Effects of Absciscic Acid and Nitric Oxide on Chilling Resistance and Activation of the Antioxidant System in Walnut Shoots In Vitro

Ningguang Dong and Jianxun Qi

Key Laboratory of Urban Agriculture (North China), Ministry of Agriculture, Beijing Engineering Research Center for Deciduous Fruit Trees, Institute of Forestry and Pomology, Beijing Academy of Agriculture and Forestry Science, Beijing 100093, People's Republic of China

Yuanfa Li

Lab of Tree Breeding and Cultivation, State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, People's Republic of China

Yonghao Chen and Yanbin Hao¹

Key Laboratory of Urban Agriculture (North China), Ministry of Agriculture, Beijing Engineering Research Center for Deciduous Fruit Trees, Institute of Forestry and Pomology, Beijing Academy of Agriculture and Forestry Science, Beijing 100093, People's Republic of China

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ABSTRACT. The roles of abscisic acid (ABA) and nitric oxide (NO) and the relationship between NO and ABA on chilling resistance and activation of antioxidant activities in walnut (*Juglans regia*) shoots in vitro under chilling stress were investigated. Walnut shoots were treated with ABA, the NO donor sodium nitroprusside (SNP), ABA in combination with the NO scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), PTIO, SNP in combination with the ABA biosynthesis inhibitor fluridone (Flu), and Flu. Their effects on chilling tolerance, reactive oxygen species (ROS) levels, and the antioxidant defense system were analyzed. The results showed that ABA treatment markedly alleviated the decreases in the maximal photochemical efficiency and survival and the increases in electrolyte leakage and lipid peroxidation induced by chilling stress, suggesting that application of ABA could improve the chilling tolerance. Further analyses showed that ABA enhanced antioxidant defense and slowed down the accumulation of ROS caused by chilling. Similar results were observed when exogenous SNP was applied. ABA in combination with PTIO or PTIO alone differentially abolished these protective effects of ABA. However, treatment with NO in combination with Flu or Flu alone did not affect the SNP-induced protective effect against CI or the activation of antioxidant activities under conditions of chilling stress. In addition, ABA treatment increased the NO content under chilling conditions, which was suppressed by the ABA biosynthesis inhibitor Flu or NO scavenger PTIO. Conversely, SNP application induced the same ABA rise observed in control plants in response to chilling. Taken together, these results suggested that ABA may confer chilling tolerance in walnut shoots in vitro by enhancing the antioxidant defense system, which is partially mediated by NO, preventing the overproduction of ROS to alleviate the oxidative injury induced by chilling.

Among the various types of environmental stress, chilling stress is one of the most important factors limiting the productivity and distribution of plants. Chilling temperatures, defined as low but not freezing temperatures (0 to 15 °C), are common in nature and can cause a complex array of cellular dysfunctions, including loss of vigor, wilting, chlorosis, sterility, and even death (Dong et al., 2014; Theocharis et al., 2012). Oxidative stress may be a significant factor involved in chilling-induced injury (Zhou and Guo, 2009). Several studies have demonstrated that exposure to low temperatures induces the accumulation of ROS, such as H₂O₂, singlet oxygen,

superoxide radicals (O₂⁻), and hydroxyl radicals (OH⁻), which could cause oxidative stress at the cellular level (Hu et al., 2017; Liu et al., 2011; Theocharis et al., 2012). To scavenge ROS and combat oxidant damage, plants have evolved an antioxidant defense system consisting of several antioxidant enzymes; i.e., superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT), and some low molecular weight nonenzymatic antioxidants, such as ascorbic acid (AsA) and reduced glutathione (GSH) (Liu et al., 2010). Membranes are the primary sites where responses to chilling-induced oxidative stress occur. Electrolyte leakage and malondialdehyde (MDA) content are often used to assess stress-induced damage in membranes (Karimi and Ershadi, 2015; Zhang et al., 2012). Chloroplasts are among the most susceptible cellular organelles, and chilling leads to a decrease in the photosystem II maximal photochemical efficiency, which is generally expressed as the ratio of variable to maximal fluorescence ratio (F_v/F_m) (Wang et al., 2016).

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¹Corresponding author. E-mail: jinhetaojht@263.net.

Abscissic acid acts as a stress signal and plays critical roles in the tolerance of plants to many stress factors (Venzhik et al., 2016). ABA induces a myriad of cellular responses in plants via complex signal transduction cascades, leading to tolerance to stress conditions (Zhang et al., 2007). In plant tissues, the increase in endogenous ABA content after exposure to cold temperatures is assumed to be associated with the subsequent increase in the chilling tolerance of plants (Karimi and Ershadi, 2015). In addition, application of exogenous ABA increased the chilling tolerance in various herbaceous plants (Guo et al., 2012; Meng et al., 2008; Zhang et al., 2007). Although ABA is typically considered a key regulator of many abiotic stress responses, the mechanism underlying plant responses to abiotic stress are not exclusively controlled by ABA signaling. Under conditions of environmental stress, ABA interacts with other endogenous signaling substances (Asgher et al., 2017; Freschi, 2013). Among these ABA-interacting substances, the gaseous free radical NO has recently attracted interest in the research community given its involvement in a number of signaling cascades controlling plant responses to abiotic stress (Fancy et al., 2017; Farnese et al., 2016).

Nitric oxide, which was first identified as a unique diffusible molecular messenger in animals, plays important roles in various plant physiological processes, including plant growth, development, and the response to abiotic stress (Fancy et al., 2017; Liu et al., 2010; Sarath et al., 2007). Because of its biological action, NO has been considered either a stress-inducing or protective agent. For example, under conditions of environmental stress, application of the NO donor SNP in plants resulted in increased tolerance to environmental stresses, thereby alleviating injury and promoting plant growth (Asgher et al., 2017). Both NO and ABA, important “stress-related” molecules, interact during certain signaling cascades triggered by environmental challenges, such as water limitation and salt stress, ultimately leading to induction of plant adaptive responses, such as stomatal closure and antioxidant defenses (Freschi, 2013; Manai et al., 2014; Neill et al., 2008). However, few studies have investigated the interaction between NO and ABA during plant responses to cold temperatures. More evidence is required regarding the existence of NO–ABA interactions in chilling resistance and antioxidant defense in plants.

Walnut, one of the most economically important crops for both its timber and edible nuts, is susceptible to CI by spring frost. Therefore, it is important to explore the mechanism underlying acclimation to cold temperatures in walnut. In the present study, walnut shoots were treated *in vitro* with ABA, SNP, the NO scavenger PTIO, and the ABA biosynthesis inhibitor Flu. First, the effects of ABA and NO on chilling resistance, ROS levels, and the antioxidant defense system were investigated in walnut shoots *in vitro* under conditions of chilling stress. The existence of NO–ABA interaction in chilling resistance and antioxidant defense in walnut shoots under conditions of chilling stress were also evaluated *in vitro*.

Materials and Methods

PLANT MATERIAL AND TREATMENTS. The walnut cultivar Jinlong 2 was cultured aseptically on proliferation medium [full-strength Driver and Kuniyuki (DKW) medium supplemented with 4.4 μM 6-benzyl-aminopurine and 4.9 nM 3-indolebutyric acid] as described previously (Pei et al., 2007). The

medium was autoclaved at 121 °C and 124 kPa for 20 min before use. Subculturing was performed every 20 d. Shoot proliferation was performed in 350-mL cylindrical bottles. The cultures were incubated at 25 $\pm$ 3 °C under a 16-h photoperiod (53 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by cool-white fluorescent tubes. As shown in Fig. 1, shoots, 4.5–5.0 cm in length, were excised and used for all investigations.

To investigate the effects of ABA or SNP on chilling tolerance in walnut shoots, walnut shoots, 4.5–5.0 cm in length, were excised and transferred into proliferation medium with ABA of different concentration (0, 100, 200, and 400 μM) or SNP of different concentration (0, 50, 100, and 200 μM). Subsequently, the treated shoots were cultured in 25 or 4 °C for 3 d. PTIO was used as the NO scavenger and Flu as the ABA biosynthesis inhibitor. To elaborate the influence of exogenously applied PTIO on the NO content or exogenously applied Flu on the ABA content, walnut shoots, 4.5–5.0 cm in length, were treated with PTIO of different concentration (0, 100, 200, and 400 μM) or Flu of different concentration (0, 50, 100, and 200 μM) and then cultured in 25 or 4 °C for 3 d.

To investigate the existence of NO–ABA interaction in chilling resistance and antioxidant defense in walnut shoots under conditions of chilling stress, the walnut shoots, 4.5–5.0 cm in length, were excised and transferred into different proliferation medium containing the following: 1) sterilized water (control), 2) 200 μM ABA, 3) 100 μM SNP, 4) 200 μM ABA + 200 μM PTIO, 5) 200 μM PTIO, 6) 100 μM SNP + 50 μM Flu, or 7) 50 μM Flu. Half of the plants in each treatment were cultured at 25 °C, the other half of plants were exposed to 4 °C. After 3 d of culture, the third leaves were sampled for

Fig. 1. *In vitro* walnut shoots of 4.5–5.0 cm height used for the investigation of effects of abscissic acid (ABA), sodium nitroprusside, 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide, and fluridone on the electrolyte leakage, malondialdehyde content, maximal fluorescence ratio, percentage survival, nitric oxide contents, and ABA contents in the leaves of walnut shoots *in vitro* after 3 d of cultivation at 25 or 4 °C.

measurement. Fresh leaves were used for electrolyte leakage and maximal photochemical efficiency measurements, and the others were immediately frozen in liquid N₂ for analysis of other physiological parameters. In addition, the percentage of plant survival for each treatment was recorded.

MEASUREMENT OF ELECTROLYTE LEAKAGE. Electrolyte leakage was determined as described by Soloklui et al. (2012) with slight modifications. Fresh leaves (0.5 g) were cut into 1-cm² pieces and placed in test tubes containing 40 mL distilled deionized water for 24 h on a shaker at 120 rpm. The electrical conductivity (EC₁) of the solutions was measured using a conductivity meter (Cond 720; WTW, Weilheim, Germany). The samples were boiled at 100 °C for 20 min to release all electrolytes and then cooled to room temperature, after which the final EC (EC₂) was measured. Electrolyte leakage was calculated using the following formula: (EC₁/EC₂) × 100.

MEASUREMENT OF MDA. Malondialdehyde content was measured according to a method described previously (Liu et al., 2011). Freeze-dried and powdered leaf mass (0.1 g) was homogenized in 5% trichloroacetic acid (TCA) and then was centrifuged at 1000 g_n for 10 min. The supernatant was mixed with the equal volume of 5% TCA containing 0.67% thiobarbituric acid (TBA). The mixture was incubated in a boiling water bath for 30 min and quickly cooled to room temperature. Optical density (OD) at 450, 532, and 600 nm was measured after centrifugation at 1000 g_n for 10 min again. MDA content was calculated using the formula: MDA (μM) = [6.45(OD₅₃₂ – OD₆₀₀) – 0.56OD₄₅₀].

ANALYSIS OF THE MAXIMAL PHOTOCHEMICAL EFFICIENCY OF PHOTOSYSTEM II. A fluorometer (FMS-1; Hansatech Instruments, Norfolk, UK) was used to measure modulated chlorophyll fluorescence. The leaves were excised and immediately mounted to the fluorometer. For dark adaptation, leaves were covered for 30 min. The initial fluorescence (F₀) was measured at a photosynthetic photon flux (PPF) density of < 0.05 μmol·m⁻²·s⁻¹, followed by a saturating pulse (3000 μmol·m⁻²·s⁻¹) to determine the maximal fluorescence (F_m). The maximal photochemical efficiency of photosystem II was estimated using the following formula: F_v/F_m = (F_m – F₀)/F_m.

RECORDING OF SURVIVAL. After 3 d of treatment, the shoots subjected to 4 °C were moved to 25 °C, and the other shoots were still kept at 25 °C. The survival of each shoot was monitored daily for 20 d. The number of shoots used was not less than 30 for each treatment.

DETERMINATION OF ABA CONTENT. ABA content was determined using the high performance liquid chromatography–mass spectrometry (HPLC-MS) method described by Gou et al. (2010). A 100-mg aliquot of freeze-dried and powdered leaf mass was extracted in 80% methanol with internal standards of [²H₂]-ABA and reduced to aqueous phase. The aqueous phase was extracted with ethyl acetate at pH 3, then with potassium phosphate buffer at pH 8.5, again into ethyl acetate at pH 3, and further purified on C₁₈ Sep-Pak and Oasis MCX SPE columns (Waters, Milford, MA). The eluant was dried and redissolved with HPLC initial solution, filtered through a 0.22-μm filter, and analyzed with a liquid chromatography–mass spectrometry system (LCQ Deca AMX, HPLC-ESI-MS; Thermo Fisher Scientific, Waltham, MA). Tandem mass spectrometry data were then analyzed using software Xcalibur 2.1 (Thermo Fisher Scientific) and quantified by reference to the internal standards using M⁺ ratios in equations for isotope dilution analysis.

DETERMINATION OF NO CONTENT. NO content was determined using the method described by Ziogas et al. (2013) with slight modifications. Freeze-dried and powdered leaf mass (0.1 g) was homogenized in cool acetic acid buffer (containing 4% zinc diacetate) at pH 3.6. The homogenates were centrifuged at 10,000 g_n for 10 min at 4 °C. The supernatant was collected. The pellet was washed by 1 mL of extraction buffer and centrifuged again as before. The two supernatants were combined and clarified with 0.1 g charcoal. After vortex and filtration, the filtrate was leached and collected. The mixture of 1 mL of filtrate and 1 mL of the Greiss reagent was incubated at room temperature for 30 min. Absorbance was measured at 540 nm. NO content was calculated by comparison with a standard curve using sodium nitrite.

DETERMINATION OF ROS PRODUCTION. The H₂O₂ content was determined according to a method described previously (Zhou et al., 2006). Freeze-dried and powdered leaf mass (0.1 g) was homogenized in 5% TCA and 0.1 g activated charcoal. Following centrifugation (10,000 g_n, 20 min, 4 °C), the supernatant was adjusted to pH 8.4 with 17 M ammonia solution and then filtered. The filtrate was divided into aliquots of 1 mL. To one of these, the blank was added 8 μg of CAT and then kept at room temperatures for 10 min. To both aliquots with and without CAT, 1 mL of colorimetric reagent was added. The reaction solution was incubated for 10 min at 30 °C. Absorbance at 505 nm was determined spectrophotometrically (model ultraviolet-2010; Hitachi, Tokyo, Japan).

The O₂⁻ production rate was measured using the method of Zhao et al. (2008) with slight modifications. Freeze-dried and powdered leaf mass (0.2 g) was homogenized in 4 mL of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5000 g_n for 10 min. The incubation mixture contained 0.9 mL of 65-mM potassium phosphate buffer (pH 7.8), 0.1 mL of 10-mM hydroxylammonium chloride and 1 mL of the supernatant. After incubation at 25 °C for 20 min, 17-mM sulphathiazole and 7-mM α-naphthyl amine were added to the incubation mixture. After reaction at 25 °C for a further 20 min, the absorbance was read at 530 nm. A standard curve with sodium nitrite was used to calculate the production rate of O₂⁻.

The content of OH⁻ was determined using the method described by Liu et al. (2011) with slight modifications. Freeze-dried and powdered leaf mass (0.1 g) was homogenized with 1.2 mL of 50-mM sodium phosphate buffer (pH 7.0) and centrifuged at 10,000 g_n for 10 min. The supernatant was mixed with 0.5 mL of 50-mM sodium phosphate buffer (pH 7.0) and 1 mL of 25-mM sodium phosphate buffer (pH 7.0) containing 2.5-mM 2-deoxyribose. The reaction was developed at 35 °C in the dark for 1 h. After adding 1 mL of 1% TBA in 0.05 M sodium hydroxide and 1 mL of acetic acid, the mixture was boiled for 30 min and immediately cooled for 10 min in ice. The production of OH⁻ was followed by measurement of absorbance at 532 nm, and the results were given as absorbance units (absorbance × 1000) per gram sample dry weight.

ANTIOXIDANT ENZYME ACTIVITY ASSAY. Freeze-dried and powdered leaf mass (0.1 g) was homogenized in 5 mL of 50-mM chilled phosphate buffer (pH 7.0, containing 1-mM AsA and 1-mM ethylenediaminetetraacetic acid). The supernatants were recovered for determination of SOD, CAT, APX, and GR activities as described previously (Guo et al., 2006; Zhang et al., 2009; Zhou et al., 2005). One unit of CAT, APX, or GR was defined as the amount of enzyme required to catalyze the conversion of 1-μmol H₂O₂ (extinction coefficient 0.0394 mm⁻¹·cm⁻¹),

AsA (extinction coefficient $2.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$), or nicotinamide adenine dinucleotide phosphate (extinction coefficient $6.22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$), respectively, within 1 min. One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of *r*-nitro blue tetrazolium chloride by 50%. Protein content was determined using Coomassie Brilliant Blue G-250 according to the method of Bradford (1976).

MEASUREMENT OF ANTIOXIDANT CONTENT. Freeze-dried and powdered leaf mass samples (0.1 g) were homogenized in 5-mL TCA at 4 °C. The homogenate was centrifuged at $13,000 g_n$ for 15 min. The supernatant was recovered and used for AsA and GSH determination according to methods described previously (Griffith, 1980; Law et al., 1983).

STATISTICAL ANALYSIS. All treatments were repeated at least three times, and all samples were analyzed three times. At least 30 shoots were included for each treatment. The data were subjected to analysis of variance, and significant differences between means were determined using Duncan's multiple-range test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

ABA OR SNP TREATMENT ENHANCES THE TOLERANCE OF WALNUT SHOOTS TO CHILLING STRESS. A preliminary experiment with different concentrations of ABA or SNP was performed to determine the optimal concentration that ABA or SNP showed the most significant effect. As shown in Fig. 2, chilling treatment caused significant increases in electrolyte leakage

Fig. 2. Effects of different abscisic acid (ABA) concentrations on electrolyte leakage (A), malondialdehyde (MDA) content (B) and maximal fluorescence ratio (F_v/F_m) (C) and different SNP concentrations on electrolyte leakage (D), MDA content (E), and F_v/F_m (F) in the leaves of walnut shoots in vitro after 3 d of cultivation at 25 or 4 °C. Values represent the means of three replicates $\pm$ SD as shown by the vertical error bars. Different letters above the bars indicate significant differences via Duncan's multiple-range test at $P < 0.05$.

and MDA content and a decrease in the F_v/F_m . Application of ABA or SNP with different concentrations had no significant effects on electrolyte leakage, MDA content, and F_v/F_m at 25 °C (Fig. 1). However, under low temperature conditions, treatment with ABA or SNP markedly alleviated the decrease in the F_v/F_m and the increases in electrolyte leakage and MDA content induced by chilling stress (Fig. 1). Among the different concentrations of ABA or SNP, 200 μM ABA, or 100 μM SNP showed the most significant alleviation effect on CI in the walnut shoots. Therefore, 200- μM ABA or 100- μM SNP was used in further experiments.

PTIO DECREASES NO CONTENT AND FLU DECREASES ABA CONTENT. As shown in Fig. 3A, the decrease of NO content was observed when varying concentrations of PTIO were applied under 25 or 4 °C. Along with the increased concentration of PTIO, there was a strong tendency toward the decrease of NO content (Fig. 3A). However, with the concentration of PTIO increased from 200 to 400 μM , there was no obvious decrease in NO content under 25 or 4 °C (Fig. 3A). Therefore, 200- μM PTIO was used in further experiments. Similarly, application of different concentrations of Flu decreased the ABA content under 25 or 4 °C (Fig. 3B). The amount of ABA was negatively correlated with the Flu concentration. Under low temperature, the ABA content dropped obviously when treated with 50 μM Flu, compared with the control treated without Flu (Fig. 3B). When the concentration of Flu increased to 100 or 200 μM , the decrease in ABA content was not obvious under 25 or 4 °C (Fig. 3B). Thus, 50- μM Flu was used in further experiments.

EFFECTS OF ABA, SNP, PTIO, AND FLU ON CHILLING TOLERANCE, ABA CONTENTS, AND NO CONTENTS. To investigate the effects of ABA, SNP, PTIO, and Flu on the tolerance of walnut shoots to chilling stress, it was necessary to evaluate the phenotypical and physiological features. Figure 4 shows the effects of different treatments on phenotypes in leaves of walnut shoots as evidenced by typical visual injury symptoms of the corresponding treatment imposed.

As shown in Fig. 5A and B, the electrolyte leakage and MDA content increased by 133.3% and 65.8% under conditions of chilling stress at 4 °C, respectively. Figure 5C shows that the F_v/F_m ratio and percentage survival decreased by 12.5% and 32% under chilling stress at 4 °C. Application of ABA or SNP significantly alleviated the increases of electrolyte leakage and MDA content and the suppression of F_v/F_m and survival induced by chilling stress (Fig. 5A–D). Under low temperature, treatment with NO scavenger PTIO or ABA synthesis inhibitor Flu alone differentially abolished those protective effects of

Fig. 3. Effects of different 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (PTIO) concentrations on nitric oxide (NO) content (A) and different fluridone (Flu) concentrations on abscisic acid content (B) in the leaves of walnut shoots in vitro after 3 d of cultivation at 25 or 4 °C. Each value represents mean of three replicates $\pm$ SD shown by vertical error bar.

Fig. 4. Phenotypes in leaves of walnut shoots in vitro subjected to different treatments for 3 d: (A) control, (B) chilling, (C) chilling + abscisic acid (ABA), (D) chilling + sodium nitroprusside (SNP), (E) chilling + ABA + 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), (F) chilling + PTIO, (G) chilling + SNP + fluridone (Flu), and (H) chilling + Flu.

Fig. 5. Effects of abscisic acid (ABA), sodium nitroprusside (SNP), 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), and fluridone (Flu) on the electrolyte leakage (A), malondialdehyde (MDA) content (B), maximal fluorescence ratio (F_v/F_m) (C), percentage survival (D), nitric oxide (NO) contents (E), and ABA contents (F) in the leaves of walnut shoots in vitro after 3 d of cultivation at 25 or 4 °C. Values represent the means of three replicates ± SD as shown by the vertical error bars. Different letters above the bars indicate significant differences via Duncan's multiple-range test at $P < 0.05$.

ABA or SNP (Fig. 5A–D). Moreover, ABA in combination with PTIO also arrested the ABA- or SNP-mediated protective effects (Fig. 5A–D). That is, in the presence of ABA in combination with PTIO, PTIO, or Flu, electrolyte leakage and MDA content increased markedly and F_v/F_m and survival decreased noticeably compared with those exposed to chilling stress alone. However, under low temperature, SNP in combination with Flu completely prevented the increased electrolyte leakage and MDA content and decreased F_v/F_m and survival induced by chilling stress (Fig. 5A–D). In addition, walnut shoots in vitro showed no obvious differences in the previously

mentioned physiological parameters among all of the treatments at 25 °C (Fig. 5A–D).

Figure 5E shows that chilling stress induced a marked increase in endogenous NO production. Under low temperature, NO content increased noticeably in the presence of SNP or ABA compared with that of chilling stress alone, whereas PTIO or Flu completely inhibited the increase in NO content (Fig. 5E). In addition, under low temperature, SNP in combination with Flu markedly increased the NO content compared with chilling stress alone, whereas ABA in combination with PTIO caused a significant decrease in NO content (Fig. 5E).

Figure 5F shows that the endogenous ABA content in walnut shoots in vitro increased by 84.4% under chilling stress at 4 °C. Under conditions of chilling stress, the application of ABA or ABA in combination with PTIO induced a marked increase in ABA content compared with chilling stress alone (Fig. 5F). However, under low temperature, SNP or PTIO treatment induced the same ABA rise observed in control plants in response to chilling (Fig. 5F). In addition, SNP in combination with Flu or Flu markedly inhibited the chilling stress-induced increase in ABA (Fig. 5F).

EFFECTS OF ABA, SNP, PTIO, AND FLU ON ROS LEVELS. Exposure of walnut shoots to low temperature in vitro led to marked increases in H_2O_2 , O_2^- , and OH^- contents. As shown in Fig. 6A–C, H_2O_2 , O_2^- , and OH^- contents increased by 175%, 62.5%, and 27.4%, respectively, under chilling stress at 4 °C. Exogenous ABA or SNP treatment significantly alleviated the increased ROS production induced by chilling stress (Fig. 6A–C). However, under low temperature condition, the H_2O_2 , O_2^- , and OH^- levels increased markedly in the presence of PTIO or Flu compared with those under only chilling stress (Fig. 6A–C). In addition, the combination of SNP with Flu also markedly reduced the increased H_2O_2 , O_2^- , and OH^- contents induced by chilling stress, whereas ABA in combination with PTIO significantly increased the contents of H_2O_2 , O_2^- , and OH^- compared with those of chilling treatment alone (Fig. 6A–C). There were no significant differences in H_2O_2 , O_2^- , or OH^- contents among all treatment groups at 25 °C (Fig. 6A–C).

EFFECTS OF ABA, SNP, PTIO, AND FLU ON ANTIOXIDANT ENZYMES AND ANTIOXIDANT CONTENTS. The data presented in Fig. 7A–D indicated that the activities of SOD and GR increased significantly under conditions of chilling stress, whereas there were no changes in CAT and APX activities. Under conditions of chilling stress, the application of ABA, SNP, or SNP in combination with Flu induced marked increases in the activities of SOD, CAT, APX, and GR (Fig. 7A–D). However, the activities of SOD, APX, and GR decreased markedly in shoots treated with ABA in combination with PTIO, PTIO, or Flu compared with those under chilling stress alone (Fig. 7A–D). In addition, the activities of the above enzymes showed no obvious differences among all of the treatments at 25 °C.

As shown in Fig. 7E and F, chilling stress caused significant increases in AsA and GSH contents. The AsA and GSH contents increased by 60% and 75.4% in walnut shoots exposed

Fig. 6. Effects of abscisic acid (ABA), sodium nitroprusside (SNP), 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (PTIO), and fluridone (Flu) on H_2O_2 content (A), superoxide radicals ($O_2^{\cdot -}$) production rate (B), and hydroxyl radicals ($OH^{\cdot}$) content (C) in the leaves of walnut shoots in vitro after 3 d of cultivation at 25 or 4 °C. Values represent the means of three replicates $\pm$ SD as shown by the vertical error bars. Different letters above the bars indicate significant differences via Duncan's multiple-range test at $P < 0.05$.

Fig. 7. Effects of abscisic acid (ABA), sodium nitroprusside (SNP), 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (PTIO), and fluridone (Flu) on the activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate peroxidase (APX) (C), and glutathione reductase (GR) (D), and the contents of ascorbic acid (AsA) (E) and reduced glutathione (GSH) (F) in the leaves of walnut shoots in vitro after cultivation at 25 or 4 °C for 3 d. Values represent the means of three replicates $\pm$ SD as shown by the vertical error bars. Different letters above the bars indicate significant differences via Duncan's multiple-range test at $P < 0.05$.

to chilling stress at 4 °C, respectively (Fig. 7E and F). Under chilling stress, treatment with ABA, SNP, or SNP in combination with Flu caused marked increases in the contents of AsA and GSH (Fig. 7E and F). However, in the presence of ABA in combination with PTIO, PTIO, or Flu, the contents of AsA and GSH decreased markedly compared with those exposed to chilling treatment alone (Fig. 7E and F). Besides, there were no significant changes in AsA or GSH contents among all treatments at 25 °C.

Discussion

Walnut, which has been cultivated for both its timber and edible nuts, is one of the most valuable and widely cultivated horticultural commodities in the world. Its nuts are a good source of flavonoids, sterols, polyphenols, and contains high amounts of essential dietary fatty acids, omega-6 and omega-3 (Ikhsana et al., 2016). However, walnut seedlings are susceptible to CI in northern China. Therefore, it is necessary to study the adaptive mechanism of walnut to chilling conditions. In the present study, walnut shoots in vitro were used to investigate the effects of ABA and NO on chilling resistance and activation of the antioxidant system under conditions of chilling stress. First, the results presented here provide theoretical basis and technical support for walnut chill-proof growing in real field conditions. Second, to reduce individual error walnut shoots in vitro that are obtained through vegetative propagation were used as experimental materials in this research. Vegetative propagation is used to produce progeny plants, which are

genetically identical to a single source plant. Currently, vegetative propagation of walnut is achieved mainly by tissue culture, grafting or cutting rooting. However, the chilling tolerance of walnut was affected by different rootstocks in grafted seedlings (Li et al., 2017). And the cutting propagation difficulty of walnut is presented as poor rhizogenesis. Finally, this study also provides a substantial base for long-term storage of walnut cultures in vitro under low temperature condition.

MECHANISMS UNDERLYING ABA-ENHANCED CHILLING TOLERANCE IN WALNUT SHOOTS IN VITRO. ABA, a recognized plant stress hormone and growth inhibitor, has been shown to be an effective plant growth regulator of induction of cold acclimation (Theocharis et al., 2012). Application of exogenous ABA has been shown to induce chilling tolerance in various herbaceous plants and some trees (Guo et al., 2012; Meng et al., 2008). However, other studies indicated either little or no effect of ABA on the development of chilling tolerance (Gusta et al., 2005). These conflicting results suggest that there may be ABA-dependent and ABA-independent pathways associated with acclimation to cold stress (Roychoudhury et al., 2013; Theocharis et al., 2012). In the present study, exogenous ABA markedly alleviated electrolyte leakage and lipid peroxidation and significantly prevented the decreased F_v/F_m and survival induced by chilling stress (Fig. 5A–D). These observations were consistent with those of other studies in *Trigonobalanus doichangensis* (Zheng et al., 2015), grapevine [*Vitis vinifera* (Karimi and Ershadi, 2015)], and *Chorispora bungeana* cells in suspension culture (Liu et al., 2011). ROS production is one of the earliest responses to abiotic stress in plants (Suzuki et al., 2012). The antioxidant defense system is rapidly induced in plant cells to combat the oxidant damage associated with ROS accumulation (Gill and Tuteja, 2010). Among the antioxidant enzyme systems, SOD is especially important because it catalyzes the removal of O_2^- , the first ROS formed after exposure to chilling stress (Jaleel et al., 2009). Other antioxidant enzymes, including CAT, APX, and GR, convert H_2O_2 into water (Farnese et al., 2016; Lázaro et al., 2013). In combination with these enzymes, nonenzymatic antioxidants, such as AsA and GSH, also play crucial roles in scavenging ROS by acting as redox buffers in plant cells (Farnese et al., 2016). AsA and GSH can scavenge O_2^- and OH^- directly and reduce H_2O_2 into water (Ordoñez et al., 2014). Correspondingly, our results showed that, in the presence of ABA, the activities of SOD, CAT, APX, and GR and the contents of AsA and GSH were much higher than those seen with chilling treatment alone, whereas the contents of H_2O_2 , O_2^- , and OH^- were decreased (Figs. 6 and 7). These findings were consistent with previous studies, which showed that ABA enhanced antioxidant defenses and slowed down the accumulation of ROS caused by chilling stress (Liu et al., 2011; Wang et al., 2013). Therefore, these results suggest that exogenous ABA may confer chilling tolerance to walnut shoots.

To clarify the physiological roles of endogenous ABA in chilling stress responses, the ABA synthesis inhibitor Flu was used alone. Our results showed that application of Flu resulted in marked damage compared with those exposed to chilling stress alone, meaning that F_v/F_m and survival declined markedly, electrolyte leakage and lipid peroxidation were significantly aggravated, ROS levels accumulated markedly, and the activities of SOD, APX, and GR and the contents of AsA and GSH decreased obviously (Figs. 5A–D, 6, and 7). Measurement

of ABA content indicated that ABA level increased noticeably under chilling stress and Flu treatment markedly inhibited the chilling stress-induced increase in ABA (Fig. 5F). These data indicate that significant increase in ABA level under chilling stress is involved in chilling tolerance in walnut shoots.

ROLE OF NO IN THE CHILLING TOLERANCE OF WALNUT SHOOTS IN VITRO. Accumulating evidence suggests that NO plays important roles in the acclimation of plants to abiotic stress (Fancy et al., 2017). Application of exogenous NO to plants has been used to investigate the effects of NO in various physiological processes (Liu et al., 2010; Zhang et al., 2011). In the present study, application of SNP effectively prevented the increased electrolyte leakage, lipid peroxidation, and F_v/F_m and survival suppression induced by chilling stress (Fig. 5A–D). These findings suggest that exogenous NO could protect walnut shoots from oxidative damage induced by chilling. NO may help plants resist oxidative injury via two pathways (Puyaubert and Baudouin, 2014). First, NO reacts directly with radical species such as O_2^- and its intermediates in lipid peroxidation, acting as an antioxidant and blocking the lipid peroxidation process (Simontacchi et al., 2013). Second, NO could serve as a signaling molecule in the activation of cellular antioxidant defense systems (Fancy et al., 2017). As illustrated in a range of physiological contexts involving NO signaling, the posttranslational modification (PTM) of proteins via NO-based mechanisms plays important roles in NO signal transduction (Puyaubert and Baudouin, 2014). The major NO-dependent PTM is nitrosylation of cysteine residues (S-nitrosylation). Several antioxidant enzymes have been shown to be S-nitrosylated in plants under conditions of abiotic stress (Romero-Puertas et al., 2013). Some studies indicated that the enzymatic activities of SOD, CAT, APX, and GR are increased by chilling stress-induced S-nitrosylation (Begara-Morales et al., 2013; Sehrawat and Deswal, 2014). In the present study, the activities of SOD, CAT, APX, and GR and the contents of AsA and GSH in the presence of SNP under chilling stress were much higher than those under chilling stress alone (Fig. 7). Application of SNP markedly reduced the accumulation of H_2O_2 , O_2^- , and OH^- induced by chilling (Fig. 6). Taken together, these observations indicate that NO, as a bioactive antioxidant, can exert a protective effect against chilling-induced oxidative injury by scavenging ROS directly or by enhancing antioxidant defense systems.

Many recent studies have indicated increased production of NO in cold-stressed plants (Liu et al., 2010; Ziogas et al., 2013). However, the NO content decreased in bell pepper (*Capsicum annuum*) exposed to chilling conditions (Airaki et al., 2012). These studies indicated a certain degree of specificity in the role of endogenous NO under conditions of chilling stress, which may depend on the plant species or type of tissue. To clarify the physiological role of endogenous NO in chilling tolerance in walnut shoots in vitro, the NO scavenger PTIO was used. Our results showed that under chilling stress, treatment with PTIO resulted in significant oxidative damage in walnut shoots in comparison with that of chilling stress alone, which means that electrolyte leakage aggravated significantly, MDA and ROS levels accumulated remarkably, and F_v/F_m , survival, the activities of SOD, APX, and GR and the contents of AsA and GSH decreased obviously (Figs. 5–7). Detection of NO content indicated that NO level increased noticeably under chilling stress and PTIO treatment markedly inhibited the chilling stress-induced increase in NO (Fig. 5E). From these results, it

is concluded that significant increase in NO level under chilling stress is involved in chilling tolerance in walnut shoots in vitro.

POSSIBLE RELATIONSHIP BETWEEN ABA AND NO IN WALNUT SHOOTS UNDER CHILLING CONDITIONS IN VITRO. There have been a number of investigations regarding the interaction between NO and ABA. In some cases, such as the induction of stomatal closure and upregulation of antioxidant enzyme gene transcription and activities, NO acts mainly as a downstream element in the ABA signaling pathway (Freschi, 2013; Zhang et al., 2007). On the other hand, ABA enhances oxidative stress tolerance by mediating ABA biosynthesis in maize leaves (Zhang et al., 2011). In addition, NO seems to counteract the effects of ABA in the regulation of seed dormancy (Sarath et al., 2007). These seemingly contradictory results may be due to the specificity of the NO-ABA interaction mechanisms, which may depend on physiological events or even the species of plant. These studies suggest that complex interactions may exist between ABA and NO in plants.

The present study indicated that prevention of NO accumulation by NO scavengers could eliminate the protective effect of exogenous ABA against CI (Fig. 5A–D). These results suggest that the protective effect of ABA may be mediated by NO. In addition, the increased NO content in ABA-treated walnut shoots under chilling conditions was suppressed by the NO scavenger PTIO or ABA synthesis inhibitor Flu (Fig. 5E), indicating that ABA triggered NO production under chilling stress. Moreover, ABA in combination with PTIO, PTIO, or Flu alone markedly blocked the activities of antioxidant enzymes, and the contents of AsA and GSH induced by ABA, while the H_2O_2 , O_2^- , and OH^- contents were also enhanced under chilling stress (Figs. 6 and 7). Meanwhile, application of exogenous NO could also play a positive role in alleviating chilling damage and activation of antioxidant activities (Figs. 5A–D and 7). Conversely, SNP application induced the same ABA rise observed in control plants in response to chilling, and NO combined with Flu or Flu alone did not affect the SNP-induced protective effect against CI or activation of antioxidant activities in walnut shoots under chilling stress (Figs. 5A–D, F, and 7). Taken together, these findings indicate that ABA induced chilling tolerance and activation of antioxidant activities by promoting accumulation of NO, i.e., NO may act as a downstream element in ABA-induced chilling tolerance and activation of antioxidant activities under chilling stress.

In conclusion, the results of the present study indicated that both ABA and NO enhanced chilling tolerance and activated the antioxidant defense system in walnut shoots under chilling stress in vitro. To our knowledge, this is the first report that ABA-triggered production of NO may play a crucial role in ABA-induced chilling tolerance by activating antioxidant defense systems and thus suppressing the accumulation of ROS in walnut shoots under low temperature conditions in vitro. NO may be a component of the ABA-induced plant acclimation response to chilling stress. However, further evidence is required to determine the complex molecular network operating under conditions of chilling stress mediated by ABA and NO. Further studies are needed to elucidate how ABA regulates NO generation under conditions of chilling stress.

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