2007). Calcium has also been shown to be essential for photo-assembly of PSII manganese clusters by preventing photo-inactivation (Chen et al., 1995).

ABA treatments led to a highly significant increase in Fv/Fm (Fig. 3), which may be attributed to the role of ABA in suppressing the increases in electrolyte leakage (Rajasekaran & Blake, 1999 and Sewelam et al., 2017), suggesting that ABA could reduce drought injury by protecting cell membranes and photosynthetic apparatus. The reported synthesis of dehydrin proteins under ABA treatment may represent another protective process induced by ABA (Landi et al., 2001).

Drought causes oxidative stress and affects the cell antioxidant activities, while Ca⁺ and ABA alleviate these effects

Drought as well as almost all abiotic stresses induce the accumulation of ROS leading to an oxidative cell damage (Luo et al., 2015), especially the oxidation of lipids in cell membranes. Results

of the present study showed a highly significant increase in lipid peroxidation levels (measured as MDA content) under PEG treatment (Fig. 4). It was reported that the level of lipid peroxidation (MDA content) increased with increasing the magnitude of the drought stress (Harish et al., 2010).

Interestingly, the present study suggested that Ca²⁺ pretreatment decreased the level of MDA in the drought treated seedlings (Fig. 4). This decrease in MDA content indicates the alleviating role of Ca²⁺ in decreasing the deleterious effects imposed by PEG. Exogenous Ca²⁺ can enhance plant drought resistance, and protect the structure of cellular membranes thus regulating the physiological responses induced by drought stress (Tuberosa et al., 2007). Results of the present work showed that the pretreatment of La³⁺ has abolished the Ca²⁺ alleviating effects on drought stressed *T. aestivum* seedlings leading to high peroxidation levels (Fig. 4). Regarding ABA, data presented herein showed that ABA decreased the MDA content. It was found that ABA is involved in membrane stability which may be achieved through ABA involvement in lipid metabolism (Zou et al., 1995). ABA may also alleviate the effect of PEG on plant as ABA interacts with membrane phospholipids to stabilize the membranes under stress conditions (Guschina et al., 2002).

Plants have developed antioxidant defense mechanisms to scavenge the free radicals and peroxides to alleviate their damaging effects. These include various enzymatic antioxidants such as catalases (CAT), and peroxidases (POX), in addition to other endogenous antioxidant compounds such as ascorbic acid (ASA) and glutathione (Li et al., 2012). CAT represents an efficient antioxidant enzyme that does not require a reductant for catalyzing dismutation reactions (Adriano et al., 2015). Our results indicated that PEG treatment has significantly increased the activity of CAT (Fig. 5). It was reported that CAT activity in *Avena* species were increased significantly with increasing the levels of water stress (Harish et al., 2010). The increase in the activity of CAT was correlated with the recorded increase of MDA content under PEG treatment.

The combined treatment of Ca²⁺ and PEG led to a highly significant reduction in the activity of catalase when compared with PEG treatment (Fig. 5). This reduction may be attributed to the role of Ca²⁺ in alleviating the damaging effects of drought

through its inhibiting role in the production of activating oxides (Tuberosa et al., 2007), as well as, its role in sustaining the membrane integrity (Lu et al., 1993). This role of Ca^{2+} may increase the cell adaptation and relief, and thus decrease the activity of catalase where there is no much ROS. On the other hand, the combined treatment of ABA and PEG resulted in a highly significant reduction in CAT activity (Fig. 5). This finding may be attributed to the role of ABA in stomatal closure and reducing water transpiration (Kim et al., 2010), as well as the role of ABA in increasing K^+ ion during oxidative stress which help in maintaining the membranes integrity (Hatata et al., 2009), and hence reducing oxidative stress. The combined pretreatment of both Ca^{2+} and ABA had synergistic relief effects resulting in a highly significant reduction in CAT activity under drought stress (Fig. 5).

The non-enzymatic antioxidant compound ascorbic acid (ASA) is involved in the cellular scavenging system controlling ROS. ASA represents the major water-soluble antioxidant in plant leaves, it can provide protection to membranes by directly scavenging the O_2^- , $\cdot\text{OH}$ and regenerating α -tocopherol from tocopheroxyl radical (Smirnoff, 2005). Data presented here showed that the PEG treatments led to increasing the ASA content in the treated seedlings indicating an active stress adaptation (Fig. 6). A marked increase of ASA was recorded under drought in rice, apple trees and mulberry (Reddy et al., 2004 and Abogadallah, 2010.). The increase in CAT activity and ASA content reported in our study under drought stress conditions, with other expected defense responses, may represent a tolerance energy cost that contribute for the reduction in plant growth during water regimes (Fig. 2).

ABA combined treatment with PEG led to an evident decrease in ASA content when compared with PEG treatment (Fig. 6). This result may be due to that ABA play an important role in the enhancement of tolerance to oxidative stress (Jiang & Zhang, 2001, 2004 and Yoshida et al., 2003).

Calcium works upstream from ABA in certain pathways activated by drought stress

It is well documented that ABA acts as a stress signal, which triggers adaptive changes in physiology and morphology of plants (Lim et al., 2015). The present data revealed that PEG

treatment induced an increase in endogenous ABA level (Fig. 7). Similar increases of ABA level were observed in *Arabidopsis thaliana* under osmotic stress (Ozfidan et al., 2012), as well as in wheat leaves under soil drought stress (Guoth et al., 2009). These findings may be attributed to the controlling role of ABA on the expression of a large set of stress-responsive genes. ABA treatment induces changes in gene expression in more than 10% of the Arabidopsis genome (Zeller et al., 2009), resulting in the increased expression of stress associated and signaling component transcripts (Yamaguchi-Shinozaki & Shinozaki, 2005). The increase in ABA content under drought or salinity stress conditions stimulates stomatal closure and the accumulation of osmo-compatible solutes, thus increasing the plant's capacity to cope with stress conditions (Seki et al., 2007 and Daszkowska-Golec & Szarejko, 2013).

As a second messenger, calcium connects the perception of different stimuli and stresses to their downstream cellular responses (Evans et al., 2005 and Dubiella et al., 2013). To check whether Ca^{2+} is working upstream or downstream from the plant hormone ABA during drought stress, the endogenous ABA levels were measured under different treatments. Our data showed that Ca^{2+} treatment has increased the endogenous ABA level exceeding the control and PEG values (Fig. 7). The results, also, showed that using La^{3+} preceding the addition of Ca^{2+} decreased the endogenous ABA level significantly. Furthermore, the level of the endogenous ABA under La^{3+} pretreatment was lower than its level under control and PEG treatment (Fig. 7) indicating that the increase of ABA under drought stress would be mediated by an endogenous increase in Ca^{2+} . This conclusion suggests that Ca^{2+} is working upstream from ABA that is induced by drought treatment in wheat seedlings. Ca^{2+} was found to participate in the process of ABA-induced drought signal transferring under PEG stress, where ABA synthesis was related with cytoplasmic Ca^{2+} concentrations (Noctor, 2006 and Scherzer et al., 2012).

However, the combined treatment of Ca^{2+} and ABA with PEG did not show synergistic effects regarding the alleviation of drought stress (Fig. 2 and 3). This may suggest that both, Ca^{2+} and ABA, may use identical signaling pathways with Ca^{2+} working upstream from ABA only in some of the many pathways activated by Ca^{2+} (Fig. 8).

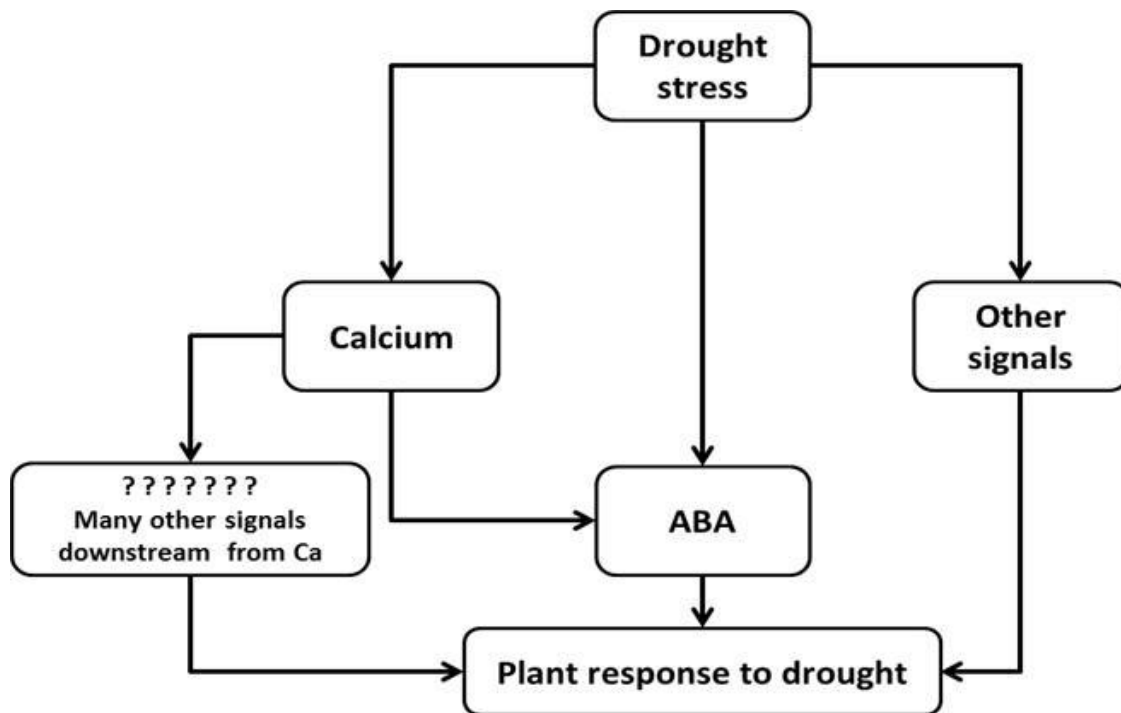


Fig. 8. A scheme presenting the expected interactions between calcium and ABA under drought stress.

The effects of drought and Ca⁺ treatments are prolonged to the yield stage

PEG application led to a marked decrease in yield parameters including the number of grains per spike and weight of 1000 grain (Table 1). It was reported that, the spike length, number of grains per spike and weight of 1000 grain weight were significantly decreased in drought stressed wheat plants (Bano et al., 2012). It was reported that the deficiency of water causes decline in yield traits of crop plants probably by disrupting leaf gas exchange which limits the assimilate translocation and dry matter partitioning (Farooq et al., 2009). Also, the disturbed nutrient uptake efficiency that frequently occurs during drought stress could lead to yield reduction (Riaz & Chowdhry, 2003). The present work showed that when wheat plants were pretreated with Ca²⁺, they produced higher yield compared to the PEG-treated ones (Table 1). These findings suggest that the alleviating effect of Ca²⁺ to drought stress is prolonged to the yield stage.

Conclusion

The work presented in this study showed that drought stress imposed by treating wheat seedlings with polyethylene glycol (PEG, 20%) has led to a significant reduction in growth of

wheat seedlings. In addition, PEG inhibited the photosynthetic activity and led to oxidative stress where it has increased the level of lipid peroxidation. Higher activities of the antioxidant enzyme catalase, together with an elevation of the antioxidant compound ascorbic acid, were recorded with the PEG treatment. Pretreating wheat seedlings with calcium chloride (Ca²⁺, 10 mM) or abscisic acid (ABA, 0.1 mM) led to alleviating the above mentioned damaging effects of PEG, but the effects of calcium were more pronounced compared to that of the ABA. The use of lanthanum chloride (LaCl₃, a calcium channel blocker) has confirmed the role of Ca²⁺ in ameliorating the drought effects on the plant. PEG treatment resulted in a significant increase in the endogenous ABA level of the treated seedlings. Interestingly, the application of Ca²⁺ increased endogenous ABA level of treated seedlings to a high extent exceeding that of PEG treatment itself suggesting that ABA requires Ca²⁺ ions for its induction, and ABA is working downstream of Ca²⁺. On the whole, the results presented here suggested that Ca²⁺ (10 mM) represents an effective agent for alleviating drought stress in plants growing in environments suffering from drought stress.

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(Received 20/4/2017;
accepted 8 /1 /2018)

دراسة التأثيرات التفاعلية للكالسيوم وحمض الأبسيسيك على بادرات القمح المُجهدة بالجفاف

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يمثل الجفاف واحداً من الإجهادات البيئية التي تسبب أضراراً بالغة لنمو وإنتاجية النباتات. يهتم هذا البحث بدراسة التأثيرات التفاعلية للكالسيوم وحمض الأبسيسيك للتخفيف من الآثار السلبية للجفاف على نمو بادرات نبات القمح. أوضحت نتائج هذه الدراسة أن معاملة بادرات القمح بمادة البولي إيثيلين جليكول (كعامل مسبب للجفاف) قد أدت إلى الكثير من الآثار السلبية على البادرات المُعالجة. من أمثلة هذه الآثار ضعف النمو، وانخفاض كفاءة عملية البناء الضوئي، وحدث أكسدة لمكونات خلايا النبات. ونتيجة لذلك ارتفع محتوى الخلايا من بعض العوامل المضادة للأكسدة مثل إنزيم الكاتاليز، وحمض الأسكوربيك. كما أوضحت النتائج أن المعالجة المسبقة بالكالسيوم أو حمض الأبسيسيك قد أدت إلى تخفيف آثار الجفاف على بادرات القمح، ولو بدرجة أقل في حالة حمض الأبسيسيك. وفيما يخص التأثيرات التفاعلية بين الكالسيوم وحمض الأبسيسيك، فقد أوضحت الدراسة أن المعاملة بالكالسيوم قد أدت إلى زيادة كبيرة في محتوى حمض الأبسيسيك بداخل النبات لدرجة أعلى من تأثير المعالجة بالجفاف نفسها. مما يقترح عمل حمض الأبسيسيك تحت تأثير الكالسيوم في مسارات الإشارات الخلوية ذات الصلة بإجهاد الجفاف. وللتأكد من دور الكالسيوم أثناء الإجهاد بالجفاف فقد تم استخدام مادة اللانثانم كلوريد التي تسبب غلق القنوات التي يمر من خلالها عنصر الكالسيوم عبر الأغشية الخلوية. حيث أن المعاملة المسبقة بهذه المادة قد أدت إلى تعطيل أدوار الكالسيوم المُستنتجة خلال هذه الدراسة. كما أوضحت الدراسة أن دور الكالسيوم في تخفيف آثار الجفاف على نباتات القمح قد امتد حتى مرحلة الحصاد. في المجمل، فإن نتائج هذه الدراسة ترصد دوراً مهماً للكالسيوم وحمض الأبسيسيك، ولو بدرجة أقل، في مسارات الإشارات الخلوية المؤدية لتخفيف آثار الجفاف على نباتات المحاصيل وتحسين إنتاجيتها.