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Effect of Nitric Oxide on Antioxidative Response and Proline Metabolism in Banana during Cold Storage

Yansheng Wang, Zisheng Luo,* Ruixue Du, Yue Liu, Tiejin Ying, and Linchun Mao

Department of Food Science and Nutrition, Zhejiang University, Hangzhou, 310058 People's Republic of China

ABSTRACT: The effect of exogenous nitric oxide (NO) on chilling injury to banana fruit was investigated. Banana fruit was treated with NO donor sodium nitroprusside of 0.05 mM at 20 °C for 10 min and then stored at 7 °C for up to 20 days. Banana fruit treated with NO sustained a lower chilling injury index and higher firmness and kept lower electrolyte leakage and malondialdehyde content than the control. Further investigation showed that NO treatment enhanced activities of guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase compared to the control. It also maintained higher ascorbic acid, reduced glutathione content, and total antioxidant capacity but reduced hydrogen peroxide and superoxide anion to lower levels compared to control fruit during storage. NO treatment significantly enhanced the accumulation of total phenolics and proline, which resulted from the increased activities of phenylalanine ammonia-lyase and Δ^1-pyrroline-5-carboxylate synthetase and decreased proline dehydrogenase activity. We proposed that the enhanced chilling tolerance induced by NO treatment may result from the reduction of oxidative stress and proline accumulation.

KEYWORDS: *nitric oxide, banana fruit, chilling injury, antioxidative enzymes, reactive oxygen species, proline*

INTRODUCTION

Banana fruit, like other tropical or subtropical fruits, is highly susceptible to chilling injury (CI). CI is a limiting factor in extending storage life and is responsible for substantial postharvest loss in many horticultural products. CI symptoms of banana fruit can occur at relatively high temperatures (e.g., approximately 12 °C), and the symptoms are aggravated at lower temperature.[1] CI symptoms of banana fruit include rapid peel browning, pitting, and failure of fruit softening.[1−3] The high chilling sensitivity leads to quality deterioration and severely reduces consumer acceptance. With extensive application of cold storage, it is important to investigate CI mechanisms in banana fruit and to optimize methods that alleviate the CI symptoms.

Oxidative stress has been demonstrated to be a significant factor in the cold-induced damage of harvested fruit.[4] The oxidative stresses are mainly caused by excess reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-). ROS could react with numerous cell components and result in lipid peroxidation, DNA damage and protein denaturation.[5] The antioxidant defense system evolved by plants to prevent ROS accumulation consists of some nonenzymatic antioxidants, such as phenolic compounds, ascorbic acid (AsA), reduced glutathione (GSH), and α-tocopherols, and some antioxidative enzymes, such as ascorbate peroxidase (APX) glutathione reductase (GR), superoxide dismutase (SOD), and peroxidase (POD).[6] The ascorbate−glutathione (ASC−GSH) cycle in this defense system has been reported to play an important role in chilling resistance of tomato fruit.[7]

Proline accumulation was observed in plants under diverse stress conditions including cold stress.[8] Increased proline content associated with enhanced chilling tolerance during cold storage has been reported in peaches and loquat fruit.[9,10] It can be inferred that proline accumulation may serve as an adaptive mechanism to low temperature in postharvest products. 1-pyrroline-5-carboxy-late synthetase (P5CS) and proline dehydrogenase (PDH) are key regulatory and rate-limiting enzymes in accumulation and metabolism of proline.[8,11] The changes of P5CS and PDH activities in cold-stored fruit, associated with their effects on proline content, may reveal the physiological significance between proline accumulation and chilling tolerance.

Various methods, such as modified atmosphere packaging, hot water, and UV−C treatment, have been demonstrated to reduce the CI of banana.[3,12,13] However, these methods are either costly or complicated to operate in practical use. Economical and convenient techniques to alleviate chilling injury of banana fruit are still in urgent demand. Nitric oxide (NO), a gaseous free radical, is known to be a signaling molecule involved in various physiology processes in plants, such as root development, flowering, reproduction, fruit ripening, and regulation of multiple plant responses toward a variety of biotic and abiotic stresses.[14−16] Recently, accumulating evidence indicated a potential role of exogenous NO treatment in alleviating chilling injury and maintaining quality of postharvest horticultural products during cold storage, including tomato fruit, mangoes, cucumbers, peach fruit, and Japanese plum.[17−22] However, the mechanism of NO in reducing chilling injury symptoms and quality deterioration has not been fully understood.

To the best of our knowledge, there has been no report on the effect of NO treatment on chilling injury in banana. In this study, the chilling injury symptoms, antioxidant enzymes, total phenolics, proline as well as proline metabolism-associated

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enzymes were evaluated. Based on the results, a possible active mechanism for the effects of NO involving the antioxidant defense system and proline accumulation is proposed.

MATERIALS AND METHODS

Fruit and NO Treatment. Banana fruit (*Musa* spp., AAA group cv. "Brazil") was transported from Hainan Province to the laboratory at Zhejiang University after being harvested at 70−80% maturity. Upon arrival, fruit were separated into fingers. Fingers (300) were randomly divided into two lots (150), comprising three replicates (50). In a preliminary study, the fruit were stored at 7 °C for 20 days after treatment with sodium nitroprusside (SNP), a NO donor that can release NO upon being in aqueous solvents. It was found that treatment with SNP of 0.05 mM showed good effect on alleviating the chilling injury in banana fruit. Fruit (50) in the first lot was immersed into a solution of 0.05 mM in a 30 L sealed vacuum container and vacuum infiltrated at low pressure (−90 kPa) for 5 min and then left in the solution at ambient pressure for 5 min (NO). Fruit in the second lot was soaked in sterile deionized water under the same conditions (control). The fingers of two lots were placed into unsealed polyethylene bags (0.04 mm) and stored at 7 °C for 20 days. Fingers were sampled at 5 d intervals for 20 d. A sample of 9 fingers from each replicate (3 × 9 per treatment) was used to take the determination of color, CI index, firmness and electrolyte leakage. Then the peels of nine banana fingers were cut into pieces, frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. The peel material was thoroughly homogenized to ensure that samples taken for analysis were representative. The experiment was repeated three times.

CI Index Assessment. CI index was assessed according to a method described by Nguyen et al., with a five-stage scale based on the intensity of surface browning.[23] 1 = no chilling injury; 2 = mild injury; 3 = moderate injury; 4 = severe injury; 5 = very severe injury. The CI index was calculated using the following formula:

$$\text{CI index} = \sum (\text{CI scale}) \times (\text{number of fruit at that scale}) / (\text{total number of fruit in the group})$$

Firmness and Color. Firmness measurements of peel and pulp were taken at the equatorial part of each banana fruit using a texture analyzer (TA-XT2i, Stable Microsystems Texture Technologies Inc., UK) fitted with a 5 mm diameter flat probe. The penetration depth was 10 mm and the test speed was 1 mm s^{-1}. Fruit firmness was recorded as N cm^{-2}.

Peel color was measured at four locations around equatorial region on each fruit using a Chroma meter (Konica Minolta, CR-400, Japan). Results were expressed as L* (lightness) and H* (hue angle).

Electrolyte Leakage and Malondialdehyde (MDA) Content. The rate of electrolyte leakage was determined using the method described by Chen et al. with modification.[1] Discs of banana peel (20) were excised with a 1 cm diameter stainless steel borer from the equatorial part of six banana fruit. The discs were washed in distilled water three times and dried on absorbent paper. Then the discs were placed into a test tube containing 20 mL of distilled water and maintained in a water bath shaker at 25 °C for 30 min. The electrical conductivity (L_0) was measured using a conductivity meter. The tubes were then heated to 100 °C for 20 min and quickly cooled to room temperature. Another reading of the electrical conductivity was taken and recorded as L_1. The rate of electrolyte leakage was expressed using the following equation: Electrolyte leakage (%) = $(L_0/L_1) \times 100\%$

The MDA concentration was assayed by the method of Chen et al. with a slight modification.1 Frozen banana peel tissues (2.0 g) were homogenized in 8 mL 50 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was collected for the measurement of MDA content. Supernatant phase (1 mL) was mixed with 3 mL of 5 g/L thiobarbituric acid in 100 g/L trichloroacetic acid. The reaction solution was held in a boiling water bath for 15 min, quickly cooled, and centrifuged at 12,000*g* for 10 min to clarify the solution. The absorbance was measured at 532, 600, and 450 nm. The MDA concentration was calculated according to the formula

$$\text{MDA content (nmol/g)} = [6.45(A_{532} - A_{600}) - 0.56A_{450}]V_tV_r/(V_sm)$$

V_t, V_r, and V_s were the total volume of the extract solution, the total volume of the reaction mixture solution, and the volume of the extract solution contained in the reaction mixture solution and m was the mass of samples. The content of MDA was expressed as nmol g^{-1} FW.

H_2O_2 Content and O_2^- Production Rate. H_2O_2 content was measured by a modification of the method of Ferguson et al.[24] Frozen banana peel tissues (1.0 g) were homogenized in 5 mL of cold acetone and centrifuged at 12,000*g* for 10 min. $TiCl_4$ in HCl (10% v/v $TiCl_4$ in concentrated HCl, 0.1 mL) and concentrated ammonia−water (0.2 mL) were added dropwise to 1.0 mL supernatant. After shaking and reacting for 5 min, the mixture was then centrifuged (12,000*g*) at 4 °C for 15 min, and the precipitates were washed repeatedly with cold acetone until the acetone was colorless. The precipitates were solubilized in 3 mL of 2M H_2SO_4 solution prior to measurement of absorbance at 415 nm. A standard curve of known concentrations of H_2O_2 was used to calculate the content of H_2O_2 in the samples. Results were expressed as μmol g^{-1} FW.

The O_2^- production rate was measured according to the method of Xu et al. with slight modifications.[25] Frozen banana peel tissues (2.0 g) were homogenized in 8 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) and centrifuged at 12,000*g* for 10 min. The incubation mixture contained 1 mL of the supernatant, 1 mL of 1.0 mM hydroxylammonium chloride, and 1 mL of 50 mM potassium phosphate buffer (pH 7.0). After incubation at 25 °C for 1 h, 1 mL of 17 mM sulphanilic acid and 1 mL of 7 mM a-naphthyl amine were added to the incubation mixture. After reaction at 25 °C for 20 min, the absorbance was read at 530 nm. A standard curve with $NaNO_2$ was used to calculate the production rate of O_2^-. The 2-fold concentration of NO_2^- was regarded as concentration of O_2^- in samples. O_2^- production rate was expressed as nmol min^{-1} g^{-1} FW.

Enzymes Measurement. Frozen banana peel tissues (2.0 g) were homogenized in 8 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM ethylene diamine tetraacetic acid and centrifuged at 12000 × *g* for 10 min. The supernatant was used for measuring activities of APX and GR. APX activity was measured according to the method of Pongprasert et al. with some modification.[13] The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized by H_2O_2. The reaction mixture contained 0.1 mL of enzyme extract, 0.1 mL of 5 mM sodium ascorbate, and 1.7 mL of sodium phosphate buffer (50 mM, pH 7.0). Then, 20 mM H_2O_2 (0.1 mL) was added to start the reaction. GR activity was assayed according to Yin et al. with slight modification.[26] The reaction mixture contained 0.1 mL of enzyme extract, 0.1 mL of 10 mM GSSG, and 1.7 mL of sodium phosphate buffer (50 mM, pH 7.0). Then, 0.1 mL of 2.4 mM NADPH was added to start the reaction. One unit of APX and GR activity was defined as the change in absorbance per minute under the assay conditions.

Frozen banana peel tissues (2.0 g) were homogenized in 8 mL of 50 mM sodium phosphate buffer (pH 8.8) and centrifuged at 12,000*g* for 10 min. The supernatant was used for measuring activities of guaiaco peroxidase (POD) and phenylalanine ammonia-lyase (PAL). POD activity was measured as previously described according to the method proposed by Omran with modification. [27] The supernatant (0.15 mL) and 0.15 mL of 20 mM H_2O_2 were added into 3 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 40 mM frozen guaiacol. One unit of POD activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per minute at 470 nm. PAL activity was measured according to the method proposed by Yingsanga et al.[28] One unit of PAL activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per hour at 290 nm.

The activities of P5CS and PDH were assayed as described by Shang et al.[9] Frozen banana peel tissues (2.0 g) were homogenized in 8 mL of 50 mM Tris-HCl buffer (pH 7.4) containing 7 mM $MgCl_2$,

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0.6 mol L^{-1} KCl, 3 mM EDTA, 1 mM DTT, and 5% (w/v) insoluble polyvinylpyrroli-done. One unit of P5CS activity was defined as the change in absorbance per minute at 340 nm. One unit of PDH activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per hour at 340 nm.

Protein concentrations in the extract were determined according to the method of Bradford using bovine serum albumin (BSA) as a standard.[29] Specific activity of the enzyme was expressed as units mg^{-1} protein.

Proline Content. The proline content was assayed according to the method of Zhao et al. with modification.[30] Frozen banana peel tissues (2.0 g) were homogenized in 5 mL of 3% (v/v) sulfosalicylic acid and centrifuged at 12,000*g* for 10 min. Glacial acetic acid (2 mL) and ninhydrin reagent (3 mL) were mixed with the supernatant (2 mL) and boiled for 30 min. Then toluene (4 mL) was added into the reaction mixture after the solution was cooled. The absorbance of the organic phase was recorded at 520 nm. A standard curve of known concentrations of proline was used to calculate the content of proline in samples. The results were expressed as μg proline per gram fresh material.

ASA Content. AsA was analyzed by using liquid chromatography on an RP-Phase with UV detection according to Xu et al. with some modifications.[31] AsA standard solutions (200, 160, 120, 80, and 40 mg/L) were prepared. Frozen banana peel tissues (5.0 g) were homogenized in 25 mL 0.1% oxalic acid for 10 min and then centrifuged (12000 × g) at 4 °C for 20 min. The supernatant (1 mL) was immediately filtered through a Millipore membrane (0.45 μm) before injection. The separation was performed on a Agilent C18 column (250 × 4.6 mm i.d.) using 0.1% oxalic acid as the mobile phase at a flow rate of 1.0 mL/min at 25 °C oven temperature, and the eluent was monitored at 243 nm. The ascorbic acid contents were expressed here as μg per gram fresh material.

GSH Content. GSH content was assayed according to the method of Guri with modification.[32] The frozen banana peel tissues (2.0 g) was homogenized with 4 mL of ice-cold 50 g/L trichloroacetic acid containing 5 mM EDTA and then centrifuged at 4 °C for 10 min at 12,000*g*. The reaction mixture contained 1.0 mL of the supernatant, 1.0 mL of 0.1 M sodium phosphate buffer (7.8), and 0.5 mL of 4 mM dithiobis-2-nitrogenzoic acid. A standard curve of known concentrations of GSH was used to calculate the content of GSH in samples. GSH content was expressed as μg g^{-1} FW.

Total Phenolics Content and Ferric Reducing Antioxidant Potential (FRAP) Assay. Frozen banana peel tissues (1.0 g) were homogenized in 8 mL of methanol and extracted for 15 h in the dark. Then the homogenate was centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was used for measuring total phenolics content and FRAP assay. Total phenolics in banana peel were determined according to the Folin-Ciocalteu procedure, using the method described by Singleton and Rossi.[33] The supernatant was allowed to react with Folin-Ciocalteu reagent and 150 g/L Na_2CO_3. Results were expressed as mg gallic acid equivalents per gram fresh material. FRAP assay was measured according to a method developed by Xu et al. with a slight modification.[34] The supernatant was diluted with methanol to a suitable concentration, and then 0.1 mL of diluted extract was added to 4.9 mL of the FRAP reagent. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC) per gram of fresh material.

Statistical Analysis. Experiments were performed according to complete randomized design. The results were expressed as the mean values ± standard deviation and analyzed by one-way analysis of variance (ANOVA). The overall least significant difference (LSD) at $P = 0.05$ was calculated and used to detect significant differences among storage temperatures and times for Figures 1−6.

RESULTS

CI Index, Fruit Color, and Firmness. CI index increased during the whole storage at 7 °C and the increase was delayed by NO treatment (Figure 1A). CI index in control fruit was 13.4% higher than treated fruit at the end of storage. Fruit color generally mirrored the changes in CI index. The overall lightness and hue angle both decreased during cold storage, but the decline was slower in NO-treated fruit than control fruit (Figure 1B,C). Significant differences of hue angle between NO-treated fruit and control fruit can be observed from day 5 to day 20.

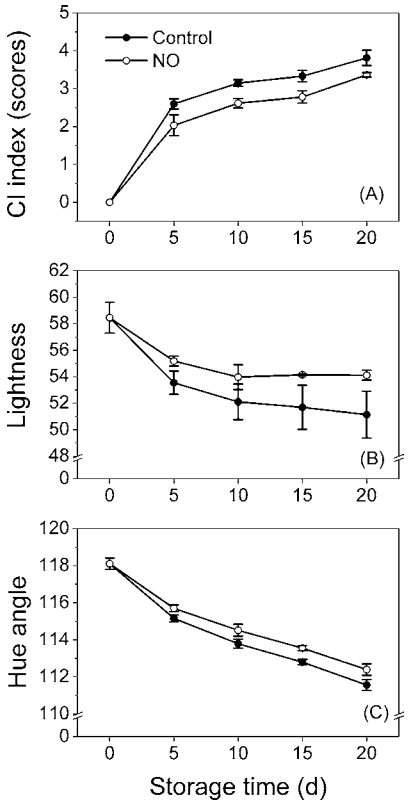

Figure 1. Effect of NO on changes in CI index, lightness, and hue angle and in banana fruit treated with H_2O (shown as control) and 0.05 mM donor SNP (shown as NO). (A) Change of CI index in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05} = 0.25$. (B) Change of lightness in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05} = 1.89$. (C) Change of hue angle in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05} = 0.43$.

Firmness of peel and pulp changed to a small extent over the 20 days of storage (Figure 2). NO treatment maintained higher fruit firmness compared to control fruit. On day 5, firmness of NO-treated fruit is 6.7% and 4.6% higher than control fruit in peel and pulp, respectively.

Electrolyte Leakage and MDA Content. Electrolyte leakage and MDA content are used to evaluate the membrane damage in banana fruit. Sharp increases were observed in both electrolyte leakage and MDA content during the first 5 days of storage, and then the increase rate became slow. Increased electrolyte leakage and MDA content associated with CI development during storage was delayed by NO treatment (Figure 3). MDA content in control fruit was 12.1% higher than treated fruit on day 20.

H_2O_2 Content and O_2^- Production Rate. The control fruit exhibited higher levels of H_2O_2 content and O_2^- production rate than NO-treated fruit. The content of H_2O_2 increased rapidly during the whole storage. NO treatment reduced the increase of H_2O_2 (Figure 4A). H_2O_2 content in control fruit was 6.7% higher than treated fruit at the end of storage.

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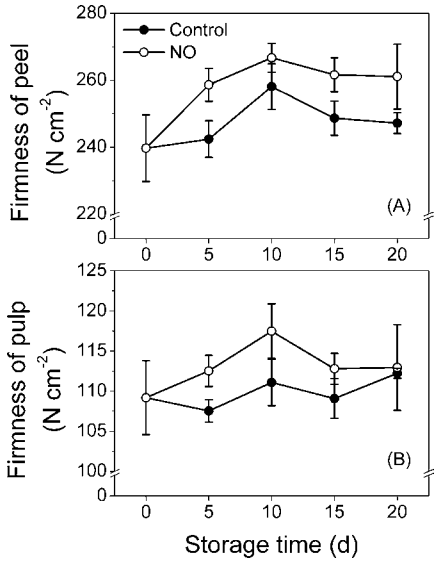

Figure 2. Effect of NO on changes in peel and pulp firmness in banana fruit treated with H_2O (shown as control) and 0.05 mM donor SNP (shown as NO). (A) Change of peel firmness in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 11.07. (B) Change of pulp firmness in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 5.30.

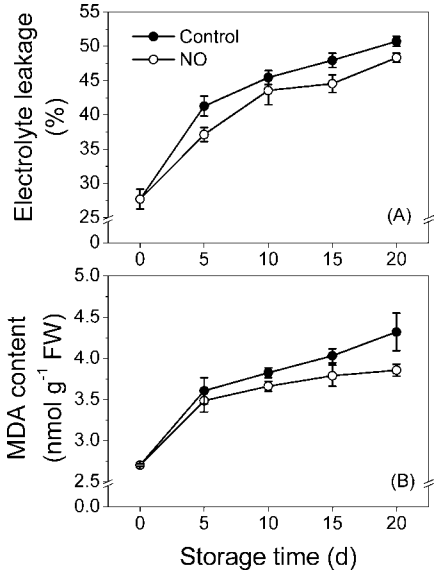

Figure 3. Effect of NO on electrolyte leakage and MDA content in banana fruit treated with H_2O (shown as control) and 0.05 mM donor SNP (shown as NO). (A) Change of electrolyte leakage in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 2.81. (B) Change of MDA content in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 0.21.

O_2^- production rate increased at first, and then quickly declined until the end of the storage. Lower O_2^- production rate was observed in NO-treated fruit than control fruit (Figure 4B). The O_2^- production rate in control fruit was 14.4% higher than treated fruit on day 10.

Activities of APX, POD, and GR and Contents of GSH and AsA. NO treatment promoted the increase of APX activity in the first 15 days of storage (Table 1). By day 15, APX activity in the control and NO-treated fruit had increased by 3.5-fold

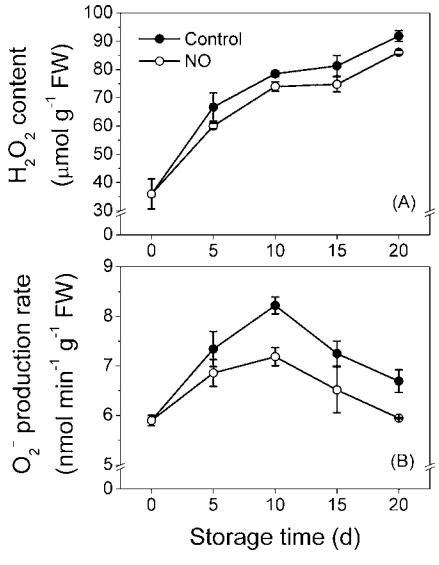

Figure 4. Effect of NO on changes in H_2O_2 content and O_2^- production rate in banana fruit treated with H_2O (shown as control) and 0.05 mM donor SNP (shown as NO). (A) Change of H_2O_2 content in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 5.78. (B) Change of O_2^- production rate in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 0.47.

and 4.5-fold, respectively. A slight decrease of APX activity was observed in treated fruit after day 15.

The increase of POD activity in response to NO treatment in the first 5 days appeared sharp in view of a modest increase in the control group (Table 1). POD activity in treated fruit on day 5 was 1.9-fold compared to that on day 0. POD activity in control fruit slightly increased during the whole storage, while a decrease was observed in treated fruit during the last period of storage.

GR activity in NO-treated fruit increased in the first 10 days of storage, and then declined. In control fruit, GR activity slowly increased to the end of storage (Table 1). Higher levels of GR activity were observed in treated fruit than control fruit from day 5 to day 10. The maximum GR activity in treated fruit was recorded on day 10, with 1.6-fold compared to control fruit.

The content of GSH in banana fruit steadily increased in the first 10 days, following a sharp accumulation to the end of storage (Table 1). NO treatment promoted the increase of GSH content. GSH content in treated fruit was 14.6% higher than control fruit on day 15.

The content of AsA in banana fruit steadily decreased during cold storage (Table 1). The decrease of AsA content was delayed by NO treatment. Significantly higher levels of AsA content can be observed in treated fruit than control fruit from day 5 to day 10.

Total Phenolics Content, PAL Activity, and Total Antioxidant Capacity (FRAP). In the NO-treated fruit, the content of total phenolics increased rapidly during the first 10 days of storage and then decreased gradually during the remainder of storage (Figure 5A). Compared to day 0, the maximum total phenolics content was recorded with 1.8-fold in treated fruit on day 10. PAL activity exhibited a similar pattern with total phenolics content during the cold storage (Figure 5B). A good positive correlation was observed in NO-treated fruit between total phenolics content and PAL activity (r =

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Table 1. Effect of NO on the Activities of APX, POD, and GR and Contents of GSH and AsA in Banana Fruit during Storage at 7 °C for 20 Days[a]

storage time	APX (units/mg protein)		POD (units/mg protein)		GR (units/mg protein)		GSH content (μmol/g FW)		AsA content (μg/g FW)	
(days)	control	NO	control	NO	control	NO	control	NO	control	NO
0	77 ± 11.7	77 ± 11.7	12.65 ± 2.24	12.65 ± 2.24	1.82 ± 0.30	1.82 ± 0.30	26.40 ± 2.00	26.40 ± 2.00	325 ± 2.50	325 ± 2.50
5	121 ± 4.9a	160 ± 23.9a	16.73 ± 1.17b	24.40 ± 1.90a	1.52 ± 0.46b	2.30 ± 0.14a	30.07 ± 1.67a	33.51 ± 2.69a	275 ± 8.95b	309 ± 3.48a
10	172 ± 12.3b	261 ± 6.0a	21.12 ± 0.84b	23.68 ± 0.64a	2.21 ± 0.37b	3.53 ± 0.40a	32.62 ± 3.36a	37.07 ± 2.60a	264 ± 3.20b	279 ± 2.99a
15	268 ± 14.3b	343 ± 14.1a	24.49 ± 1.93a	21.63 ± 1.13a	2.85 ± 0.34a	2.90 ± 0.21a	59.96 ± 4.23b	68.73 ± 3.00a	265 ± 9.06a	272 ± 6.41a
20	402 ± 26.1a	300 ± 18.90b	26.66 ± 1.83a	20.42 ± 1.29b	3.52 ± 0.10a	2.65 ± 0.40b	101.07 ± 8.00a	90.07 ± 4.33a	262 ± 6.30a	269 ± 6.30a

[a]Banana fruit were treated with H_2O (shown as Control) and 0.05 mM donor SNP (shown as NO), then stored at 7 °C for 20 days. Means in a row followed by different lower case letters for each parameter are significantly different by t test at $P = 0.05$. Data are accompanied by standard deviation of the means ($n = 3$).

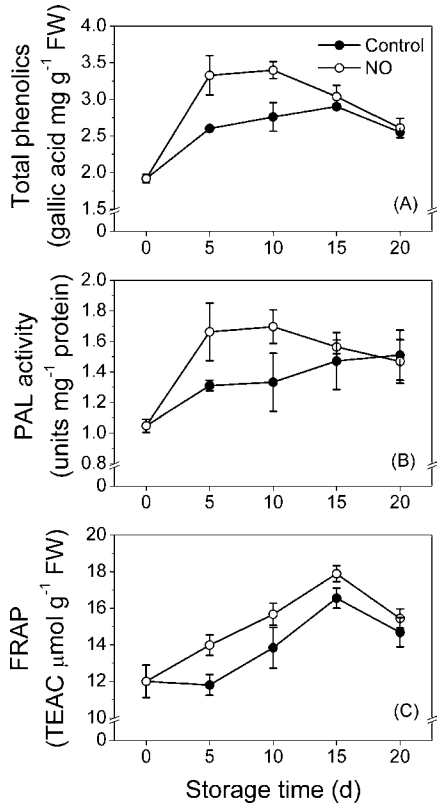

Figure 5. Effect of NO on changes in total phenolics content, PAL activity, and total antioxidant capacity (FRAP) in banana fruit treated with H_2O (shown as Control) and 0.05 mM donor SNP (shown as NO). (A) Change of total phenolics content in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05}$ = 0.30. (B) Change of PAL activity in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05}$ = 0.29. (C) Change of total antioxidant capacity (FRAP) in banana fruit during storage at 7 °C. Data are presented as means ± SD ($n = 3$), $LSD_{0.05}$ = 1.20.

0.96). After 15 days of storage, no significant differences were observed between the control and NO-treated fruit.

The total antioxidant capacity showed an increase during the first 15 days of storage and then a sharp decrease for the rest of the storage period (Figure 5C). NO-treated fruit displayed a higher level of total antioxidant capacity compared to that of the control. On day 15, the total antioxidant capacity in treated fruit was 8.1% higher than control.

Proline Content and Activities of P5CS and PDH. Proline content in fruit of both treatments increased during the first 10 days of storage and then declined. NO treated fruit had a higher proline content than that of the control fruit (Figure 6A). However, there were no significant differences between the proline content of NO treated and control fruit at the end of storage.

P5CS activity was higher in NO treated fruit than in control fruit (Figure 6B). Enzyme activity increased first and then declined until the end of storage. The P5CS activity in treated fruit was 84.7% higher than that in the control fruit on day 10.

Banana fruit had the highest PDH activity at the initial storage period. NO treatment accelerated the decrease of PDH activity (Figure 6C). At the end of storage, PDH activity in the control fruit was 1.7-fold compared to that in the NO treated fruit. No major shifts in PDH activity were observed in banana fruit over the storage period.

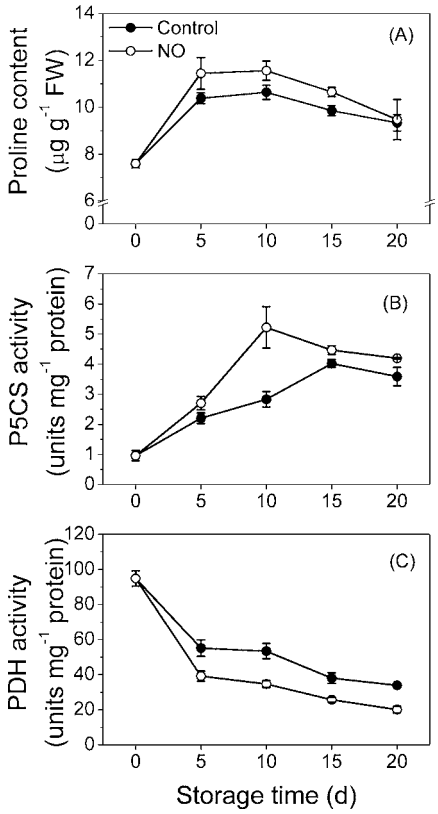

Figure 6. Effect of NO on changes in proline content and P5CS, PDH activities in banana fruit treated with H_2O (shown as control) and 0.05 mM donor SNP (shown as NO). (A) Change of proline content in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 0.78. (B) Change of P5CS activity in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 0.52. (C) Change of PDH activity in banana fruit during storage at 7 °C. Data are presented as means ± SD (n = 3), $LSD_{0.05}$ = 5.41.

DISCUSSION

NO is an important signal involved in plant responses to biotic and abiotic stress.[14,15] Accumulating evidence suggests a potential role of NO against chilling stress in postharvest products.[17−22] The mechanisms of exogenous NO's effect on reducing CI symptoms of fruit including reduction of ethylene production and respiration, improved antioxidant defense system and regulation of certain cold-related gene expression.[17−22] Chilling temperature induced elevated levels of ROS, which may cause damage to cellular components and disrupting metabolic function.[35] NO can protect plants against oxidative damage by directly scavenging ROS and enhancing activities of antioxidant enzymes.[14,15] NO treatment enhanced the activities of POD, APX and GR, while inhibited H_2O_2 accumulation and O_2^- production rate (Table 1; Figure 4). Total antioxidant capacity as indicated by FRAP assay was also enhanced in NO-treated fruit (Figure 5C). Similar results are obtained in tomato fruit and cucumbers.[15,18] POD may protect plants against stress-induced damage by scavenging H_2O_2.[36] APX and GR are two important antioxidant enzymes involved in the ASC−GSH cycle, which plays an important role in chilling resistance of fruit.[7] APX utilizes AsA as an electron donor for scavenging H_2O_2.[37] GR catalyzes the reduction of oxidized glutathione to GSH, which can regenerate AsA through the ASC-GSH cycle.[37] NO treatment promoted the activities of antioxidant enzymes in banana fruit, leading to a higher scavenging ability of ROS and thus reduced the oxidative damage under chilling stress. The present research suggested that the enhanced antioxidant defense system, as indicated by promoted antioxidant enzyme activities and ROS scavenging ability, may account for the chilling tolerance induced by NO.

Proline, a proteinogenic amino acid with an exceptional conformational rigidity, can stabilize cellular homeostasis, protect protein integrity and trigger specific gene expression, which can be essential for plant recovery from stress.[34] Except for acting as an alternative to directly scavenge ROS radicals, proline can also protect ROS scavenging enzymes and activate alternative detoxification pathways.[38] Proline increased activities of ASC−GSH cycle enzyme, such as APX and GR, protecting cultured tobacco cells against salt stress.[39] Previous reports have indicated a positive correlation between proline content and chilling tolerance in postharvest products. For example, during the cold storage of peach fruit, proline accumulation contributed to the enhanced chilling tolerance by exogenous γ-aminobutyric acid treatment.[9] Cao et al. also found that MeJA treatment increased proline content in loquat fruit, which was associated with the resistance of fruit to CI.[10] In the present study, a higher level of proline, associated with enhanced chilling tolerance was determined in NO-treated fruit during cold storage (Figure 6A). Similar results were obtained in NO-treated tomato fruit under chilling stress.[17] NO played an important role in the cold acclimation-induced increase in freezing tolerance by modulating proline accumulation in *Arabidopsis*.[30] It can be inferred that the alleviation of CI symptoms in banana fruit by NO treatment may be due to the high proline content. The elevated proline content induced by NO treatment may take a part in maintaining high activities of antioxidant enzymes, and scavenging ROS radicals thus reduce oxidative damage and protect fruit from chilling stress.

The metabolism of proline in plants has been well documented.[8,11,38] P5CS is the key regulatory and rate-limiting enzyme in the biosynthesis process of proline, while PDH catalyzes the first and rate-limiting step of proline degradation.[8,11] Cold acclimation-induced NO may get involved in the accumulation of proline through rapid up-regulation of expression of the P5CS1 gene and down-regulation of the PDH gene, thus contributing to enhanced freezing tolerance in *Arabidopsis*.[29] NO treatment promoted the increase of P5CS activity in banana fruit during the initial 10 days of storage, and then delayed the decrease of P5CS activity till the end of the storage (Figure 6B). The decrease of PDH activity was also inhibited by NO treatment (Figure 6C). Similar results have been obtained in peaches and loquat fruit with γ-aminobutyric acid and MeJA treatment respectively during cold storage.[9,10] Our study indicated that proline accumulation was closely related to activities of P5CS and PDH. The higher proline in banana fruit with NO treatment may be due to enhanced proline synthesis and reduced proline degradation.

Phenolic compounds, GSH and AsA, are important non-enzymatic antioxidants against oxidative damage of ROS in plants.[6] Many phenolic compounds in plant tissues, such as flavonoids, tannins, and lignin precursors, may work as potential antioxidants to scavenging ROS.[6] PAL is considered to be the principal enzyme in the biosynthesis of phenolics.[40] Nitric oxide fumigation stimulated PAL activity and phenolic accumulation and enhanced antioxidant activity of mushroom.[41] Yin et al. reported that exogenous NO stimulated PAL activity and accumulation of phenolics in sweetpotato root

under wound stress.[26] In our research, the increase in total phenolics was associated with higher PAL activity in NO-treated fruit (Figure 5A,B). Similar to total phenolics, the content of GSH is positively correlated with GR activity in fruit treated with NO (Table 1). GSH is a key nonenzymatic antioxidant that can scavenge ROS either directly or indirectly by participating in the ASC−GSH cycle. [37] In *Chorispora bungeana* suspension cultured cells, exogenous NO promoted GR activity as well as GSH content against chilling-induced oxidative damage.[42] NO treatment increased the activities of PAL and GR, which may account for the higher levels of total phenolics and GSH. The decrease of AsA in banana fruit was delayed by NO treatment (Table 1). AsA can directly scavenge ROS and act as an electron donor to APX for scavenging H_2O_2 involved in the ASC−GSH cycle.[37] NO treatment maintained high AsA and total phenolics content of longan fruit during storage, which was in agreement with our research.[43] The enhanced total antioxidant capacity, as indicated by FRAP assay, may be partially attributed to the accumulation of total phenolics, GSH and AsA. The results revealed that NO treatment enhanced antioxidant defense system partially by promoting biosynthesis of nonenzymatic antioxidants, leading to high chilling-tolerance in banana fruit.

In conclusion, this study indicates a possible role of NO in inducing chilling tolerance of banana fruit during cold storage. The effect of NO treatment may be attributed to its ability to enhance ROS scavenging ability and proline contents in banana fruit, which is due to the increased antioxidant enzymes and P5CS activities and decreased PDH activity. In addition, accumulation of total phenolics may also get involved in the enhanced antioxidant system against oxidative stress during cold storage. These results suggest a new strategy to alleviate chilling injury of banana fruit by applying NO treatment.

AUTHOR INFORMATION

Corresponding Author

*E-mail: luozisheng@zju.edu.cn. Phone: +86-571-88982162.

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ABBREVIATIONS USED

APX, ascorbate peroxidase; AsA, ascorbic acid; ASC−GSH, ascorbate−glutathione; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NO, nitric oxide; O_2^-, superoxide anion; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; PAL, phenylalanine ammonia-lyase; PDH, proline dehydrogenase; POD, guaiacol peroxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside

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