Journal of Agricultural Science; Vol. 7, No. 11; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Hydrogen Peroxide Acts as a Signaling Molecule for the Methyl Jasmonate-Induced Antioxidant Defense in Wheat Callus to Promote Enhanced Drought Tolerance

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Received: July 29, 2015 Accepted: August 25, 2015 Online Published: October 15, 2015

doi:10.5539/jas.v7n11p99 URL: http://dx.doi.org/10.5539/jas.v7n11p99

Abstract

Drought stress is a major challenge in agriculture, causing severe loss of crops throughout the world. Methyl jasmonate (MeJA) regulates a variety of plant developmental process and responds to many biotic and abiotic stresses. To explore the physiological mechanisms of drought stress mitigated by exogenous MeJA, we subjected callus of wheat (*Triticum aestivum* L.) to drought induced medium after pretreatment with 10 ml sterile solution that contained the following singly or in combinations: MeJA (0, 0.25, and 2.5μM) and the lipoxygenase (LOX) inhibitor salicylhydroxamic acid (SHAM; 0 and 0.5 mM) for 24 hours. The endogenous jasmonic acid (JA) content, activity of LOX, hydrogen peroxide (H_2O_2) content, antioxidant enzymes activities, levels of reactive oxygen species (ROS), content of malondialdehyde (MDA), and cell viability were measured. The results showed that exogenous MeJA induced the activity of LOX and resulted in rapid increase in endogenous JA levels in a concentration-dependent manner; JA declined gradually after reaching its maximum. The level of H_2O_2 increased with increases in LOX activity and endogenous JA level, which are involved in the octadecanoid signaling pathway. However, SHAM inhibited the LOX activity, the endogenous JA level, and the accumulation of H_2O_2 in wheat callus. In addition, the activities of several antioxidant enzymes increased after MeJA-pretreatment, but this effect was also inhibited by SHAM. The cumulative results suggest that H_2O_2 was generated through the octadecanoid signaling pathway, and some antioxidant defense genes were provoked by the accumulation of H_2O_2. Therefore, we inferred that H_2O_2 might act as a signaling molecule for the MeJA-induced antioxidant defense. Drought stress can be improved by MeJA-pretreatment, leading to decrease in endogenous MDA and ROS contents, which are normally induced by drought conditions. Both drought stress and exogenous MeJA-pretreatment increased in LOX activity, endogenous JA level, and antioxidant enzymes. Combinatorial treatments showed an apparent synergistic effect on the activities of these antioxidant enzymes and resulted in improved cell viability. These results confirmed the hypothesis that H_2O_2 acts as a signaling molecule for the MeJA-induced antioxidant defense and could alleviate the negative effects of drought stress on wheat callus.

Keywords: methyl jasmonate, hydrogen peroxide, drought stress, antioxidative capacity, wheat calls

1. Introduction

Methyl jasmonate (MeJA) is considered to be a type of essential plant growth regulator which has a vital role in regulating many aspects of plant-development process in response to biotic and abiotic stresses (Cheong et al., 2003; Kazan et al., 2008; Avanci et al., 2010). It is reported that MeJA, as a volatile compound, was first identified in the flowers of *Jasminum grandiflorum*. Besides, it has been found in a number of many other plants in recent years. MeJA and jasmonic acid (JA), which are both termed as jasmonates (JAs), play key roles in regulating the growth and development of plants including seed germination, root growth, blossoming, fruit

ripening, and senescence (Creelman et al., 1997; Wasternack, 2007).

In order to maintain at a normal condition and adapt to the stressful environments, plants have evolved comprehensive adaptive response mechanisms. For instance, the higher levels of JA is found in *Asparagus officinalis* spear tips, *Carica papaya* seedlings, as well as *Pinus pinaster* plants, when plants were exposed to drought stress (Gapper et al., 2002; Mahouachi et al., 2007; Pedranzani et al., 2007). Moreover, exogenous applications of MeJA can strengthen the drought-tolerance in plants in dry condition caused by polyethylene glycol (Li et al., 1998) and natural drought (Wang, 1999). The accumulation of JAs induces the enzymatic and non-enzymatic mechanisms to clear the Reactive oxygen species (ROS) and minimize the damage (Wang, 1999; Wang et al., 2008; Shan et al., 2010). ROS are produced in plants in response to a wide variety of abiotic and biotic stresses. The accumulation of ROS may affect many cellular components and trigger plant defense signals immediately (Breusegem et al., 2001). ROS and particularly H_2O_2 are deemed to be signaling molecules in environmental stress response, since they induce the expression of a variety of defense-related genes (Foyer et al., 2005). H_2O_2 is involved in many signaling pathways in plants. It can act as a partial signal or a diffusion signal to induce cells to react defensively (Wu et al., 1995; Wu et al., 1997; Alvarez et al., 1998). H_2O_2 has varying effects on plant cells depending on its concentration and cellular location (Desikan et al., 2004). For instance, MeJA alleviate the influence of drought by promoting stomatal closure, which was induced by generation of H_2O_2 (Suhita et al., 2004). Moreover, it has been reported that the increase of H_2O_2 in the cells of some drought-resistant plants might be more related to a structural role than to oxidative damage (Munné-Bosch et al., 2001; Jubany-Marí et al., 2009). In addition, a number of studies focused on physiological function of exogenous H_2O_2 which enabled plants to tolerate many kinds of abiotic stresses (Li et al., 2009; Gao et al., 2010; Ishibashi et al., 2011; Zhang et al., 2011). ROS is considered to be a part of stress-induced signal transduction, and H_2O_2 regulates numerous expressions of genes and complex signaling pathways.

It is important to clarify how plants adapt to drought stress and maintain growth, development, and productivity during stress periods, because understanding the mechanisms would help to against further loss of yield and in breeding for drought stress (Yin et al., 2006). Despite the extensive research on the response of plant either to drought stress or MeJA alone, there is limited understanding about the interactive effect. This research aims to investigate how MeJA regulates drought tolerance at physiological levels under drought stress, providing new knowledge about the relationship between exogenous MeJA and antioxidant metabolism in plants.

2. Materials and Methods

2.1 Plant Material and in vitro Culture

The experiments were conducted at the science and technology station, Henan Agricultural University (34°47′N, 113°38′E). Callus used in this study were derived from semi-winter wheat (*Triticum aestivum* L.) sources. Seeds of Zhoumai18 were obtained from Zhoukou Academy of Agricultural Sciences. It was grown in field conditions with recommended cultural practices. Calluses were obtained as described earlier with a slight modification (Qureshi et al., 1989). All inflorescences were tagged at the onset of anthesis and, depending upon the need, whole spikes were harvested 14 days post-anthesis. For callus induction, kernels were surface-sterilized for 1 min in 70% ethanol and they were rinsed four times with sterile deionised water, followed by treatment in 0.1% mercuric chloride (w/v) for 6 min, then they were rinsed four times with sterile deionised water. Afterwards, aseptic immature embryos were dissected and cultured in the gnotobasis. The embryos (40 per plate) were aseptically separated from endosperm and placed with the scutellum upwards on 20 ml inductive culture medium. Embryos were incubated for 20 days at 25 °C in the dark in a constant temperature incubator and the obtained callus were transferred onto the same fresh medium without abscisic acid (ABA) for an additional period of 20 days. After this period, 10 ml sterile solution contained the following singly or in combinations: MeJA (0, 0.25 and 2.5 μM, Sigma) and salicylhydroxamic acid (SHAM, a inhibitor of LOX) (0 and 0.5 mM, Sigma) was added for 24 hours. Parts of the callus of uniform size (diameter ranking from 7 to 10 mm) were harvest depending upon the need. Detailed treatments were conducted as follows: 0 μM MeJA (CK), adding 10 ml sterile deionized water on the surface of the medium; 0.25 μM and 2.5 μM MeJA, adding 10 ml sterile solution containing 0.25 μM and 2.5 μM MeJA, respectively; 0 μM MeJA + SHAM, adding 10 ml sterile solution containing 0.5 mM SHAM; 0.25 μM MeJA + SHAM, adding 10 ml sterile solution containing 0.25 μM MeJA and 0.5 mM SHAM; 2.5 μM MeJA + SHAM, adding 10 ml sterile solution containing 2.5 μM MeJA and 0.5 mM SHAM. Then, the rest of callus of uniform size were placed on the inductive culture medium (-0.15 MPa, without ABA) and the drought induced medium (-1.25 MPa, without ABA) for 5 days, then the callus were harvested for assays.

The inductive culture medium consisting of MS salt (Murashige et al., 1962) supplemented with 30 g l^{-1} sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 7 g l^{-1} agar (Phytagel; Sigma) and was adjusted to pH 5.7 before

autoclaving for 20 min at 120 °C and 150 KPa, when temperature of the autoclaved medium reduce to about 50 to 60 °C, sterile abscisic acid (ABA; Sigma) were added to the autoclaved medium and maintain its concentration to 0.5 mg l^{-1}.

The drought induced medium was prepared according to Paul et al. (2006) method with a slight modification. Polyethylene glycol 6000 (PEG 6000, Kermel), a high-osmotic stresses producer, was dissolved in a sterilized basal media (MS salt with 5 mM MES buffer), and PEG final concentration was adjusted to 500 g l^{-1} followed by adjustment of pH to 5.7. This solution was added on the top of solid medium (1:1, v/v) which was the same to the inductive culture medium, then incubated overnight (15 h), in order to ensure the PEG molecule could diffuse well into the solid medium. The water potential of inductive culture medium and drought induced medium were -0.15 ± 0.013 and -1.25 ± 0.018 MPa (n = 10), respectively, which were measured by using Psypro (C-52, wescor, USA).

2.2 Extraction and Determination of JA Content

Extraction and quantification of JAs were carried out according to methods previously described by Tan et al. (2012) with a little modification. Samples (500 mg) were ground to powder with liquid nitrogen and extracted three times with 2 ml of 80% pre-cooling methanol (v/v) (containing 0.01% ascorbic acid as an antioxidant) overnight at 4 °C. After centrifugation (8,000 × g for 10 min), 5 ml of extracts were concentrated to the aqueous phase with N_2 and adjusted to pH 3.0 with 0.4 M citric acid, and partitioned twice with equal volumes of ethyl acetate. The combined acidic ethyl acetate phase was concentrated until dry with N_2, then dissolved in 0.4 ml methanol and stored at -20 °C before analysis. JA was quantified using HPLC-MS system (QTRAP 4000, AB SCIEX, USA), with JA (Sigma) as the external standards.

2.3 Enzyme Extraction and Assay Procedures

After ground with liquid nitrogen in a small mortar and pestle, the powder was then extracted with the appropriate buffer. Protein content was extracted by deionized water, and then measured by measured using Coomassie brilliant blue G-250 method; Bovine serum albumin grade V (Sigma) was used as a standard (Bradford, 1976).

LOX was determined spectrophotometrically by measuring the formation of conjugated dienes at 234 nm under 25 °C using linoleic acid (Sigma) as substrate following the method (Axelrod et al., 1981). Samples weighing 500 mg were homogenized in 5 ml of 25 mM Tris-HCl buffer (pH 7.5). After centrifugation at 20,000× g for 15 min at 4 °C, the supernatant was collected as the crude enzyme solution. Then, 0.1 ml enzyme extract was mixed with 2.4 ml substrate stock solution which contained 0.2 M K-phosphate buffer, 5 mM Na_2HPO_4, 2.5 mM linoleic acid, 0.25% Tween 20. Absorbance was measured at 234 nm at 30 s intervals up to 5 min using spectrophotometer (TU-1810, Persee, China) at room temperature.

SOD was assayed by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) chloride (Beauchamp et al., 1971); one unit of the SOD activity was defined as the amount of enzyme required to inhibit reduction of NBT by 50%. Samples weighing 500 mg were homogenized in 5 ml of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). After centrifugation at 12,000× g for 20 min at 4 °C, the supernatant was collected as the crude enzyme solution. A 3 ml of reaction mixture contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 0.1 ml of enzyme extract and 2 μM riboflavin was prepared. Riboflavin was added at the end. The reaction mixture was exposed to 15 watt fluorescent tubes and the decrease in the absorbance of the reaction mixture was measured at 560 nm using spectrophotometer (TU-1810, Persee, China) at room temperature.

POD activity was determined specifically with guaiacol at 470 nm (Egley et al., 1983). Samples weighing 500 mg were homogenized in 5 ml of 50 mM K-phosphate buffer (pH 5.5). After centrifugation at 12,000× g for 20 min at 4 °C, the supernatant was collected as the crude enzyme solution. A 3 ml of reaction mixture contained 2.9 ml of 50 mM K-phosphate buffer (pH 5.5), 1 ml of 0.6 M H_2O_2, 1 ml of 50 mM guaiacol and 0.1 ml crude enzyme solution was prepared. Absorbance was measured at 470 nm at 30 s intervals up to 5 min using spectrophotometer at room temperature.

CAT activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm (Cakmak et al., 1992). Samples weighing 500 mg were homogenized in 5 ml of 25 mM Tris-HCl buffer (pH 7.5). After centrifugation at 12,000× g for 20 min at 4 °C, the supernatant was collected as the crude enzyme solution. A 1.5 ml of reaction mixture contained 1 ml of 100 mM K-phosphate buffer (pH 7.0), 0.4 ml of 200 mM H_2O_2 and 0.1 ml of crude enzyme solution was prepared. Absorbance was measured at 240 nm at 30 s intervals up to 3 min using

spectrophotometer at room temperature.

GR activity was measured spectrophotometrically by measuring the decline of NADPH at 340 nm (Grace et al., 1996). Samples weighing 500 mg were homogenized in 5 ml of 25 mM K-phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone (PVP) and 0.2 mM EDTA. After centrifugation at 12,000×g for 20 min at 4 °C, the supernatant was collected as the crude enzyme solution. The reaction mixture contained 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, 0.1 mM K-phosphate buffer (pH7.8), and 0.15 ml crude enzyme solution was prepared. The reaction was started by the addition of NADPH. Absorbance was measured at 340 nm at 15 s intervals up to 2 min using spectrophotometer at room temperature.

2.4 Hydrogen Peroxide (H_2O_2) Contents, the Formation Rate of Superoxide Anion Radical ($O_2.^-$)

H_2O_2 was assayed by potassium iodide method (Velikova et al., 2000). The oxidation product was measured at 390 nm. The amount of H_2O_2 formed was computed from the standard curve made earlier with known concentrations of H_2O_2. Samples weighing 200 mg were homogenized in 2 ml of 0.1% trichloroacetic acid solution. After centrifugation at 12,000×g for 20 min at 4 °C, a 1.5 ml of reaction mixture contained 1 ml of 1 M potassium iodide and 0.5 ml supernatant was prepared. The reaction mixture was measured at 390 nm after 5 min at room temperature.

The formation rate of $O_2.^-$ was measured by using sulfanilamide method (Elstner et al., 1976). The absorbance was measured at 530 nm using spectrophotometer (TU-1810, Persee, China) at room temperature, and the formation rate of $O_2.^-$ was calculated from a standard curve of $NaNO_2$ reagent. Samples weighing 400 mg were homogenized in 2 ml of 65 mM K-phosphate buffer (pH 7.8). After centrifugation at 12,000× g for 20 min at 4 °C, a 3 ml of reaction mixture contained 1.5 ml of 65 mM K-phosphate buffer (pH 7.8), 0.5 ml of 10 mM hydroxylamine hydrochloride and 1 ml supernatant were incubated 20 min at 25 °C. Taking 2 ml of reaction mixture mentioned above, mixed with 2 ml of 17 mM sulfanilic acid and 2 ml of 7 mM 1-naphthylamine, then, the mixture were incubated 30 min at 30 °C and was measured at 530 nm at room temperature.

2.5 Determination of Malonaldehyde (MDA) Contents, Cell Viability

MDA was assayed by using thiobarbituric acid method (Dhindsa et al., 1981). Samples weighing 200 mg were homogenized in 4 ml of 10% trichloroacetic acid. After centrifugation at 12,000× g for 15 min at 4 °C, a 2 ml of reaction mixture contained 1 ml of 0.6% thiobarbituric acid and 1 ml supernate was incubated 30 min at 95 °C. The reaction mixture was measured at 450 nm, 532 nm and 600 nm at room temperature.

Cell viability was determined by using triphenyltetrazolium chloride (TTC) reduction method. Extracted formazan was quantified spectrophotometrically at 487 nm (Lutts et al., 2004). Samples weighing 50 mg of fresh tissue were quickly rinsed in deionised water containing 0.05% Tween 20 and incubated at 30 °C in darkness in tubes containing 5 ml of 50 mM K_2HPO_4 (containing 0.5% TTC, pH 7.0) for 15 h. Samples were then filtered on filter paper, rinsed with deionised water, and incubated in 3 ml ethanol 94% at 80 °C during 5 min under gentle agitation (80 rpm) to ensure homogenisation during the extraction. After centrifugation at 5000× g during 1 min, extracted formazan was quantified spectrophotometrically at 487 nm at room temperature. The viability index is defined as the absorbance measured per gram of fresh tissue.

2.6 Statistics

The experiment was repeated at least six times and gave similar trends. The SPSS software (SPSS v19.0, Chicago, IL, USA) was used for statistical analyses. The data are presented as means ± standard errors (SE) from six independent experiments.

3. Results

3.1 Time Course of JA Content

Time course of JA content induced by exogenous MeJA was shown in two phases (Figure 1). Once the wheat callus were exposed to exogenous MeJA, endogenous JA content increased rapidly in a concentration-dependent manner. JA reached its maximum accumulation at 3 and 6 h in the concentrations of 0.25 and 2.5 μM, respectively, whose values were 10.7 and 14.4 ng g^{-1} (FW). After this point, JA content declined gradually. Endogenous JA content was steady in the control treatment (0 μM). The process of endogenous JA accumulation was significantly inhibited by 0.5 mM of SHAM; when the callus were treated with SHAM only, endogenous JA content (8.18 - 8.5 ng g^{-1}) decreased below that of the control treatment (8.31 - 8.71 ng g^{-1}).

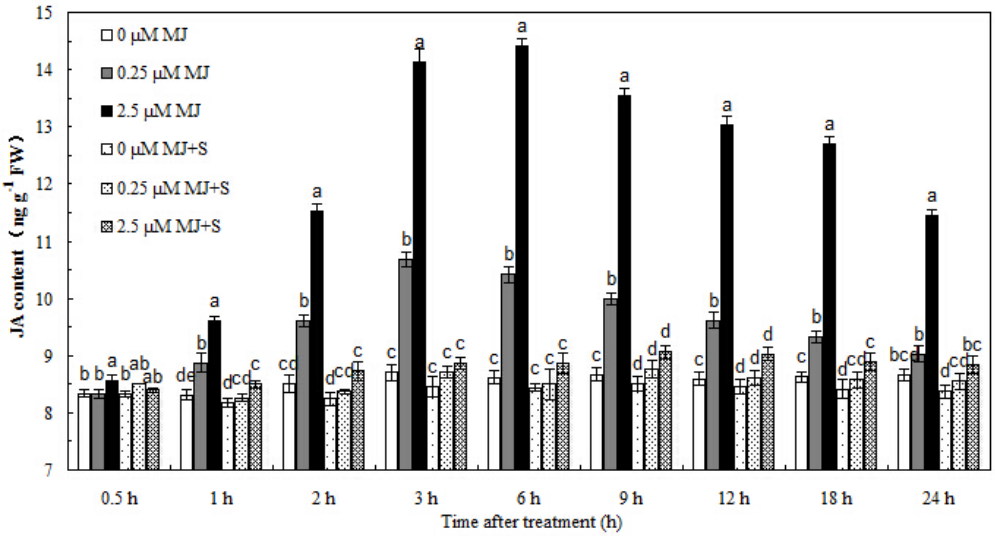

Figure 1. The time course of jasmonic acid contents in wheat callus treated by methyl jasmonate alone or together with salicylhydroxamic acid. Samples were collected at 0.5, 1, 2, 3, 6, 9, 12, 18, and 24 h after treatment. The data represent the mean ± SE from six independent experiments of treatments at the $P < 0.05$ level

3.2 Time Course of LOX Activity and H_2O_2 Content

To evaluate the changes in LOX activity after the MeJA-pretreatment, portions of wheat callus were harvested 6, 12, 18, and 24 h after MeJA was applied alone or together with SHAM (Figure 2(A)). The treatment of different concentrations of MeJA significantly increased the LOX activity at 12, 18, and 24 h after the MeJA application ($P < 0.05$). However, only higher concentrations (2.5 μM) significantly increased the LOX activity at an earlier time point ($P < 0.05$, 6 h after MeJA application). SHAM application significantly inhibited the MeJA-activated LOX activity at all MeJA concentrations and SHAM application only showed the lowest levels of LOX activity, whose values were 31.3-32.6 (nmol hydroperoxyde min^{-1} mg^{-1} protein). Thus, all treatments with both MeJA and SHAM showed no difference in LOX activities ($P > 0.05$, Figure 2(A)).

To further confirm the role of H_2O_2 during exogenous MeJA-pretreatment, we measured the changes in endogenous H_2O_2 levels of wheat callus (Figure 2(B)). Similar to the LOX activities, exogenous MeJA significantly increased the endogenous H_2O_2 levels at 12, 18, and 24 h after MeJA application ($P < 0.05$). Samples of MeJA applied together with SHAM had no effect on endogenous H_2O_2 levels ($P > 0.05$), except at high MeJA concentration ($P < 0.05$, 2.5 μM); SHAM application only showed the lowest levels (6.8-7.4 μmol g^{-1}) of H_2O_2. H_2O_2 levels of treatment with lower concentration (0.25 μM) of MeJA increased 9%, 65%, and 84% in the production of H_2O_2, after 12, 18, and 24 h, respectively, compared with the control (Figure 2(B)). H_2O_2 levels of treatment with high concentration (2.5 μM) of MeJA increased 66%, 114%, and 151% in the production of H_2O_2, after 12, 18, and 24 h, respectively, compared with the control (Figure 2(B)). However, MeJA-pretreatments had no effect on other ROS, such as $O_2^{\cdot -}$ (data not shown).

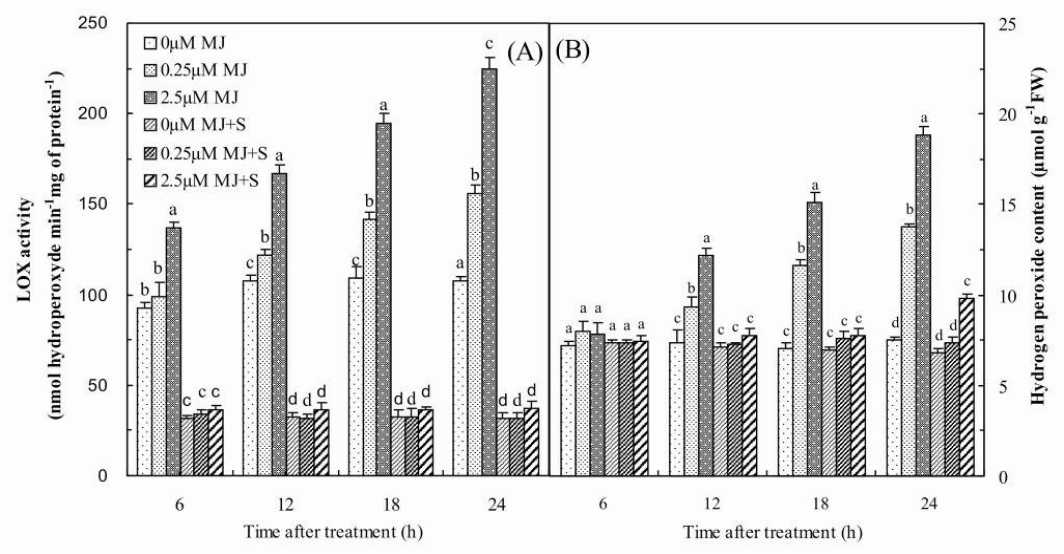

Figure 2. The time course of lipoxygenase activity in wheat callus treated by methyl jasmonate alone or together with salicylhydroxamic acid. Samples were collected at 6, 12, 18, and 24 h after treatment. The data represent the mean ± SE from six independent experiments of treatments at the $P < 0.05$ level. Different letters indicate significant difference among treatment at the 0.05 significance level

3.3 Effects of MeJA-Pretreatment on Antioxidant Enzymes

MeJA-pretreatment at higher concentrations significantly increased the antioxidant enzyme activity at 24 h ($P < 0.05$). Total superoxide dismutase (SOD) activities showed no significant changes for all concentrations of MeJA ($P > 0.05$), except at the higher MeJA concentration (2.5 μM; Figure 3(A)). Total peroxidase (POD) activities increased significantly after MeJA pretreatment ($P < 0.05$); however, application of SHAM reduced total POD activity significantly ($P < 0.05$), except at the higher MeJA concentration (2.5 μM) (Figure 3(B)). Total activities of catalase (CAT) and glutathione reductase (GR) also increased after MeJA treatment ($P < 0.05$, Figures 3(C) and 4(D)). When the callus were treated with SHAM only, the activity of SOD, POD, CAT, and GR were 21.1, 7.8, 0.3, and 0.1 (units mg^{-1} protein), which were significantly decrease compared to MeJA treatment except SOD activity in lower concentration (0.25 μM). Overall, the MeJA-treated wheat callus exhibited a slight increase in total SOD activity levels and a significant increase in the activities of other antioxidant enzymes; SHAM suppressed the MeJA-activated antioxidant enzyme activities (Figure 3).

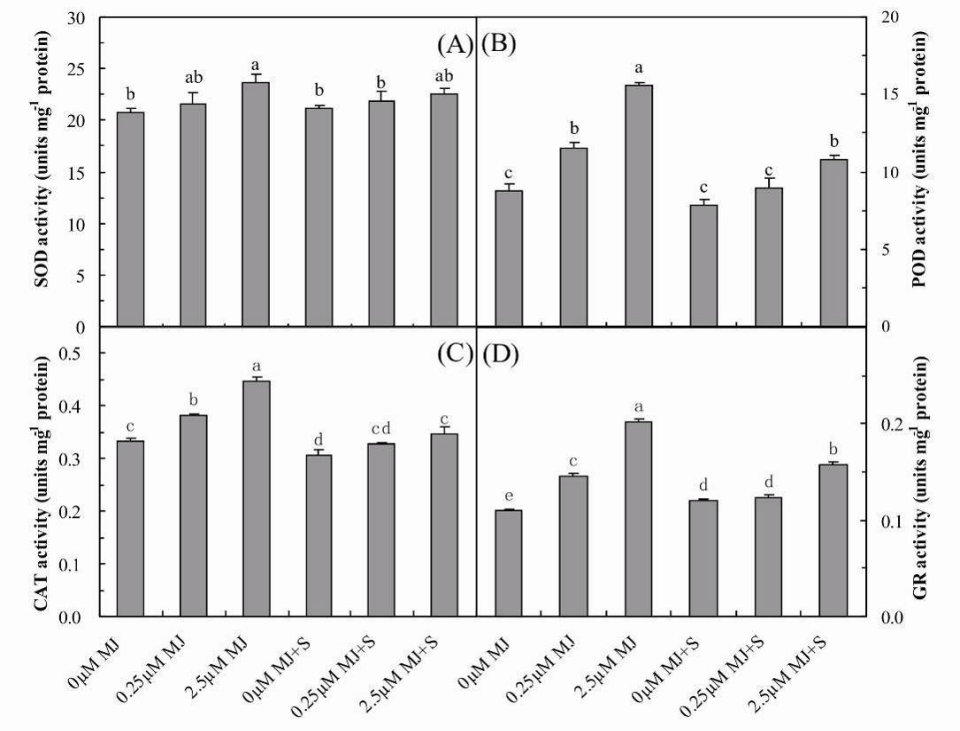

Figure 3. Effects of methyl jasmonate-pretreatment on antioxidant enzyme activities. The activities of superoxide dismutase (A), peroxidase (B), catalase (C) and glutathione reductase (D) were measured at 24 h after treatment with MeJA alone or together with SHAM. The data represent the mean ± SE from six independent experiments of treatments at the $P < 0.05$ level. Different letters indicate significant difference among treatment at the 0.05 significance level

3.4 Effects of MeJA-Pretreatment on LOX Activity and JA Content after Drought Stress

To understand role of MeJA-pretreatment under drought stress, we assayed LOX activity and JA content after 5 d drought stress. Drought induced condition significantly enhanced the activity of LOX ($P < 0.05$) after MeJA-pretreatments (Figure 4(A)). LOX activities were synergistically activated by MeJA pretreatment and drought induced condition in wheat callus. SHAM significantly inhibited the activities of LOX (Figure 4(A)). SHAM application only showed the lowest levels in control and drought induced mediums, whose values were 34.0 and 55.1 (nmol hydroperoxyde min^{-1} mg^{-1} protein).

There were significant changes in endogenous JA contents between control and drought induced conditions ($P < 0.05$; Figure 4(B)). The effect of MeJA-pretreatment lasted throughout the drought induced condition and resulted in increased endogenous JA contents in a concentration-dependent manner. JA content was synergistically induced by MeJA-pretreatment and drought induced medium. In addition, JA accumulation was also inhibited by SHAM, and only the higher MeJA concentration (2.5 μM) could significantly increase the endogenous JA content ($P < 0.05$). SHAM application only showed the lowest levels in control and drought induced mediums, whose values were 5.4 and 6.9 ng g^{-1}. LOX activity and JA content, respectively, which showed a good correlation when these two results were combined.

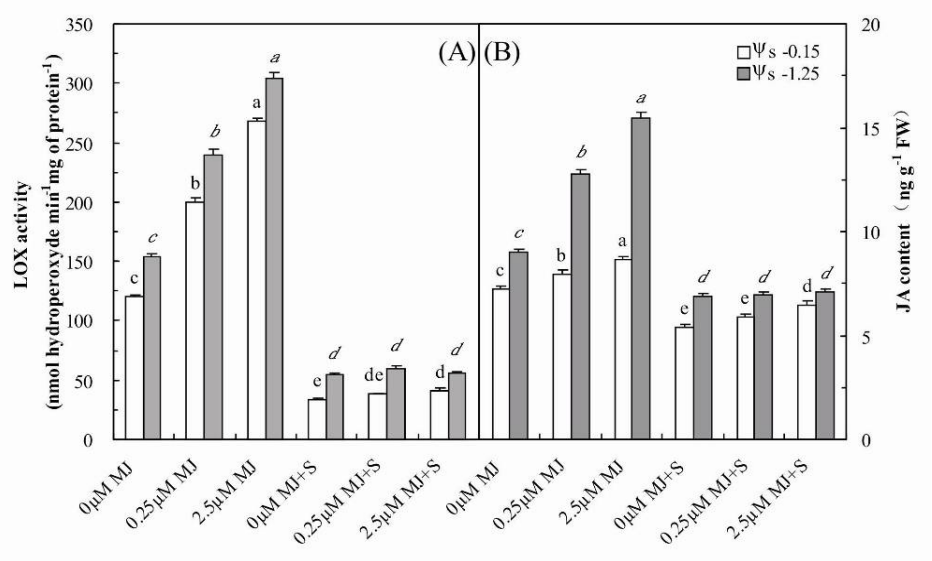

Figure 4. Effects of methyl jasmonate-pretreatment on lipoxygenase activity (A) and jasmonic acid content (B) in wheat callus after the methyl jasmonate-pretreatment, the callus were further exposed to drought induced medium for 5 days. The data represent the mean ± SE from six independent experiments of treatments at the $P < 0.05$ level. Different lower-case letters and its italics indicate significant difference between normal (ψ_s -0.15 MPa) and drought induced (ψ_s -1.25 MPa) treatment, respectively, at the 0.05 significance level

3.5 Effects of MeJA-pretreatment on antioxidant enzymes after drought stress

There were significant differences ($P < 0.05$) in the SOD activity in wheat callus between the normal and drought induced groups at higher concentrations of MeJA-pretreatment (Figure 5(A)). After pretreatment with MeJA at higher concentrations, the SOD activities were elevated additively by MeJA-pretreatment and drought induced condition ($P < 0.05$). Therefore, drought did not change the SOD activity at lower concentration of MeJA-pretreatment ($P > 0.05$), but higher concentrations of MeJA-pretreatment together with drought induced condition significantly increased SOD activity ($P < 0.05$). SHAM application only inhibited the activities of SOD to a minor extent for all treatments of MeJA with or without drought, whose values were 21.3 and 22.6 (units mg^{-1} protein), respectively.

Compared to the normal group, the POD activities were higher ($P < 0.05$) in the wheat callus of the drought induced group, and this trend did not change significantly ($P > 0.05$) by adding SHAM treatment (Figure 5(B)). When the callus were treated with SHAM only, the activity of POD were 8.5 and 9.8 (units mg^{-1} protein), respectively in control and drought induced treatments. Both MeJA-pretreatment and drought induced treatment increased the POD activity significantly ($P < 0.05$); the POD activity in the MeJA-pretreatment plus drought induced treatment was the highest ($P < 0.01$) among all treatments.

In comparison to the control, the CAT activities of wheat callus were significantly higher ($P < 0.05$) in both drought non-induced and drought induced groups ($P < 0.05$; Figure 5(C)). When the callus were treated with SHAM only, the activity of CAT were 0.3 and 0.4 (units mg^{-1} protein), respectively in drought non-induced and drought induced treatments. Among all treatment groups, the CAT activity in the MeJA-pretreatment plus drought induced condition was the highest ($P < 0.05$).

The activity of GR (Figure 5(D)) in the wheat callus of the drought induced group was higher ($P < 0.05$) than those in the control group. GR activity in the MeJA-pretreatment group was significantly higher ($P < 0.05$) than those in the control. When the callus were treated with SHAM only, the activity of GR were 0.122 and 0.123 (units mg^{-1} protein), respectively in normal and drought induced treatments. Both drought induced treatments and MeJA-pretreatment increased ($P < 0.05$) the activity of GR in wheat callus. The activities of the GR in the MeJA-pretreatment plus drought induced treatment were the highest ($P < 0.05$).

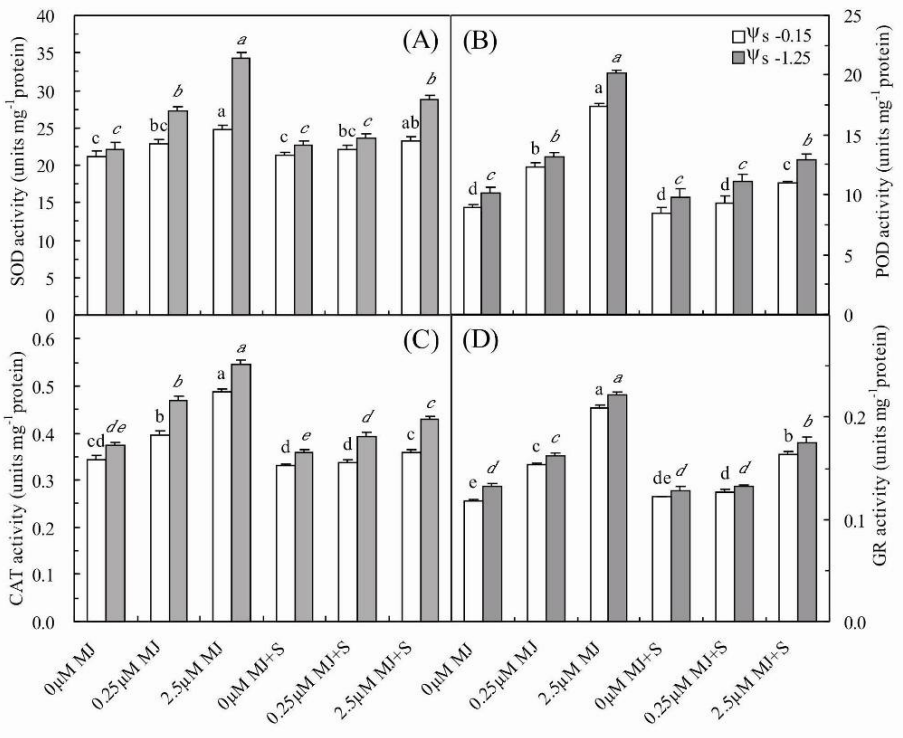

Figure 5. Effects of methyl jasmonate-pretreatment on superoxide dismutase (A), peroxidase (B), catalase (C) and glutathione reductase (D) in wheat callus. After the methyl jasmonate-pretreatments, the callus were further exposed to drought induced medium for 5 days. Values are the mean of six replicates and vertical bars are SE. Different lower-case letters and its italics indicate significant difference between normal (ψ_s -0.15 MPa) and drought induced (ψ_s -1.25 MPa) treatment, respectively, at the 0.05 significance level

3.6 Effects of MeJA-Pretreatment on Reactive Oxygen Species (ROS) after Drought Stress

To further understand the roles of ROS (including H_2O_2 and $O_2^{\cdot-}$) with drought-induced responses of MeJA-pretreatment, the content of endogenous H_2O_2 and the formation rate of $O_2^{\cdot-}$ were determined. H_2O_2 and $O_2^{\cdot-}$ were significantly affected by drought induced condition ($P < 0.05$; Figure 6). MeJA-pretreatment had no significant effect on H_2O_2 in the normal group, except at the higher concentration of MeJA plus SHAM treatment, which showed a significant decline. MeJA-pretreatment suppressed the generation of H_2O_2. However, adding SHAM partly arrested this effect. In addition, MeJA-pretreatment significantly inhibited the formation of $O_2^{\cdot-}$ both in the normal and drought induced groups. Adding SHAM did not affect the formation of $O_2^{\cdot-}$ except in the higher concentration of MeJA treatment in the normal condition. SHAM application only showed the highest levels of H_2O_2 and $O_2^{\cdot-}$ in normal and drought induced treatments, whose values were 9.5, 16.1 μmol g^{-1} and 0.7, 0.8 nmol min^{-1} g^{-1}, respectively.

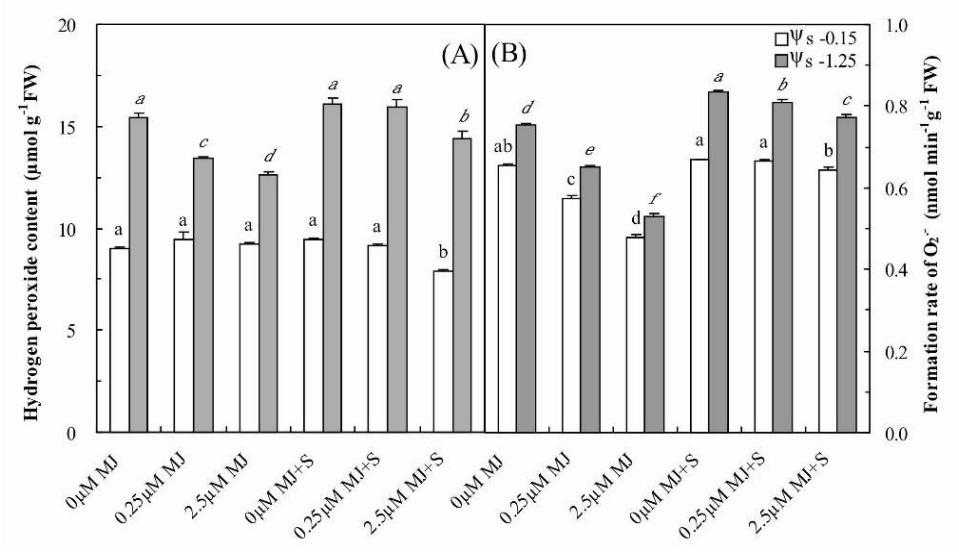

Figure 6. Effects of methyl jasmonate-pretreatment and drought stress on hydrogen peroxide (A), Superoxide anion radical (B) in wheat callus. After the methyl jasmonate-pretreatments, the callus were further exposed to drought induced medium for 5 days. Values are the mean of six replicates and vertical bars are SE Different lower-case letters and its italics indicate significant difference between normal (ψ_s -0.15 MPa) and drought induced (ψ_s -1.25 MPa) treatment, respectively, at the 0.05 significance level

3.7 Effects of MeJA-Pretreatment on MDA Content and Cell Viability after Drought Stress

Membrane destabilization is generally attributed to lipid peroxidation, which results in the accumulation of malondialdehyde (MDA), showing the damage to the cell membranes. Therefore, we determined the MDA levels to investigate the extent of the damage to cell membranes of wheat callus caused by drought induced condition. Drought induced treatment increased the MDA contents in the wheat callus in this study (Figure 7(A)). In the drought induced group, the MDA level decreased in the treatments with lower concentration of MeJA. However, higher concentrations of MeJA gave the opposite effect because there was too much lipid peroxidation. When the callus were treated with SHAM only, the MDA contents were 8.9 and 29.2 (nmol g^{-1}), respectively in normal and drought induced treatments. Drought induced condition increased the MDA content significantly ($P < 0.05$), while MeJA-pretreatment with 0.25 μM concentration decreased the MDA content ($P < 0.05$).

Furthermore, drought induced condition significantly reduced the TTC reduction ($P < 0.05$; Figure 7(B)). In the normal group, there was no significant effect on TTC reduction, except at the higher concentration of MeJA. In the drought induced group, lower concentration of MeJA significantly ($P < 0.05$) alleviated the decline of TTC reduction, which was induced by drought induced medium. When using SHAM, there was no difference of TTC reduction ($P > 0.05$) in the normal group, and SHAM suppressed the alleviation of TTC reduction in lower concentration of MeJA in the drought induced group. When the callus were treated with SHAM only, the TTC reduction values were 2.0 and 0.8 (ΔOD at 487 nm g^{-1}), respectively in normal and drought induced treatments.

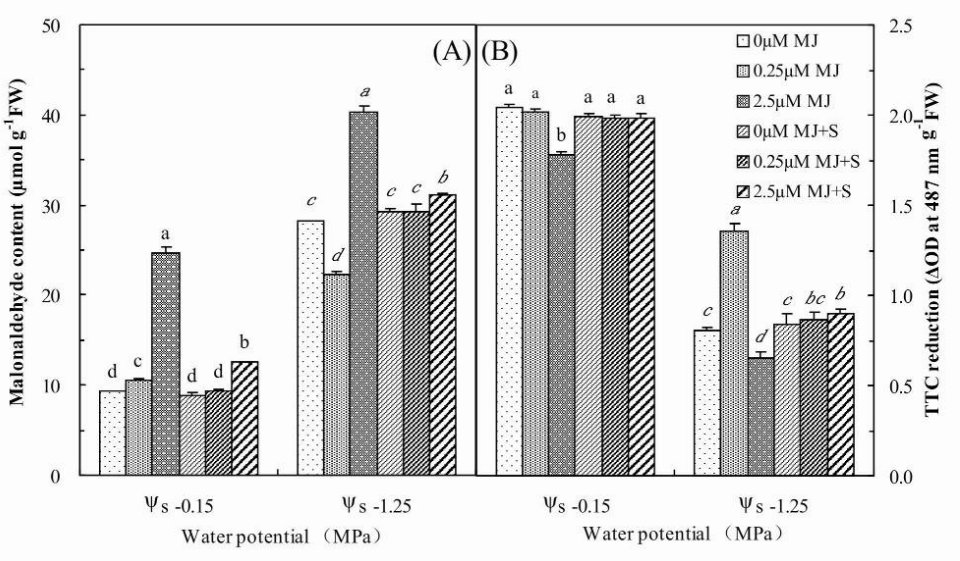

Figure 7. Effects of methyl jasmonate-pretreatment and drought stress on malondialdehyde contents (A) and triphenyltetrazolium chloride reduction (B) in wheat callus. After the methyl jasmonate-pretreatments, the callus were further exposed to drought induced medium for 5 days. Values are the mean of six replicates and vertical bars are SE. Different lower-case letters and its italics indicate significant difference between normal (ψ_s -0.15 MPa) and drought induced (ψ_s -1.25 MPa) treatment, respectively, at the 0.05 significance level

4. Discussion

JAs are synthesized in plants via the octadecanoid pathway (Wasternack, 2007). Some special signals lead the linolenic acid to releasing from the membrane lipid, and then the free-linolenic acid was oxygenated to form 13 (S)-hydroxy linolenic acid (13-HPOT) by LOX, which is considered to be the first step of the biosynthesis of JA. Subsequently, 13-HPOT is translated into JAs. The whole process was defined as the octadecenoic acid pathway (Rangel et al., 2002). The MeJA-pretreatment regulates a series of expression of genes that were involved in JA biosynthesis, cell-wall formation, secondary metabolism, besides encoding stress-protective and defense proteins (Cheong et al., 2003). This signaling molecule led to an increase in LOX activity as well as endogenous JA content (Figure 1). Time course experiments (Figure 2) showed that the LOX activity was clearly detectable in the wheat callus after 6 h exposed to MeJA and increased continuously for the next 18 h. Furthermore, SHAM, which is an efficient inhibitor of JA biosynthesis, could suppress LOX activity and gene expression (Knöfel et al., 1984; Gao et al., 2003; Yang et al., 2011). It was reported that SHAM inhibit LOX activity by 90% and prevent the synthesis of the vegetative storage protein mRNA (Macrì et al., 1994; Staswick et al., 1991). Throughout the current experiment, SHAM significantly inhibited the activity of LOX of wheat callus. In addition, the peak of JA accumulation were induced by exposing the wheat callus to exogenous MeJA in the concentration of 0.25 and 2.5 μM for 3 and 6 h, respectively; biosynthesis of JA was suppressed by the application of SHAM in contrast. Similar results were reported in *Gladiolus hybridus*; LOX activity, and endogenous MeJA content in corms steadily decreased with the increasing concentration of SHAM (Lian et al., 2011). What is important was the content of H_2O_2 increased continuously with the increases of LOX activity and endogenous JA content (Figure 1, 2, and 4), suggesting a link between these responses (Jubany-Marí et al., 2009). In addition, our research corroborated previous findings that plant LOX is strongly induced by exogenous MeJA (Feussner et al., 1995; Avdiushko et al., 1995; Royo et al., 1996; Heitz et al., 1997). In this study, it was shown that LOX of wheat callus were induced by exogenous application of MeJA (Figure 2(A)). Similar result was reported that MeJA was also a potent inducer of LOX accumulation in barley leaves as demonstrated by Western blot analysis (Feussner et al., 1995), and was a signaling molecule that induced H_2O_2 production in plants (Jubany-Marí et al., 2009). Therefore, these observations suggest that the exogenous MeJA must play a vital role in the generation of signals (such as H_2O_2) that were regulated by JA via the octadecanoid pathway; then H_2O_2, as a second signaling molecule, must induce a series of signaling pathways, such as antioxidant defense genes.

ROS play important roles in metabolic activities of organisms; they are generated in plant tissues and organs during plant growth and development and also in response to environmental and biotic stresses (Dangl et al., 1996; Greenberg, 1996; Pennell et al., 1997). H_2O_2 acts as a signaling molecule to regulate the transduction of

stress signals (Foyer et al., 1997). In the normal condition, intracellular H_2O_2 is maintained at a certain level (Veljovic-Jovanovic et al., 2001; Karpinski et al., 1999). In response to some signals, such as MeJA, plant cells would accumulate H_2O_2, potentially damaging cell membranes (Xu et al., 2006) and leading to cell death (Molassiotis et al., 2006). To prevent or alleviate the ROS effects, plants possess an antioxidant defense system including enzymatic and non-enzymatic mechanisms to scavenge ROS and minimize their harmful effects. We found that exogenous MeJA-pretreatment increased SOD, POD, CAT, and GR activity, regardless of whether wheat callus were subjected to drought or not (Figures 3 and 5). SHAM not only had an inhibitory impact on the LOX activity (Figure 2(A)), but also suppressed the activities of these antioxidant enzymes (Figures 3 and 5). Meanwhile, H_2O_2 content was also suppressed by SHAM (Figure 2(B)). These results suggested that there is a link among LOX, H_2O_2 and antioxidant enzymes. During drought induced treatments, LOX, JA and these antioxidant enzymes were also induced by MeJA-pretreatment which had an additive effect with drought stress (Figures 4 and 5). In addition, MeJA-pretreatment resulted in decreasing the levels of ROS (H_2O_2 and $O_2^{\cdot-}$) in plant under drought induced condition (Figure 6). What is particularly interesting was inhibitory effects of SHAM still exists during drought stress (Figures 4 and 5). To investigate whether MeJA-pretreatment induced membrane protection is accomplished by preventing lipid peroxidation, we detected the MDA content, which is produced when polyunsaturated fatty acids in the membrane undergo peroxidation (Liu et al., 2012). The results indicated that the higher activity of these antioxidant enzymes in MeJA-pretreated wheat callus coincided with low levels of ROS and MDA. Nevertheless, the higher concentration of MeJA induced the excessive LOX activity, which would generate a lot of ROS, and result in cell membrane disruption and increased MDA content (Figure 7(A)). In addition, 2, 3, 5-triphenyltetrazolium chloride (TTC) is used to assay activity of dehydrogenase which has a high redox potential. Since TTC can be inserted into the mitochondrial respiration chain, its reduction stands for mitochondrial activity (Zapata et al., 1991). In the present study, cell viability (TTC reduction) was almost opposite to the MDA content. It is hypothesized that the 0.25 μM MeJA in the study can reduce lipid peroxidation by modulating the relative amounts of ROS and increasing the ability of wheat callus to maintain cell viability during drought stress (Figure 7(B)). However, the higher concentration of MeJA-pretreatment induced excessive generation of ROS, which would break the balance of ROS metabolism, and result in acceleration of senescence. It was probably because LOX involve in lipid peroxidation of membrane; its activity was induced by MeJA, and subsequently the higher concentration of MeJA induced higher LOX activity, as a consequence the lipid peroxidation of membrane would be promoted inordinately (Gardner, 1995).

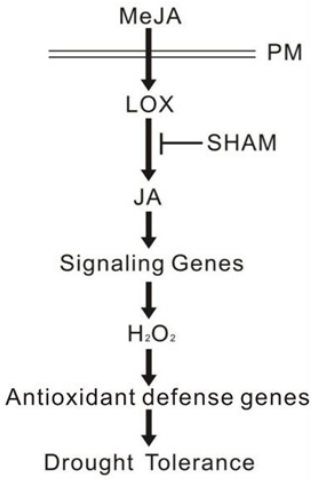

Figure 8. A proposed model for the MeJA mediated regulation of drought tolerance in wheat callus

In this model, exogenous MeJA promotes LOX activity, which was involved in octadecanoid pathway. The resulting level of endogenous JA increased. Consequently, the expression of signaling genes was activated, which promoted the generation of H_2O_2. H_2O_2 acted as the secondary signal, activating the expressions of the defensive genes to enhance drought tolerance. MeJA, Methyl Jasmonate; PM, Plasma Membrane; LOX, Lipoxygenase; SHAM, Salicylhydroxamic Acid; JA, Jasmonic Acid.

It is clear that the JA signaling pathway is connected to other signaling pathways, constituting a complex

regulatory network. In the present study, a hypothesis was put forward according to known signal transduction pathway that regulate MeJA-induction of signaling and antioxidant defense genes (Figure 8). Upon pretreatment by MeJA, the octadecanoid pathway would be activated, up-regulating the synthesis of signaling pathway genes, such as LOX gene which would induce the accumulation of JAs (Feussner et al., 1995; Avdiushko et al., 1995; Royo et al., 1996; Heitz et al., 1997). Then, the time course of generation of JA correlates with the accumulation of H_2O_2 which acts as a signaling molecule to induce antioxidant defense (Jubany-Marí et al., 2009; Alvarez et al., 1998), thereby increasing drought tolerance. However, these pathways were inhibited by SHAM. MeJA-induced activation of antioxidant enzymes was H_2O_2-dependent, and the tolerance of drought stress was conceivably linked to ROS (H_2O_2) signaling.

5. Conclusion

In conclusion, the findings of this study suggest that exogenous MeJA is able to increase LOX activity and increase endogenous JA content in a concentration-dependent manner; the level of H_2O_2 also elevated, thereby stimulating the activity of antioxidant enzymes. Consequently, drought-stress tolerance was improved, resulting in declining levels of ROS and MDA, and improved cell viability. This work establishes the relationship between MeJA-pretreatment and drought-stress tolerance, and offers a possible solution to improve tolerance to drought-stress in future.

Acknowledgements

This research was financially supported by National Natural Science Foundation of China (31401323 and U1204314), National Science and Technology Support Program (2013BAC09B01) and Fund of Henan University of Science and Technology (09001814).

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