Interactive effects of abscisic acid and nitric oxide on chilling resistance and active oxygen metabolism in peach fruit during cold storage

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Abstract

BACKGROUND: Cold conditions can accelerate the production of reactive oxygen species (ROS), and excessive ROS may attack biological macromolecules, disrupt related signal pathways, induce oxidative stress and influence plant metabolism. The cross-talk between nitric oxide (NO) and abscisic acid (ABA) and the inhibitions by NO or ABA on oxidative damage have been reported in fruits. However, there are few reports about the roles of NO–ABA interactions in chilling stress and antioxidant defense in fruits during cold storage. This study was conducted to investigate the roles of NO, ABA and interactions between NO and ABA in response to chilling stress on peach fruit (*Prunus persica* (L.) Batsch, cv. 'Xintaihong').

RESULTS: Treatments with $15 \mu mol \, L^{-1}$ NO, $100 \mu mol \, L^{-1}$ ABA and $15 \mu mol \, L^{-1}$ NO $+ 5 \, mmol \, L^{-1}$ sodium tungstate solution could reduce ROS content, alleviate lipid peroxidation and enhance antioxidant enzyme activities and antioxidant capacities. However, treatments with $5 \mu mol \, L^{-1}$ potassium 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), $5 \, mmol \, L^{-1}$ sodium tungstate and $100 \mu mol \, L^{-1}$ ABA $+ 5 \mu mol \, L^{-1}$ c-PTIO differentially blocked these protective effects and significantly increased ROS content and lipid peroxidation of peaches under low-temperature conditions.

CONCLUSIONS: Application of exogenous ABA could increase the resistance to cold-induced oxidative stress by enhancing the efficiency of enzymatic and non-enzymatic systems, which were partially mediated by NO.
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Keywords: nitric oxide; abscisic acid; antioxidative metabolism; reactive oxygen species; peach

INTRODUCTION

Peach is popular with consumers around the world due to its delicious taste and nutritious richness. However, it is highly perishable after harvest. When stored at ambient temperature, peaches ripen, senesce and rot quickly.[1] Refrigerated storage is one of the most widely used techniques for the preservation of peach fruit, which can retard these processes effectively and prolong the storage period of peach fruit. However, peach fruit easily suffers from chilling injury between 2.2 and 7.6 °C.[2] Cold conditions can accelerate the production of reactive oxygen species (ROS), and excessive ROS may attack biological macromolecules, disrupt related signal pathways, induce oxidative stress and influence plant metabolism.[3] Plants have developed detoxification strategies to maintain balanced levels of ROS to eliminate these toxic substances and alleviate oxidative stress.[4]

Nitric oxide (NO), as a diffusible signal molecule in plants, is involved in many physiological processes, including ripening and senescence, as well as responses to biotic and abiotic stress.[5,6] Owing to its biological role, NO is considered a stress-inducing or protective agent. Under cold conditions, short-term application of low concentrations of NO can effectively alleviate chilling injury and delay the postharvest period of various fruits and vegetables. Previous research has shown that exogenous NO

and its donors, such as sodium nitroprusside, can retain cold stored peach fruit quality.[7,8] Furthermore, studies have indicated that cold can induce the generation of endogenous NO, which in turn provides a major push toward alleviating chilling injury symptoms via regulating antioxidative metabolism in fruit.[9] Moreover, studies have shown that ROS and NO can interact in plant cells.[10,11] However, research regarding the protective mechanism of NO in fruit under cold conditions is still limited.

Abscisic acid (ABA), an important signaling molecule, plays key roles in the resistance of plants to many stresses.[12] Application of exogenous ABA under normal conditions can cause similar types of stress, while its application under adverse conditions can

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improve plant defense mechanisms.[13] Therefore, exogenous ABA may be an effective substance for improving the resistance of peaches to chilling injury during cold storage. In many situations, short-term application of proper concentrations of ABA can effectively alleviate chilling injury, thereby delaying the postharvest period of various fruits and vegetables. For example, exogenous ABA can improve the antioxidant capacity and photosynthesis of grape leaves to alleviate cold stress,[14] as well as increase chilling resistance of zucchini.[15] Although ABA is generally regarded as a key regulator of many abiotic stresses, the mechanism of plant stress on abiotic reactions is not completely controlled by ABA signaling.[12] Under ambient pressure conditions, other endogenous signaling substances can interact with ABA. For example, NO has recently attracted the interest of researchers because it is involved in many signal cascades of ABA that control plants responses to abiotic stresses.

In recent years, the interactions between NO and ABA have been studied, and NO is now considered as an important participant in ABA-mediated signaling pathways. NO participates downstream in the effects of ABA on processes such as stomata movement and oxidative tolerance.[16–18] However, there have been few studies of the roles of NO–ABA interactions in chilling stress and antioxidant defense in horticultural plants. Thus, the study reported here was conducted to examine the effects of exogenous NO and ABA on ROS levels and antioxidative metabolism.

MATERIALS AND METHODS

Plant material
Peaches (*Prunus persica* (L.) Batsch, cv. 'Xintaihong') were harvested at physiological maturity (soluble solids content (SSC), $11.8 \pm 0.01\%$; firmness, 109.2 ± 1.48 N) from a local orchard in Xintai, Shandong Province, China. Peaches selected had uniform size and color and were free from mechanical injury and disease and transported to the laboratory within 2 h. Before analysis, samples were pre-cooled at 0 °C for 24 h.

Experimental design
After pre-cooling at 0 °C for 24 h, 30 fruits were randomly sampled and expressed as 0 day. The remaining pre-cooled fruits were randomly divided into seven batches, each batch containing 100 fruits. The peaches were soaked in one of the following solutions for 10 min: (1) distilled water (control); (2) $15 \, \mu\text{mol L}^{-1}$ NO solution; (3) $100 \, \mu\text{mol L}^{-1}$ ABA (Solarbio, Beijing) solution; (4) $5 \, \mu\text{mol L}^{-1}$ potassium 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; Sigma, USA) solution; (5) $5 \, \text{mmol L}^{-1}$ sodium tungstate (Solarbio, Beijing) solution; (6) $100 \, \mu\text{mol L}^{-1}$ ABA + $5 \, \mu\text{mol L}^{-1}$ c-PTIO solution; or (7) $15 \, \mu\text{mol L}^{-1}$ NO + $5 \, \text{mmol L}^{-1}$ sodium tungstate solution. In the $100 \, \mu\text{mol L}^{-1}$ ABA + $5 \, \mu\text{mol L}^{-1}$ c-PTIO and $15 \, \mu\text{mol L}^{-1}$ NO + $5 \, \text{mmol L}^{-1}$ sodium tungstate treatments, samples were pre-treated with $5 \, \mu\text{mol L}^{-1}$ c-PTIO or $5 \, \text{mmol L}^{-1}$ sodium tungstate for 10 min and subsequently treated with $100 \, \mu\text{mol L}^{-1}$ ABA or $15 \, \mu\text{mol L}^{-1}$ NO for 10 min. After treatment, the fruits were dried and stored at 0 °C and relative humidity (RH) 95% for up to 5 weeks. At each time point, 30 fruits were sampled for each treatment. After measuring the chilling index (CI index), firmness, SSC, peel color (*L**) and electrolyte leakage, the fruit was cut into cubes of approximately $2 \, \text{cm} \times 2 \, \text{cm}$ in size and immediately frozen in liquid nitrogen, and then stored at −80 °C until analysis for selected parameters as described below. All experiments were performed for three biological replicates.

The concentrations of NO, c-PTIO, ABA and sodium tungstate were selected based on the results of previous reports.[7,19–21] The NO solutions were prepared using the method described by Jing *et al.*[7]

Determination of CI index, firmness, SSC and peel color
The CI indexes of ten fruits were evaluated subjectively according to a scoring index, where no browning = 0, slight (less than 5% browning) = 1, moderate (5–25%) = 2, moderately severe (25%–50%) = 3, extreme (>50%) = 4. The following formula was used to calculate the browning index: chilling index = $\sum$(fruit score × fruit number of this score)/(5 × number of total fruit) × 100%.

The firmness of peaches was measured with an Edelberg GY-4 fruit firmness tester (Shanghai Shandu Co. Ltd, China). The diameter of the probe was 11 mm, and the pressure peak was recorded. Nine fruit from each treatment were selected, with each peach individually tested. The results are presented as mean ± SE ($n = 3$) and expressed as newtons (N).

The experiment was repeated in triplicate with nine fruits each time, and SSC of peaches was measured using a hand refractometer (Shanghai Cany Precision Instrument Co. Ltd, China), and was expressed as °Brix.

The peel color of peaches was measured with a colorimeter (Konica Minolta Sensing, Inc., USA). Nine fruit from each treatment were selected, with each peach individually tested. The results are presented as mean ± SE ($n = 3$) and expressed as lightness (*L**) and hue angle value.

Determination of ROS species
The superoxide radical ($O_2^{\bullet -}$) generation rate was determined as described by Li *et al.*,[22] after the peach mesocarp (5 g) was homogenized with $65 \, \text{mmol L}^{-1}$ potassium phosphate buffer (at a ratio of 1:3, pH 7.8). The $O_2^{\bullet -}$ generation rate was expressed as $\mu\text{mol kg}^{-1} \, \text{min}^{-1}$ on a fresh weight basis (FW). The content of hydrogen peroxide (H_2O_2) was measured using the method described by Patterson *et al.*,[23] to analyze peach mesocarp (5 g) that had been homogenized with pre-cooled acetone (at a ratio of 1:3). The content of H_2O_2 was expressed as mmol kg^{-1} FW. Finally, the rate of hydroxyl radical ($^{\bullet}$OH) production was measured according to the method developed by Liu *et al.*,[4] using peach mesocarp (5 g) that had been homogenized with $50 \, \text{mmol L}^{-1}$ sodium phosphate buffer (at a ratio of 1:3, pH 7.0). The content of $^{\bullet}$OH was expressed as absorbance units (absorbance × 1000) per gram FW.

Measurement of electrolyte leakage and malondialdehyde (MDA)
The electrolyte leakage was determined using a DDS-307 conductometer (Leici, Shanghai, China). Fifteen fruit discs (about 1 mm thick, 5 cm in diameter) were dipped in distilled water (40 mL), and the conductivity (P_0) was recorded immediately. After 10 min, the conductivity was recorded again as P_1. The discs were then boiled for 10 min (the solution was readjusted to a volume of 40 mL to offset the evaporation). As the solution cooled to ambient temperature, the final conductivity (P_2) was measured. The following formula was used to calculate the electrolyte leakage: electrolyte leakage = $(P_1 - P_0)/(P_2 - P_0) \times 100\%$.

The content of MDA was measured using the method described by Guo *et al.*,[24] using peach mesocarp (2 g) that had been homogenized with the pre-cooled 0.5% (w/v) trichloroacetic acid (at a ratio of 1:6). The MDA content was expressed as mmol kg^{-1} FW.

Table 1. Chilling index (CI index), firmness, soluble solids content (SSC) and peel color (L^*) in cold-stored peaches subjected to various treatments

Treatment	Time (weeks)	CI index (%)	Firmness (N)	SSC (%)	L^*
0 week	0	0	109.20 ± 1.48[a]	11.8 ± 0.01[a]	80.3 ± 0.68[a]
CK	1	18.1 ± 0.21[b]	93.74 ± 1.03[c]	11.6 ± 0.12[c]	77.3 ± 1.65[ab]
NO		17.3 ± 0.12[c]	97.47 ± 0.82[b]	12.3 ± 0.08[b]	79.3 ± 0.74[a]
c-PTIO		18.9 ± 0.03[a]	93.67 ± 0.46[c]	11.0 ± 0.23[e]	76.0 ± 1.78[ab]
NO + tungstate		17.5 ± 0.26[c]	97.32 ± 0.55[b]	12.2 ± 0.01[b]	78.5 ± 0.25[a]
ABA		17.6 ± 0.11[c]	101.70 ± 1.33[a]	13.2 ± 0.13[a]	78.6 ± 0.76[a]
Tungstate		19.3 ± 0.17[a]	91.30 ± 1.66[cd]	11.1 ± 0.11[de]	74.1 ± 0.75[b]
ABA + c-PTIO		19.0 ± 0.09[a]	89.40 ± 1.06[d]	11.5 ± 0.24[cd]	76.6 ± 0.88[ab]
CK	2	23.5 ± 0.29[c]	87.73 ± 0.95[c]	12.3 ± 0.15[cd]	75.0 ± 0.79[b]
NO		21.4 ± 0.26[d]	91.10 ± 0.90[b]	13.1 ± 0.12[ab]	78.1 ± 0.76[a]
c-PTIO		25.8 ± 0.24[b]	83.63 ± 1.41[d]	11.8 ± 0.20[de]	74.9 ± 0.53[b]
NO + tungstate		21.0 ± 0.29[d]	89.98 ± 0.40[bc]	13.0 ± 0.07[ab]	77.2 ± 0.87[a]
ABA		21.6 ± 0.40[d]	97.50 ± 0.81[a]	13.4 ± 0.23[a]	77.4 ± 0.55[a]
Tungstate		26.8 ± 0.12[a]	79.30 ± 1.20[e]	12.6 ± 0.23[bc]	74.7 ± 0.22[b]
ABA + c-PTIO		25.6 ± 0.45[b]	84.20 ± 1.36[d]	11.7 ± 0.27[e]	74.1 ± 0.44[b]
CK	3	32.9 ± 0.46[d]	78.87 ± 0.95[b]	13.9 ± 0.32[ab]	73.5 ± 0.87[c]
NO		28.8 ± 0.26[e]	84.95 ± 1.01[a]	13.7 ± 0.10[b]	76.3 ± 0.37[ab]
c-PTIO		42.0 ± 0.52[a]	73.77 ± 0.96[c]	12.4 ± 0.17[c]	72.2 ± 0.35[c]
NO + tungstate		29.4 ± 0.17[e]	85.27 ± 1.41[a]	13.6 ± 0.16[b]	77.8 ± 0.88[a]
ABA		29.3 ± 0.25[e]	85.99 ± 2.42[a]	14.6 ± 0.24[a]	75.4 ± 0.55[b]
Tungstate		39.4 ± 0.38[b]	73.07 ± 1.01[c]	12.8 ± 0.15[c]	72.9 ± 0.38[c]
ABA + c-PTIO		38.1 ± 0.69[c]	75.37 ± 0.32[bc]	12.6 ± 0.30[c]	72.3 ± 0.21[c]
CK	4	48.3 ± 0.91[c]	71.71 ± 1.24[b]	15.3 ± 0.29b	70.7 ± 1.27[bc]
NO		37.6 ± 0.36[e]	80.39 ± 1.49[a]	16.2 ± 0.33[a]	74.1 ± 0.64[abc]
c-PTIO		58.3 ± 0.75[a]	68.64 ± 0.84[b]	13.5 ± 0.20[c]	64.5 ± 1.88[d]
NO + tungstate		37.3 ± 0.47[e]	79.87 ± 1.89[a]	16.1 ± 0.43[ab]	74.9 ± 1.74[ab]
ABA		39.6 ± 0.85[d]	79.01 ± 1.94[a]	15.2 ± 0.09[b]	76.8 ± 0.52[a]
Tungstate		53.7 ± 0.41[b]	68.18 ± 1.59[b]	13.6 ± 0.16[c]	68.9 ± 3.32[cd]
ABA + c-PTIO		53.4 ± 0.46[b]	68.93 ± 1.86[b]	12.9 ± 0.24[c]	70.5 ± 0.82[bc]
CK	5	72.6 ± 0.38[c]	63.89 ± 1.03[b]	13.1 ± 0.12[c]	69.7 ± 0.95[bc]
NO		57.7 ± 0.70[e]	70.96 ± 0.77[a]	15.4 ± 0.30[a]	73.0 ± 0.26[ab]
c-PTIO		81.3 ± 0.66[a]	58.95 ± 1.41[c]	10.7 ± 0.29[e]	66.0 ± 1.07[c]
NO + tungstate		58.6 ± 0.57[e]	68.44 ± 0.86[a]	15.5 ± 0.31[a]	73.5 ± 0.99[a]
ABA		61.6 ± 0.52[d]	70.85 ± 0.83[a]	14.7 ± 0.12[b]	74.2 ± 1.55[a]
Tungstate		75.6 ± 0.37[b]	59.64 ± 2.01[bc]	11.4 ± 0.23[d]	67.6 ± 1.59[c]
ABA + c-PTIO		74.6 ± 0.45[b]	59.49 ± 2.35[bc]	11.4 ± 0.10[d]	68.1 ± 1.05[c]

Values are the means ± SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

Measurement of antioxidant content

For analysis of the antioxidant contents, peach mesocarp (5 g) was homogenized with 15 mL of extract buffer containing metaphosphoric acid solution (5%) and ethylenediaminetetraacetic acid (EDTA; 1 mmol L^{-1}). Extracts were centrifuged at $12\,000 \times g$ at 4 °C for 20 min and the supernatant was then collected for measurement of the following indicators. Total ascorbate (AsA plus DHA) and reduced ascorbate (AsA) contents were measured using the 2,2′-bipyridyl spectrophotometric method described by Zhou et al.[25] Additionally, the content of oxidized ascorbate (DHA) was computed by subtracting the AsA content from the AsA plus DHA content and expressed as mg kg^{-1} FW. Total glutathione (GSH plus GSSG) and oxidized glutathione (GSSG) contents were measured using the 5,5-dithiobis(2-nitrobenzoic acid) method described by Dong et al.[26] The content of reduced glutathione (GSH) was determined by subtracting the GSSG content from the GSH plus GSSG and expressed as mg kg^{-1} FW.

Assay of antioxidative enzymes

For analysis of the antioxidant enzymes, the peach mesocarp (5 g) was homogenized with 15 mL of sodium phosphate buffer (50 mmol L^{-1}, pH 7.8) containing AsA (2 mmol L^{-1}), EDTA (0.2 mmol L^{-1}) and crosslinking polyvinylpyrrolidone (2%). Extracts were centrifuged at $12\,000 \times g$ at 4 °C for 20 min and the supernatant was then collected for measurement of the following indicators. The superoxidase dismutase (SOD) activity was measured by observation of the suppression of photochemical reduction by nitroblue tetrazolium chloride (NBT) according to the method of Yang et al.[27] One unit (U) of SOD activity was defined as the amount of enzyme that caused 50% inhibition of NBT. The
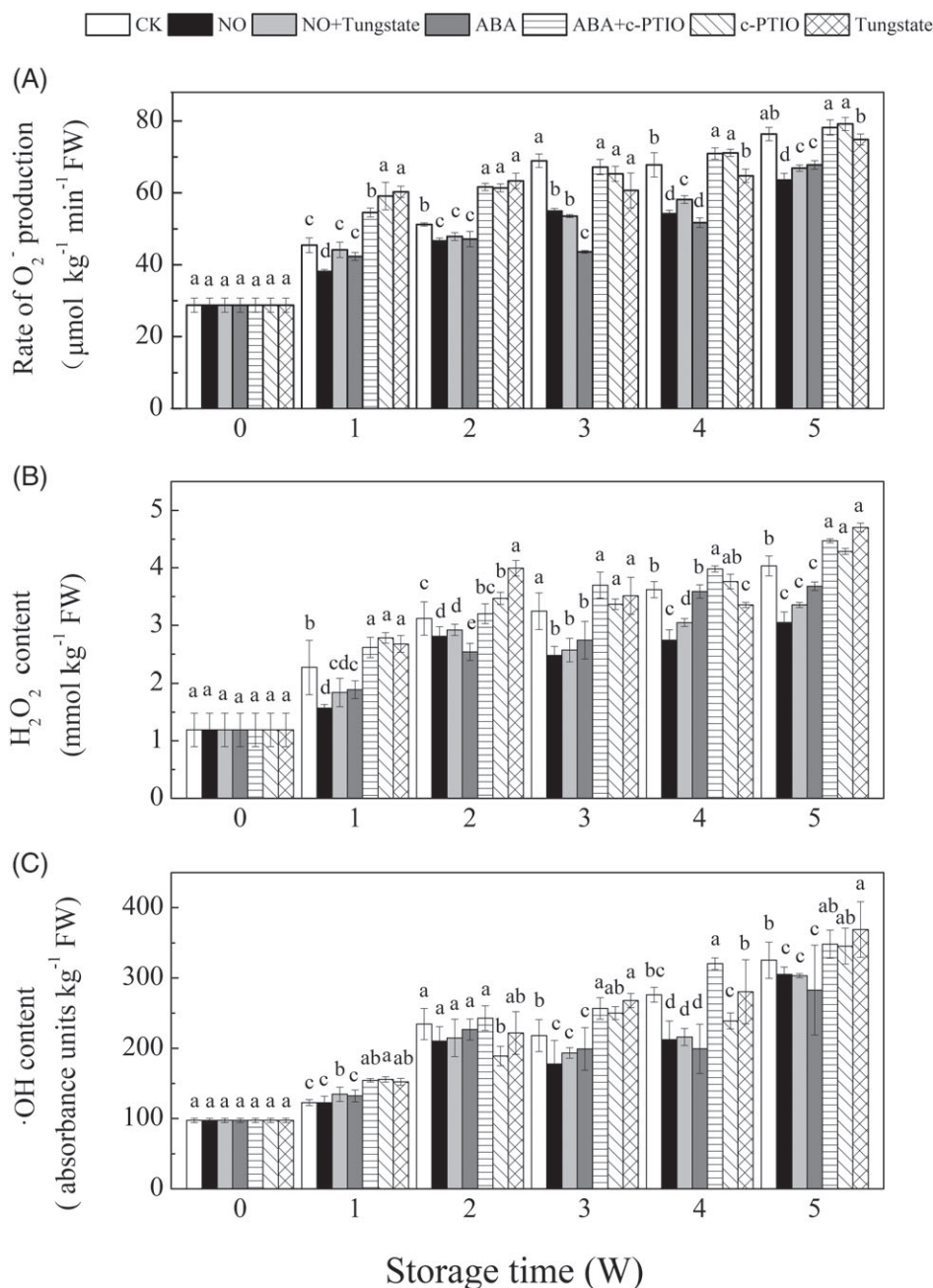
Figure 1. Superoxide radical ($O_2^{\bullet-}$) production rate (A), hydrogen peroxide (H_2O_2) content (B) and hydroxyl radical ($^{\bullet}OH$) content (C) in cold-stored peaches subjected to various treatments. Values are the means $\pm$ SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

peroxidase (POD) activity was measured using guaiacol as a substrate according to the method described by Talaat,[28] based on observed changes in the absorbance at 470 nm (A_{470}). The enzyme activity (1 U) was defined as the change in absorbance at A_{470} in 1 min. Ascorbate peroxidase (APX) was measured according to the method of Nakano and Asada,[29] and the enzyme activity (1 U) was defined as the rate of ascorbate oxidation at A_{290} in 1 min. Glutathione reductase (GR) was measured as described by Nakano and Asada,[29] and the enzyme activity (1 U) was defined as the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at A_{340} in 1 min. Monodehydroascorbate reductase (MDHAR) activity was measured using the method described by Hossain

et al.,[30] and the enzyme activity (1 U) was defined as the change in absorbance at A_{292} in 1 min. Dehydroascorbate reductase (DHAR) activity was measured using the method described by Nakano and Asada,[29] and the enzyme activity (1 U) was defined as the changes in absorbance at A_{265} in 1 min. These enzymes were all expressed as U kg^{-1} FW.

Determination of NO and ABA content

NO content was measured using a Nitric Oxide Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The content of NO was expressed as μmol kg^{-1} FW. ABA content in peach mesocarp was measured by applying an ELLSA Kit (Qingdao
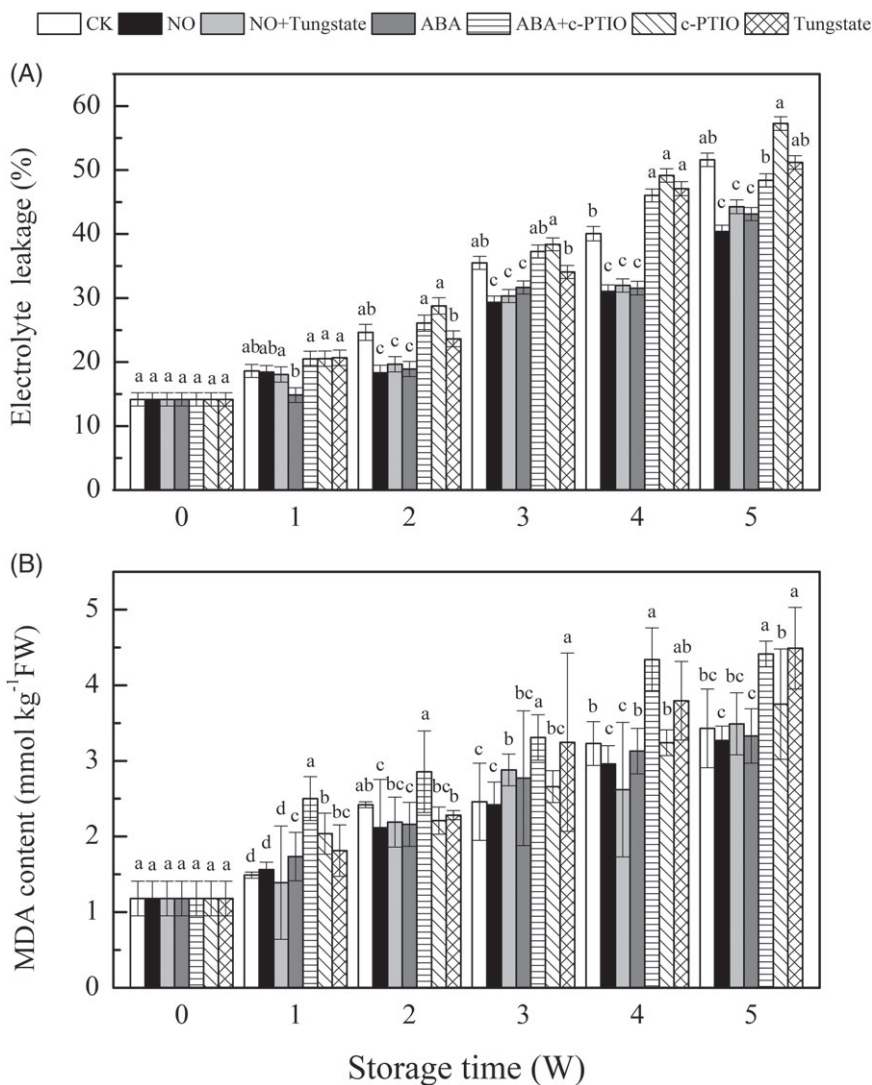
Figure 2. Electrolyte leakage (A) and MDA content (B) in cold-stored peaches subjected to various treatments. Values are the means ± SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

Jisskang Biotechnology Co. Ltd, Qingdao, China). The content of ABA was expressed as µg kg^{-1} FW.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Separation of the averages was performed using Duncan's multiple range test. Differences at $P < 0.05$ were considered significant. Data were expressed as the mean ± standard deviation (SD) of three replicates.

RESULTS

Changes in CI index, firmness, SSC and peel color

To investigate the effect of different treatments on the chilling injury and the quality of fruit, we assessed the CI index, firmness, SSC and peel color of fruit during cold storage. As evident from Table 1, the CI index of peach fruit in all treatments increased during cold storage. When compared with the control, exogenous NO, ABA or NO in combination with sodium tungstate significantly inhibited increase the CI index of fruit.

However, c-PTIO and sodium tungstate each led to differential increases in CI index, when compared with the NO or ABA treatment. Furthermore, ABA in combination with c-PTIO also blocked the ABA-mediated protective effect, while it significantly increased the CI index by 7.9–34.8% during the cold storage period.

Firmness and peel color of peaches in all treatments decreased gradually during cold storage (Table 1). Compared with the control, exogenous NO, ABA or NO in combination with sodium tungstate treatment significantly retarded the decrease in firmness and also exerted positive effects on the peel color. Conversely, the levels of firmness and peel color decreased significantly in peaches treated with c-PTIO or sodium tungstate when compared with those subjected to NO or ABA treatment alone. Moreover, ABA plus c-PTIO significantly decreased the level of firmness by 12.1–16.0% and the peel color by 2.5–8.2% during cold storage period when compared to the ABA treatment.

As evident from Table 1, SSC in peach fruit increased, peaking on the fourth week and then decreasing gradually over time during cold storage. When compared with the control, exogenous NO, ABA or NO in combination with sodium tungstate markedly

increased SSC in peaches in the first, second and fifth week. In contrast, c-PTIO and sodium tungstate led to differential decreases in SSC of 9.5–30.6 and 5.9–22.4%, respectively, when compared with the NO or ABA treatment. Meanwhile, ABA plus c-PTIO significantly decreased SSC during cold storage period when compared to the ABA treatment alone.

Changes in $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ contents

The $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ content in peaches increased markedly throughout the cold storage period (Fig. 1). When compared with the control, exogenous NO, ABA or NO in combination with sodium tungstate significantly alleviated increases in the $O_2^{\bullet-}$ production rate and H_2O_2 content in peaches during cold storage. In contrast, the contents of $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ increased significantly in peaches treated with c-PTIO or sodium tungstate when compared with those subjected to NO or ABA treatment alone. Furthermore, ABA in combination with c-PTIO significantly increased the contents of $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ in peaches by 15–54, 10–38 and 7–60%, respectively, throughout the cold storage period when compared with the ABA treatment ($P < 0.05$).

Changes in electrolyte leakage and MDA content

Similar to the $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ contents, the MDA content and electrolyte leakage in all peaches increased significantly throughout the cold storage period (Fig. 2). When compared with the control, application of NO, ABA or NO in combination with sodium tungstate significantly alleviated the increase in electrolyte leakage of peaches in the second, third, fourth and fifth week of storage. However, c-PTIO and sodium tungstate led to differential increases in electrolyte leakage of 11–58 and 7–49%, respectively, when compared with the NO or ABA treatment. Moreover, ABA in combination with c-PTIO also blocked the ABA-mediated protective effect, while it significantly increased the MDA content and electrolyte leakage by 19–44 and 12–45%, respectively, during the cold storage period.

Changes in SOD and POD activities

As shown in Fig. 3(A), the SOD activity in all peaches under different treatments decreased gradually over time during cold storage. When compared to treatment with NO or ABA alone, application of c-PTIO or sodium tungstate markedly decreased the SOD activity in peaches during the first 3 weeks (1–3 weeks) of cold storage. Especially in the first week, SOD activity in NO- or ABA-treated peaches was much higher than that of peaches treated with c-PTIO or sodium tungstate. However, SOD activity in peaches treated with ABA plus c-PTIO was 14–21% lower than that in peaches treated with ABA during the first 3 weeks (1–3 weeks) of cold storage.

As shown in Fig. 3(B), POD activity in peach fruit increased, peaking in the third week and then decreasing gradually over time during cold storage. When compared with the control, exogenous NO, ABA or NO in combination with sodium tungstate markedly increased POD activity of peaches in the first, second and fifth week. When compared to treatment with NO or ABA alone, application of c-PTIO or sodium tungstate decreased POD activity in peaches during storage. Especially in the third week, the POD activity in peaches treated with c-PTIO or sodium tungstate was only about 67 and 53% of that of peaches treated with NO or ABA alone, respectively. Additionally, POD activity in peaches treated with ABA in combination with c-PTIO was significantly lower than that in peaches treated with ABA throughout the cold storage period.

Changes in APX and GR activities

As shown in Fig. 4, exogenous NO or ABA alone could enhance the APX and GR activities of peaches, and the peaks of these enzyme activities appeared in the third week for NO-treated peaches, which was 1 week later than that for peaches treated with ABA. When compared to treatment with NO or ABA alone, treatment with c-PTIO or sodium tungstate alone significantly reduced the APX and GR activities in the first 3 weeks (1–3 weeks). Additionally, NO in combination with sodium tungstate maintained the APX and GR activities in peaches under cold stress, and had a protective effect similar to treatment with NO alone. In contrast, ABA plus c-PTIO significantly decreased the activity of APX by 10–27% and the GR activity by 10–26% during cold storage period when compared to the ABA treatment.

Changes in MDHAR and DHAR activities

Application of NO or ABA alone exerted positive effects on the activity of these enzymes in peaches during cold storage (Fig. 5). Similar effects were also found in response to treatment with NO in combination with sodium tungstate. When compared to treatment with ABA, application of sodium tungstate significantly decreased the activity of MDHAR by 17 and 29% in the second and third week, and DHAR activity by 6 and 7% in the third and fifth week, respectively. Moreover, DHAR activities of peaches treated with c-PTIO were 20, 7 and 9% lower than those of peaches treated with NO in the second, fourth and fifth week, respectively ($P < 0.05$). Furthermore, treatment with ABA plus c-PTIO markedly decreased the MDHAR and DHAR activities in peach fruit in the third and fifth week, respectively, when compared to treatment with ABA alone ($P < 0.05$).

Changes in AsA and GSH contents and redox status in peaches

The contents of antioxidants (AsA and GSH) and the ratios of AsA to DHA significantly increased in peaches treated with NO (Table 2). When compared to treatment with NO, application of c-PTIO decreased the contents of GSH and AsA, as well as the ratios of GSH to GSSG in peaches. In addition, the content of antioxidants and the ratios of cellular redox status in peaches treated with NO plus sodium tungstate were slightly lower than those in peaches treated with NO alone during the entire experiment.

The content of antioxidants and the ratios of cellular redox status in peaches treated with ABA alone were markedly higher than those of the control throughout the experimental process (Table 2). When compared to treatment with ABA alone, treatment with ABA plus c-PTIO significantly decreased the content of AsA and GSH by 11–26 and 20–34%, respectively. Additionally, the ratios of cellular redox status in peaches treated with ABA plus c-PTIO were 11–50 and 49–71% lower than those of peaches treated with ABA. During storage, treatment with sodium tungstate decreased AsA content by 4–19%, GSH content by 7–39%, the ratio of AsA to DHA by 12–37% and the ratio of GSH to GSSG by 21–58% when compared to treatment with ABA ($P < 0.05$).

Changes in NO and ABA contents

The NO and ABA contents in peaches were assessed to confirm the effect of different treatments on endogenous NO and ABA contents. Treatment with NO or ABA noticeably increased the content of NO in the first, second and fourth week compared with the control (Fig. 6(A)). In contrast, c-PTIO or sodium tungstate completely inhibited the increase in NO content when
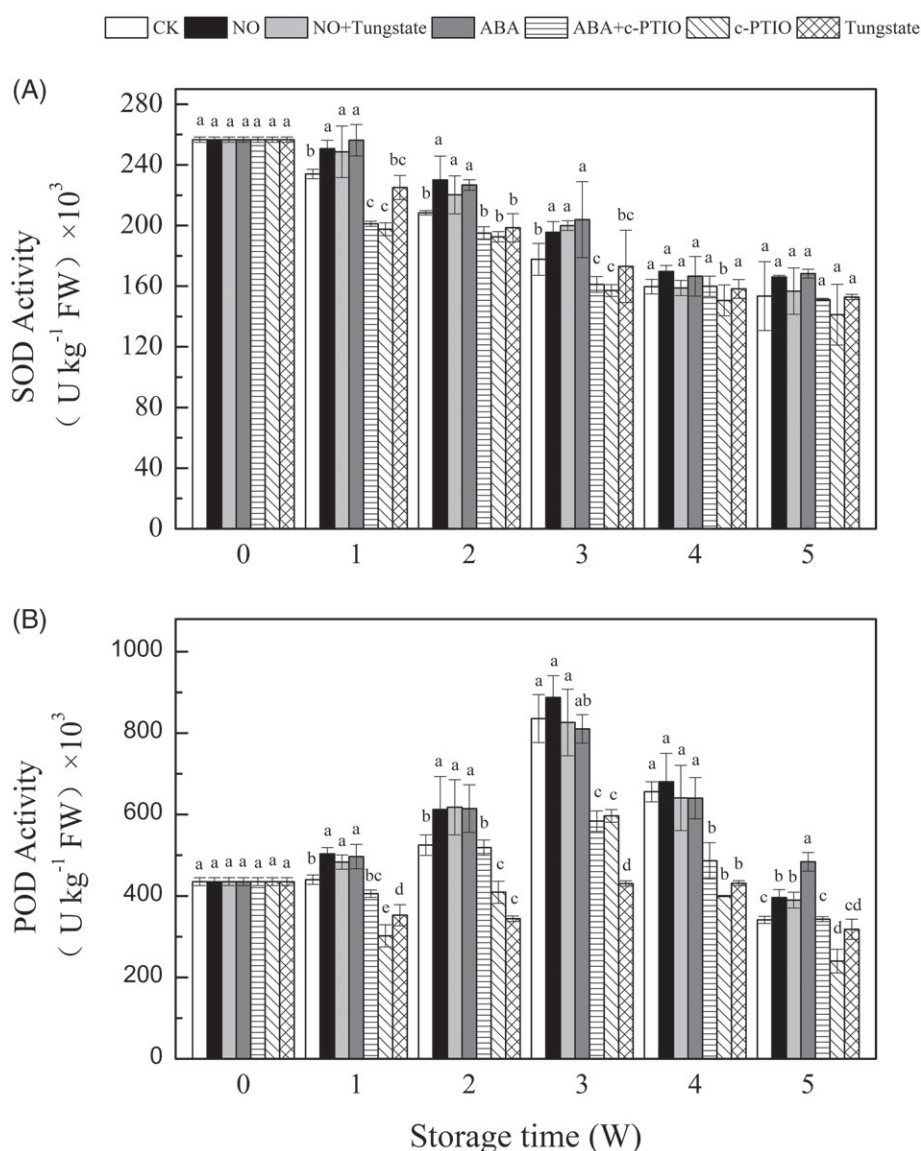
Figure 3. SOD (A) and POD (B) activities in cold-stored peaches subjected to various treatments. Values are the means $\pm$ SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

compared with NO or ABA treatment alone. In addition, the content of NO in peaches treated with NO plus sodium tungstate was markedly increased compared to peaches treated with control alone (1–4 weeks). However, treatment with ABA plus c-PTIO caused a significant decrease in NO content, when compared to treatment with ABA.

Application of ABA or ABA plus c-PTIO alone induced a significant increase in ABA content compared with the control during the entire experiment (Fig. 6(B)). However, NO plus sodium tungstate or sodium tungstate markedly inhibited the chilling stress-induced increase in ABA content. Moreover, NO or c-PTIO had little effect on ABA content, when compared with the control.

DISCUSSION
Both NO and ABA are involved in many botanic physiological and stress-resistance processes.[31,32] Low temperature could induce endogenous NO and ABA production to relieve chilling injury symptoms of fruit during storage.[9,33] Firmness is an important quality factor in many fruits, and the decrease in firmness during storage is primarily due to the destruction of cell wall structure and the depolymerization of pectin, which are catalyzed by the action of polygalacturonase and pectin methylesterase.[34] In our current study, it is worth pointing out that exogenous NO or ABA treatment was effective in reducing CI and delaying peach fruit softening. Similar results have been found in other cold-sensitive fruits such as Hami melon,[35] papaya,[34] zucchini[15] and sweet cherry.[21] SSC and peel color are essential factors affecting fruit flavor and appearance and can be affected by fruit senescence. In this study, SSC in peach fruit increased, peaking on the fourth week and then decreased gradually over time during cold storage. Wannabussapawich *et al.*[36] reported that the increase in SSC during fruit ripening was attributed to the increased activity of enzymes responsible for the hydrolysis of starch to soluble sugars. Similar results have been found in other fruits such as mango[36] and plum.[37] In this study, NO or ABA could lead to differential increases in SSC contents compared with the control. The primary reason for this was that NO or ABA
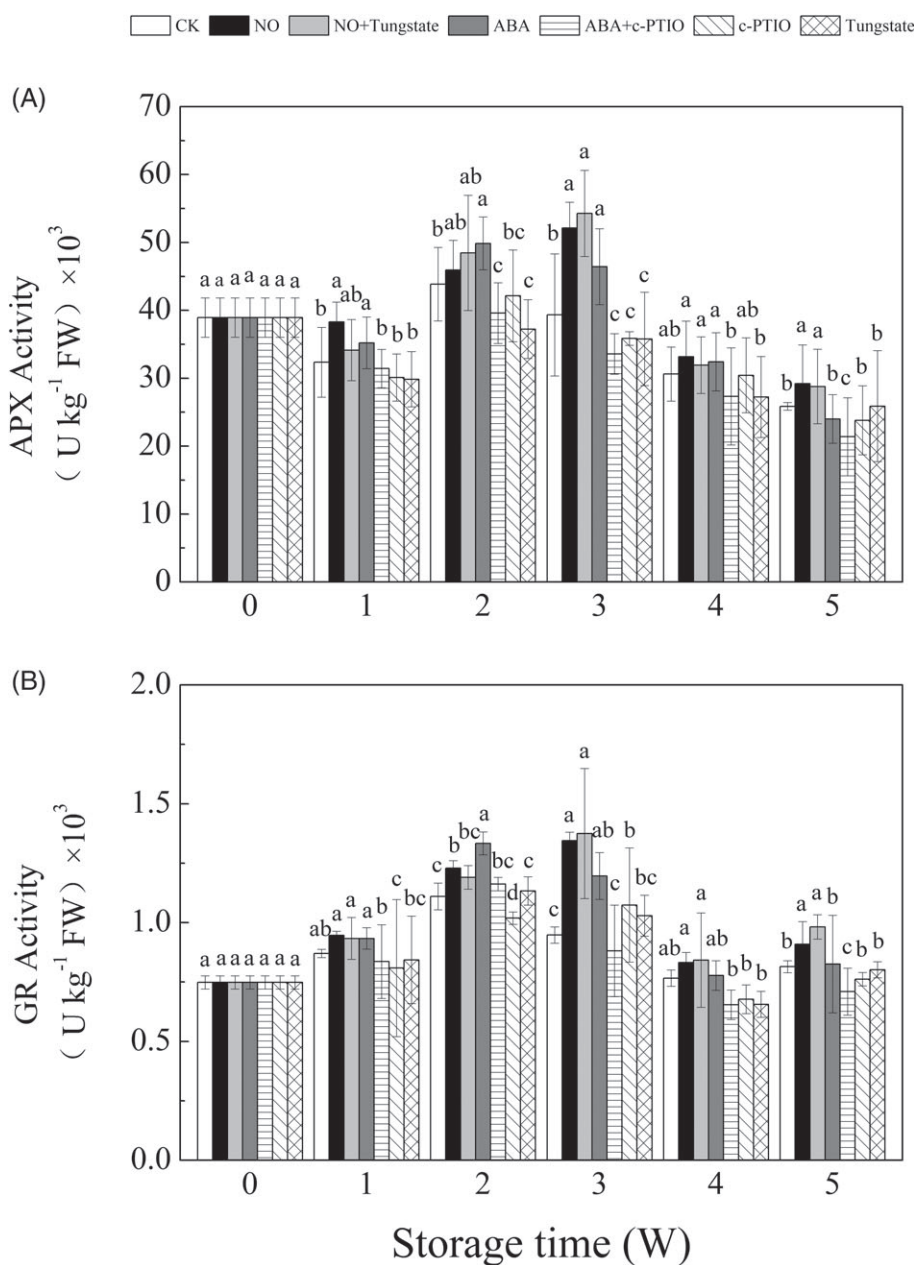
CK   NO   NO+Tungstate   ABA   ABA+c-PTIO   c-PTIO   Tungstate

Figure 4. APX (A) and GR (B) activities in cold-stored peaches subjected to various treatments. Values are the means ± SD (*n* = 3). Values with different letters within the same sampling date are significantly different (*P* < 0.05).

might inhibit the respiration of peach fruit, reduce its physiological metabolism and slow down the degradation of soluble solids. Similar results have been found in other treatment such as fruit wax and thiabendazole combined with 1-MCP.[38] In addition, as observed in the present study, NO or ABA could effectively maintain high levels of peel color, and could be effective for controlling CI and maintaining the quality of peach fruit during cold storage.

It is well known that membrane lipid peroxides and ROS are important factors influencing oxidative stress levels. Up to the present, one hypothesis that has been widely proposed is that cold-induced membrane rigidification probably is a primary cold sensor in both prokaryotes and plants.[39,40] Membrane fluidity and integrity can be indirectly reflected by MDA content and electrolyte leakage. High MDA content and electrolyte leakage are widely considered as important indicators of cell membrane

damage.[41] In this study, MDA content and electrolyte leakage of the control peaches increased quickly, while NO or ABA treatment significantly inhibited the increase of electrolyte leakage and also exerted positive effects on the MDA content in peaches (Fig. 2). These results suggested that NO and ABA could delay the process of membrane lipid peroxidation and maintain the fluidity and integrity of the cell membrane, thus reduce the effects of cold stress. Moreover, as observed in the present study, electrolyte leakage and MDA increased in peaches under cold stress, as did $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ contents (Figs 1 and 2). These changes indicated that significant oxidative stress existed in the tissues studied. This often occurs because of the accumulation of ROS, particularly H_2O_2, which is a powerful oxidant that can oxidize thiol residues and cause damage to host cells. H_2O_2 is an essential component of ROS in organisms. It can lose electrons easily and
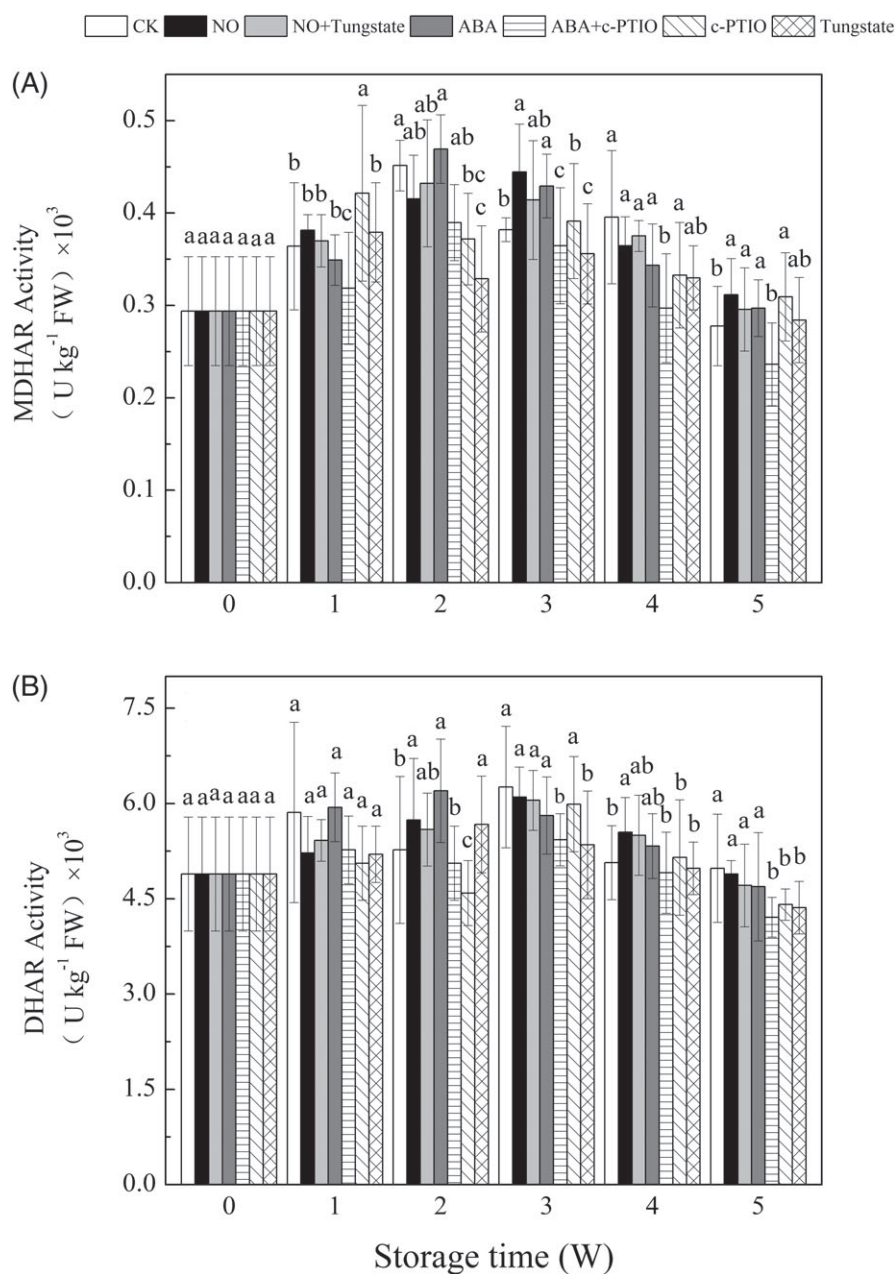
Figure 5. MDHAR (A) and DHAR (B) activities in cold-stored peaches subjected to various treatments. Values are the means ± SD (*n* = 3). Values with different letters within the same sampling date are significantly different (*P* < 0.05).

has a strong oxidizing ability, which will damage the biomembrane system.[42,43] In this study, NO or ABA significantly inhibited the accumulation of H_2O_2 and $O_2^{\bullet-}$ in peaches during cold storage and reduced the oxidative damage to peach fruit.

Many studies have reported that ROS and membrane lipid peroxide levels increase in plants under cold conditions.[44–46] However, these compounds can also exert toxic effects; therefore, they must be tightly regulated by protein antioxidants and/or small molecules, so that any increases are brief and followed by rapid returns to non-harmful levels.[25] Accordingly, plant cells have developed antioxidant defense systems to prevent damage from ROS. Among the enzymatic antioxidant defense systems, SOD provides the first line of defense by converting $O_2^{\bullet-}$ into H_2O_2 and O_2, after which H_2O_2 is converted into H_2O by POD and the AsA–GSH cycle.[47,48] In the AsA–GSH cycle, APX plays a crucial

role in catalysis of the removal of H_2O_2, while other enzymes are primarily responsible for providing APX with substrates by forming AsA and GSH. Non-enzymatic systems also play an important role in the elimination of free radicals. Specifically, high contents of antioxidants and ratios of cellular redox status help to determine itself (cellular redox status), and may have a direct impact on many basic cellular processes. AsA and GSH can directly remove $O_2^{\bullet-}$ and $^{\bullet}OH$ and reduce H_2O_2 into water, thus alleviating the oxidative injury induced by all kinds of stressors.[49] In the present study, treatment with NO led to increases in the activities of SOD, POD, APX and GR to varying degrees relative to the control treatment, and these increases lasted throughout the cold storage (Figs 3 and 4). The primary reason for this was that NO has a strong affinity for iron-containing enzymes. As a result, NO could take part in a series of resistant physiological

Table 2. AsA content, AsA/DHA ratio, GSH content and GSH/GSSG ratio in cold-stored peaches subjected to various treatments

Treatment	Time (weeks)	AsA	AsA/DHA	GSH	GSH/GSSG
0 week	0	68.9 ± 0.23[a]	5.98 ± 0.89[a]	128.9 ± 2.13[a]	8.98 ± 0.72[a]
CK	1	59.7 ± 0.05[d]	4.08 ± 0.41[b]	97.75 ± 1.12[d]	9.74 ± 0.54[c]
NO		63.7 ± 0.01[bc]	4.22 ± 0.30[b]	138.68 ± 0.14[a]	11.54 ± 0.87[b]
c-PTIO		61.7 ± 0.09[cd]	3.80 ± 0.48[b]	78.09 ± 0.63[f]	5.12 ± 0.09[d]
NO + tungstate		64.9 ± 0.08[ab]	7.36 ± 0.99[a]	122.78 ± 0.36[c]	10.15 ± 0.53[bc]
ABA		66.2 ± 0.13[a]	6.08 ± 0.24[a]	130.97 ± 1.21[b]	13.68 ± 0.51[a]
Tungstate		54.1 ± 0.06[e]	3.28 ± 0.07[b]	79.03 ± 0.67[f]	5.74 ± 0.24[d]
ABA + c-PTIO		48.7 ± 0.06[f]	3.54 ± 0.31[b]	85.97 ± 0.45[e]	5.40 ± 0.09[d]
CK	2	53.5 ± 0.01[c]	3.04 ± 0.19[c]	73.39 ± 0.05[d]	9.30 ± 0.59[a]
NO		61.8 ± 0.14[ab]	6.12 ± 0.59[a]	109.57 ± 1.21[a]	10.97 ± 1.29[a]
c-PTIO		52.6 ± 0.02[c]	3.09 ± 0.20[c]	61.03 ± 0.40[f]	4.50 ± 0.02[bc]
NO + tungstate		63.2 ± 0.03[a]	6.78 ± 0.38[a]	102.41 ± 1.04[ab]	10.79 ± 0.11[a]
ABA		60.6 ± 0.09[b]	4.50 ± 0.46[b]	81.45 ± 1.30[c]	8.79 ± 0.65[a]
Tungstate		49.1 ± 0.03[d]	3.72 ± 0.14[bc]	68.96 ± 0.81[de]	5.74 ± 0.24[b]
ABA + c-PTIO		46.4 ± 0.04[e]	2.66 ± 0.11[c]	64.26 ± 2.20[ef]	3.76 ± 0.41[c]
CK	3	56.7 ± 0.07[c]	6.23 ± 0.44[c]	77.78 ± 1.75[c]	3.31 ± 0.25[c]
NO		65.4 ± 0.06[a]	10.32 ± 0.50[a]	98.42 ± 1.79[ab]	3.81 ± 0.34[c]
c-PTIO		50.2 ± 0.03[e]	2.37 ± 0.19[d]	78.63 ± 1.26[c]	8.77 ± 0.62[a]
NO + tungstate		62.8 ± 0.07[b]	6.00 ± 0.20[c]	99.72 ± 1.39[a]	7.24 ± 0.29[b]
ABA		58.3 ± 0.01[c]	8.15 ± 0.40[b]	93.54 ± 0.85[b]	9.87 ± 0.37[a]
Tungstate		52.5 ± 0.01[d]	2.71 ± 0.07[d]	78.81 ± 2.33[c]	4.01 ± 0.20[c]
ABA + c-PTIO		43.5 ± 0.06[f]	3.37 ± 0.23[d]	69.40 ± 1.70[d]	2.81 ± 0.29[c]
CK	4	52.2 ± 0.01[c]	10.25 ± 0.91[a]	71.20 ± 0.90[de]	4.12 ± 0.25[c]
NO		55.9 ± 0.08[a]	4.80 ± 0.46[bc]	92.42 ± 2.96[a]	8.16 ± 1.27[a]
c-PTIO		46.9 ± 0.01[de]	3.03 ± 0.11[c]	60.49 ± 4.79[f]	4.48 ± 0.52[c]
NO + tungstate		53.5 ± 0.02[bc]	3.65 ± 0.40[bc]	86.15 ± 3.76[ab]	6.42 ± 0.60[ab]
ABA		53.8 ± 0.10[b]	4.29 ± 0.44[bc]	81.00 ± 1.75[bc]	8.41 ± 1.03[a]
Tungstate		45.4 ± 0.01[e]	3.87 ± 0.35[bc]	74.55 ± 1.39[cd]	6.64 ± 0.01[ab]
ABA + c-PTIO		47.5 ± 0.03[d]	5.11 ± 0.24[b]	63.36 ± 0.76[ef]	4.22 ± 0.08[c]
CK	5	46.8 ± 0.01[c]	3.12 ± 0.17[d]	87.59 ± 3.32[b]	11.21 ± 2.21[ab]
NO		48.5 ± 0.08[b]	5.12 ± 0.46[b]	99.72 ± 2.29[a]	9.05 ± 1.80[ab]
c-PTIO		49.9 ± 0.04[b]	9.12 ± 0.19[a]	86.87 ± 1.16[b]	8.59 ± 0.87[b]
NO + tungstate		51.7 ± 0.01[a]	5.39 ± 0.05[b]	97.03 ± 0.04[a]	11.95 ± 0.96[a]
ABA		48.5 ± 0.06[b]	4.95 ± 0.49[bc]	83.33 ± 1.24[b]	8.03 ± 0.88[b]
Tungstate		42.5 ± 0.05[d]	2.65 ± 0.11[d]	63.90 ± 2.29[c]	4.09 ± 0.37[c]
ABA + c-PTIO		37.4 ± 0.01[e]	3.40 ± 0.13[cd]	66.18 ± 0.45[c]	3.03 ± 0.12[c]

Values are the means ± SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

responses by regulating the activity of APX-related enzymes as well as other enzymes containing hemichrome iron.[50] Meanwhile, early reports demonstrate that the activities of SOD, POD, APX and GR are increased by chilling stress-induced *S*-nitrosylation. In plant tissues, NO may react with thiol proteins, resulting in the formation of *S*-nitrosothiols. *S*-Nitrosothiols is considered as intracellular antioxidant glutathione, which may react with NO, resulting in the formation of *S*-nitrosoglutathione.[51,52] Indeed, increasing evidence confirms that the intracellular redox state plays a crucial role in the ability of plants to overcome biotic and abiotic stresses. Maintaining a high level of reduction power is essential for plants to quench excess ROS.[53,54] The application of NO enhanced the efficiency of non-enzymatic systems in peach fruit during cold storage (Table 2). Such changes could maintain the redox state of ROS in peaches, thus reducing the oxidative

injury under cold conditions. Furthermore, it has been reported that high concentrations of NO accelerate the development of chilling symptoms of tomato fruit.[55] Therefore, an appropriate content of NO is conducive to maintain the stability of physiological and biochemical processes of plants, while high levels of NO are harmful. The present study showed that treatment with 15 μmol L^{-1} NO solution significantly alleviated the chilling injury and softening symptoms of peach fruit during cold storage. These results are consistent with those of a previous report.[7]

Studies have shown that the application of exogenous ABA under normal conditions can cause similar stress, but its application under adverse conditions can improve plant defense mechanisms.[13] These conflicting results suggest that there may be ABA-dependent and ABA-independent pathways that lead to adaptation to cold stress. The production of ROS has developed
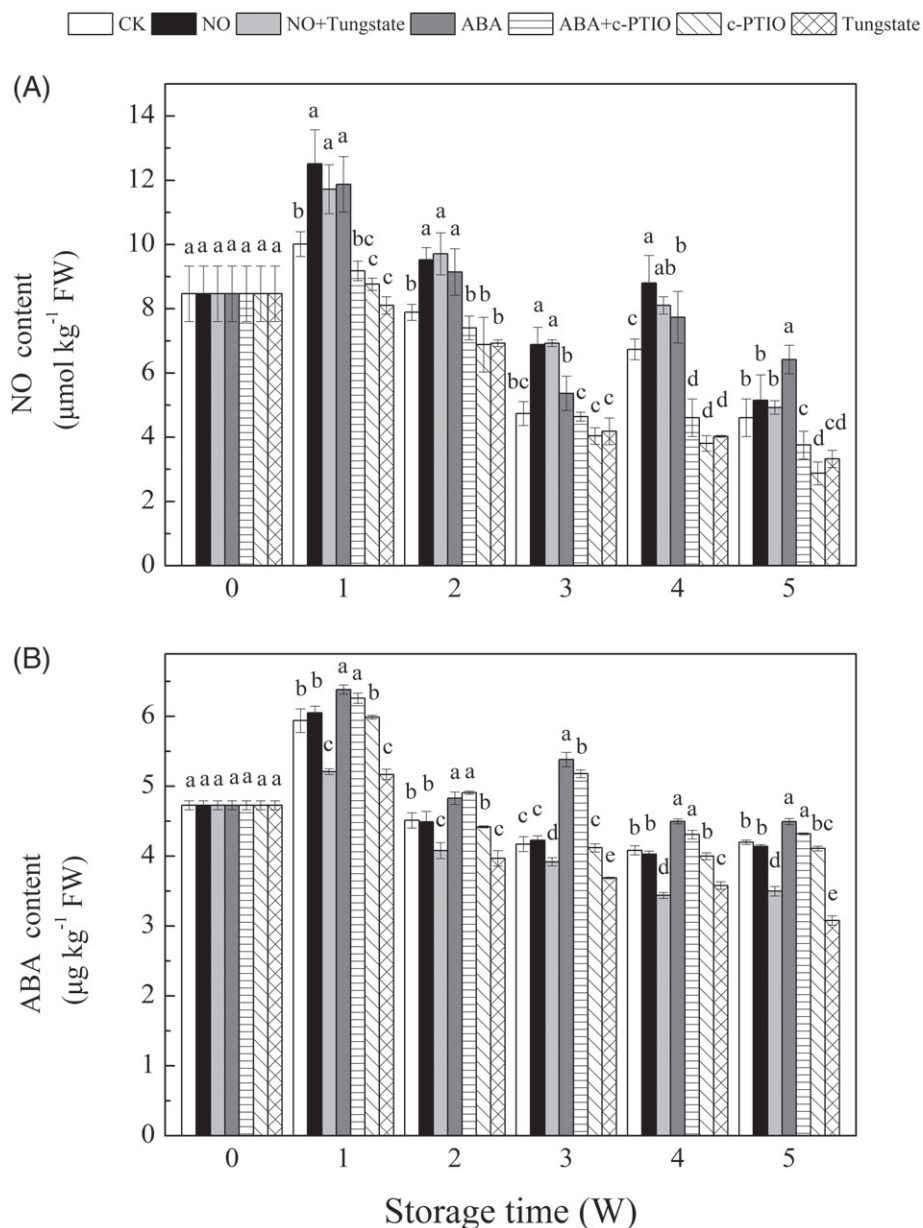
Figure 6. NO (A) and ABA (B) contents in cold-stored peaches subjected to various treatments. Values are the means ± SD ($n = 3$). Values with different letters within the same sampling date are significantly different ($P < 0.05$).

into one of the most important responses to abiotic stress in plants, and the antioxidant defense system is rapidly induced in plant cells to attenuate the accumulation of ROS-related oxidant damage. ABA has also been regarded as an enhancer of antioxidant defense against ROS produced during stress conditions.[15] The results of the present study showed that application of exogenous ABA could enhance the efficiency of enzymatic systems (especially those of SOD, POD and APX) to varying degrees relative to the control treatment (Figs 3 and 4). These findings were similar to those of previous studies,[56] that indicated ABA could enhance antioxidant defenses and relieve increases in ROS induced by chilling conditions. The present study indicated that the application of ABA could enhance the efficiency of non-enzymatic systems during the cold storage of peaches (Table 2). Such changes allow the plant to maintain a high ability to reduce ROS in fruit and lead to alleviation of cold stress.

Many recent studies have indicated that cold can induce the generation of endogenous NO or ABA, which in turn contribute to the alleviation of chilling injury symptoms via regulating antioxidative metabolism in fruit.[9,33] These studies have shown that changes in endogenous NO or ABA have certain degrees of specificity under cold stress. In this study, c-PTIO, as a NO scavenger,[7] and sodium tungstate, as an ABA synthesis inhibitor,[15] were used to verify the physiological role of endogenous NO or ABA in cold-storage peaches. c-PTIO or sodium tungstate could lead to differential increases in CI index and obviously increase the contents of ROS as well as electrolyte leakage, resulting in significant oxidative injury to peaches compared with that of NO or ABA alone (Figs 1 and 2; Table 1), while the enzyme activities were lower than those of NO or ABA treatment alone (Figs 3–5). Furthermore, the contents of antioxidants and the cellular redox status in peaches treated with c-PTIO or sodium tungstate during cold storage were also lower
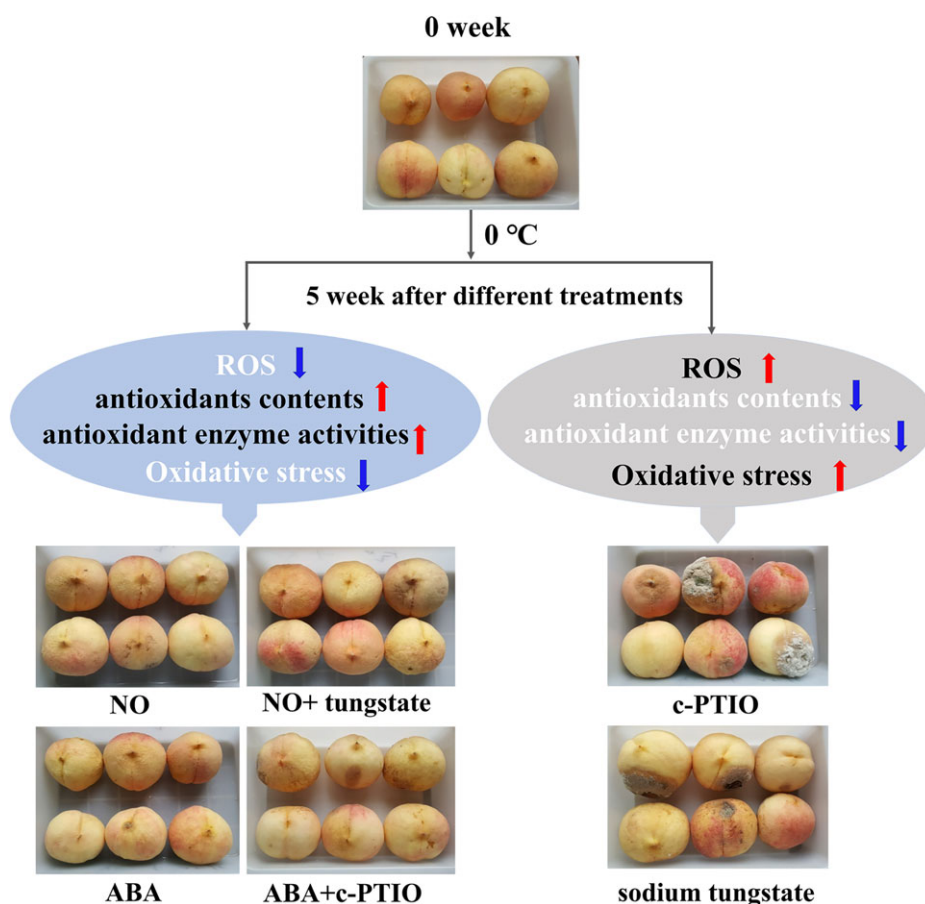
Figure 7. Proposed model for roles of NO, ABA and interactions between NO and ABA in response to chilling stress in peach fruit (*Prunus persica* (L.) Batsch, cv. 'Xintaihong').

than those in peaches treated with NO or ABA alone (Table 2). c-PTIO or sodium tungstate completely inhibited the increase in NO or ABA content compared with NO or ABA treatment alone (Fig. 6). These findings indicated that NO and ABA played important roles in the regulation of peaches during cold storage.

Previous studies have provided some evidence of cross-talk between NO and ABA. In certain circumstances, such as the induction of stomatal closure and upregulation of antioxidant enzyme gene transcription and activities, NO mainly acts as a downstream factor of the ABA signaling pathway.[52,57] Moreover, in warm-season C₄-grasses, NO and ABA also seem to interact extensively with each other to control ROS.[58] However, the combination of ABA and c-PTIO had an effect opposite to that of ABA in a study by Zhou *et al.*,[59] who reported that NO is involved in ABA-induced antioxidant activities in *Stylosanthes guianensis*. It is worth noting that, in that study, ABA increases the activities of APX, CAT and SOD in *S. guianensis*, but its effects are reversed in the presence of the NO scavenger c-PTIO. Moreover, this study demonstrates that the increase in NO content in ABA-treated shoot is reversed in the presence of the NO scavenger. It is suggested that ABA could trigger the production of endogenous NO, and the endogenous NO could mediate the activities of ABA-induced enzymes. c-PTIO, as NO scavenger, could scavenge all or parts of the endogenous NO induced by ABA, and then lead to these reverse effects. These findings also indicated that complex interactions between NO and ABA occur in plants.

The results of this experiment showed that NO scavengers could counteract the protective effects of exogenous ABA against CI

index (Table 1), ROS contents (Fig. 1) and lipid peroxidation (Fig. 2). These results suggested reversely that NO could mediate the protective effects of ABA. The results were consistent with those of previous reports.[12,59] Moreover, the increased NO content in ABA-treated peach during cold storage was inhibited by the NO scavenger or ABA synthesis inhibitor sodium tungstate (Fig. 6). These findings were similar to those of previous studies,[59,60] indicating that ABA could trigger NO production. ABA in combination with c-PTIO, c-PTIO or sodium tungstate alone markedly blocked the activities of antioxidant enzymes and the contents of antioxidants (Figs 3–5; Table 2). Meanwhile, application of exogenous NO could elevate the chilling tolerance in peach fruit by enhancing the antioxidant defense system (Figs 3–5; Table 2), while NO combined with sodium tungstate did not affect the NO-induced protective effects against activation of antioxidant activities in peaches under chilling stress (Figs 3–5; Table 2). Conversely, NO had little effect on ABA content under cold conditions (Fig. 6). In summary, these results indicated that NO could regulate the activation of antioxidant activity induced by ABA in peaches during cold storage (Fig. 7), and NO could act as a downstream element in ABA-induced chilling tolerance.

CONCLUSIONS

Exogenous NO and ABA alleviated oxidative stress in response to cold by increasing the efficiency of enzymatic and non-enzymatic systems. Both ABA and NO could serve as signaling molecules to activate cellular antioxidant defense

systems to alleviate cold stress, and NO could mediate the ABA-induced and ABA-regulated plant adaptive responses to cold stress.

CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

ACKNOWLEDGEMENTS

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