Coronatine Modulated the Generation of Reactive Oxygen Species for Regulating the Water Loss Rate in the Detaching Maize Seedlings

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Abstract: Coronatine (COR), a structural and functional mimic of jasmonates, is involved in a wide array of effects on plant development and defense response. The present study aims to investigate the role of COR, in counteracting drought stress by modulating reactive oxygen species (ROS) homeostasis, water balance, and antioxidant regulation in detached maize plants. Our results showed that COR can markedly decrease the water loss rate, but the antioxidants diphenyleneiodonium chloride (DPI) and dimethylthiourea (DMTU) eliminate the effect of water loss induced by COR. Using the dye 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) loaded in the maize epidermis guard cells, it is observed that COR could increase ROS production, and then antioxidants DPI and DMTU decreased ROS production induced by COR. In addition, the expression of ZmRBOHs genes, which were associated with ROS generation, was increased by COR in levels and ZmRBOHC was highly expressed in the epidermis guard cells. Moreover, COR-treated plants increased H2O2 and O2−· accumulation, antioxidant enzyme activities in control plants, while COR relieved the ROS accumulation and antioxidant enzyme activities under PEG treatment. These results indicated that COR could improve maize performance under drought stress by modulating ROS homeostasis to maintain water loss rate and antioxidant enzyme activities.

Keywords: coronatine; drought; antioxidant defense; reactive oxygen species (ROS); maize

1. Introduction

The frequency and duration of extreme weather events are increasing as a result of global climate change, which declines crop yields and enhances worldwide yield variability for threatening food security [1]. Among extreme weather events, drought stress is widely recognized as the most abiotic stress limiting crop productivity, and also one of the major problems of future climate change [2,3]. Moreover, drought stress adversely impacts physiological and biochemical processes in plant cells to repress plant growth and development [4,5]. For instance, drought stress can disorder plant tissue water status, decline photosynthetic efficiency and assimilate allocation, and repress nutrients uptake and translocation, which leads to growth retardation and low productivity [6]. Meanwhile, drought stress induces the generation of reactive oxygen species (ROS) and lipid peroxidation, resulting in oxidative damage to cellular components for inhibiting normal growth or even causing plant death [7,8].

Because of drought stress as the most severe factor limiting crop productivity worldwide, improving drought tolerance in crops is very essential to maintaining or increasing crop production for ensuring food security [9]. Plants responding to drought stress are
complex and involve diverse and multiple physiological and molecular mechanisms [6,10]. Moreover, plants have evolved and developed sophisticated strategies to deal with drought stress, and various strategies are achieved to improve drought tolerance in crop production [11,12]. Among the various strategies, priming has been proved as an important approach to enhance plant stress tolerance by prior exposure to chemical agents or abiotic stressors [13–15]. For instance, drought priming can improve drought tolerance to the subsequent drought stress by modulating photosynthesis and stress defense in crops [16–18]. Additionally, several studies have demonstrated that exogenous chemical-induced priming can enhance drought tolerance in plant, such as osmopriming (mannitol or PEG) in cotton [19], hydrogen sulfide priming in wheat [20], abscisic acid priming in Arabidopsis [21], nitric oxide priming in rice [22], and hydrogen peroxide (H$_2$O$_2$) seed priming in Cakile maritima [23].

Coronatine (COR) is a phytotoxin produced by Pseudomonas syringae and mimics the form and function of jasmonic acid (JA) and jasmonoyl-L-isoleucine [24,25]. COR can induce chlorophyll degradation leading to leaf diffusing chlorosis, stimulate ethylene and anthocyanin production, and promote root growth retardation and hypertrophy [24,26,27]. Moreover, COR has similar activity to JA while not identical [24]. For instance, COR induces higher phytoalexin production compared to JA in rice [28]. Meanwhile, several studies indicated that COR acts in a dose-dependent manner in plants, thus a high concentration of COR can be a virulence factor in plant disease development while a low concentration of COR can enhance plant tolerance to environmental stresses [26,29,30]. In addition, exogenously applied COR improves drought tolerance in rice [31], cauliflower [32], soybean [33], chickpea [34], tobacco [35], and maize [36].

Several reports have demonstrated that COR can restrain the foliar stomatal closure for promoting the entry of P. syringae pv. tomato into foliar tissue [37], while suppresses NADPH oxidase-dependent ROS production to inhibit the closure of stomata induced by the flagellin-derived peptide flg22 [30]. Inversely, COR induces the accumulation of light-dependent ROS during P. syringae pv. tomato pathogenesis [38]. These opposite phenomena may be due to COR functions in a dose-dependent manner. Likewise, ROS such as hydrogen peroxide (H$_2$O$_2$) is considered a toxic agent for cell structures while as a potential signal molecule in plants responded to abiotic and biotic stresses [39,40]. Furthermore, exogenous H$_2$O$_2$ enhances stress tolerance through activating transcription factors and signaling proteins in plants subjected to abiotic stresses [41–43]. Although previous studies demonstrated exogenous application of COR improving drought tolerance in crops [31,33,36], it is still unclear how COR modulates ROS production in plants responses to drought stress.

Maize is a staple food and forage cereal crop in many parts of the world. Drought stress affects maize seedling establishment [44], vegetative growth [45], root growth [46], photosynthetic activity [47], reproductive growth [48], and is the most severe abiotic factor limiting maize productivity globally [49]. Hence it is essential to improve drought tolerance by various strategies in maize production [50]. Likewise, pretreatment of plants with COR regulates antioxidant enzyme activities and lipid peroxidation to alleviate drought stress damage in maize seedlings [36]. However, it is still unclear how COR modulates ROS production in plants that responded to drought stress. The objectives of this study are to investigate the role of COR application in leaf water loss, ROS production, and antioxidant system in detached maize leaves and protoplast system under drought stress, and explore the physiological and molecular mechanism of action of COR in ROS dynamic characteristic for enhancing drought tolerance in maize.

2. Materials and Methods

2.1. Plant Growth and Treatments

Maize seeds (Zea mays L. cv. Zhengdan 958) were surface-sterilized for 10 min with 75% (v/v) ethanol solution and rinsed with sterilized distilled water five times. Seeds were then sown in a plastic box (120 × 80 × 10 cm deep) containing a mixture of vermiculite and
commercial garden soil (1:1; v/v). Plants were placed in a greenhouse under 30 °C/20 °C and 14 h/10 h day/night conditions. When the second leaves were fully expanded, plants were collected and used for investigations [51]. The whole detached plants were used to test the water loss rate. After having identified the best COR concentration and a series of treatments, the first leaves were used to histochemical detection of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \cdot \), generation, antioxidant enzyme activities, and stomatal aperture.

The plants were excised at the base of the stem and placed in distilled water for 1 h to eliminate wound stress, based on the method described by Ma [52]. Then cut ends of the stems were placed in 10 mL tubes wrapped with aluminum foil containing 0.1, 0.01, 0.001, 0.0001 \( \mu \)M coronatine for 12 h, under continuous light intensity of 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). After treatment with different concentrations COR, the stems were subjected to 10% (w/v) polyethylene glycol (PEG 6000) solution, and weighed at intervals of one hour for 8 h to calculate the water loss rate of the detached plants after. To study the effects of the inhibitors diphenyleneiodonium chloride (DPI) and dimethylthiourea (DMTU), the detached plants were pre-treated with distilled water as control and 0.001 \( \mu \)M COR for 12 h, then treated with 2.5 mM DMTU, or 2.5 \( \mu \)M DPI for 8 h, respectively, and then subjected to 10% (w/v) polyethylene glycol (PEG 6000) solution. To calculate the water loss rate, the detached plants were weighted every hour for 8 h. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. After treatments of detached maize plants, the second leaves were sampled and immediately frozen in liquid \( \text{N}_2 \) for further analysis.

To obtain the etiolated maize plants for protoplast isolation, seeds were germinated under light for 3 days (1 cm shoots visible) and then moved to a darkroom until the second leaf was about 15 cm (for about 10 days at 25 °C). Only the middle section (4–10 cm from the tip) of the second leaves were collected and cut into 0.5–1 mm strips for the following protoplast isolation.

### 2.2. RNA Isolation and Real-Time Quantitative PCR Analysis

Total RNA was isolated from leaves using QIAGEN RNeasy Plant Mini Kit according to the manufacturer’s instructions. Approximately 2 \( \mu \)g of total RNA was reverse transcribed using oligo d (T) 18 primer and M-MLV reverse transcriptase (Takara Bio Inc., Shiga, Japan) at 42 °C for 1 h. Real-time quantitative RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, CA, USA) using SYBR Premix EX TaqTM (Takara, Japan). All the primers were designed by DNAMAN program and listed in supplementary Table S1. About 1 \( \mu \)L of cDNA diluted 10 folds and 1.5 \( \mu \)L of primers with a concentration of 10 \( \mu \)M (amplification efficiency greater than 95%) were added to a 20 \( \mu \)L reaction system. After 40 cycles, \( \beta \)-tubulin gene was chosen as an internal control to normalize data [53]. An analysis was performed to monitor primer-dimer formation and amplification of gene-specific products according to the manufacturer’s instructions. The relative quantification method was used to evaluate quantitative variation between replicates.

### 2.3. In Vivo Detection of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \cdot \)

\( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \cdot \) were visually detected in the leaves of plants using 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) as substrates [54]. Briefly, plants were excised at the base of stems with a razor blade and pre-treated with 0.001 \( \mu \)M COR for 12 h. Next, plants were transferred to 10 mL tubes containing 1 mg mL\(^{-1}\) solution of DAB (pH 3.8) and 0.1% NBT (pH 6.5) for 8 h under light at 25 °C. After these treatments, maize plants were subjected to 10% (w/v) polyethylene glycol (PEG 6000) solution for 5 h. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. The first and second leaves were decolorized in boiling ethanol for 15 min. This treatment decolorized the leaves except for the deep brown and purple polymerization products produced by the reaction of DAB with \( \text{H}_2\text{O}_2 \) and NBT with \( \text{O}_2^- \cdot \). After cooling, the leaves were kept in fresh ethanol and photographed.
2.4. Biochemical Parameters Analysis

The detached maize plants were treated with 0.001 µM COR and distilled water for 12 h, respectively, then subjected to 10% \((w/v)\) polyethylene glycol (PEG 6000) for 5 h. The second leaves were sampled for physiological and biochemical parameters analysis. The crude extract was obtained by grinding 0.5 g maize leaf with 4 mL pre-cold acetone. After centrifugation, the supernatants were used to determine the content of \(\text{H}_2\text{O}_2\) by monitoring the absorbance at 415 nm of the titanium-peroxide complex following the method described by Brennan [55]. Absorbance values were calibrated to a standard curve generated with known concentrations of \(\text{H}_2\text{O}_2\) to the leaf extracts as an internal standard.

For analysis of the \(\text{O}_2^-\cdot\) content, the total soluble protein was extracted from the labeled leaves of PEG-treated and distilled water-treated plants using 50 mM phosphate buffer (pH 7.8), and protein content was determined using Coomassie Brilliant Blue G-250 according to the method of Bradford [56]. The supernatants were recovered for determinations of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity, and \(\text{O}_2^-\cdot\) content as described previously [53, 57]. The SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride, and the activity was expressed in enzyme units per mg of protein. The POD activity was determined by the guaiacol oxidation method, and its enzyme activity was expressed as an increase in absorbance \(\text{min}^{-1}\ \text{mg}^{-1}\) protein. The CAT activity was estimated by monitoring the disappearance of \(\text{H}_2\text{O}_2\) by recording the decline in absorbance at 240 nm. The reaction of hydroxylamine and \(\text{O}_2^-\cdot\) produced \(\text{NO}_2^-\), which was developed by adding sulfanilic acid and \(\alpha\)-naphthylamine. The absorption at 530 nm of the sample quantitatively correlates with the \(\text{O}_2^-\cdot\) amount.

2.5. Stomatal Aperture Measurement

To study the effects of COR on water loss, the detached plants were pre-treated with distilled water as control and 0.001 µM COR for 12 h, respectively. Then they were treated with 2.5 mM dimethylthiourea (DMTU), 2.5 µM diphenyleneiodonium chloride (DPI) for 8 h. After treatments of detached maize plants, the second leaves were sampled and immediately fixed in FAA buffer (4% paraformaldehyde and 5% glutaraldehyde). Stomata were examined using a Desktop scanning electron microscope (TM3000) and stomatal aperture was determined by measuring the width and length of the stomata using ImageJ software. At least five leaves were chosen for each treatment and 10 observations were recorded from each leaf. Stomatal aperture was determined as pore width/length.

2.6. Isolation of Maize Protoplasts and ROS Measurement

Maize protoplasts were isolated from leaves using the method described previously [58] with minor modifications. The enzyme solution [0.4 M mannitol, 20 mM MES (pH 5.7), 20 mM KCl, 2% \((w/v)\) Cellulase R10 (Yakult), 0.2% Macerozyme R10 (Yakult)] was heated at 55 °C for 7 min and cooled to room temperature before adding 10 mM CaCl\(_2\), 5 mM \(\beta\)-mercaptoethanol, and 0.1% bovine serum albumin (BSA) solution. The middle section of the second leaves (4 cm to 10 cm from the tip) were cut into 0.5 mm strips with razor blades and gently immersed into the enzyme solution and incubated under vacuum pressure (25 mm Hg) for 30 min. The cut leaves were subjected to continuous digestion for another 3 h in the dark. After adding 10 mL of W5 solution [(154 mM NaCl, 125 mM CaCl\(_2\), 2 mM MES (pH 5.7), 5 mM KCl], the enzyme solution containing protoplasts was filtered with a 35-µm nylon mesh. Next, the solution was spin at 100 × g to pellet the protoplast in a round-bottomed tube for 3 min, washed once with W5 solution and kept on ice for at least 30 min. The protoplasts were resuspended in a MMG solution [0.4 M Mannitol, 4 mM MES (pH 5.7), 15 mM MgCl\(_2\)] to the final concentration at \(2 \times 10^5\) mL\(^{-1}\). \(2',7'\)-dichlorofluorescein diacetate (H2DCF-DA) was added to the protoplast solution at a final concentration of 50 µM for 20 min in the dark. W5 solution was added to wash the protoplasts for four times to remove the excess H2DCF-DA. Finally, the protoplasts were resuspended with 2 mL MMG solution for the following experiment. About 10 µL
of H2DCF-DA-treated protoplast cell solution was applied to the slides and treated with 0.001 µM COR. The fluorescence signal was collected once every two minutes for a total of 14 min. Meanwhile, 2.5 mM DMTU, and 2.5 µM DPI were added to the control group and COR-treated group for 2 min, respectively. The fluorescence in protoplast was detected with a confocal microscope (λEx/λEm = 504/529 nm, Zeiss LSM 710 META, Oberkochen, Germany), and the fluorescence intensities were analyzed using ImageJ software and calculated as the average value of those obtained by scanning over 30 protoplasts from three different experiments.

2.7. Determination of ROS Production in Maize Guard Cells

The production of H$_2$O$_2$ was detected by an H2DCF-DA staining assay as described previously (Hua et al. 2012). Epidermal strips were taken from maize leaves of 7 day-old seedlings. The epidermal strips without mesophyll cells were soaked in MES buffer (10 mM MES-KOH (pH 6.5), 10 mM KCl, and 50 μM CaCl$_2$) for 3 h at 22 °C to eliminate the excess of ROS generated during the operation. Then, 50 μM H2DCF-DA (Sigma-Aldrich, cat. No. D6883) was added to the buffer. The samples were cultured in the dark for 20 min and then washed five times with ddH$_2$O to remove the excess H2DCF-DA. Finally, the epidermal strips were stored in MES buffer. Next, the epidermal strips were treated with 0.001 µM COR, 0.001 µM COR + 2.5 mM DMTU, and 0.001 µM COR + 2.5 µM DPI for 5 min respectively. The fluorescence in guard cells was detected with a confocal microscope (Zeiss LSM 710 META). The fluorescence intensities were analyzed using ImageJ software. About 20 guard cells were assessed per sample, and the experiment was independently performed three times.

2.8. Statistical Analysis

The data were statistically analyzed using the SPSS software (SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) was calculated for multiple comparisons, and Student’s unpaired $t$-tests were used for pairwise comparisons. $p$-values < 0.05 were considered significant.

3. Results

3.1. COR Altered the Dynamics of Water Loss in Detached Seedlings

To investigate the effect of COR on water loss in maize plants, the detached two-leaf stage seedlings that responded to different COR concentrations were analyzed to determine water loss. Regardless of COR-treated or control seedlings, the rate of water loss was increased with the extension of time after the start of the water stress (Figure 1). The effects of different concentrations of COR on water loss of detached seedlings varied in a dose-dependent manner. At concentrations increased from 0.0001 to 0.1 µM COR, the rate of water loss decreased by 2.7 to 9.2% in 0.0001 to 0.01 µM COR-treated seedlings compared to control during 8 h of PEG treatment, while 0.1 µM COR increased the rate of water loss by 5.1% compared to 0 µM COR. Moreover, 0.001 µM COR-treated seedlings presented a significantly lower rate of water loss than 0 µM COR. Next, we use this concentration in the next experiments.
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Figure 1. Water loss rates of detached maize plants treated with gradient concentration of COR under PEG treatment. Plants were pre-treated with 0, 0.1, 0.01, 0.001, 0.0001 µM COR for 12 h, then water loss of isolated detached plants was monitored every 1 h over 8 h with 10% PEG treatment. Values are the mean ± SD ($n = 8$), asterisks indicate significant differences ($p < 0.05$) between 0 and 0.001 µM using Student’s t-test.

3.2. COR Regulated ROS Accumulation, Antioxidant Enzyme Activities, and Stress-Induced Gene Expression in Seedlings Exposed to Drought Stress

To further analyze the effect of COR on ROS accumulation in plants subjected to drought stress, the accumulation of $H_2O_2$ and $O_2^\cdot$ in leaves were determined by histochemical detection. The well-watered plants treated with COR showed higher intensities of DAB and NBT staining than that of well-watered plants in the first or second leaves. Moreover, there is an enhanced staining of the water-stressed plants, while with the addition of COR, the color intensity was reduced compared to PEG treatment (Figure 2A,C). Furthermore, COR induced the accumulation of $H_2O_2$ and $O_2^\cdot$ higher by 29.5 and 27.3% compared to the well-watered plants. Although PEG treatment significantly enhanced the accumulation of $H_2O_2$ and $O_2^\cdot$, the $H_2O_2$ and $O_2^\cdot$ contents were reduced by 24.1 and 15.2% by water-stressed in presence of COR compared to water-stressed plants (Figure 2B,D).

In general, enzymes such as SOD, POD, and CAT are very important for scavenging the enzyme system to eliminate the detrimental effect of ROS in plants under abiotic stress. Under well-watered conditions, COR increased the activities of SOD and POD by 19.1 and 14.6% compared to the control, while it showed slightly different levels of CAT activities compared to the control, but the difference is not significant (Figure 3). Moreover, PEG treatment significantly increased SOD, POD, and CAT activities in all plants, and SOD, POD, and CAT activities were also higher in COR-treated plants compared to the control plants under drought stress.

$ZmDREB2A$ regulates the drought-responsive genes and upregulated in response to water stress [59], $ZmcAPX$ encoding a cytosolic isoform of ascorbate peroxidase [60], and $ZmCAT1$ encoding catalase 1 [61] were tested to explore whether COR regulated the expression of stress-related responsive genes for improving drought tolerance in maize seedlings. COR significantly increased the expression levels of $ZmDREB2A$ under drought stress conditions (Figure 4A). However, there was no significant difference in the values of gene expression level between COR-treated and control plants under well-watered conditions. Similarly, the expression of $ZmcAPX$ and $ZmCAT1$ were also induced by drought stress, while COR improved greatly the expression levels of $ZmcAPX$ and $ZmCAT1$ compared to the only drought-stressed plants (Figure 4B,C). There was no significant difference between COR-treated and control plants under well-watered conditions except for $ZmcAPX$ at 1 h.
Figure 2. H$_2$O$_2$ and O$_2^-$ accumulation in leaves of maize plants after treatments with COR in well-watered and drought plants. (A) Histochemical detection of H$_2$O$_2$ and (B) O$_2^-$ with nitroblue tetrazolium (NBT) in maize leaves. The second leaves were homogenized, and (C) H$_2$O$_2$ content and (D) O$_2^-$ were assayed by spectrophotometry. CK and COR indicated the well-watered plants and well-watered plants treated with COR, respectively. PEG and PEG+COR indicate the water-stressed plants and water-stressed plants in presence of COR, respectively. Values are the mean ± SD (n = 3), and different letters indicate significant differences (p < 0.05) among the treatments. Bars = 2 cm.

Figure 3. Activities of SOD (A), POD (B), CAT (C) in leaves of maize seedlings under 10% PEG treatment. CK and COR indicated the well-watered plants and well-watered plants treated with COR, respectively. PEG and PEG+COR indicate the water-stressed plants and water-stressed plants in presence of COR, respectively. Values are the mean ± SD (n = 3), and different letters indicate significant differences (p < 0.05) among the treatments.
Figure 4. Expression analysis of ZmDREB2A (A), ZmcAPX (B), and ZmCAT1 (C) in leaves of COR-treated and control plants under well-watered conditions and PEG treatment. The gene expression levels of COR-treated and control plants are shown relative to the expression of control plants grown under well-watered conditions at the corresponding time. CK and COR indicated the well-watered plants and well-watered plants treated with COR, respectively. PEG and PEG+COR indicate the water-stressed plants and water-stressed plants in presence of COR, respectively. One hour CK-treatment as calibrator samples. Values are the mean ± SD (n = 3), and different letters indicate significant differences (p < 0.05) among the treatments.

3.3. COR Induced NADPH Oxidase of the ROS Signaling Gene Network, Modulated Stomatal Closure and Water Loss

Plant NADPH oxidases, termed Rboh (respiratory burst oxidase homologue) catalyze the production of superoxide. ZmRBOHs genes encode NADPH oxidases in maize plants. To investigate the effect of COR on the NADPH oxidase-dependent ROS production in maize seedlings leaves, the expression of ZmRBOH-A-C genes were analyzed. The expression levels of ZmRBOH-A with COR could not be significantly induced compared to the control except at 24 h and for ZmRBOH-B, only at 3 h and 24 h (Figure 5A,B). Furthermore, ZmRBOH-C gene expression was significantly induced by COR at 3 to 12 h after treatments (Figure 5C).

Figure 5. Expression patterns of ZmRBOH-A (A), ZmRBOH-B (B), and ZmRBOH-C (C) in maize leaves exposed to COR. Relative expression levels of ZmRBOH-A (A), ZmRBOH-B (B), ZmRBOH-C (C) genes, analyzed by real-time quantitative PCR, are presented as values relative to CK at 0 h, defined as 1, after normalization to β-tubulin transcript levels. Values are means ± standard error (n = 3), and different letters indicate significant differences (p < 0.05) among the treatments.

To determine whether COR-induced ROS production could modulate stomatal closure and water loss, several ROS manipulators, such as an inhibitor of NADPH oxidase (DPI), and a scavenger of H2O2 (DMTU) were applied. The water loss rate was increased by DMTU compared to WS in the detached seedlings, while greater by 7.8% in DMTU-treated seedlings in comparison to WS during 8 h of PEG treatment (Figure 6A). As expected, DMTU treatment eliminated the effect of water loss induced by COR, and the rate of water loss by DMTU associated with COR presented no significant difference in WS. A similar change was observed in the DPI-treated seedlings. DPI enhanced water loss rate by 8.0% compared to WS during 8 h of PEG treatment, and also eliminated the effect of water loss induced by COR (Figure 6B).
Figure 6. Water loss rate in antioxidants-treated detached maize plants. (A) Water loss rate of the pre-treated maize plants with distilled water and COR, subsequently exposed to dimethylthiourea (DMTU) and (B) diphenyleneiodonium chloride (DPI) for 8 h. Then subjected to 10% (w/v) polyethylene glycol (PEG 6000) solution. WS and COR indicated the distilled water-treated and COR-treated plants under PEG treatment conditions, respectively. DMTU/DPI and DMTU+COR/DPI+COR indicate the DMTU/DPI-treated and DMTU+COR/DPI+COR-treated plants under PEG treatment, respectively. The experiments were repeated three times with similar results. Each data point represents mean ± SE (n = 6), asterisks indicate significant differences (p < 0.05) between COR and DMTU/DPI treatment using Student’s t-test.

To observe whether COR and ROS are related to the regulation of stomatal action inhibiting the activation of NADPH oxidase in well-watered plants. Here, the stomatal apertures of COR-treated leaves were smaller by 20.0% compared to the control, while DMTU increased the stomatal apertures by 10.8% compared to control (Figure 7A). Moreover, DMTU associated with COR could not significantly regulate the stomatal apertures compared to control. Similarly, DPI enhanced the stomatal apertures by 11.5% in comparison to the control (Figure 7B). However, DPI associated with COR could not significantly regulate the stomatal apertures compared to the control.

Figure 7. Effect of COR on the stomatal aperture in maize leaves treated with DMTU (A) and DPI (B). The experiments were repeated three times with similar results. Values are means ± standard error (n = 10), and different letters indicate significant differences (p < 0.05) among the treatments.

3.4. Dynamic Accumulation Characteristics of COR-Induced ROS Production

Since stomatal closure is accompanied by increased ROS level in the guard cell [62], ROS production was monitored in maize leaves or protoplasts treated with COR and the fluorescent probe dye 2’,7’-dichlorofluorescein diacetate (H2DCF-DA). It was observed that the relative ROS fluorescence intensity was increased by 45.3% in guard cells of COR-treated leaves compared to the control. At the same time, DPI and DMTU as the ROS inhibitors, could inhibit the effect of COR-induced ROS production (Figure 8A). Moreover, DMTU and DPI associated with COR decreased the relative ROS fluorescence intensity by 53.3 and 49.0% compared to COR only, while by 32.1 and 25.8% compared to the control.
In the maize protoplast system, COR rapidly activated ROS production. The relative ROS fluorescence intensity was increased by 28.8 to 47.3% in COR-incubated protoplast compared to the control at 2 to 14 min (Figure 8B). Moreover, DPI and DMTU significantly decreased the relative ROS fluorescence intensity compared to the control without COR-priming treatment (Figure 8C). COR increased the relative ROS fluorescence intensity higher by 23.4% relative to the only DPI-incubated protoplasts, and by 19.2% compared to the only DMTU incubated protoplasts.

4. Discussion

Drought stress widely threatens plant growth, development, and crop yield [9]. When exposed to drought stress, plants undergo a massive change in cellular process to cope with dehydration, such as reduced water potential, stomatal closure, membrane stability, osmotic adjustment, antioxidant accumulation, and reactive oxygen species (ROS) scavenging [63]. Maize is an important cereal crop known to be sensitive to drought stress, which negatively impacts vegetative growth, biomass production, and the formation of reproductive organs, which prevents maize plants from performing at their maximum potential and can threaten their survival [1]. In the present study, COR was applied in maize plants aiming to improve drought tolerance. Indeed, COR-primed plants showed lower water loss in comparison to the control in detached maize seedlings, which indicated that COR had higher intracellular water-holding capacity compared to the control in detached seedlings subjected to drought stress. Similar previous studies demonstrated that COR can help different plant species to keep high leaf water content in maize [36], soybean [33], and cauliflower [32] under drought stress. It has been shown that the pleiotropic action of COR happens in a concentration-dependent manner. Furthermore, COR as a virulence factor promotes plant disease development while COR at low concentration can improve plant stress tolerance [26,29,30]. In the present study, COR also showed a dose-dependent effect on water loss in detached maize plants, where 0.1 µM COR enhanced the water loss rate compared to the control, and 0.001 µM COR significantly reduced the water loss rate. Accordingly, the appropriate concentration of COR application could maintain better water status in maize plants exposed to drought stress. Our results are similar to the previous studies where it was shown that micro-molar concentration of COR can play an important role in plant abiotic stress resistance, such as enhancing drought tolerance in soybean and tobacco [33,35], salt-resistance in cotton [31], heat resistance in winter wheat [29], and cold tolerance in cucumber [64].

Drought stress is shown to enhance the ROS production and cause ROS-associated injury [65,66]. Moreover, ROS plays a dual role in plant response to abiotic stresses acting as toxic by-products of stress metabolism, and is also an important signal transduction molecule [42]. In the present study, the accumulation of ROS including H$_2$O$_2$ and O$_2$ was enhanced by PEG treatment, and COR could reduce ROS accumulation in plants.
exposed to PEG treatment. In addition, our results were very similar for DAB staining and NBT staining suggesting that COR application could suppress ROS production, and enhance antioxidant scavenging capacity in plants subjected to drought stress. As expected, the activities of antioxidant enzymes of SOD, POD, and CAT were enhanced by drought stress, and COR could improve its activities under drought stress. Consistent with these results, COR application can increase the activities of antioxidant enzymes in rice [31], soybean [33], and cauliflower [32] under drought stress. Indeed, we showed that COR could significantly up-regulate the expression of the antioxidant enzyme genes ZmcAPX and ZmCAT1 under drought stress, and remarkably increased the expression of stress-related responsive gene ZmDREB2A in leaves of plants subjected to drought stress. Furthermore, the drought tolerance is improved by the constitutive or stress-inducible expression of ZmDREB2A in Arabidopsis [59] and OsDREB2A in soybean [67]. These results indicate that COR regulates the expression of stress-related responsive genes, improving drought tolerance in maize seedlings.

It is well documented that, at high concentrations, COR promotes stomatal reopening through the E3 ligase subunit COI1, a key component of the jasmonic acid signaling pathway, which allows the invasion of plant-pathogens to the leaf interior [68–70]. Inversely, at low concentrations, COR can reduce water loss by keeping high relative water content to improve drought tolerance in crops [31,33,36], suggesting that COR might modulate stomatal actions to reduce water loss in crops under drought stress. Here, COR significantly reduced the stomatal apertures in leaves compared to the control. Consistent with this, the rate of water loss in COR-treated leaves was less than that of the control. Moreover, COR has remarkable structural and functional homology with MeJA [71,72], while the biological activity of COR is higher than that of MeJA [73]. Thus, MeJA has been known to increase ROS accumulation in guard cells and for inducing stomatal closure. Besides, MeJA-induced stomatal closure can be suppressed by DPI as an NADPH oxidase inhibitor and catalase [43,74,75]. It is worth noting that COR promoted the accumulation of H$_2$O$_2$ and O$_2$−· in maize leaves compared to control under well-watered conditions. Meanwhile, DPI and DMTU as a trap for H$_2$O$_2$ reduced the effect of water loss induced by COR, while weakened the stomatal actions induced by COR, which suggested that COR reduced the water loss in detached plants possibly through the ROS signaling pathways to regulate stomatal actions.

In plant cells, ROS are key secondary signals involved in the regulation of stomatal closure [69,76], and ROS production is mediated by specific enzymes including NADPH oxidase and peroxidases in response to various stimuli [77,78]. Genetic and pharmacological studies have demonstrated that MeJA induces ROS generation in guard cell through NADPH oxidase activation and subsequently induces ROS signaling for modulating stomatal movements [79,80]. In the present study, COR could induce ROS accumulation in the guard cells demonstrated by the use of fluorescent probe H2DCF-DA. Moreover, the expression of ZmRBOHs genes, associated with ROS generation was increased by many levels. ZmRBOHA and ZmRBOHB induced by COR was moderate, and the expression of ZmRBOHC was highly expressed in the epidermis guard cells. Combining with the protoplast system results, COR could rapidly activate ROS production in maize protoplast, while DPI and DMTU significantly suppressed the effect of ROS production induced by COR. These results suggested that COR could induce ROS production through regulating ZmRBOHs genes in the guard cells of maize. Based on the research stated above, we propose a possible mechanism for COR to regulate water loss in detached maize leaves: COR could induce ROS production through regulating ZmRBOHs genes in the guard cells. Thus, COR reduced the water loss in detached plants possibly through the ROS signaling pathways to regulate stomatal actions of maize. When plants are subjected to drought stress, COR regulated the expression of stress-related responsive genes and enhanced antioxidant scavenging capacity for improving drought tolerance in maize seedlings. In summary, COR could modulate ROS homeostasis to maintain water loss rate and antioxidant enzyme activities to improve maize performance under drought stress.
5. Conclusions

COR can significantly decrease the water loss rate, but the antioxidants DPI and DMTU eliminate the effect of water loss induced by COR. COR could intensify the ZmRBOHC expression level and increase ROS production in the maize epidermal guard cells and protoplasts, antioxidants DPI and DMTU decreased ROS production which are induced by COR. Moreover, COR-treated plants increased H$_2$O$_2$ and O$_2^•$ accumulation, antioxidant enzyme activities in the control plants, while COR reduced the ROS accumulation and antioxidant enzyme activities under PEG treatment. Overall, COR could improve maize performance under drought stress by modulating ROS homeostasis to maintain water loss rate and antioxidant enzyme activities.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11070685/s1, Table S1: List of primers used in this study.

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