

INTERACTIVE EFFECTS OF ABSCISIC ACID (ABA) AND DROUGHT STRESS ON THE PHYSIOLOGICAL RESPONSES OF WINTER WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

Water deficit is confirmed to be the most destructive factor in abiotic stress, which can inhibit crop growth. Here we examined the effect of exogenous applied 10 μ M abscisic acid (ABA) on the physiological and biochemical responses to water deficit in winter wheat (*Triticum aestivum* L.) over a 12-day period at seedling stage. ABA application by root irrigation was transported to the aboveground part to increase the leaf ABA accumulation, which led to the decrease of leaf stomatal conductance (G_s) to reduce water loss. Drenching the soil with ABA triggered an early closure of stomata in the leaves of winter wheat, resulting in broadened the soil water content (SWC) threshold range of non-hydraulic root-sourced signal (nHRS). Exogenous application of ABA also decreased the leaf hydrogen peroxide (H_2O_2) content and increased SOD, CAT and POD activities, leading to reduce the damage of free radicals accumulation on the structure of cell membrane system in wheat plants. Collectively, these results suggested that exogenously applied ABA under drought stress confer growth promoting and stress relieving effects on wheat plants.

Key words: Abscisic acid; Drought stress; Non-hydraulic root-sourced signal; Wheat.

Introduction

Water deficit is confirmed to be the most destructive factor in abiotic stress, which can limit crop yield and quality. Wheat (*Triticum aestivum* L.) is one of the main grain crops, which is extensively cultivated all over the world, especially in drought conditions (Cheng *et al.*, 2016). Crops have formed many complex mechanisms to deal with water deficit in the long-term evolution, including reducing water loss by closing stomata, osmotic adjustment, accumulation of antioxidative enzymes and synthesis of substance (Wang *et al.*, 2008; Zhou *et al.*, 2015).

Many researches have described many physiological and molecular mechanisms of crop responses to water deficit. An early response is to close the stomata by promoting the biosynthesis of abscisic acid (ABA) without causing a significant change in leaf water status under water shortage (Lv *et al.*, 2019; Du *et al.*, 2012). This phenomenon is usually defined as non-hydraulic root-sourced signal (nHRS) which is considered as a unique positive early warning for plants to cope with water deficit (Davies *et al.*, 2005; Xiong *et al.*, 2006). ABA is an important regulator factor of plant response to water shortage (Davies *et al.*, 2005; Zhang & Davies, 1990). Plant roots sensed the drying soil and produced signals, and transmitted them up the xylem to regulate leaf stomatal closure, so as to adjust plant water efficiency by reducing plant transpiration (Saradadevi *et al.*, 2017; Xiong *et al.*, 2006). The improved leaf ABA concentration was related to the decreased leaf stomatal conductance (G_s) under water deficit has been confirmed in wheat (Caverzan *et al.*, 2016; Du *et al.*, 2013). The transient increase of ABA level can also stimulate many downstream stress responses under water-stressed (Hossain *et al.*, 2012), such as inducing and expressing a great many specific drought resistance genes and protective proteins, so as to improve the drought

resistance ability of plants (Reddy *et al.*, 2004). These drought stress response genes and proteins were primarily involve with signal transduction and transcriptional regulation, antioxidant enzyme system and reactive oxygen species (ROS) scavenging (Shinozaki *et al.*, 2007). Water deficit led to excessive ROS formation, which damaged to the structure and function of cell membrane system, the synthesis of proteins and other macromolecules in plants (Impa *et al.*, 2012). Consequently, plants need to rapidly eliminate the accumulation of ROS to protect cells from oxidative damage under water deficit.

Plants have a protective enzyme system under water deficit, which is composed of many enzymes and reducing substances, among which superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are main antioxidant enzymes, which can eliminate the excess free radicals and prevent accumulation in plants, so as to improve the tolerance of plants to drought (Hossain *et al.*, 2012). It was found that ABA was one of the important substances to reduce oxidative damage by activating antioxidant defense mechanism in maize seedlings under water deficit (Jiang & Zhang, 2002; Wei *et al.*, 2015). Under water deficit, ABA content and water status in plant leaves played a crucial role in regulating the balance between ROS and anti-oxidant defense system, which was also related to the production of nHRS (Jangpromma *et al.*, 2010). In particular, the production of ABA and ROS can induce the expression of stress response genes related to osmoregulation synthesis, antioxidant and ROS scavenger accumulation, so as to prevent plants from damage caused by water deficit (Koh *et al.*, 2015; Hu & Xiong, 2014). However, when drought stress continued to increase beyond the tolerance of plants, the antioxidant enzyme system collapsed, leading to the accumulation of ROS and the normal metabolism of cells was destroyed, thus the growth of plants was inhibited (Wei *et al.*, 2015).

In the present study, we applied ABA by root irrigation to evaluate whether it involved in leaf *Gs* and LRWC, the production of nHRS, and the equilibrium between ROS and antioxidant enzyme system in winter wheat at seedling stage under water deficit.

Materials and Methods

Plant materials and growth conditions: The experiment was carried out in a controlled plant growth chamber in Anhui Agriculture University, Hefei, China. The growth conditions of the plant were controlled by the photoperiod 14h (07:00-21:00 h BST), photo flux density $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, and day/night temperature 25/20°C. The winter wheat (*Triticum aestivum* L) cultivar, Changwu 131 was selected as experiment material in this experiment. Seeds were obtained from the Northwest Agriculture and Forestry University, Xi'an, China. The selected wheat seeds which had good quality with full grain and neat consistency were disinfected with 2% sodium hypochlorite solution for 20 min, and then flushed with ultra-pure water for 4-5 times. After that, the wheat seeds were sown in plastic pots with diameter of 26 cm and height of 30 cm filled with 2.5 kg of mixture sieved soil (dry soil: vermiculite = 2:1, v/v) which was sampled from the upper 300 mm cultivated layer of a field site at Nongcuiyuan Experiment Station of Anhui Agriculture University, Hefei, China. Then dried first, screened out stones and gravels through a 4 mm sieve and mixed with vermiculite. The filed capacity (FC) was calculated before sowing and 1 L ($5.42 \text{ g L}^{-1} \text{ NH}_4\text{NO}_3$ and $2.32 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$) were will mixed with the soil. Fifteen wheat seeds were sown in 3-4 cm soil in each plastic pot and thinned to ten plants 15 days after sowing (DAS).

Water-stressed and abscisic acid (ABA) treatment: The soil water content (SWC) of all pots were weighted and watered 2-3 days to maintain at 90% FC until water stress treatment were carried out on 30 DAS: (i) one third of these pots were weighed and watered every day to keep the SWC close to 90% FC (well-watered, WW); (ii) another one third of these pots stopped watering, so that the SWC decreased slowly for the subsequent 12d (water-stressed, WS); (iii) the remaining one third of the pots were also not watering and treated with ABA (Sigma-Aldrich, Steinheim, Germany) one day before the imposition of the watering treatments (water-stressed and ABA-treated, WS+ABA). ABA was dissolved with distilled water and sprayed into the soil ($10 \mu\text{M}$ ABA soil concentration) (Du *et al.*, 2013). Therefore, there were three treatments: WW (control), WS and WS+ABA. Once the water stress treatment was imposed, SWC, leaf stomatal conductance (*Gs*) and leaf relative water content (LRWC) of all pots were measured regularly every day.

The wheats were sampled six times during the water-stressed treatment: H1, the first day after water-stressed; H2, the *Gs* decreased significantly but no decreasing of LRWC found; H3, 6 days after water-stressed; H4, LRWC decreased significantly; H5, 10 days after water-stressed; and H6, permanent wilting was observed. The permanent wilting was recognized as the leaf began to wilt in the daytime, but failed to recover overnight after rehydration (Fan *et al.*, 2008). Each treatment consisted of

three biological replicates. After the determination of leaf *Gs* and LRWC, the fully expanded leaves were collected, frozen in liquid N_2 immediately, and stored in an ultra-low temperature refrigerator at -80°C for the subsequent physiological and biochemical analysis. All Pots were weed free and moved every 2-3 days to minimize the impact of location effects.

Determination of soil water content (SWC)

According to Xiong *et al.*, (2006), SWC was calculated as follows:

$$\text{SWC} = (W_t - W_d - W_e - W_p) / (W_d \times \text{FC}) \times 100\%$$

W_t was temporary pot weight; W_d was dried soil weight; W_e was weight of empty pot weight; W_p was fresh plants weight in each pot; FC was field capacity.

Leaf stomatal conductance (*Gs*): It was measured by LI-6400 portable photosynthesis system (LiCor Company, USA). A fully unfolded leaf was selected from per pot and measured under good light exposure at 09.00 to 11.00 am every day from sufficient water supply (90% FC) to permanent wilting nearby 12 days after water was withheld.

Leaf relative water content (LRWC): It was also measured from sufficient water supply (90% FC) to permanent wilting nearby 12 days after water stress. The most expanded youngest leaves were chosen to weigh immediately for fresh weight (FW) from completely randomly selected wheats. Saturated weight (DW) was measured as the plants were soaked with distilled water in dark for 8 h, then dried the surface water with absorbent paper. Dry weight (DW) was measured as the plant leaves after drying in an oven at 80°C for 24h. The LRWC was calculated as follows:

$$\text{LRWC} = [(\text{FW} - \text{DW}) / (\text{SW} - \text{DW})] \text{ (Xiong } et al., 2006)$$

Hydrogen peroxide (H_2O_2) content and activities of antioxidant enzymes: The 0.5 g fresh leaves were fully ground with liquid N_2 , added with 5 ml pre-cooled 50 mM phosphate buffer (PBS) (pH 7.8) containing 1% polyvinyl pyrrolidone to grind into homogenate. The homogenate was fully shaken and extracted at 4°C for 1 h. The supernatant was taken and sub packed after centrifugation at $12000 \text{ g } 4^\circ\text{C}$ for 20 min, and stored at -20°C for determination of hydrogen peroxide and enzyme assays (Xi *et al.*, 2010). The following indicators were measured with the kits produced by the Nanjing Jiancheng Bioengineering Institute.

Total soluble protein: Was determined based on the amino-binding of the anions on the Coomassie brilliant blue dye to the protein molecules, which makes the solution of Coomassie brilliant blue change from brown to blue, and was calculated by the absorbance of 595 nm.

Hydrogen peroxide (H_2O_2): Content was mainly through the interaction with molybdcic acid to form a complex, which was calculated by its absorption at 450 nm.

Catalase (CAT): Activity was determined by reaction of decomposing H_2O_2 to stop rapidly after adding ammonium molybdate which reacted with the remaining H_2O_2 to form a kind of yellowish complex, and calculated by its absorption at 405 nm.

Total superoxide dismutase (SOD): Activity was measured by repression of enzyme extract on superoxide anion free radicals, which reduced the formation of nitrite, resulting in the absorbance value at the wavelength of 550 nm lower than that of the control. The amount of SOD corresponding to the inhibition of 50% of SOD in 1 ml of reaction solution per mg of plant leaf protein is a SOD activity unit.

Peroxidase (POD): Activity was determined by the principle of catalyzing hydrogen peroxide reaction with peroxidase, and calculated by measuring the change of absorbance at 420 nm.

Activities of antioxidant enzymes were expressed as units (s) (mg^{-1}) protein.

Leaf ABA content: The 0.5 g fresh leaves were fully ground with liquid N_2 , added with 5 ml pre-cooled 80% methanol solution containing 1mM butylated hydroxytoluene to be fully ground into homogenate. The homogenate was transferred to another clean tube, fully shaken and extracted overnight at 4°C. After centrifugation at 1000 g 4°C for 20 min, the supernatant was changed to another clean tube, and then 1ml of the extract was added to the precipitation and extracted for another 1 h. After 15 min at the same condition, the supernatant was combined in the same tube and the volume was recorded, and the remaining residue was discarded. The combined supernatants passed through C18 chromatographic columns (Millford, MA, USA), pre-balanced with 10 ml 80% methanol, then washed with 5 ml 80% methanol after treatment. The liquid after passing the column was transferred into a new centrifuge tube, vacuum concentrated or dried by nitrogen to remove the methanol. The residues were fully dissolved with 1.5 ml PBS containing 0.1% Tween 20 and 0.1% gelatin (pH 7.5), and then measured by enzyme-linked immunosorbent assay (ELISA) produced by China Agricultural University, Beijing, China (Bollmark *et al.*, 1988; He 1993).

Statistical analyses

All statistical analyses were performed using SPSS 17.0 program by one-way analysis of variance (ANOVA). Significant differences among different treatments were presented at $p < 0.05$. The 'broken stick' regression was carried-out for calculate the threshold of SWC.

Results

The SWC and leaf ABA content of winter wheat: By weighing and watering the pots every day, the soil water content (SWC) in WW treatment was always close to 90% FC, while the SWC in WS and WS+ABA treatments gradually decreased from 90% FC at the beginning to ~20% FC for 12 days after water-stressed, which began to decrease rapidly and then decreased slowly in the last 4 days (Fig. 1).

Withholding water increased leaf ABA concentration in WS and WS+ABA treatment from 20.5 and 23.1 nmol g^{-1} FW at the beginning (90% FC) to 60.5 and 63.1 nmol g^{-1} FW when the SWC decreased to ~20% FC on the 12th day after water-stressed, respectively (Fig. 2). Exogenous ABA application significantly promoted ABA accumulation in leaves, especially when the SWC decreased between 65% FC and 30% FC (Fig. 2). The leaf ABA concentration between WS and WS+ABA treatment had no significant differences when the SWC decreased to ~20% FC (Fig. 2).

Effect of ABA on leaf conductance (G_s) and leaf relative water content (LRWC): Leaf G_s of WW-treated wheats always maintained at 0.12-0.14 $\text{mol m}^{-2}\text{s}^{-1}$, however, G_s of WS and WS+ABA plants decreased with the time after the soil withheld (Fig. 3A). Drenching the soil with ABA significantly changed leaf G_s ($p < 0.05$). Leaf G_s of WS+ABA treatment decreased on the fourth day after water was withheld and was 1 day earlier than WS treatment (Fig. 3A). This suggested that ABA caused an early decrease in leaf G_s , which increased the sensitivity of wheats to water stress. However, the LRWC did not change significantly when G_s decreased at this point ($p > 0.05$) (Fig. 3B). The leaf G_s continued to decrease as the soil dried and then LRWC gradually began to decline (Fig. 3B). The LRWC decreased significantly on the ninth day after water was withheld in both WS and WS+ABA treatment, but WS+ABA treatment was 6.0% higher than WS treatment (Fig. 3B).

The nHRS is generally defined as when leaf G_s decreases significantly while plant leaf water status does not change significantly during the gradual drying soil. Compared with WW, the SWC thresholds of leaf G_s and LRWC were calculated by linear-plateau functions (Fig. 4A, 4B). Leaf G_s and LRWC decreased with SWC when SWC was lower than a threshold value. The fitting results showed that SWC thresholds for leaf G_s were 62.4% and 75.5% (Fig. 4A) and for LRWC were 34.5% and 36.7% (Fig. 4B) in WS and WS+ABA treated plants, respectively (Fig. 4C). According to the calculation, the SWC threshold ranges of nHRS in WS and WS+ABA treatments were 27.9% FC and 38.8% FC, respectively (Fig. 4C). Exogenous ABA application significantly broadened the SWC threshold ranges of nHRS. Therefore, exogenous ABA could decrease the leaf G_s to reduce leaf water loss by broadening the SWC threshold ranges of nHRS, so as to improve the drought resistance of winter wheat. The results showed that exogenous ABA triggered an early closure of stoma in the leaves of winter wheat, resulting in a decrease in leaf G_s .

Hydrogen peroxide (H_2O_2) content and actives of antioxidant enzymes: Soil drying increased leaf H_2O_2 concentration slightly when the soil dried to 40%-50% FC and then increased quickly when SWC decreased below 35% FC (Fig. 5). Exogenous applied ABA induced the accumulation of H_2O_2 when SWC decreased below 50% FC, but it was different from that of WS treatment plants (Fig. 5). The leaf H_2O_2 content in WS+ABA treatment was lower than that of WS treatment under the same soil water gradient, especially when SWC decreased to 25% FC, which decreased by 14 mmol gprot^{-1} (Fig. 5). Therefore, the application of ABA inhibited the leaf H_2O_2 concentration of wheat under drought stress.

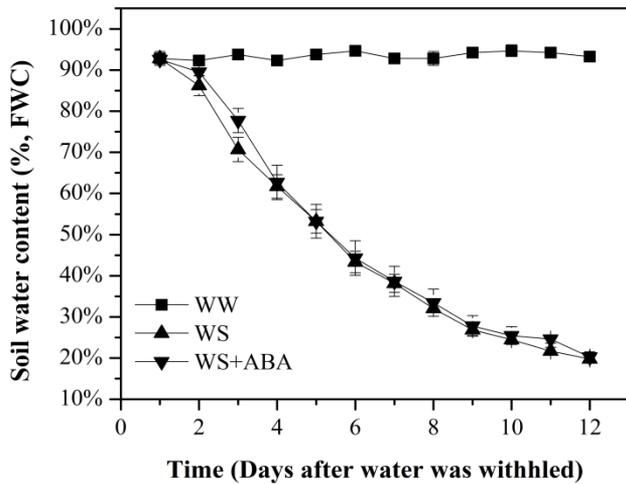


Fig. 1. Changes in soil water content (SWC) of winter wheat in well-watered (WW) treatment, water-stressed (WS) treatment and drenched soil with ABA under water-stressed (WS+ABA) treatment with time after water was withheld. Values are means \pm standard error of the mean ($n = 3$).

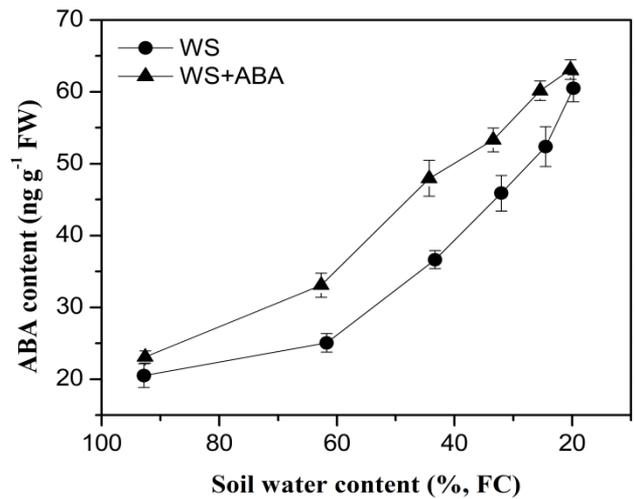


Fig. 2. Changes of leaf ABA concentration of winter wheat after drenched with 10 μ M ABA and without ABA during progressive soil drying. Values are means \pm standard error of the mean ($n = 3$).

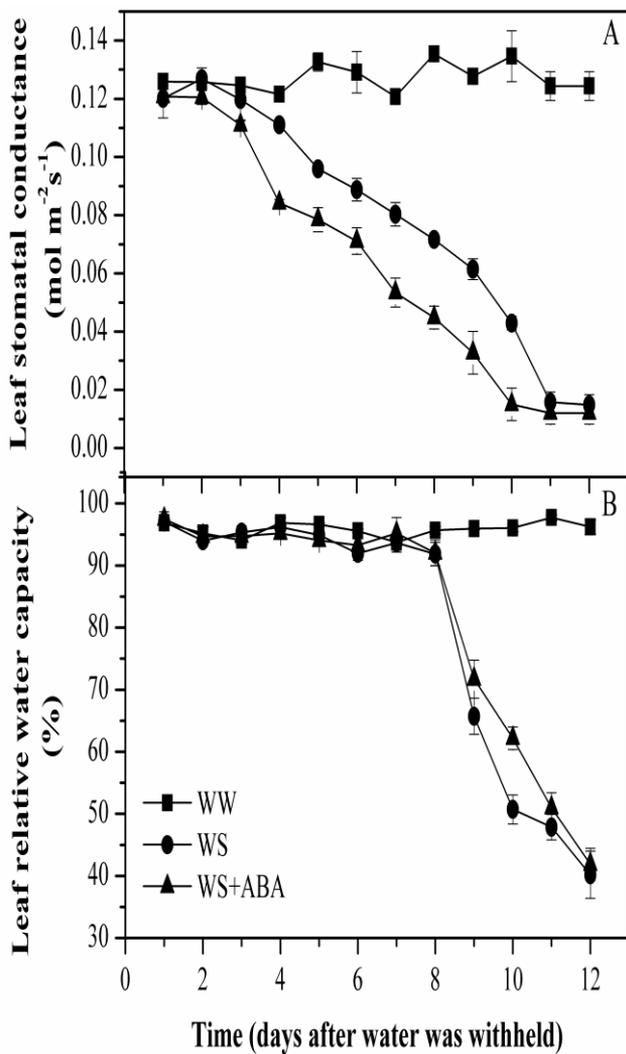


Fig. 3. After treated with 10 μ M ABA, stomatal conductance (G_s) and leaf relative water content (LRWC) of winter wheat changed with time (days after water was withheld) under well-watered (WW) and water-stressed (WS). Values are means \pm standard error of the mean ($n = 3$).

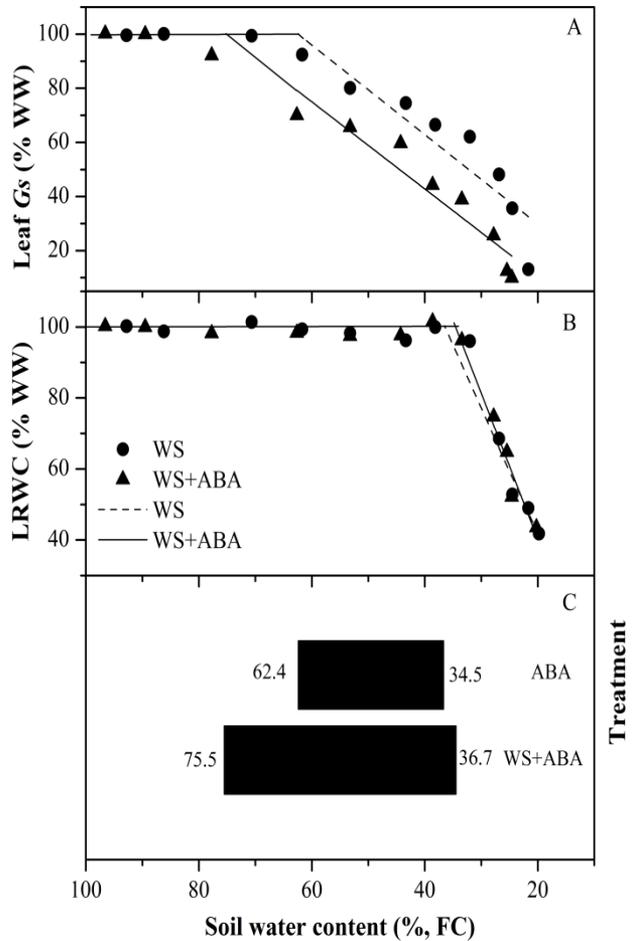


Fig. 4. Relationships between stomatal conductance (G_s , % WW) and soil water content (SWC, % field capacity, FC) (A); leaf relative water content (LRWC, % WW) and SWC (% FC) (B), and the changes in SWC threshold between the threshold for a decrease in G_s and the threshold for a decrease in LRWC for winter wheat (C). Arrows indicate the threshold values which are summarized in C. In C, values left the black bars are the threshold values for a decrease in G_s from A and those rights of the black bars are the threshold values for a decrease in LRWC from B.

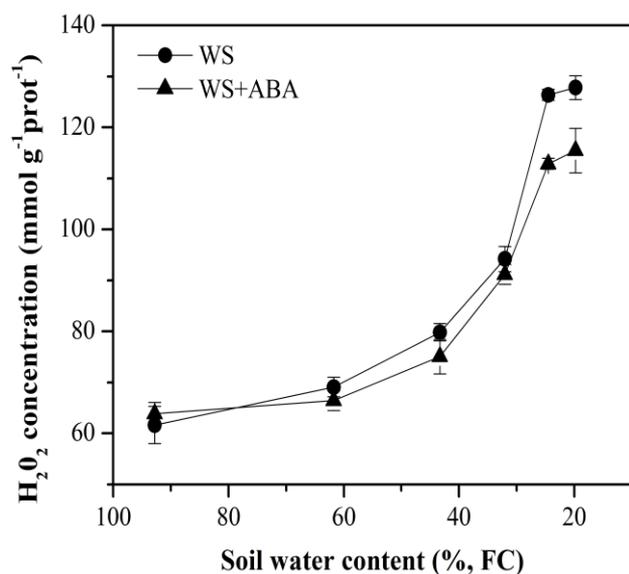


Fig. 5. The effects of exogenous ABA and without ABA on hydrogen peroxide concentration (H_2O_2) in leaves of winter wheat during progressive soil drying. Values are means \pm standard error of the mean ($n=3$).

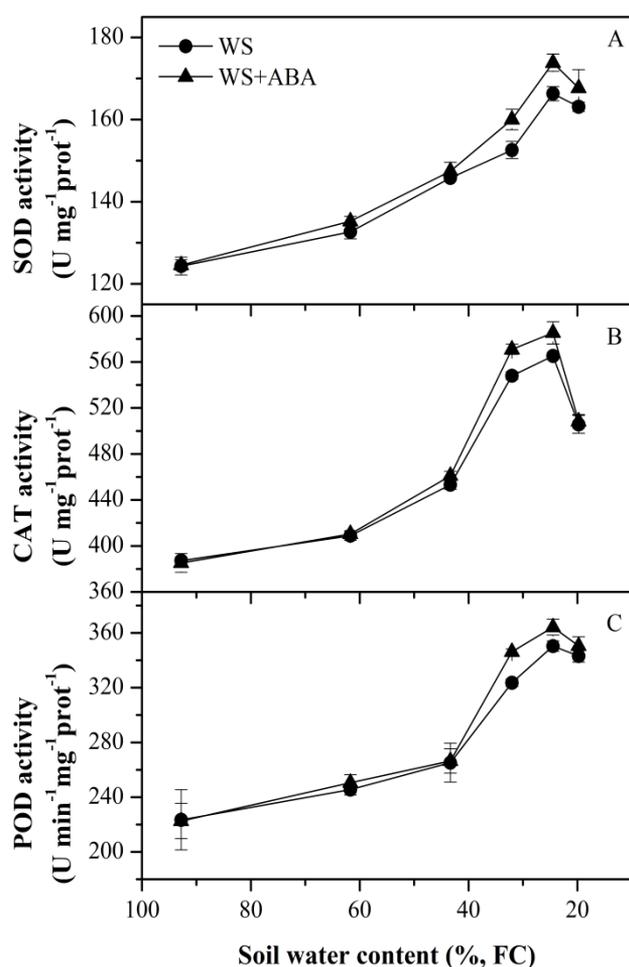


Fig. 6. The effects of exogenous 10 mM ABA (ABA+WS) and without ABA on the activities of the antioxidant enzymes: (A) superoxidase dismutase (SOD), (B) catalase (CAT), and (C) peroxidase (POD) in leaves of winter wheat during progressive soil drying. Values are means \pm standard error of the mean ($n=3$).

The SOD, CAT and POD activities in leaves had no significant changes when SWC was above 50% FC, but increased sharply at lower SWC, especially when SWC was below 35% FC (Fig. 6). Drenching the soil with ABA increased the activities of SOD, CAT and POD in leaves when SWC decreased below 40% FC (Fig. 6). However, there was no significant changes of SOD, CAT and POD activities between WS and WS+ABA treated plants when SWC decreased to \sim 20% FC (Fig. 6).

Discussion

The study was conducted to insight the effect of ABA on the physiological and biochemical responses to water deficit in winter wheat. Many researches have shown that the chemical signals of root synthesis can be transported to the above-ground part of plants over a long distance to regulate the change of leaf *Gs* under mild drought stress (Xiong *et al.*, 2006; Du *et al.*, 2012; Batool *et al.*, 2019a). ABA, as one of the root signaling substances, played an crucial role in regulating wheat response to water-stressed (Ren *et al.*, 2007; Saradadevi *et al.*, 2017). Soil drying can induce plant roots to produce a large amount of ABA, which can be transported to the above-ground part along with transpiration of xylem juice (Parent *et al.*, 2009). It was mainly responsible for regulating stomatal closure in non-hydraulic root signaling stage, and also can reduce the growth rate of plant canopy (Ren *et al.*, 2007). Other results have also confirmed upward ABA can regulate stomatal guard cells and reduce plant transpiration (Du *et al.*, 2013; Saradadevi *et al.*, 2017). ABA synthesized may interact with other substances in xylem sap to regulate leaf stomatal closure (Chen *et al.*, 2018). The present study confirmed exogenous applied ABA increased the leaf ABA content of wheat and promoted the production of nHRS earlier than without ABA treatment (Figs. 1, 4). Our results witnessed the application of ABA in root irrigation promoted the ABA content in leaves, which resulted in the early closure of stomata in leaves, and thus broadening the threshold range of soil moisture under nHRS. ABA can maintain the leaf water status by increasing the stomatal behavior of the leaves. Exogenous applied ABA further affects the sensitivity of winter wheat to leaf water content, and has a long-term regulatory effect on stomatal behavior of winter wheat, and also affects stomatal response to drought especially after nHRS stimulation (Ma *et al.*, 2015). Therefore, ABA, as one of the root signaling substances, played a key role in inducing stomatal closure and nHRS production in winter wheat under water deficit.

Many studies have confirmed that water deficit induced ROS formation in plants, such as the hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) (Liu *et al.*, 2009; Borges *et al.*, 2014). H_2O_2 played an crucial role in signal transduction of leaf guard cells. The excessive accumulation of H_2O_2 in plants damaged the cell membrane, protein and other macromolecular substances, led to the inhibition of the plant normal growth (Li *et al.*, 2011). The results confirmed that exogenous applied ABA increased the leaf H_2O_2 content of wheat when SWC decreased below 50% FC. The leaf H_2O_2 concentration in WS+ABA treatment was lower than that of WS treatment

under the same soil water gradient, especially when SWC decreased to 25% FC. Exogenous applied ABA alleviated the damage of wheat seedlings by reducing the leaf H₂O₂ concentration under water shortage.

The effect of ABA in plants under drought has been widely studied, mainly focusing on its physiological and molecular mechanisms (Ferrandino and Lovisolo, 2014; Li *et al.*, 2018). Application of exogenous ABA increased leaf SOD, CAT and POD activities of plants, so as to enhance the ability of scavenging free radicals in plants under water-stressed (Wang *et al.*, 2011; Zhang *et al.*, 2015). Drenching the soil with ABA improved SOD, CAT and POD activities compared to without ABA treatment, especially when SWC decreased below 40% FC (Fig. 6), which inhibited the leaf H₂O₂ concentration of wheat under drought stress (Fig. 5). Therefore, ABA can maintain the balance of ROS and antioxidant system. The results were similar to other experiments conducted in wheat under drought (Cheng *et al.*, 2016; Batool *et al.*, 2019b). Many studies have confirmed the changes of antioxidant enzyme activities under drought stress may be related to the expression of stress genes (Fang *et al.*, 2016; Zhang *et al.*, 2015). Our results will provide some important information for further molecular research.

Conclusions

Exogenous application ABA increased the leaf ABA concentration, decreased the leaf stomatal conductance (*G*_s) and the leaf relative water content (LRWC) under drought stress. During the water stress, the nHRS of winter wheat caused by ABA application appeared at a higher soil water capacity (SWC), which widened the SWC threshold range of nHRS. Drenching the soil with ABA also increased SOD, CAT and POD activities and reduced the hydrogen peroxide (H₂O₂) formation, so as to maintain the balance of active oxygen, protect the membrane structure, and improve the ability of wheats to resist drought stress. Our results can provide particular information on the molecular mechanisms of ABA regulating nHRS generation in plants under drought stress.

Author contribution statement: Hai-Yan Kong conducted the experiment, analyzed the data and drafted the manuscript. Nudrat Aisha Akram modified the manuscript and improved the language of the manuscript. Juan Qin and Zhen Zhang helped in conducting the experiment.

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