

# Priming With The Green Leaf Volatile (Z)-3-Hexeny-1-yl Acetate Enhances Drought Resistance in Wheat Seedlings

**Ximei Li**

Qingdao Agricultural University

**Yuan Ji**

Qingdao Agricultural University

**Yuting Sheng**

Qingdao Agricultural University

**Linshan Sheng**

Qingdao Agricultural University

**Weiwei Guo**

Qingdao Agricultural University

**Huifang Wang**

Qingdao Agricultural University

**Yumei Zhang** (✉ [zhangcui2003@163.com](mailto:zhangcui2003@163.com))

Qingdao Agricultural University <https://orcid.org/0000-0001-5969-3831>

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## Research Article

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# Abstract

It was reported that green leaf volatiles play vital roles in multiple plant biotic and abiotic stresses, however, their functions in drought resistance have not been determined. The present study was to investigate the possible role of (Z)-3-hexeny-1-yl acetate (Z-3-HAC), a kind of green leaf volatile, in alleviating wheat drought stress and the underlying physiological mechanisms governing this effect. Seedlings of a drought-resistant variety were primed with 100  $\mu\text{M}$  Z-3-HAC at the four-leaf stage before drought treatment. Morphological analysis showed that the primed seedlings grew better and possessed higher biomass accumulation in both shoot and root under drought stress. Additionally, exogenous Z-3-HAC significantly increased the total root length, total root surface area, and total root volume of the seedlings under drought stress. Physiological measurements showed that the primed seedlings possessed higher relative water content (RWC), net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), maximal photochemical efficiency of photosystem II (PSII) (Fv/Fm), photochemical activity of PSII (Fv'/Fm'), total chlorophyll content, activities of the antioxidant enzymes, and osmolyte accumulation under drought conditions. Furthermore, relative electrolyte conductivity (REC), intercellular CO<sub>2</sub> concentration (Ci), reactive oxygen species (ROS) accumulation, and malondialdehyde (MDA) content were significantly lower than in non-primed seedlings. Lastly, principal component analysis (PCA) indicated that Z-3-HAC protects wheat seedlings against damage from drought stress mainly through antioxidant and osmoregulation systems.

## Introduction

Wheat is an important food crop widely distributed in the world. With the global warming, the worldwide drought problem has become increasingly serious, and has become one of the most important factors restricting agricultural production (Hirschi et al. 2011; Mueller and Seneviratne 2012). According to statistics, global arid and semi-arid regions account for about 43% of cultivated land (Kong et al. 2009; Zhao et al. 2016), while 70% of global wheat planting is distributed in arid and semi-arid regions (Yang 2012). Drought can cause various physiological and biochemical changes of wheat, such as decrease of relative water content (RWC) and increase of relative electrical conductivity (REC) (Altaf et al. 2021). Photosynthetic efficiency is also affected, including decrease of net photosynthetic rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs), and increase of intercellular CO<sub>2</sub> concentration (Ci) (Hungsaprug et al. 2019). Besides, reactive oxygen species (ROS) would accumulate, which then would result in oxidative damage of membrane structure (Xing et al. 2016; Xu et al. 2016). All above will affect wheat overall growth seriously, and then cause yield reduction ultimately. It was reported that drought stress reduced average wheat yield by 17~70% (Nouri-Ganbalani et al. 2009).

Of course, wheat has evolved various mechanisms to response drought stress. In response to ROS accumulation under drought stress, ROS scavenging system is activated, which make the ROS level reach a new steady state (Lee et al. 2009). The antioxidant system plays a major role in this process, which mainly relies on increase of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), etc (Shah and Nahakpam 2012). In addition, osmoregulation substances would

accumulate to counter water uptake difficulty caused by drought. It has been widely reported that soluble organic solutes, such as proline (Pro) (Verbruggen and Hermans 2008), betaine (Ashraf and Foolad 2007), free amino acids (FAA) (Rai 2002), total soluble proteins (TSP) (Amini and Ehsanpour 2005), and total soluble sugars (TSS) (Chaves et al. 2002; Martínez et al. 2004), would accumulate to varying degrees under drought stress. Besides, inorganic ions also play important roles in osmotic adjustment during drought resistance, such as  $K^+$  and  $Cl^-$  (Jones et al. 1980).

As all known, drought resistance is controlled by multiple genes, and many drought-responsive genes have been reported. *TaPIMP1* enhanced wheat drought resistance by activating defense and stress-related genes in ABA and SA signaling pathways (Zhang et al. 2012). *TaASR2D* improved drought resistance of *Brachypodium distachyon* by regulating the ABA biosynthesis pathway and redox homeostasis system (Yoon et al. 2021). All these theoretical studies have laid important foundation for cultivation of drought-resistant varieties, and there has been some excellent varieties with outstanding drought resistance. The practice showed that excellent cultivation and management measures could fulfill variety potential and increase production. And application of exogenous plant growth regulators has been proved to be an effective method to improve crop drought resistance (Peleg and Blumwald 2011). Up to now, salicylic acid (Canales et al. 2019), proline (Semida et al. 2020), melatonin (Liu et al. 2021), abscisic acid (Zhang et al. 2020b), and jasmonic (Abeed et al. 2021) have been reported to play roles in plant drought resistance. To ensure the healthy and sustainable development of agriculture, more eco-friendly plant growth regulating substances that confer crop drought resistance should be found.

Green leaf volatiles (GLVs), an important group of volatile organic compounds (VOCs) that are emitted by plants under stress (Heil and Bueno 2007), could prime plant defense against insect herbivore attacks (Engelberth et al. 2004). Among the major components of GLVs, (Z)-3-hexeny-1-yl acetate (Z-3-HAC) plays a pivotal role (Ameye et al. 2015). Later, Ameye et al. (2015) demonstrated that preexposure to Z-3-HAC primed wheat for enhanced defense against *Fusarium graminearum*. Subsequently, researches regarding priming by Z-3-HAC in response to plant abiotic stresses emerged. Cofer et al. (2018) reported that exogenous Z-3-HAC alleviated the damage of cold stress to maize seedlings. Tian et al. (2019) found that exogenous Z-3-HAC effectively alleviated the inhibitory effect of salt stress on peanut seedlings. In view of these findings, we speculate that Z-3-HAC may also play a role in plant drought resistance. Therefore, the present study was designed to elucidate whether exogenous application of Z-3-HAC could enhance drought resistance in wheat seedlings. Then physiological regulation mechanisms of Z-3-HAC involved in wheat drought resistance were conducted. This effort not only furthers our understanding of the role that Z-3-HAC plays in plant abiotic stress, but also lays theoretical foundation for wheat drought resistance improvement.

## Materials And Methods

### Plant materials

The drought-resistant variety “Lumai 21” was used in this study. Seeds were sterilized and germinated following the methods described by Zhang et al. (2016). Three days later, uniform seedlings were carefully moved to hydroponic boxes (inner length of 35 cm, width of 25 cm, and height of 15 cm, 48 seedlings/box) filled with 8 L Hoagland solution. Totally, 12 boxes were set up in this experiment. Then all of them were transferred to artificial climate-controlled room with 22 °C/18 °C (day/night), 16 h/8 h (light/dark), 50% humidity, and 1,200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD). Replace of Hoagland solution was conducted every 5 days. Lastly, the four-leaf period seedlings with uniform size were selected for subsequent experiments.

## **Experimental design**

The detailed information of Z-3-HAC is available in the previous report (Tian et al. 2019). The 12 boxes of seedlings were randomly divided into two batches. One batch was foliar applied with 100  $\mu\text{M}$  Z-3-HAC (Z-3-HAC was dissolved in 95% (v/v) ethanol as stock solution) twice with an interval of 3 days, while the other batch was treated with distilled water with the same amount of ethanol. According to our previous experiments (data not shown), the relatively moderate concentration of 100  $\mu\text{M}$  Z-3-HAC was the most effective. After pretreatment for 7 days, half of the seedlings treated with Z-3-HAC and distilled water were exposed to drought stress. Detailly, 20% PEG (200 g polyethylene glycol/L) was applied to simulate drought stress, and it was achieved by 3 visits that 5% at the 1<sup>st</sup> day, 15% at the 2<sup>nd</sup> day and 20% at the 3<sup>rd</sup> day. Finally, there were four treatments: control (water + water without PEG), Z-3-HAC (Z-3-HAC + water without PEG), PEG (water + water with PEG), and Z-3-HAC + PEG (Z-3-HAC + water with PEG). Morphological and physiological parameters were measured 6 days after the onset of drought stress, with three independent biological replications for each treatment.

## **Measurement of biomass and root morphology**

Firstly, one representative seedling for each treatment was selected to photograph. Then 9 randomly chosen seedlings for each treatment, with every 3 seedlings as a repeat, were used to measure fresh and dry weight according to the method of Tian et al. (2019). Besides, the fresh roots washed twice by distilled water, with 3 independent biological replicates for each treatment, were scanned using a two-lens scanning system (V700, SEIKO EPSON COPR., Japan) according to the method of Jiang et al. (2017). Then the obtained data were analyzed using the WinRHIZO Pro software (Version 2012b, Regent Instruments Inc., Canada). At the same time, one representative picture of root morphology for each treatment was kept and displayed.

## **Measurement of RWC and REC**

The leaf relative water content (RWC) was measured basically according to the method of Jensen et al. (2000). RWC was calculated by  $\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$ . FW means fresh weight of excised leaves, DW means dry weight of oven-dried leaves, and TW means turgid weight of soaked leaves. The relative electrolyte conductivity (REC) was measured according to the method of Griffith and McIntyre (1993). REC was calculated by  $\text{REC (\%)} = \text{C1} / \text{C2} \times 100$ . C1 is the conductivity of newly excised

leaf samples after briefly rinsing and 12 h soaking at room temperature using deionized water. And C2 is the conductivity measured after that the solution is boiled for 30 min and cooled afterwards.

### **Determination of gas exchange parameters, chlorophyll fluorescence parameters and total chlorophyll content**

Determination of gas exchange parameters was conducted between 9:00 am and 11:00 am using the portable photosynthesis system (Li-COR 6800, Lincoln, NE, USA). And the conditions in the leaf chamber were the same as described by Tian et al. (2019). Chlorophyll fluorescence was determined using the imaging pulse amplitude modulation (PAM) fluorometer (IMAG-MAXI; Heinz Walz, Effeltrich, Germany), as described in detail by Ahammed et al. (2013). Then, the maximal photochemical efficiency of photosystem II (PSII) ( $F_v/F_m$ ), the photochemical activity of PSII ( $F_v'/F_m'$ ), the quantum efficiency of PSII photochemistry ( $\Phi_{PSII}$ ), and the non-photochemical quenching ( $NPQ$ ) were calculated according to the formulas as described by Kramer et al. (2004). Besides, the representative  $F_v/F_m$  image from each treatment was exported and shown. For the determination of the total chlorophyll content, fresh leaves were firstly treated in accordance with the previous methods (Tian et al. 2019), then they were measured based on the method of Lichtenthaler and Wellburn (1983). Notably, the ultraviolet-visible spectrophotometer (UV3200, Mapada Instruments Co., Ltd., China) was applied throughout this study.

### **Histochemical staining and quantitative assay of $H_2O_2$ and $O_2^-$ , as well as measurement of lipid peroxidation**

Histochemical staining and concentration of  $H_2O_2$  were visually detected and determined, respectively, according to the methods described by Tian et al. (2019). In addition, histochemical staining analysis and concentration measurement of  $O_2^-$  were also conducted basically according to the methods of Tian et al. (2019). As all known, the lipid peroxidation level was usually determined by quantifying the equivalents of malondialdehyde (MDA), which was calculated based on the absorbance values of the red adduct at 450, 532, and 600 nm according to the method of Hodges et al. (1999).

### **Extraction and analysis of activity of antioxidant enzymes**

Sample preparation and enzyme extraction were conducted according to the method of Tian et al. (2019) with minor modifications. The phosphate buffer (25 mM HEPES-NaOH, pH 7.8) consisted of the following components: 20% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbic acid (AsA), 5 mM  $MgCl_2$ , 1 mM reduced ascorbic acid (GSH) and 1 mM dithiothreitol (DTT). Superoxide dismutase (SOD) activity was assessed by its ability to inhibit the photochemical reduction of NBT at 560 nm (Stewart and Bewley 1980). Peroxidase (POD) activity was assayed at 470 nm using the guaiacol–hydrogen peroxide method (Cakmak and Marschner 1992). Catalase (CAT) activity was assayed based on the oxidation of  $H_2O_2$  and measured as a decline at 240 nm (Patra et al. 1978). Ascorbate peroxidase (APX) activity was determined based on the oxidation of ascorbate and measured as a decline at 290 nm (Nakano and Asada 1981).

## Contents of TSS, Fru, FAA and Pro

Preparation of leaf samples and obtain of their extractions in this study were also conducted according to the method of Tian et al. (2019). Then total soluble sugar (TSS) content was measured using anthrone method (Buyssse and Merckx 1993). Fructose (Fru) content was measured using resorcinol method (Buyssse and Merckx 1993). Free amino acids (FAA) content was assessed by the ninhydrin reaction at 570 nm according to the method of Moore and Stein (1954). Proline (Pro) content was assessed by the ninhydrin colorimetry at 520 nm according to the method of Bates (1973).

## Statistical analysis

All data collected were statistically analyzed using one-way ANOVA with the SPSS statistical software package (Version 22.0, SPSS Inc., Chicago, IL, USA). Duncan's test ( $p < 0.05$ ) was performed to evaluate the difference of each treatment. Principal component analysis (PCA) was carried out according to the method of Sun et al. (2018).

## Results

### Effects of exogenous Z-3-HAC on seedling growth, plant biomass and root morphology under drought stress

As shown in Fig. 1a, wheat seedlings primed with Z-3-HAC grew significantly better than those un-primed in both normal and drought stress conditions. In detail, shoot fresh weight and dry weight of seedlings primed with Z-3-HAC were respectively 33.64% and 28.70% higher than those un-primed under normal growth conditions (Fig. 1b and c). Drought stunted wheat seedling growth as indicated by the significant decreases in shoot fresh weight and dry weight by 45.50% and 23.15%, respectively (Fig. 1b and c). However, priming of Z-3-HAC significantly mitigated the adverse effects of drought stress as indicated by no significant difference between the control and "Z-3-HAC + PEG" treatments (Fig. 1b and c). Similar results were obtained in root fresh weight and dry weight analysis. Root fresh weight and dry weight of seedlings primed with Z-3-HAC were respectively 50.33% and 47.83% higher than those un-primed under normal growth conditions (Fig. 1d and e). Drought stress decreased root fresh weight and dry weight by 42.14% and 23.91%, respectively (Fig. 1d and e). And priming of Z-3-HAC made no significant difference of root fresh weight and dry weight exist between the control and "Z-3-HAC + PEG" treatments (Fig. 1d and e).

As root plays an important role in crop drought resistance, root morphology was investigated detailly. Intuitively, roots of seedlings with Z-3-HAC priming showed a clear advantage regardless of normal growth or drought stress conditions (Fig. 2a). Quantitative data of root morphological parameters showed that exogenous application of Z-3-HAC significantly increased total root length, total root surface area and total root volume compared with the non-drought stressed control (Fig. 2c-e). The total root length, total root surface area, and total root volume were decreased by 53.88%, 48.51%, and 43.61%, respectively, under drought stress (Fig. 2c-e). Whereas, the application of Z-3-HAC before drought stress

increased them by 67.60%, 41.77%, and 41.33%, respectively, compared to the drought stressed control (Fig. 2c-e). However, no significant difference was observed between treatments in root average diameter (Fig. 2b).

### **Effects of exogenous Z-3-HAC on RWC and REC under drought stress**

It was showed that exogenous application of Z-3-HAC had no effect on the RWC and REC of wheat seedlings under normal growth conditions (Fig. 3). Compared with the control, drought stress significantly decreased RWC by 27.62% (Fig. 3a), while significantly increased REC by 196.35% (Fig. 3b). Whereas, compared with the drought stressed control, the application of Z-3-HAC increased the RWC by 14.47% (Fig. 3a), while significantly decreased the REC by 32.36% (Fig. 3b).

### **Effects of exogenous Z-3-HAC on gas exchange parameters, chlorophyll fluorescence parameters and total chlorophyll content under drought stress**

Firstly, there is no obvious difference of photosynthetic indexes between seedlings primed with and without Z-3-HAC under normal growth conditions (Fig. 4). Seedlings treated with only drought stress displayed significant decreases of 72.45% in  $P_n$  (Fig. 4a), 23.29% in  $C_i$  (Fig. 4b), 94.34% in  $G_s$  (Fig. 4c), and 85.98% in  $T_r$  (Fig. 4d), respectively. Exogenous Z-3-HAC significantly reduced the adverse effects of drought stress on seedlings. It was specifically indicated by significant increases of  $P_n$  by 101.60%,  $G_s$  by 366.67% and  $T_r$  by 210.14%, respectively, while a significant decrease of  $C_i$  by 17.28%.

Drought stress significantly decreased  $F_v/F_m$  by 29.11%. Again,  $F_v/F_m$  was significantly increased by 30.36% when seedlings were previously primed with Z-3-HAC (Fig. 5a).  $F_v/F_m$  status in different treatments was also indicated by pseudo color images of the leaves (Fig. 5a). Similarly, drought stress significantly decreased the other chlorophyll fluorescence parameters, such as  $F_v'/F_m'$ ,  $\Phi PSII$ , and  $NPQ$ , by 35.71%, 24.44%, and 57.14%, respectively (Fig. 5b-d). However, only  $F_v'/F_m'$  was significantly increased by 33.33% (Fig. 5b), while  $\Phi PSII$  (Fig. 5c) and  $NPQ$  (Fig. 5d) showed no change, when seedlings were previously primed with Z-3-HAC. Besides, drought stress significantly decreased chlorophyll content by 72.81%, while exogenous Z-3-HAC increased it by 140.86% (Fig. 5e).

### **Effects of exogenous Z-3-HAC on ROS accumulation and lipid peroxidation under drought stress**

Firstly, the accumulation of  $H_2O_2$  and  $O_2^-$ , two representative ROS, was detected by histochemical method.  $H_2O_2$  and  $O_2^-$  accumulated slightly following the application of Z-3-HAC under normal growth conditions, and seriously following the treatment of only drought stress (Fig. 6a and b). Notably, the  $H_2O_2$  and  $O_2^-$  accumulation of seedlings under drought stress was largely reduced by the exogenous Z-3-HAC (Fig. 6a and b). All these were basically confirmed by the quantitative data. Drought stress significantly increased  $H_2O_2$  and  $O_2^-$  by 58.33% and 79.39%, respectively, while exogenous Z-3-HAC decreased them by 15.79% and 9.79%, respectively, compared with only drought stress (Fig. 6c and e). The lipid peroxidation of wheat seedlings was detected according to the accumulation of MDA. Drought stress significantly

increased MDA content by 24.00%. Consistent with the effect of Z-3-HAC on ROS accumulation, exogenous Z-3-HAC significantly reduced the MDA content by 26.30% under drought stress conditions (Fig. 6d).

### **Effects of exogenous Z-3-HAC on antioxidant metabolism and osmolytes accumulation under drought stress**

Under normal growth conditions, exogenous application of Z-3-HAC showed almost no effect on the activities of SOD, POD, CAT and APX (Fig. 7). Under drought stress, the activities of all these four antioxidant enzymes increased significantly, SOD by 22.29%, POD by 50.33%, CAT by 228.08%, and APX by 200.07%, respectively. And exogenous Z-3-HAC further significantly increased activities of SOD by 16.01%, POD by 24.14%, CAT by 37.78%, and APX by 24.44% (Fig. 7).

Low molecular weight organic compounds, such as TSS, Fru, FAA and Pro, are the main components of osmotic substances in plants. Under normal growth conditions, exogenous application of Z-3-HAC significantly increased the concentrations of TSS by 20.62%, Fru by 18.29%, FAA by 113.12% and Pro by 57.11%, respectively (Fig. 8). Under drought stress conditions, concentrations of TSS were significantly increased by 26.80%, FAA by 216.87% and Pro by 151.38%, respectively. While concentration of Fru was slightly increased by 10.49% (Fig. 8). As shown in Fig. 8, exogenous Z-3-HAC significantly increased TSS by 17.48%, and Pro by 13.92%, respectively, compared with only drought stress. However, concentration of Fru was just slightly increased by 11.70%, while concentration of FAA was significantly decreased by 12.49% instead.

### **Principal component analysis**

A principal component analysis (PCA) integrating all the information of four treatments was performed. The two components of PCA collectively explained 95.54% of data variability (Fig. 9). The first PC (PC1) accounted for 73.58% of the total qualitative variation and had REC, APX, CAT, Pro, POD, SOD and FAA with high negative loadings (Fig. 9a). The second PC (PC2) accounted for 21.96% of the total qualitative variation and had Fru, RDW, SDW and TSS with high positive loadings (Fig. 9b). SOD, POD, CAT, APX, TSS, FAA, Pro and Fru were located toward the negative end of PC1 axis and the positive end of PC2 axis in the second quadrant (Fig. 9b). It corresponded to the result of "Z-3-HAC + PEG" (Fig. 9a). In conclusion, the antioxidant enzymes and osmoregulation substances were the most important factors in response to Z-3-HAC under drought stress.

## **Discussion**

### **Exogenous Z-3-HAC improved drought resistance of wheat**

Many previous studies have shown that plant GLVs are rapidly produced after injury to resist biotic stress (Yan and Wang 2006; Heil 2014; Tanaka et al. 2018). Afterwards, it was reported that Z-3-HAC enhanced defense against *Fusarium graminearum* in wheat (Ameye et al. 2015), alleviated cold stress in maize

(Cofer et al. 2018), and reduced salt stress in peanut (Tian et al. 2019). However, there is still no report regarding the pivotal role for Z-3-HAC in plant drought stress response. In the present study, it was showed that, under drought stress conditions, both growth and biomass accumulation of wheat seedlings previously primed with Z-3-HAC were dramatically improved compared with those non-primed (Fig. 1). The results indicated that Z-3-HAC could play a role in plant drought resistance, which expands the application field of Z-3-HAC.

Root morphological properties are crucial to water and nutrients absorption, as well as gas exchange, which play vital roles in avoiding dehydration, promoting carbon assimilation, and improving yield potential under drought stress (Gewin 2010; Kell 2011; Lopes et al. 2011; Palta et al. 2011). As expected, exogenous Z-3-HAC significantly increased total root length, total root surface area and total root volume, compared with drought stress alone (Fig. 2), which supported the results about wheat seedling growth and biomass accumulation, and proved the important role of root system in drought resistance again.

### **Exogenous Z-3-HAC increased RWC and decreased REC of wheat under drought stress**

Leaf RWC and REC are closely related to drought resistance (Farooq et al. 2009). The RWC exhibits the leaf water status and the REC estimates the leaf cell membrane stability in stressed plants (Petrov et al. 2018). Zhang et al. (2020a) reported that, under drought stress, better water balance (higher leaf RWC) and better cell membrane stability (lower leaf REC) were important to improve wheat drought resistance. In keeping with these findings, our results indicated that drought stress led to a significant decline of RWC and a significant increase of REC in wheat seedling leaves (Fig. 3), while exogenous Z-3-HAC increased RWC and decreased REC (Fig. 3), which was conducive to maintain the integrity of cytoplasmic membrane. In support of the RWC data, the significant increase accumulation of TSS and Pro was also observed in the “Z-3-HAC + PEG” treatment (Fig. 8). However, there is no significant increase of Fru and significant decrease of FAA. The above indicated that TSS and Pro might play main roles in increasing leaf RWC of seedlings under “Z-3-HAC + PEG” treatments, while not Fru and FAA.

### **Exogenous Z-3-HAC alleviated the inhibition of drought on photosynthesis in wheat**

Photosynthesis is the fundamental metabolic process determining crop growth and yield, but it is strongly inhibited by drought stress (Pandey and Shukla 2015). Consistent with previous theories, our results demonstrated that Pn, Ci, Gs and Tr were all significantly disrupted by drought stress (Fig. 4). Notably, Pn, Gs and Tr were reverted significantly by exogenous Z-3-HAC (Fig. 4a, c and d). Nevertheless, a considerably steeper reduction of Ci was detected in the “Z-3-HAC + PEG” treatment (Fig. 4b). It is generally considered that photosynthesis limitation is divided into stomatal and nonstomatal factors (Farquhar and Sharkey 1982). In this study, significant decrease of Ci under drought stress may be caused by significant decrease of Gs, then resulting in significant decrease of Pn. Whereas, priming of Z-3-HAC prompted stomata reopen and made a nonstomatal limitation condition, which made Pn resume rapidly, then resulted in a decrease of Ci.

In this paper, leaf TSS content was significantly increased under “Z-3-HAC + PEG” treatment, compared with “PEG” treatment (Fig. 8a), making it likely that the enhanced photosynthesis by Z-3-HAC might be partly attributed to the acceleration of carbon metabolites (Paul and Pellny 2003). Additionally, TSS and Pro, important osmotic adjustment substances, play important roles in maintaining osmotic pressure and normal metabolism under drought stress (Sanders and Arndt 2012). The further accumulation of these osmolytes was observed in the “Z-3-HAC + PEG” treatment (Fig. 8), in principle, could help to decrease membrane permeability and maintain organelle integrity, which was of great significance to ensure the normal photosynthesis.

Chlorophyll is an important material base for photosynthesis, content of which could reflect the photosynthetic performance to a certain extent (Cui et al. 2019). A significant higher total chlorophyll content of seedlings under the “Z-3-HAC + PEG” treatment was observed, compared with those under the “PEG” treatment (Fig. 5e), partly explaining the higher Pn of seedlings under the “Z-3-HAC + PEG” treatment (Fig. 4a). In addition, chlorophyll fluorescence parameters have been widely considered to intrinsically reflect the photosynthetic performance (Xia et al. 2009; Ivanov and Bernards 2016). Significant  $F_v/F_m$  and  $F_v / F_m$  improvement of seedlings under the “Z-3-HAC + PEG” treatment indicated that Z-3-HAC could alleviate the damage to photosystem under drought stress (Fig. 5a and b). However, there was no increase of  $\Phi_{PSII}$  and  $NPQ$  (Fig. 5c and d), suggesting that improvement of photosynthesis induced by Z-3-HAC was not by way of accommodating lower NADPH demand or dissipating light energy.

### **Exogenous Z-3-HAC reduced the ROS level of wheat seedlings under drought stress through the antioxidant system**

Drought stress destroys the balance between light energy capture and light energy utilization (Chaitanya et al. 2003), which then inhibits photoreaction. Subsequently, formation of NADPH, which is product of NADP reduction by electrons, is greatly reduced, resulting a decrease of Calvin cycle. Finally, photosynthetic efficiency decreases. At the same time, some of the electrons are shunted off to Mehler reaction, changing  $O_2$  to  $O_2^-$  (Mittler et al. 2004; Wiese et al. 1998). It is well accepted that SOD is an important scavenging factor of superoxide free radicals, which can transform  $O_2^-$  to  $H_2O_2$  (Li et al. 2015). As all known, both  $H_2O_2$  and  $O_2^-$  are representative ROS. Generally, the production and elimination of ROS in plant cells are in dynamic equilibrium (Zhang et al. 2019), and the accumulation of ROS is a double-edged sword (Tian et al. 2019). Detailly, excessive accumulation of ROS aggravates membranous peroxidation, whereas moderate induction of ROS might be a crucial signal to alert plants to response biotic or abiotic stress (Mittler et al. 2004; Neill et al. 2002; Miller et al. 2008; Baxter et al. 2014; Qi et al. 2017; Waszczak et al. 2018). In the present study, the accumulations of  $H_2O_2$  and  $O_2^-$  were both determined using histochemical allocation and chemical quantitative analysis methods. It was showed that drought stress caused an excessive accumulation of ROS, resulting in membranous peroxidation, as indicated by high content of MDA (Fig. 6d). Whereas, exogenous Z-3-HAC significantly reduced the ROS

level, which might become a signaling substance to stimulate ROS scavenging system. It was confirmed by MDA decrease of seedlings under the “Z-3-HAC + PEG” treatment (Fig. 6d).

As mentioned above, SOD, POD, CAT and APX are major components of the antioxidant system (Shah and Nahakpam 2012). Under drought stress, exogenous Z-3-HAC induced a significant increase of SOD (Fig. 7a), which transformed  $O_2^-$  of great capacity to  $H_2O_2$  of less harm (Li et al. 2015). Then,  $H_2O_2$  was broken down to  $H_2O$  rapidly by POD and CAT (Menconi et al. 1995; Wang et al. 2009), which were also significantly accumulated in the “Z-3-HAC + PEG” treatment (Fig. 7b and c). Besides, accumulated APX (Fig. 7d) oxidized ascorbic acid to dehydroascorbic acid and  $H_2O$ , avoiding  $H_2O_2$  formation directly (Asada 1999). All these led to decline of ROS and MDA (Fig. 6). We therefore speculated that significant accumulation of antioxidase in the “Z-3-HAC + PEG” treatment eliminated ROS effectively, then reduced membranous peroxidation. Ultimately, exogenous Z-3-HAC improved wheat drought resistance.

## Conclusions

In conclusion, Z-3-HAC could effectively protect wheat seedlings against damage from drought stress, as directly reflected by that wheat seedlings primed with Z-3-HAC grew better, accumulated higher biomass, and possessed stronger roots. Physiological analysis showed that it may be caused by higher RWC, Pn, Gs, Tr, Fv/Fm, Fv'/Fm', total chlorophyll content, and lower REC, Ci, ROS accumulation, MDA content. Further analysis indicated that it works mainly through antioxidant and osmoregulation systems. Totally speaking, the present effort has certain theoretical significance for wheat drought resistance improvement.

## Declarations

## Author contributions

Ximei Li analyzed the data and drafted the manuscript. Yuan Ji performed most of the experiments. Yuting Sheng participated in part of the physiological measurements. Linshan Sheng participated in part of the data analysis. Weiwei Guo proofed the manuscript. Huifang Wang gave many advices on the manuscript structure. Yumei Zhang designed the experiments. All authors read and approved the final manuscript. In addition, the authors report no declarations of interest.

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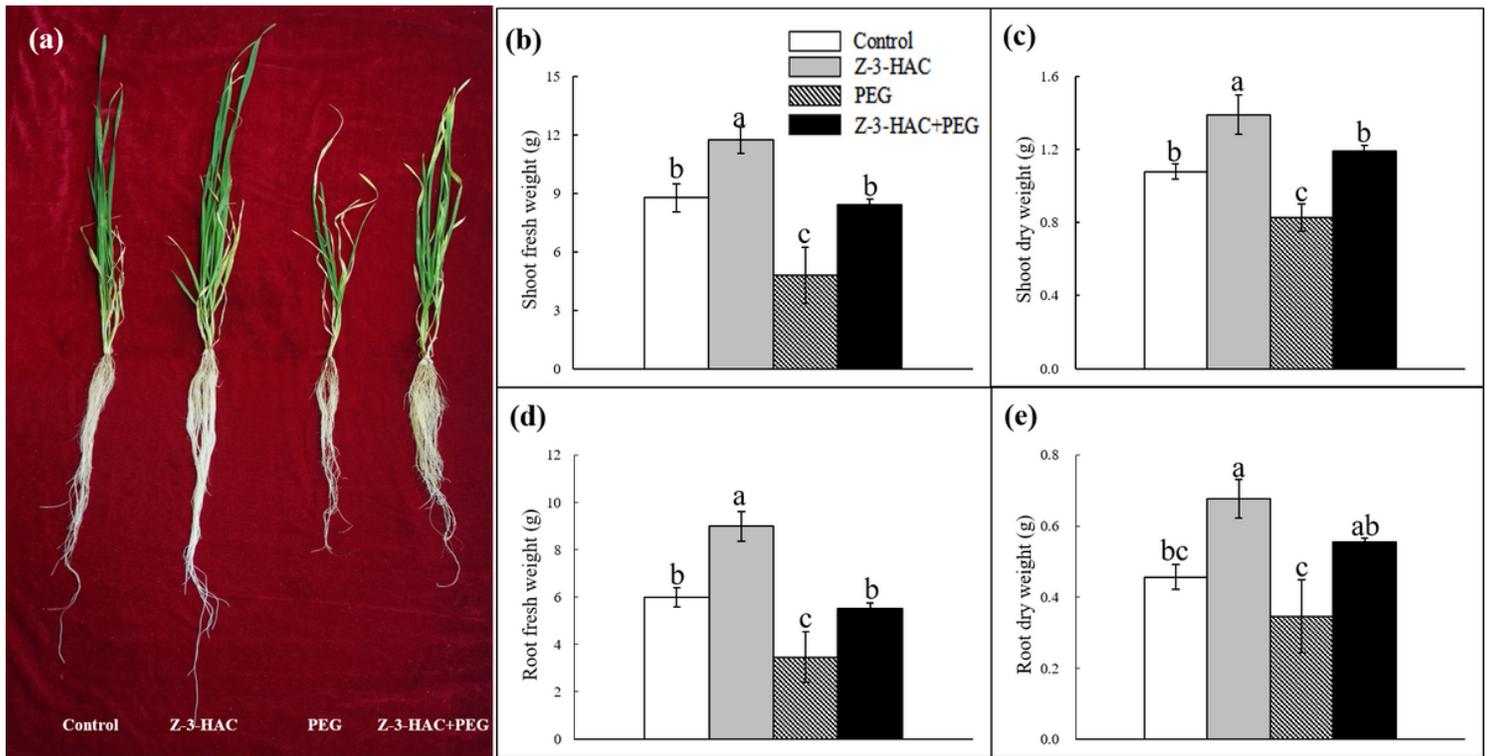
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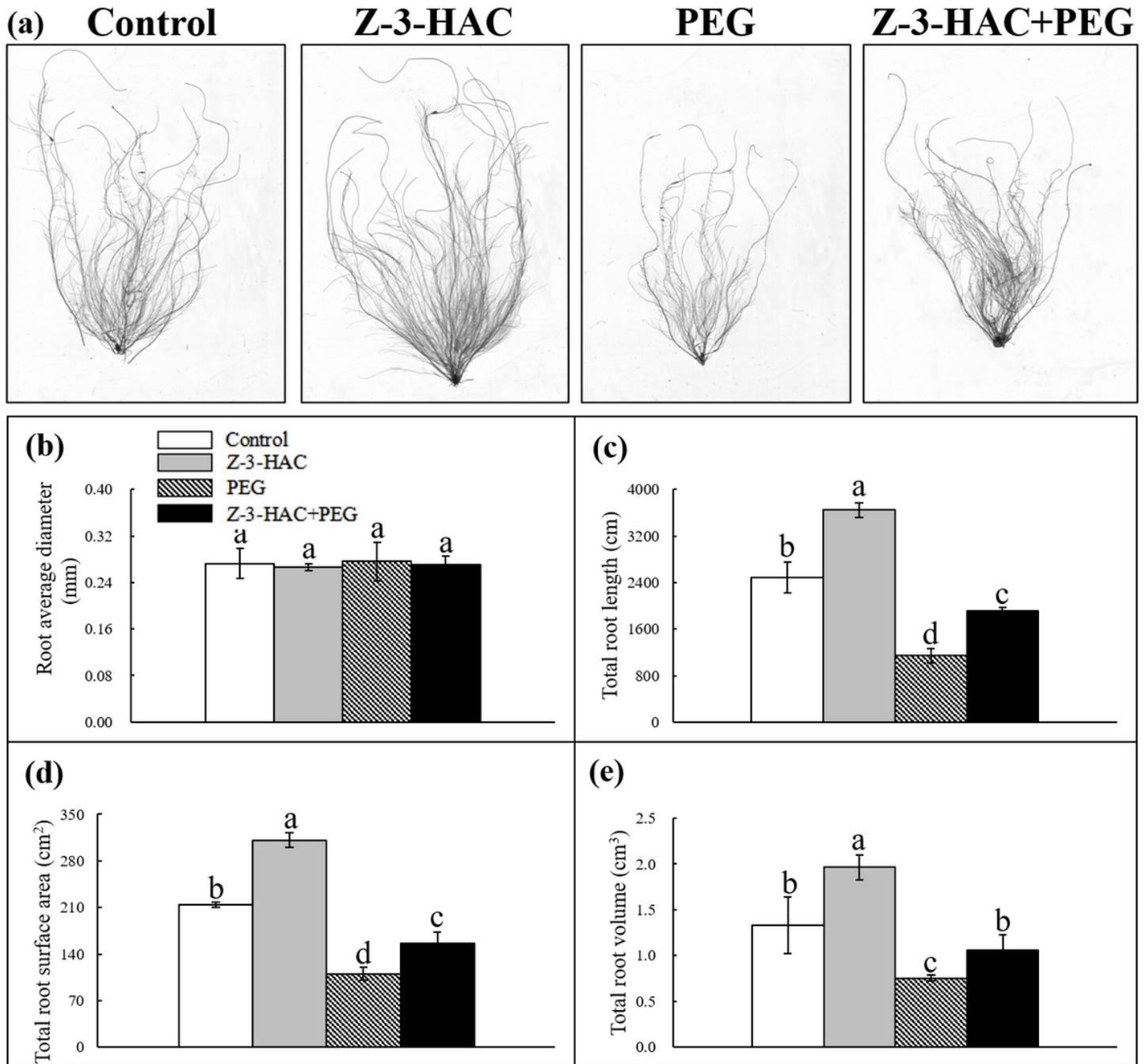
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## Figures



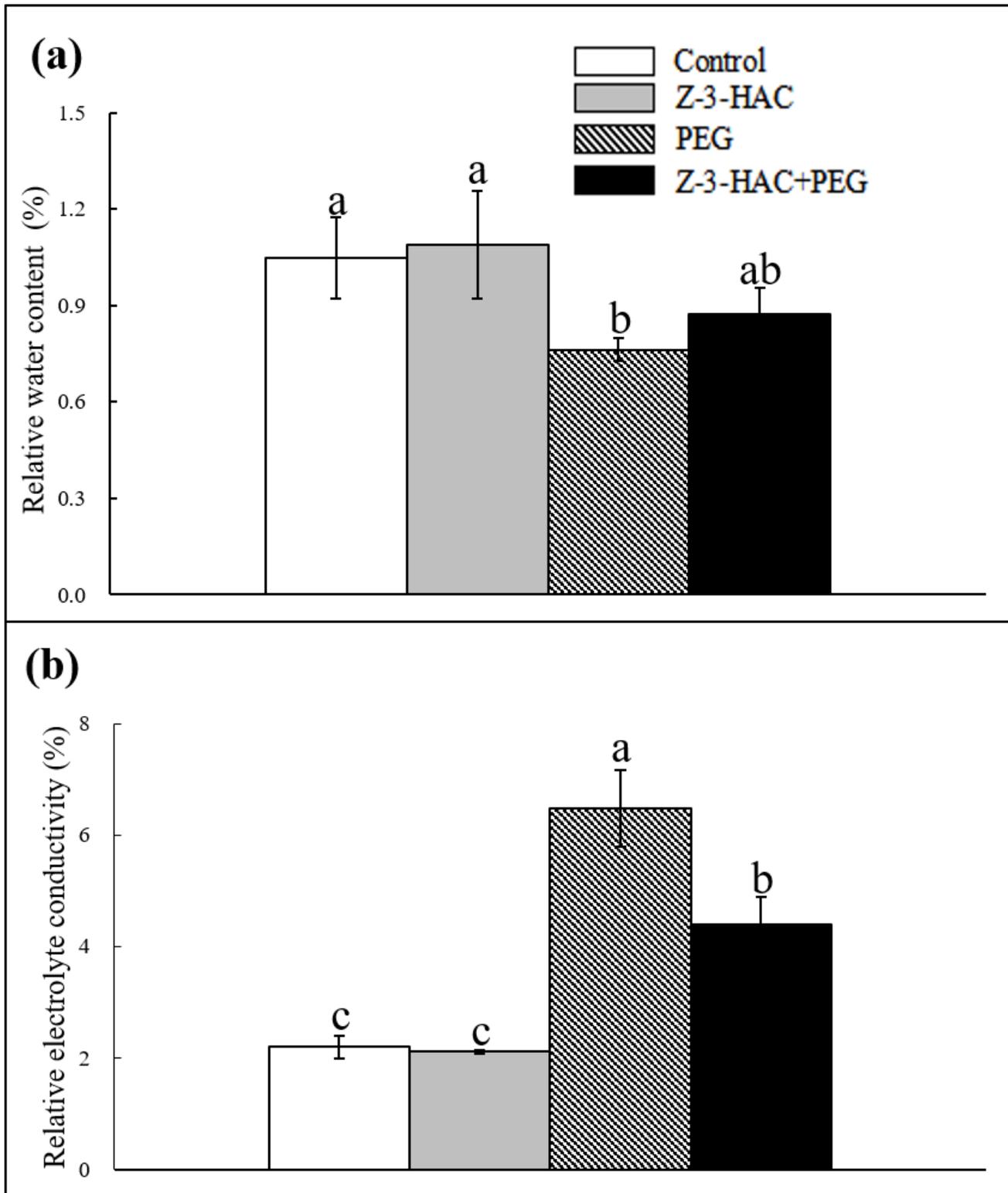
**Figure 1**

Exogenous Z-3-HAC application conferred drought resistance of wheat seedlings. (a) Growth of wheat seedlings under normal growth/drought stress conditions with or without Z-3-HAC priming, (b) shoot fresh weight, (c) shoot dry weight, (d) root fresh weight, and (e) root dry weight. Bars are the standard deviations (SD) of three independent replicates ( $n = 3$ ). Error bars labels with different letters indicate significant differences at  $p < 0.05$  between treatments according to Duncan's test. The same below



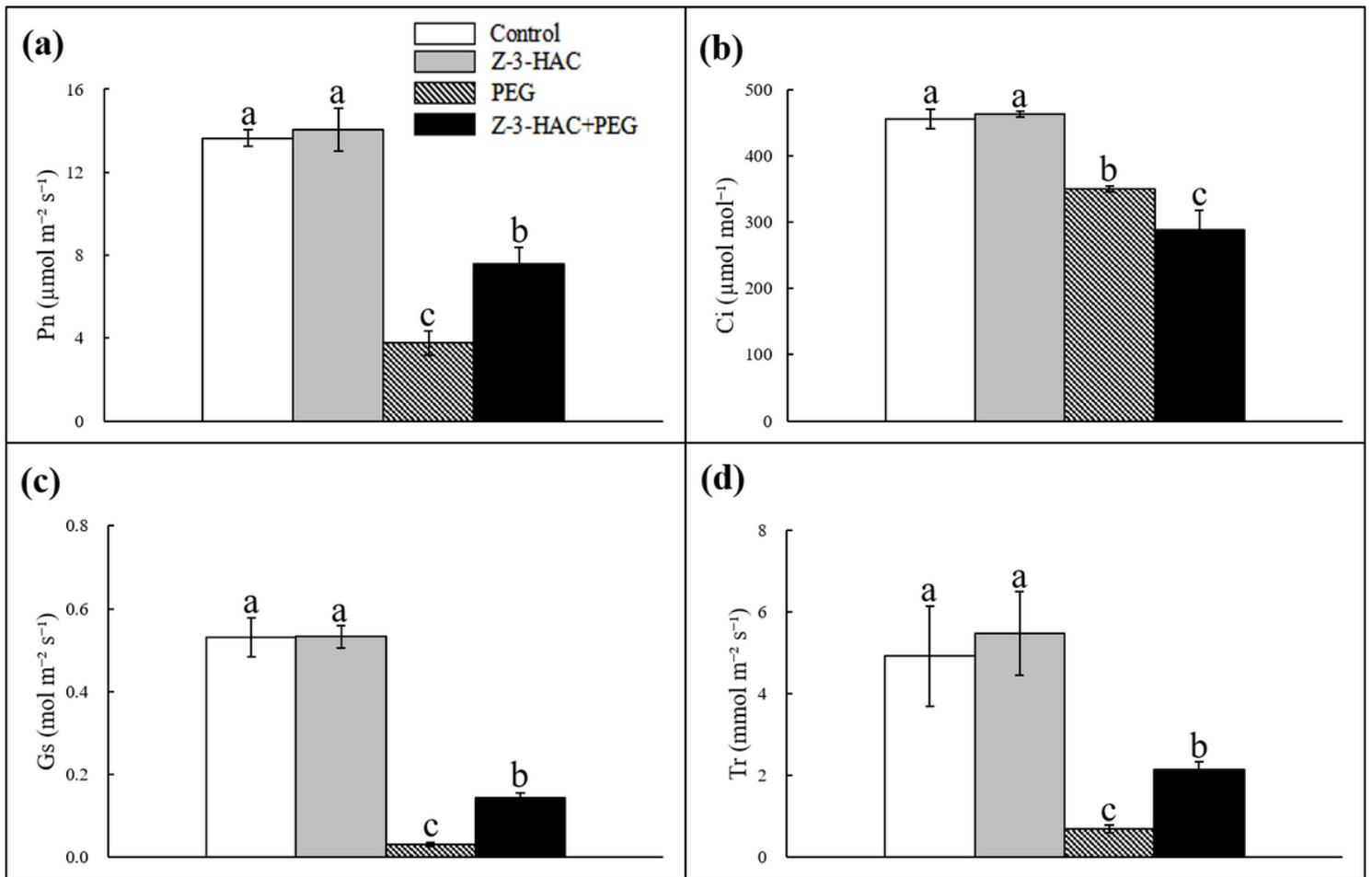
**Figure 2**

Effects of Z-3-HAC on root morphology of the wheat seedlings under drought stress. (a) Representative picture for each treatment, (b) root average diameter, (c) total root length (RL), (d) total root surface area (RSA), and (e) total root volume (RV)



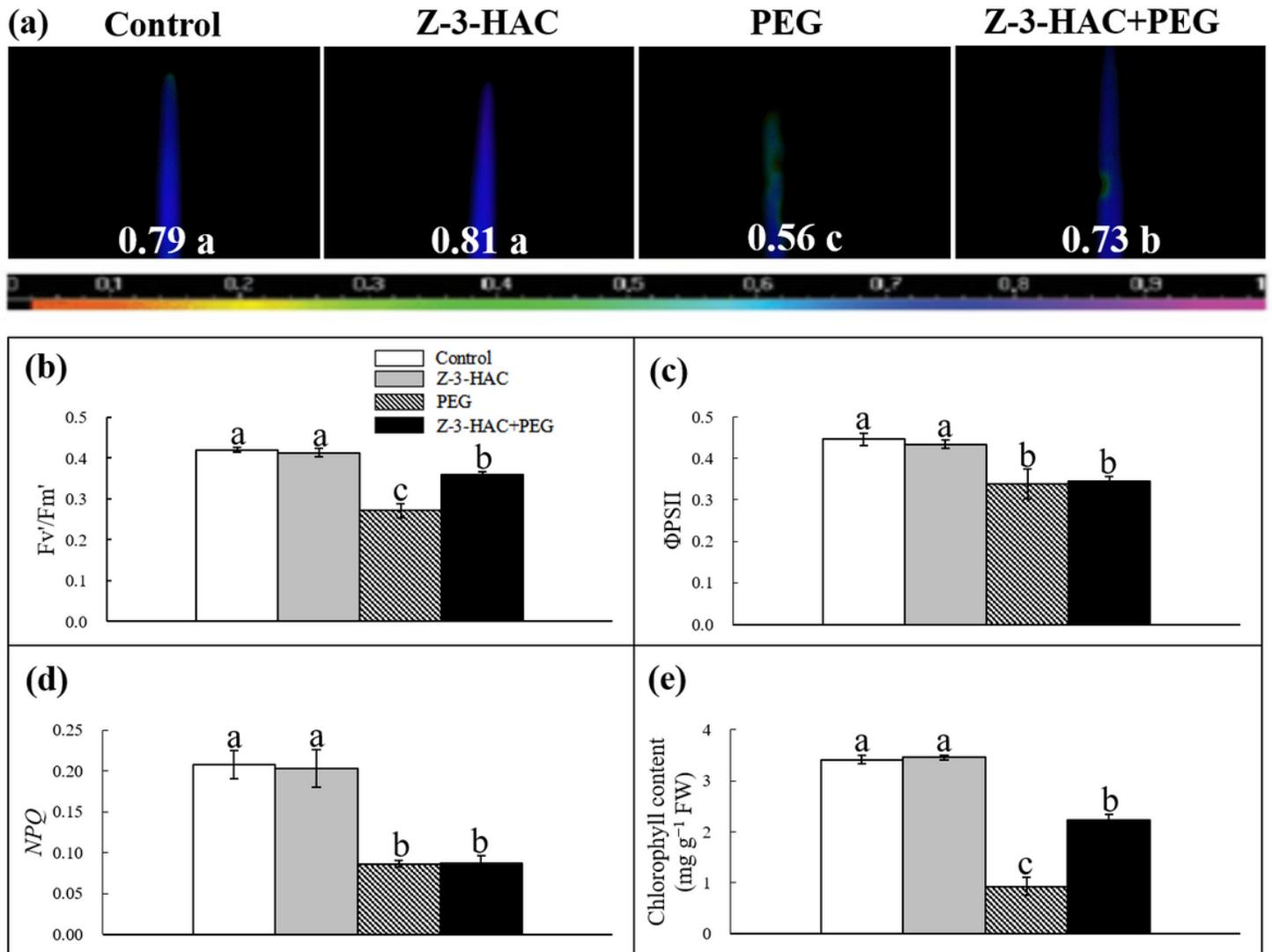
**Figure 3**

Effects of Z-3-HAC on RWC (a) and REC (b) of the fully expanded leaves in wheat seedlings under drought stress



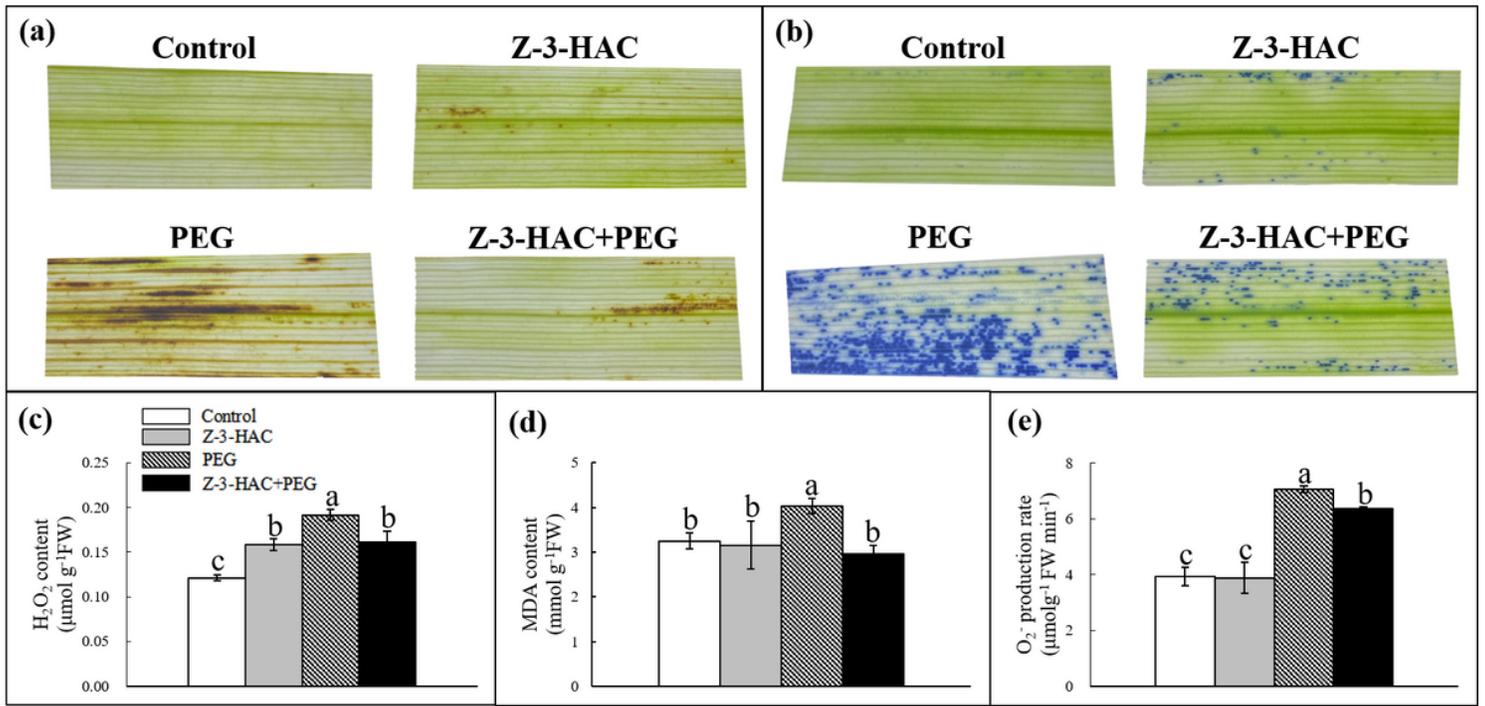
**Figure 4**

Effects of Z-3-HAC on gas exchange parameters of the fully expanded leaves in wheat seedlings under drought stress. (a) Net photosynthetic rate (Pn), (b) intercellular CO<sub>2</sub> concentration (Ci), (c) stomatal conductance (Gs), and (d) transpiration rate (Tr)



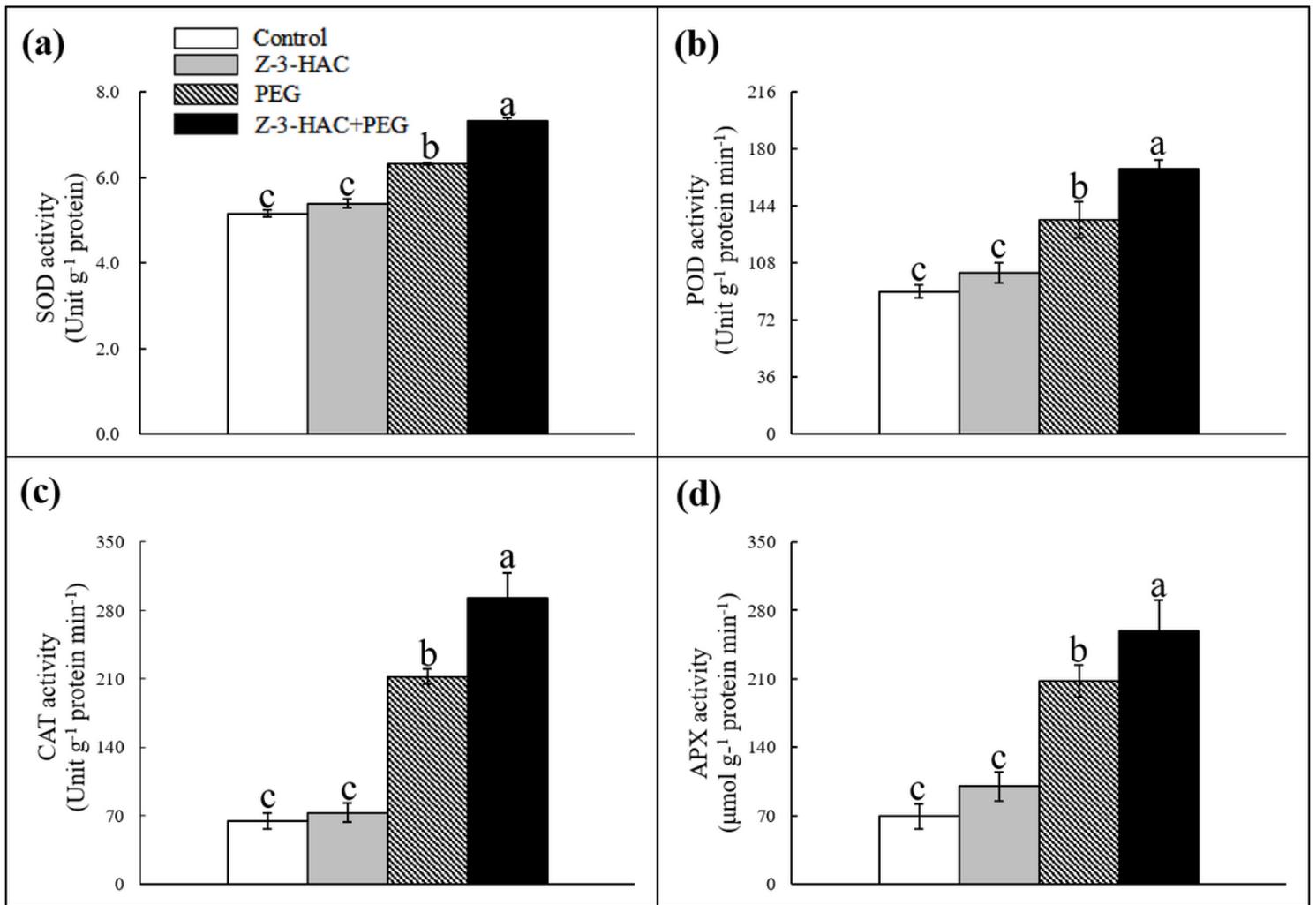
**Figure 5**

Effects of Z-3-HAC on chlorophyll fluorescence parameters and total chlorophyll content of the fully expanded leaves in wheat seedlings under drought stress. (a) The maximal photochemical efficiency of photosystem II (PSII) ( $F_v/F_m$ ). The false color code depicted at the bottom of the image ranges from 0 (black) to 1 (purple). The  $F_v/F_m$  values are depicted at the bottom of each image. (b) the photochemical activity of PSII ( $F_v'/F_m'$ ), (c) the quantum yield of PSII ( $\Phi_{PSII}$ ), (d) the nonphotochemical quenching (NPQ), and (e) the total chlorophyll content expressed in  $\text{mg g}^{-1}$  fresh weight



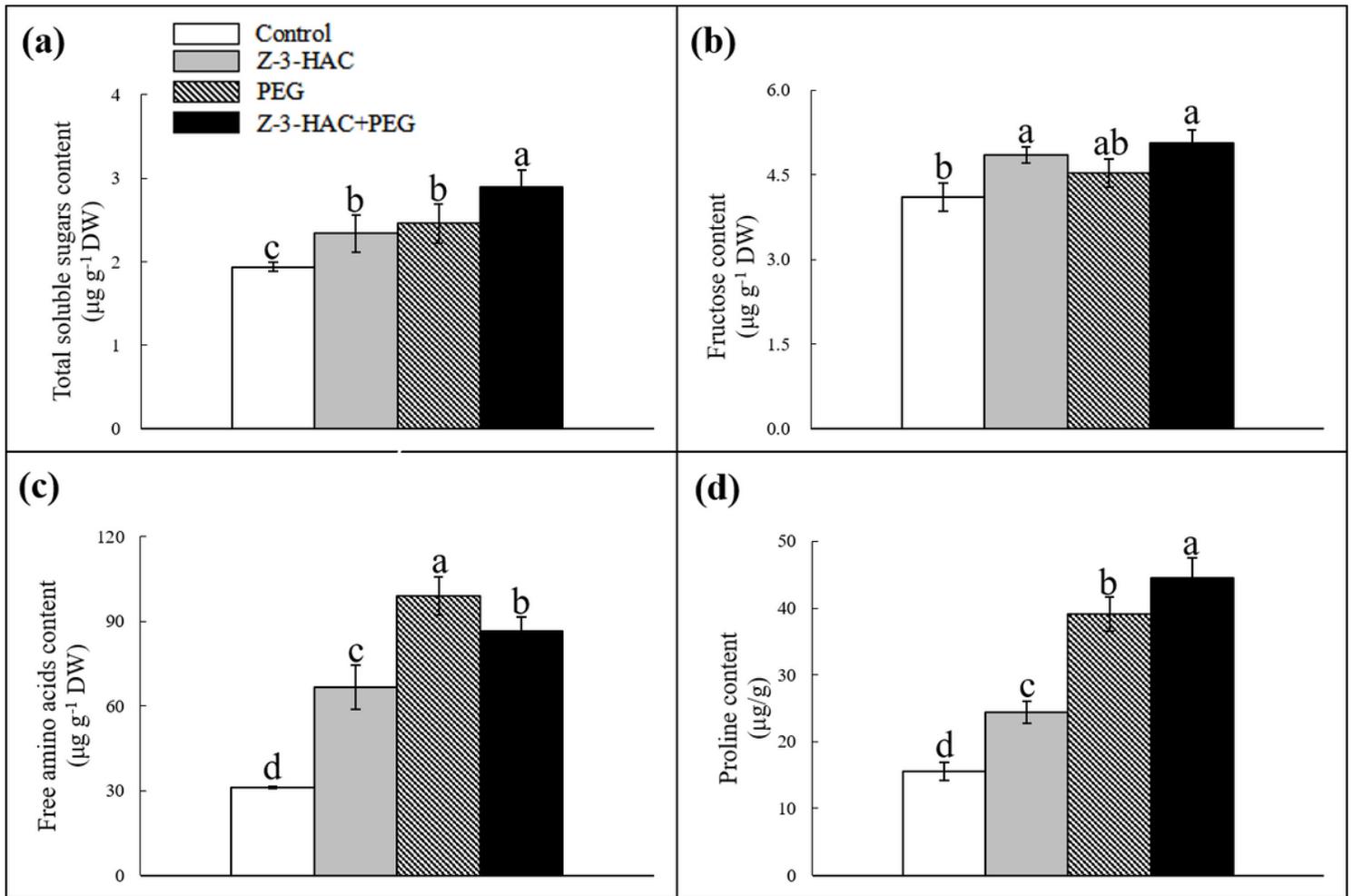
**Figure 6**

Effects of Z-3-HAC on the accumulation of H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, and content of MDA of the fully expanded leaves in wheat seedlings under drought stress. (a) DAB staining reaction reflecting H<sub>2</sub>O<sub>2</sub> accumulation, (b) NBT staining reaction reflecting O<sub>2</sub><sup>-</sup> accumulation, (c) H<sub>2</sub>O<sub>2</sub> content, (d) MDA content, and (e) O<sub>2</sub><sup>-</sup> content



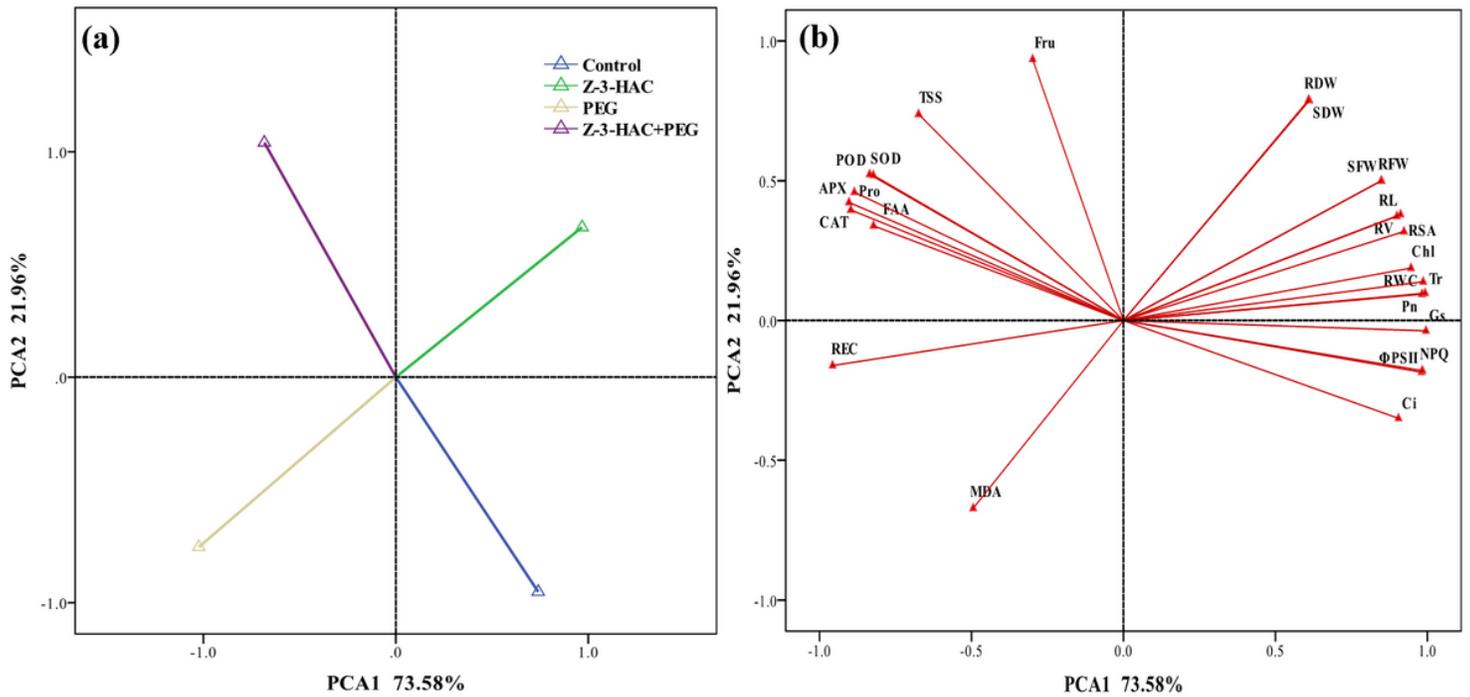
**Figure 7**

Effects of Z-3-HAC on the activities of the antioxidant enzymes of the fully expanded leaves in wheat seedlings under drought stress. (a) superoxide dismutase (SOD), (b) peroxidase (POD), (c) catalase (CAT), and (d) ascorbate peroxidase (APX)



**Figure 8**

Effects of Z-3-HAC on osmolytes concentrations of the fully expanded leaves in wheat seedlings under drought stress. (a) total soluble sugars (TSS), (b) fructose (Fru), (c) free amino acids (FAA), and (d) proline (Pro)



**Figure 9**

(a) Statistical analysis score diagram for four treatments based on all parameters. (b) Statistical analysis score diagram of the detailed parameters. Direction represents the correlation between features and length represents the strength of correlation. Partial abbreviations: SFW, shoot fresh weight; SDW, shoot dry weight; RFW, root fresh weight; RDW, root dry weight; RL, total root length; RSA, total root surface area; RV, total root volume; Chl, total chlorophyll content