Arch Biol Sci. 2018;70(2):379-386 https://doi.org/10.2298/ABS170722056L

20-Hydroxyecdysone protects wheat seedlings from salt stress

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Received: July 22, 2017; **Revised**: November 14, 2017; **Accepted**: December 11, 2017; **Published online**: December 22, 2017

Abstract: 20-Hydroxyecdysone (20E), a molting hormone of insects, is the most abundant phytoecdysteroid (PE) produced by plants, where it represents a protective molecule against insect herbivores. The objective of the investigation is to determine the effect of 20E on the growth, physiological and biochemical characteristics and the transcript levels of antioxidant enzymes of wheat seedlings under salt stress. Our results showed that exogenous 20E was able to significantly alleviate salt-induced oxidative damage in wheat seedlings. It most likely acts by decreasing the concentration of malondialdehyde (MDA) and the rate of superoxide radical ($O_2^{\bullet-}$) generation, and by increasing the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), concentrations of ascorbic acid (AsA) and glutathione (GSH), and the gene expression levels of *SOD*, *POD*, *CAT*. It was suggested that foliar spraying with 20E could efficiently protect wheat seedlings against salt-induced oxidative stress. The results also show that 20E had a positive physiological effect on the growth of salt-stressed seedlings. This is the first report dealing with the effect of 20E pretreatment in the enhancing of wheat seedling tolerance under salt stress.

Key words: *Triticum aestivum*; 20-hydroxyecdysone; salt stress; oxidative stress; antioxidative system

INTRODUCTION

20-Hydroxyecdysone (20E) is one of the phytoecdysteroids (i.e. analogs of insect steroid hormones) found in the rhizomes, root and stems of many plants. 20E is also the ecdysteroid hormone of arthropods and many other invertebrates, where it regulates several physiological processes [1]. In plants, phytoecdysteroids, being physiologically active compounds, may affect morphological and physiological processes and protect plants against phytophagous insects and nematodes [2-5]. 20E has attracted much attention for its plant growth regulatory, cytotoxic and antioxidant properties [2,6]. Hu et al. [7] found that 20E could protect against oxidative stress-induced neuronal injury by scavenging free radicals and modulating NF-κB and JNK pathways. Lamhamdi et al. [8] have also shown that 20E treatment had a positive effect on the growth and metabolism of wheat seedlings against lead stress. However, little is known about the antioxidative effect of 20E on the seedlings of wheat (*Triticum aestivum* L.), one of the most commercial crops in the world, under both normal and salt-stressed conditions. Furthermore, it remains unclear whether 20E plays a significant protective role in ameliorating the influence of salt stress on wheat seedlings by increasing its salt tolerance.

Salinity is one of the major environmental factors that severely limit the growth and yield of crop plants worldwide because most crop plants are sensitive to salinity caused by high concentrations of salts in the soil [9-11]. Salt stress can induce several morphological, physiological and metabolic responses, and the accumulation of reactive oxygen species (ROS) in plant cells, which show toxicity after conversion to hydrogen peroxide (H_2O_2) [12,13]. To mitigate and repair the damage initiated by various ROS, plants have developed a wide range of nonenzymatic and enzymatic defense mechanisms to detoxify free radicals and help protect them from destructive oxidative stress [14]. Salt-tolerant plants, in addition to regulating ion and water homeostasis, should also possess an efficient antioxidant system for the effective removal of ROS. Studies have shown that salt tolerance is often correlated with a more efficient antioxidant

system [9,10,14]. Therefore, enhancing the activities of antioxidant enzymes and contents of nonenzymatic antioxidants in plants is necessary to counteract many environmental stressors, and can confer enhanced tolerance to unfavorable growth conditions.

The aim of this study was to investigate the role of 20E on wheat seedlings under salt stress by examining the transcript levels and enzymatic activities of SOD, POD, CAT and APX, the concentration of photosynthetic pigments, of AsA and GSH, and membrane injury. Results from this study are fundamental for understanding the antioxidative effect of 20E on wheat seedlings under salt stress.

MATERIALS AND METHODS

Plant materials and salinity treatments

Wheat seed samples of *Triticum aestivum* L. cv Zhoumai No.9023 were harvested from the Henan Academy of Agricultural Sciences. Growth was controlled by fluorescent illumination (800 μmol $m^{-2}·s^{-1}$) set to provide a 12 h photoperiod, at 60% humidity and 25°C/18°C (day/night temperatures, respectively). Hoagland solution served as a growth medium in Petri dishes (18 cm in diameter). The seeds were sterilized with 0.01% $HgCl_2$ for 2 min, followed by running water for another 10 min. One-week-old homogenous seedlings (with fully expanded leaves) served as experimental material for treatment with 20E and NaCl. Treatments were as follows: (i) distilled water (control); (ii) 150 mM NaCl; (iii) pretreatment with 3 μM 20E alone; (iv) pretreatment with 3 μM 20E, followed by 150 mM NaCl. 20E was purchased from Sigma-Aldrich (St. Louis, MO, USA). For 20E treatment, 3 μM of 20E was sprayed over the leaves two times at two-day intervals, while control plants received the same amount of distilled water. Three days after commencing the 20E treatments, 150 mM of NaCl was added to the Hoagland solution for seedlings with or without 20E treatment. The concentrations of 20E were selected based on a preliminary experiment, performed using different concentrations of 20E (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 μM), to examine where significant growth stimulation would be observed. The concentration of 3 μM of 20E was subsequently selected (data not shown). Five Petri dishes containing 80 seedlings each were regarded as a treatment group, and all experiments were independently repeated at least three times. On the 4th day of exposure to salt stress, wheat seedlings were collected and frozen instantly in liquid nitrogen and stored at -80°C until further use.

Analysis of growth and biomass

Root and shoot dry weights were estimated after oven-drying at 65°C to a constant weight. Plant height and root length were also gauged with a ruler.

Determination of malondialdehyde and hydrogen peroxide

Malondialdehyde (MDA) concentration was assayed using the thiobarbituric acid method described by Predieri et al. [15]. Wheat seedlings (0.2 g fresh weight – FW) were homogenized in phosphate buffer (50 mM, pH 7.8) and centrifuged for 10 minutes at 8000 × g. One mL of the supernatant was mixed with 2.5 mL of thiobarbituric acid for 20 min in a boiling water bath and cooled immediately on ice. Absorbance of the supernatant was measured at 532 nm and 600 nm after 5 min centrifugation at 10000 × g. After subtracting the nonspecific absorbance (600 nm), MDA concentration was measured by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and the results were expressed in μmol MDA g^{-1} FW.

Hydrogen peroxide was estimated according to Sergiev et al. [16]. Wheat seedlings (0.3 g FW) were homogenized in an ice bath with 3 mL 3% (w/v) trichloroacetic acid and centrifuged at 12000 × g for 15 min. One mL of the supernatant was added to 1 mL of 100 mM of potassium phosphate buffer (pH 7.0) and 2 mL of 1 M KI. The absorbance was measured at 390 nm and the content of H_2O_2 was calculated based on a standard curve of a known concentration of H_2O_2.

Determination of the rate of production of the superoxide radical ($O_2^{\bullet-}$)

The rate of $O_2^{\bullet-}$ production was measured according to Elstner and Heupel's [17] modified protocol. First, homogenized seeds (0.2 g) were mixed with phos-

phate buffer (1 mL, 50 mM, pH 7.8) and centrifuged at 10000 × g for 10 min. Next, 0.5 mL of phosphate buffer (50 mM, pH 7.8) and 0.1 mL of hydroxylamine hydrochloride (10 mM) were applied to 0.5 mL of supernatant for 1 h at 25°C. Finally, 1 mL of sulfanilamide (17 mM) and 1 mL α-naphthylamine (7 mM) were added to the reaction for another 20 min. The rate of $O_2^{\bullet-}$ generation was determined by the absorbance at 530 nm. Sodium nitrite was used as a standard solution to calculate the production rate of $O_2^{\bullet-}$.

Determination of antioxidative enzyme activities

For determination of antioxidant enzyme activities, 0.5 g homogenized seeds and 3 mL of ice-cold phosphate buffer (pH 7.8, containing 1 mM EDTA) were mixed and centrifuged at 15000 × g for 20 min (4°C). After centrifugation, the supernatant was used as a crude extract in which the activities of SOD, POD, CAT and APX were estimated.

SOD (EC1.15.1.1) activity was determined by the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) [18]. The reaction solution was composed of phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (130 mM), NBT (0.75 mM), riboflavin (0.02 mM) and enzyme extract (0.1 mL). The reaction was initiated by exposure with riboflavin to two 40 W fluorescent lamps, and then terminated by removing after 10 min away from the light source. Calibration standards were made using the same procedure but without the addition of the supernatant. Absorbance was measured at 560 nm and was expressed as U mg^{-1} (FW).

CAT (EC1.11.1.6) activity was measured as the absorbance at 240 nm for H_2O_2 decomposition [19]. The reaction solution was composed of phosphate buffer (50 mM, pH 7.8), EDTA (0.1 μM), H_2O_2 (0.1%), and enzyme extract (0.1 mL). The decomposition of H_2O_2 was detected by measuring the decrease in absorbance at 240 nm for 3 min, and quantified by its molar extinction coefficient (36 mM^{-1} cm^{-1}). One unit of catalase activity was defined as a change of 0.01 absorbance min^{-1} by the enzyme extract.

POD (EC 1.11.1.7) activity was determined by the rate of formation of tetraguaiacol at 470 nm [20]. Absorbance was measured at 470 nm for 5 min by adding the enzyme extract (0.02 mL) to the reaction mixture, which contained guaiacol solution (0.02 mL) and hydrogen peroxide solution (0.01 mL) in 3 mL of phosphate buffer solution (pH 7.0). Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 mM^{-1} cm^{-1}).

APX (EC 1.11.1.1) activity was measured according to the method of Nakano and Asada [21]. The 3-mL reaction mixture was composed of phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), ascorbate (0.5 mM), H_2O_2 (0.1 mM) and enzyme extract (0.1 mL). Activity was recorded as the decrease in absorbance at 290 nm for 3 min and the concentration of oxidized ascorbate was calculated using the molar extinction coefficient of 2.8 mM^{-1}·cm^{-1}.

Determination of ascorbate, glutathione and soluble protein concentrations

Wheat seedlings (0.2 g) were centrifuged at 15000 × g for 15 min after grinding in ice-cold 5% (w/v) trichloroacetic acid. Then, 0.2 mL of supernatant was added to 2.6 mL NaH_2PO_4 (pH 7.7) and 0.2 mL 5, 5-dithiobis (2-nitrobenzoic) (DTNB). After 5 min at 30°C, the absorbance was measured at 412 nm and the reduced glutathione (GSH) content was calculated based on the standard curve [22].

Homogenized seeds (0.2 g) in ice-cold metaphosphoric acid (2 mL 10%) were centrifuged at 15000 × g for 10 min. After centrifugation, to 0.5 mL of supernatant was added 1 mL of citric acid-phosphoric acid buffer (pH 2.3) and 1 mL of 2, 6-dichlorophenol indophenol (30 mg·L^{-1}). Absorbance was measured at 524 nm after 30 s. AsA concentration was determined as described [23]. The soluble protein content in wheat seedlings was measured according to the procedure of Bradford [24] using bovine serum albumin as a standard.

Total RNA extraction, cDNA synthesis and real-time quantitative PCR

Total RNA from wheat seedlings exposed to different treatments was isolated using Concert Plant RNA Reagent (Invitrogen, USA) according to the manufac-

turer's instructions. Total RNA quantitation, cDNA synthesis and qRT-PCR were performed according to a previously described method [25]. Ten μg of total RNA were treated with RNase-free DNase I (Promega), and 2 μg were reverse-transcribed into cDNA using random hexamers and M-MLV Reverse Transcriptase (Promega, USA). After reverse transcription, the products of each reaction were diluted 5 times to avoid potential primer interference in the qRT-PCR reaction. Quantitative RT-PCR was performed using SYBR® qPCR Mix (Toyobo, Japan) in a 20-μL reaction volume, containing 2 μL of diluted cDNA as template and 300 nM of each primer. Triplicate reactions were carried out using a Rotor-Gene 3000 real-time PCR detection system (Qiagen). Gene-specific primers for catalase (*CAT*), peroxidase (*POD*), superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), and the reference gene tubulin were used to amplify amplicons specific for wheat. The used primers are given in Table S1, and they were designed according to wheat EST sequences of candidate proteins available in the NCBI.

Statistical analysis

The experiments were repeated three times and each data point was the mean of six replicates. All obtained data were statistically analyzed by two-way analysis of variance (ANOVA), and statistical significance of the means were compared by Duncan's multiple range tests at 5% probability level using SPSS software. In all figures, the error bars represent standard errors of the means. The effects of NaCl, 20E and their interaction (20E×NaCl) in terms of activity of antioxidant enzymes, content of MDA, $O_2^{\bullet-}$, GSH, AsA, protein and growth parameters are presented in Table 1 according to two-way ANOVA.

RESULTS

Effect of 20E on growth parameters of wheat seedlings under NaCl stress

Fig. 1 presents plant height, root length, shoot dry weight (DW) and root DW in wheat seedlings exposed to 150 mM NaCl stress for 4 days with or without 3 μM of 20E. It can be observed that there was a significant decrease in plant height, root length, shoot DW and root DW in wheat seedlings exposed to NaCl stress as compared with the control. When the 20E pretreatment was combined with 150 mM of NaCl, root length and root DW were significantly enhanced in wheat seedlings, but plant height and shoot DW were not. 20E treatment alone did not significantly change plant height, shoot DW and root DW in wheat seedlings, with the exception of root length.

Table 1. Two-way analysis of variance (ANOVA) for NaCl, 20E, and their interaction (ES×NaCl) for morphological and physiological parameters in wheat seedlings under NaCl stress.

Dependent variable	NaCl	Independent variable 20E	20E×NaCl
MDA	7.30*	5.98*	2.11 ns
O•— 2	28.50**	46.64***	44.67***
H_2O_2	43.20**	18.56*	20.36*
SOD	35.58**	83.29***	69.50***
POD	91.21***	9.97*	24.10**
CAT	0.91 ns	7.79*	10.04*
APX	41.25***	2.95 ns	13.09*
GSH	0.29 ns	49.96***	2.16 ns
ASA	0.18 ns	9.85*	6.63*
Protein	13.44*	0.98 ns	2.24 ns
Plant height	11.73*	1.97 ns	4.97*
Root length	30.23**	10.79*	17.55*
Shoot dry weight	6.03*	5.19*	20.86*
Root dry weight	5.35*	12.13*	5.82*

Numbers represent F values at 5% level. Ns – not significant. *P<0.05; **P<0.01; ***P<0.001

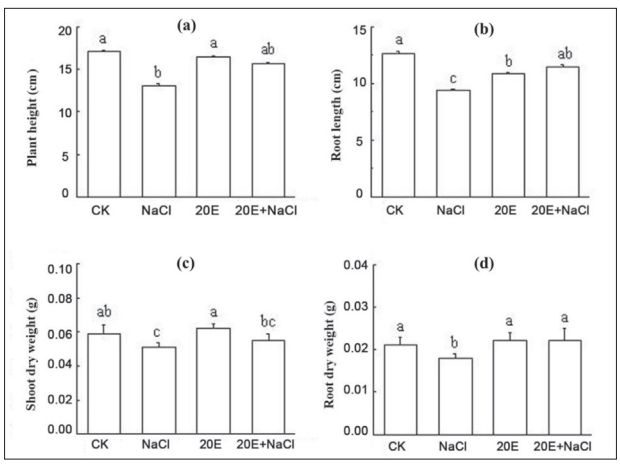

Fig. 1. Effect of exogenous 20E on plant height **(a)**, root length **(b)**, shoot DW **(c)** and root DW **(d)** in wheat seedlings under NaCl stress for 4 days. Different treatment were as follows: distilled water (control, CK), 3 μM 20E, 150 mM NaCl, 3 μM 20E + 150 mM NaCl (20E + NaCl). Bars are means ± standard error of 30 replicates. Means with different letters above bars were significantly different at 0.05 level according to Duncan's multiple range test.

Effect of 20E application on the MDA and H_2O_2 contents and the production rate of $O_2^{\bullet-}$ in wheat seedlings under NaCl stress

The contents of MDA and H_2O_2 and the production rate of O•— 2 in wheat seedlings were affected by the salt stress and 20E application (Table 1, Fig. 2). Compared to the control, NaCl stress alone resulted in significant increases in MDA, H_2O_2 content and the production rate of O•— 2 in wheat seedlings by 22%, 54% and 35%, respectively. However, the exogenous 3-μM 20E treatment significantly alleviated the salt-induced accumulation of MDA and H_2O_2, and decreased the production rate of O•— 2 in wheat seedlings (P<0.05). When the 20E-treated seedlings were compared to the control, the content of MDA, H_2O_2 and the production rate of O•— 2 were not affected.

Effect of 20E on antioxidant enzyme activities in wheat seedlings under NaCl stress

To investigate the antioxidant role of 20E against NaCl stress, the activities of antioxidant enzymes were determined. As shown in Fig. 3, when compared to the control, NaCl stress alone significantly decreased the activities of SOD and CAT (P<0.05) in wheat seedlings, although it induced increases in POD and APX activities by 15% and 13%, respectively. In contrast, a more remarkable increase (P<0.05) in the activities of SOD, POD and CAT were observed in the seedlings treated with exogenous 20E combined with 150 mM NaCl (Fig. 3). Fig. 3 also shows that the treatment with 20E alone had little effect on the activities of SOD, CAT and APX, with the exception of POD.

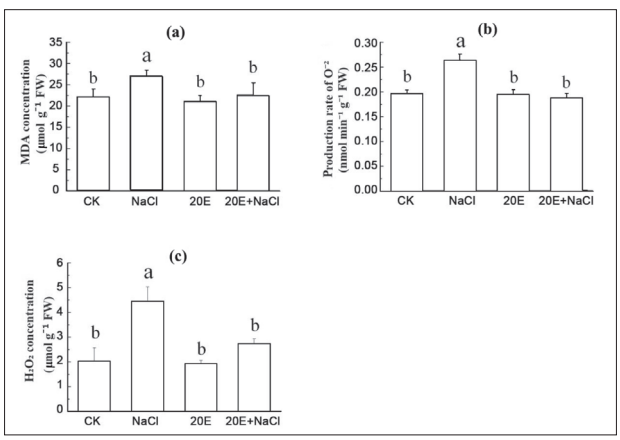

Fig. 2. Effects of exogenous 20E on the content of MDA **(a)**, H2O2 **(b)** and the production rate of O2•– **(c)** in wheat seedlings under NaCl stress for 4 days. Different treatment were: distilled water (control, CK), 3 μM 20E, 150 mM NaCl, 3 μM 20E + 150 mM NaCl (20E + NaCl). Bars are means ± standard error of 6 replicates. Means with different letters above the bars were significantly different at 0.05 level according to Duncan's multiple range test.

Effect of 20E on gene expression in wheat seedlings under NaCl stress

Real-time PCR analysis was performed to determine the expression levels of four antioxidant enzyme genes, *SOD*, *POD*, *CAT* and *APX*, in wheat seedlings under 150-mM-NaCl stress for 4 days with or without 20E treatment. Consistent with the enzyme activities, *SOD* and *CAT* expression levels were significantly downregulated by 150-mM NaCl stress when compared with the control (Fig. 4). In contrast, significant

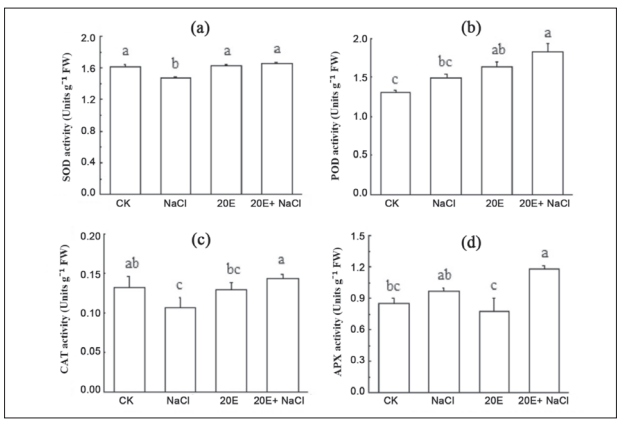

Fig. 3. Effect of exogenous 20E on the activities of SOD **(a)**, POD **(b)**, CAT **(c)** and APX **(d)** in wheat seedlings under NaCl stress for 4 days. See notes to Fig. 2.

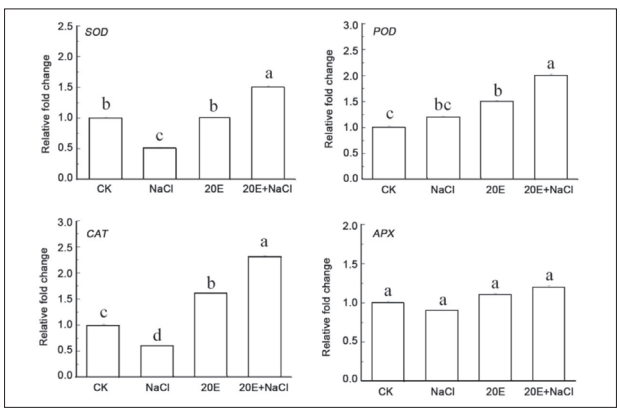

Fig. 4. Effect of 20E treatment on SOD, POD, CAT and APX transcript levels in wheat seedlings exposed to 150 mM NaCl stress for 4 days. See notes to Fig. 2. The mRNA expression levels in the control were arbitrarily set at 1.

Table 2. Effect of 20E treatment on GSH, AsA and protein concentrations in wheat seedlings exposed to NaCl stress for 4 days (mg g^{-1} FW).

Treatment	GSH concentration (μmol g^{-1} FW)	ASA concentration (μmol g^{-1} FW)	Protein concentration (mg g^{-1} FW)
CK	0.749±0.018b	0.733±0.001b	11.19±0.79b
NaCl	0.661±0.085c	0.602±0.003c	9.14±0.85c
20E	0.990±0.022a	0.761±0.001a	13.74±1.07a
20E+NaCl	1.029±0.022a	0.773±0.003a	14.07±2.84a

Different treatments were: distilled water (control, CK), 3 μM 20E, 150 mM NaCl, 3 μM 20E + 150 mM NaCl (20E + NaCl). Data are means ± standard error of 6 replicates. Different letters within the columns indicate significant differences at 0.05 level according to Duncan's multiple range test.

upregulation of *SOD*, *POD* and *CAT* gene expression was observed in seedlings treated with 20E and 150 mM of NaCl. However, *APX* transcript levels did not vary significantly, irrespective of the presence or absence of 20E treatment or NaCl stress. In addition, the 20E treatment alone had a significantly effect on *POD* and *CAT* transcript levels, but not *SOD* gene expression, when compared to the control.

Effect of 20E on GSH, AsA and protein contents in wheat seedlings under NaCl stress

Salt stress caused significant decreases ($P<0.05$) in the concentrations of GSH, AsA and protein in wheat seedlings (Table 2), while the same concentrations in 20E-treated seedlings were significantly enhanced as compared to 150-mM-NaCl stress alone. Table 1 also shows that 20E alone had a significant influence on GSH, AsA and protein compared to the control.

DISCUSSION

Salt stress is known to cause significant reduction in plant growth, manifesting as decreased leaf area, leaf length and root and shoot DW [9]. In the present study, a significant reduction in the growth of plant height, root length, shoot DW and root DW of wheat seedlings was observed under salt stress. This result is in agreement with Li et al. [9], who showed that salinity caused a marked reduction in the growth parameters of wheat. However, it was observed that exogenous 20E treatment significantly increased the root length and root DW of wheat seedlings under salt stress when compared to corresponding untreated plants. A similar result was reported by Bakrim et al. [5], who found that 20E exogenously applied to the seeds of tomato induced morphological and biochemical modifications, including effects on root and shoot length in the seedlings. A significant increase ($P<0.05$) in wheat seedling growth under salt stress (root DW and root length) was observed under exogenous 20E treatment, which was concomitant with the decreased production rate of $O_2^{\bullet-}$ and MDA concentration. The high MDA decrease points to changes in membrane fluidity and damage to membrane proteins, such as inactivation of receptors, enzymes and ion channels. MDA has been considered a marker for membrane lipid peroxidation in various crops [9,10,25]. Hu et al. [1] showed that 20E protected against oxidative stress-induced neuronal injury by scavenging free radicals and decreasing MDA concentration. Lamhamdi et al. [8] also reported that 20E treatment notably decreased MDA concentration in wheat seedlings under lead stress. Here, we also found that exogenous 20E treatment significantly reduced the increase in MDA, H_2O_2 concentration and the production rate of $O_2^{\bullet-}$ in wheat seedlings under salt stress, suggesting that the application of exogenous 20E could ameliorate the effect of lipid peroxidation and facilitate maintenance of membrane functions.

The reactive nature of ROS makes them potentially harmful to cellular components. However, ROS in plants are eliminated by an efficient ROS-scavenging system. There is evidence that the alleviation of oxidative damage and increased resistance to salinity and other environmental stresses are usually associated with an efficient antioxidative system [9,26]. Yasmeen et al. [27] suggested that an efficient antioxidant defense system provided by moringa leaf extract might play an important role in the tolerance of wheat seedlings to salt stress. Lamhamdi et al. [8] also showed that exogenous application of 20E significantly increased the activities of SOD, POD and APX in wheat seedlings exposed to lead stress. A similar result was found here, where the activities of the ROS-scavenging enzymes, SOD, POD and CAT, were significantly increased in 20E-treated wheat seedlings under salt stress, suggesting that the spraying 20E on leaves enhanced the activities of antioxidant defense enzymes and conferred salt tolerance to wheat seedlings. Moreover, exogenous 20E induced the synthesis

of antioxidant metabolites that provided an additional neutralization of the toxic effects of salt-stress-generated ROS. The decreased contents of chlorophyll, AsA and protein in *C. Vulgaris* cells treated with lead were restored by the co-application of 20E [28]. We also observed that 20E significantly increased the contents of GSH and AsA in salt-stressed wheat seedlings. The activities of enzymes and antioxidant metabolites in salt-stressed wheat seedlings were enhanced by 20E application, which was consistent with 20E reducing the rate of $O_2^{\bullet-}$ generation and MDA content in wheat seedlings. These results indicated that 20E alleviated salt-induced membrane injury by increasing the ROS-scavenging ability, suggesting that 20E could strongly protect wheat seedlings from salt-stress damage.

There is relatively little information on the effect of 20E on the regulation of antioxidant enzyme activities and antioxidant concentrations during plant adaptation to salt stress. We showed that one of the effects of a pretreatment with 20E on oxidative stress is increased activities of antioxidant enzymes. Analysis of changes in the expression of antioxidant enzymes at the transcriptional levels has not been reported. To determine whether salt-induced stress was capable of regulating the expression of antioxidant genes, we performed a quantitative RT-PCR-based assay. We showed that *Cu/Zn-SOD*, *POD* and *CAT* were upregulated in plants exposed to 20E pretreatment combined with 150 mM NaCl, when compared to salt stress alone. This was consistent with the increased activities of SOD, POD, CAT in wheat seedlings. Upregulation of antioxidant enzymes by brassinosteroids is the consequence of enhanced expression of the gene *det2*, which enhanced the resistance to oxidative stress in *Arabidopsis* [29]. In agreement with these results, Qiu et al. [25] reported that the expression pattern for the catalase gene (*CAT1*) correlated with its enzyme activity. These results suggest that enhanced antioxidant enzymatic gene expression contributed to the increase in the activities of SOD, POD and APX, resulting in enhanced protection against oxidative damage induced by salt stress.

CONCLUSION

Application of 20E significantly ameliorated the adverse effects of salt stress by upregulating the expression of *SOD*, *POD* and *CAT* genes and raising the enzymatic activities of SOD, POD and CAT. 20E also increased AsA and GSH concentrations and decreased the concentration of MDA and the rate of $O_2^{\bullet-}$ generation. These results support the hypothesis that foliar spraying with 20E can enhance the tolerance of wheat seedlings to salt stress. Further investigations into the roles of 20E will help us to better understand the antioxidative effect of 20E on wheat seedlings under salt stress.

Funding: This research was funded by the National Nature Science Foundation of China [81274076] and the Key Projects of Henan Province Colleges and Universities [17A180026].

Author contributions: Jinting Li and Xueping Han designed the study and revised the manuscript. Xueping Han, Li Tang, Can Wang, Weiyu Zhang and Jianhui Ma performed the sample collection, analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

Conflict of interest disclosure: The authors declare that they have no competing interests.

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Supplementary Data

Table S1. Primer sets used for real-time PCR in the study

Gene	NCBI accession No.	Sequence (5'–3')	Product (bp)
SOD	JQ613154.1	F:CATTGTCGATAGCCAGATTCCTTT AGTCTTCCACCAGCATTTCCAGTA	138
POD	AF387866.1	F: GACCAGGTGCTCTTCAACAACGAC R: TAGCCGTAGGTCAATCACGAGTTC	188
CAT	GU984379.1	F: TTTGATGGGAGTCTTGTGCTTGTG R: ACGGTGAGGGAGTTGTCGTTGTT	119
APX *Tublulin*	AY513262.1 DQ435660.1	F: AAAACCACCTACTGCCACCCTATC R: AGCATTCGCTCCATGACTCAACT F: CCGTGGTGATGTTGTGCCAAAGGA R: CGACGACACTGGTGGAGTTGGAGA	148 200