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Contribution of polyamines metabolism and GABA shunt to chilling tolerance induced by nitric oxide in cold-stored banana fruit

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ABSTRACT

Effect of exogenous nitric oxide (NO) on polyamines (PAs) catabolism, γ-aminobutyric acid (GABA) shunt, proline accumulation and chilling injury of banana fruit under cold storage was investigated. Banana fruit treated with NO sustained lower chilling injury index than the control. Notably elevated nitric oxide synthetase activity and endogenous NO level were observed in NO-treated banana fruit. PAs contents in treated fruit were significantly higher than control fruit, due to the elevated activities of arginine decarboxylase and ornithine decarboxylase. NO treatment increased the activities of diamine oxidase, polyamine oxidase and glutamate decarboxylase, while reduced GABA transaminase activity to lower levels compared with control fruit, which resulted the accumulation of GABA. Besides, NO treatment upregulated proline content and significantly enhanced the ornithine aminotransferase activity. These results indicated that the chilling tolerance induced by NO treatment might be ascribed to the enhanced catabolism of PAs, GABA and proline.

1. Introduction

Banana fruit, as a typical tropical fruit, are highly sensitive to chilling injury (CI). CI symptoms in banana fruit seriously reduce commercial fruit quality and consumer acceptance (Jiang, Joyce, Jiang, & Lu, 2004). Nitric oxide (NO) is considered to be an important signaling molecule involved in various plant responses to biotic and abiotic stresses (Arasimowicz & Floryszak-Wieczorek, 2007). Endogenous NO is suggested to act as an important role in postharvest fruit in response to cold stress (Xu, Dong, Zhang, Xu, & Sun, 2012). Recently, the application of exogenous NO treatment on postharvest fruit to enhance chilling tolerance has gained a growing interest (Zaharah & Singh, 2011). According to our previous study, NO treatment could reduce chilling symptoms of cold-stored banana fruit, which may be attributed to the enhanced antioxidant system and promoted proline metabolism (Wang et al., 2013). However, the specified mechanism of NO-induced chilling tolerance needs to be further explored.

A possible linkage may exist between polyamines (PAs) and NO in plants (Wimalasekera, Tebartz, & Scherer, 2011). In recent years, the important role of PAs in the regulation of plant abiotic stress tolerance has been well established (Gupta, Dey, & Gupta, 2013). PAs mainly includes putrescine (Put), spermidine (Spd) and spermine (Spm), which can be found widespread in the plant tissues. The biosynthesis of PAs in plants starts with the decarboxylation of ornithine or arginine, which is catabolized by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) respectively to yield Put (Hussain, Ali, Ahmad, & Siddique, 2011). Spd and Spm can be synthesized from Put by the sequential addition of aminopropyl groups (Alcazar et al., 2010). Polyamine degradation is catalyzed by diamine oxidase (DAO) and polyamine oxidase (PAO) (Gupta et al., 2013; Shelp et al., 2012). It is reported that PAs could induce rapid biosynthesis of NO in *Arabidopsis thaliana* seedlings, suggesting a potential interplay between PAs and NO (Tun et al., 2006). Previous studies claimed that NO may act downstream of PAs in plant under abiotic stress (Arasimowicz-Jelonek, Floryszak-Wieczorek, & Kubis, 2009). Due to the fact that both PAs and NO are multifunctional molecules involved in plant stress response, it can be claimed that the enhanced chilling tolerance induced by NO might be associated with the coordinated biosynthesis of both PAs and NO.

Polyamine metabolism is closely linked to other compounds such as γ-aminobutyric acid (GABA) and proline, all known to be involved in stress tolerance (Gupta et al., 2013). Put can be converted into GABA catalyzed by DAO (Gupta et al., 2013; Yang,

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Chen, & Gu, 2011). GABA, a four carbon nonprotein amino acid, can rapidly accumulate in response to a variety of biotic and abiotic stresses (Shelp et al., 2012). In plants, GABA is mainly produced through a short pathway called the GABA shunt. GABA and the shunt pathway are involved in various physiological processes, including regulation of osmotic and cytosolic pH, bypass of the tricarboxylic acid (TCA) cycle, mediation of C:N balance, defense against insects, protection against oxidative stresses and functions in signal transduction (Fait, Fromm, Walter, Galili, & Fernie, 2008). The first step of this shunt is the irreversible decarboxylation of glutamate to GABA via glutamate decarboxylase (GAD). Through the canalization of GABA transaminase (GABA-T), GABA is reversibly converted to succinic semialdehyde, which in turn is reduced to succinate via succinic semialdehyde dehydrogenase and enters into the TCA cycle. GABA shunt and polyamine degradation are two main pathways of GABA synthesis in plant, with GAD and DAO as the most important rate-limiting enzymes respectively (Shelp et al., 2012). Although the polyamine degradation pathway is involved in the biosynthesis of GABA, the GABA shunt is supposed to be the main pathway for GABA biosynthesis (Hyun, Eom, Jeun, Han, & Kim, 2013).

Some amino acids, such as proline, play an important role in plant responses to stress (Rai, 2002). In higher plants, proline can be synthesized from either glutamate or ornithine (Ruiz et al., 2002). As a key intermediate in the biosynthesis of Put and proline, ornithine may play a regulatory role in the metabolism of these metabolites (Hussain et al., 2011). Polyamine metabolism is closely related to the formation of various signaling molecules and metabolites that involved in plant stress responses (Alcazar et al., 2010). Based on the fact that PAs, proline and GABA share some substrates, it is suggested that a common signal may trigger all these pathways in a coordinated manner. To our knowledge, this is the first report that exogenous NO treatment can alleviate CI by activating the catabolism pathways of PAs, GABA and proline in postharvest horticultural products. The objective of this study was to investigate the effect of NO treatment on PAs, GABA and proline catabolism in postharvest banana fruit in response to cold stress.

2. Materials and methods

2.1. Fruit and treatments

Banana fruits (*Musa* spp., AAA group cv. 'Brazil') were transported from Hainan Province to the laboratory at Zhejiang University at 70–80% mature green stage. Upon arrival, fruit were separated into fingers, and selected for uniformity of size, color and absence of damage.

Fingers (360) were randomly divided into two groups of 180, comprising three replicates of 60. Fingers in the first group were immersed into 0.05cmM sodium nitroprusside (SNP), a NO donor that can release NO upon in aqueous solvents, in a 30 L sealed vacuum container and vacuum infiltrated at low pressure (10 kPa) for 5 min (NO). SNP at 0.05 mM concentration was selected to alleviate the chilling injuries of cold-stored banana fruit based on our preliminary research (data not shown). Fingers treated with sterile deionized water were regarded as control (Control). After treatment, banana fingers were placed into unsealed polyethylene bags (0.04 mm) and stored at 7 °C for 20 days. Samples were collected at a period of 5 days. The peel of ten banana fingers were selected and 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. Three independent replicates were conducted.

2.2. CI index assessment

CI index was determined according to the method developed by Nguyen, Ketsa, and van Doorn (2003) with a five-stage scale.0: no chilling injury; 1: mild injury; 2: moderate injury; 3: severe injury; 4: very severe injury. The following formula was used to calculate the CI index:

CI index = $\sum$ (CI scale) × (number of fruit at that scale)/(total number of fruit in the group).

2.3. Endogenous NO content determination

Endogenous NO content was determined according to the method described by Zhang, Shen, Li, Meng, and Sheng (2011) with slight modification. Frozen samples (2.0 g) were homogenized in 5 mL of cold phosphate buffered saline (50 mM, pH 7.4) containing 1 mM DTT, 1 mM $MgCl_2$. After centrifuged at 12,000*g* for 20 min, the supernatant was collected to measure NO content according to the instruction of the NO assay kits (Nanjing Jiancheng Bioengineering Institute, PR China). Absorbance was measured at 550 nm against a blank prepared with distilled water. NO was calculated by comparison to a standard curve of nitrite ion. NO content was expressed as mM per gram fresh weight (FW).

2.4. NOS (nitric oxide synthetase) activity assay

NOS activity assay was performed according to Zhang et al. (2011) with slight modification. Frozen samples (2.0 g) were homogenized in 5 mL phosphate buffered saline (50 mM, pH 7.4) containing 1 mM EDTA, 10 mM ethylene glycol bis 2-aminoethyl ether) etraacetic acid, 1 mM leupeptin, 1 mM PMSF and 1% PVP. The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was collected and used for assays. The NOS activity was determined by measuring NO production with an NOS colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Absorbance was obtained at 530 nm against a blank prepared with distilled water. One unit of the enzyme activity was defined as the amount catalyzing the formation of 1 nmol NO per min. The NOS activity was expressed as units per mg of protein.

Protein in the enzyme extract was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. Specific activity of the enzyme was expressed as units per mg protein.

2.5. PAs content determination

Frozen samples (2.0 g) were homogenized in 5 mL 5% (w/v) cold perchloric acid. After at 12,000*g* for 20 min at 4 °C, the supernatant was collected for PAs assays. PAs in the supernatant was benzoylated using the method of Zhang, Shen, Li, Meng, & Sheng (2013a, 2013b). 2 mL chilled diethyl ether was added to extract the benzoylated PAs. The ether phase (1 mL) was collected and evaporated to dryness under a stream of air. The residue was then dissolved in 500 μL methanol and filtered through 0.45 m membrane filters. Aliquots (20 μL) were analyzed by HPLC (LC-2010A, Shimadzu Corporation, Kyoto, Japan) equipped with a reverse-phase Luna 5u C18 column (4.6 mm × 250 mm, Phenomenex, Torrance, CA) and eluted with 64% methanol at a flow rate of 0.8 mL/min. The PAs were detected using a UV detector at 254 nm and quantified by comparing the results with benzoylated standards. Results were expressed as nmol per gram fresh weight.

2.6. Proline content determination

The proline content was determined using the method of Zhao et al. (2009) with modification. Frozen samples (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 2 mL supernatant, and then boiled for 30 min. After the solution was cooled, 4 mL toluene was added into the reaction mixture. The reading of organic phase absorbance was taken at 520 nm. Proline content was calculated using a standard curve of known concentrations of proline. The results were expressed as μg proline per gram fresh material.

2.7. Assay of ornithine aminotransferase (OAT) activity

OAT activity assay was performed according to Cao, Cai, Yang, and Zheng (2012) with slight modification. Frozen samples (2.0 g) were ground in 5 mL potassium phosphate buffer (100 mM, pH 7.9), contained 15% glycerol, 1 mM EDTA, and 10 mM 2-mercaptoethanol. After centrifuged at 12,000*g* for 20 min, the supernatant was collected for OAT assay. The reaction mixture contained 0.2 M Tris–HCl buffer (pH 7.8), 46.8 mM ornithine, 12.5 mM α-ketoglutarate and 0.125 mM NADH. One unit of OAT activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per hour at 340 nm.

2.8. Assay of ADC and ODC activities

ADC and ODC activities were determined using the method of Hu et al. (2012) with modifications. Fresh samples (2.0 g) were homogenized in 5 mL potassium phosphate buffer (100 mM, pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 25 mM ascorbic acid, 1 mM pyridoxal phosphate (PLP), 5 mM EDTA, and 0.1% polyvinylpyrrolidone (PVPP). The homogenate was at 12,000*g* for 20 min at 4 °C, and the supernatant was collected for enzyme assays.

The reaction mixtures contained 100 mM Tris–HCl buffer (pH 7.5), 5 mM EDTA, 50 mM PLP, 5 mM DTT, and 0.3 mL supernatant. After incubation at 37 °C for 2 min, 0.2 mL of 25 mM L-arginine (pH 7.5) or 0.2 mL of 25 mM L-ornithine was added into the mixture for ADC or ODC determination, respectively. Then the mixtures were further incubated at 37 °C for 60 min, perchloric acid (PCA) was added to terminate the reaction until the final concentration of PCA was 5% (PCA was added at the start of the reaction as blanks). Finally, the mixtures were centrifuged at 12,000*g* for 5 min and the supernatant was collected. 0.5 mL supernatant was mixed with 1 mL 2 mM NaOH and 10 μL benzoyl chloride, and then the mixture was stirred continuously for 20 s. After incubation at 37 °C for 30 min, 2 mL saturated NaCl solution were added to stop the reaction. After centrifugation at 1500*g* for 5 min, 2 mL ether was added. 1 mL the ether phase was evaporated to dryness and redissolved in 2 mL of 60% methyl alcohol. One unit of ADC or ODC activity was defined as the amount of enzyme that produced 1 μmol agmatine or Put per minute at 37 °C, respectively. Results were expressed as units per milligram protein.

2.9. Assay of DAO and PAO activities

DAO and PAO activities were determined according to Gao et al. (2011) with some modification. Frozen samples (2.0 g) were homogenized in 5.0 mL potassium phosphate buffer (100 mM, pH 6.5). The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was used for enzyme assays. The reaction mixture contained 2.0 mL potassium phosphate buffer (100 mM, pH 6.5), 0.2 mL 4-aminoantipyrine/N,N-dimethylaniline reaction solution, 0.1 mL horseradish peroxidase (250 U/mL), and 0.5 mL of the enzyme extract. The reaction was initiated by the addition of Put (20 mM) for DAO and Spd + Spm (20 mM each) for PAO determination. A change of 0.001 in absorbance per minute at 555 nm was considered one unit of the enzyme activity.

2.10. GABA content determination

GABA content was determined using the method of Deewatthanawong, Rowell, and Watkins (2010b) with slight modification. Frozen samples were ground in liquid nitrogen to a fine powder, and 1.0 g of the ground powder was extracted in 1 mL methanol for 10 min at room temperature. The mixture was air-dried under a stream of nitrogen, and then dissolved in 1 mL 70 mM lanthanum chloride followed by 15 min of shaking. After centrifugation at 12,000*g* for 5 min, 0.8 mL supernatant was transferred to a new tube, and mixed with 0.16 mL 1 M potassium hydroxide. The mixture was shaken for 5 min, and centrifuged at 12,000*g* for 5 min. The supernatant was collected for GABA determination.

GABA content was determined using GABase (Sigma, St. Louis, MO). The 1 mL reaction solution contained 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate, 0.6 mM $NADP^+$, 0.1 unit GABase and 500 μL supernatant. After incubation at room temperature for 30 min, the reduction of NADP to NADPH was monitored at 340 nm. The results were calculated according to a standard curve established with known amounts of GABA, and expressed as μg per gram fresh weight (FW).

2.11. Assay of GAD and GABA-T activities

GAD and GABA-T activities were determined according to the method of Deewatthanawong, Nock, and Watkins (2010a) with slight modification. Frozen samples (2.0 g) were homogenized on an ice bath with 5 mL Tris–HCl buffer (100 mM, pH 9.1), containing 10% (v/v) glycerol, 1 mM PMSF, 0.5 mM pyridoxal phosphate (PLP), 1 mM DTT, and 5 mM EDTA. The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was collected for the GAD and GABA-T assay.

For GAD assay, the reaction mixtures consisted 0.1 M potassium phosphate buffer (pH 5.8), 40 μM PLP and 3 mM glutamate and 0.1 mL supernatant. After incubating at 30 °C for 60 min, 0.1 mL of 0.5 M hydrochloric acid was added into the mixture to stop the reaction. GABA content was analyzed as mentioned above. Enzyme activity was calculated by measuring the amount of GABA produced. One unit of GAD activity was defined as the amount of enzyme catalyzing the formation of 1 μg GABA per hour.

For GABA-T assay, the reaction mixtures consisted 50 mM Tris–Cl (pH 8.2), 10% (v/v) glycerol, 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP, 16 mM GABA, 4 mM pyruvate and 0.2 mL supernatant. After incubating at 30 °C for 60 min, 0.1 mL of 100 mM sulfosalicylic acid was added into the mixture to stop the reaction. Enzyme activity was calculated by measuring the amount of alanine produced using the enzymatic reaction of alanine dehydrogenase. The 1 mL reaction mixture contained 50 mM sodium carbonate buffer (pH 10), 1.5 mM NAD^+, 0.02 unit of L-alanine dehydrogenase and 0.1 mL terminated sample. After incubating at 25 °C for 10 min, the absorbance was read at 340 nm. One unit of GABA-T activity was defined as the amount of enzyme catalyzing the formation of 1 μg alanine per hour.

2.12. Statistical analysis

Experiments were performed according to a completely randomized design. All statistical analyses were carried out using the SPSS18.0 statistical software (SPSS Inc., Chicago, IL, USA). Data 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 treatments.

3. Results

3.1. CI index, endogenous NO content and NOS activity

All banana fruit developed CI symptoms after 5 days of cold storage, which increased with storage time, as indicated by sustained rise of CI index (Fig. 1A). The CI index was significantly reduced in NO-treated fruit ($P < 0.05$). On day 10, CI index in control fruit was 46.5% higher than the treated fruit.

Endogenous NO content steadily increased during the whole storage (Fig. 1B). Compared to control, NO-treated fruit exhibited a higher level of NO content after 10 days of cold storage ($P < 0.05$). As shown in Fig. 1C, NOS activity in control and treated fruit both increased rapidly and reached a maximum value on day 10. NOS activity in fruit with NO treatment was significantly higher than that of control ($P < 0.05$). At the end of storage, NOS activity in NO-treated fruit was 26.9% higher than that of control.

3.2. PAs content

Put in both control and treated fruit increased during the first 15 days of storage period, following a slight decrease (Fig. 2A).

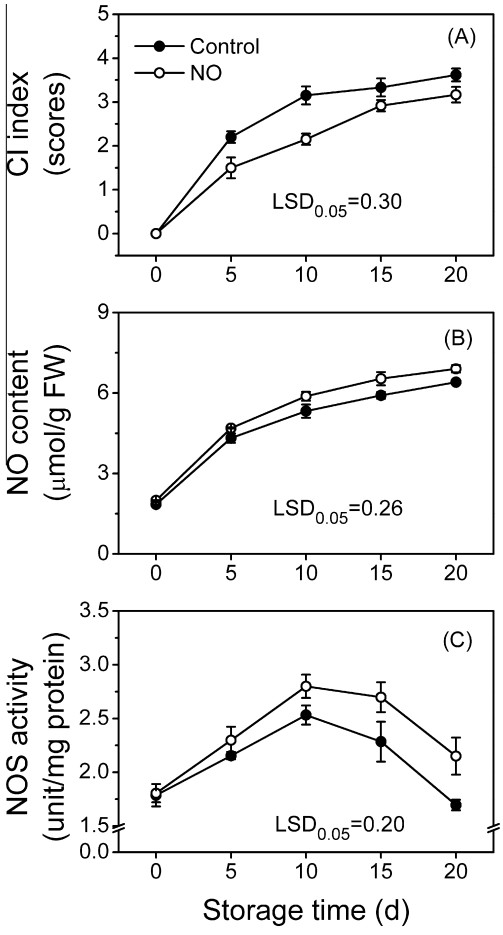

Fig. 1. Effect of NO treatment on CI index (A), endogenous NO content (B) and NOS activity (C) of banana fruit during cold storage. Values are the means ± SD of triplicate assays.

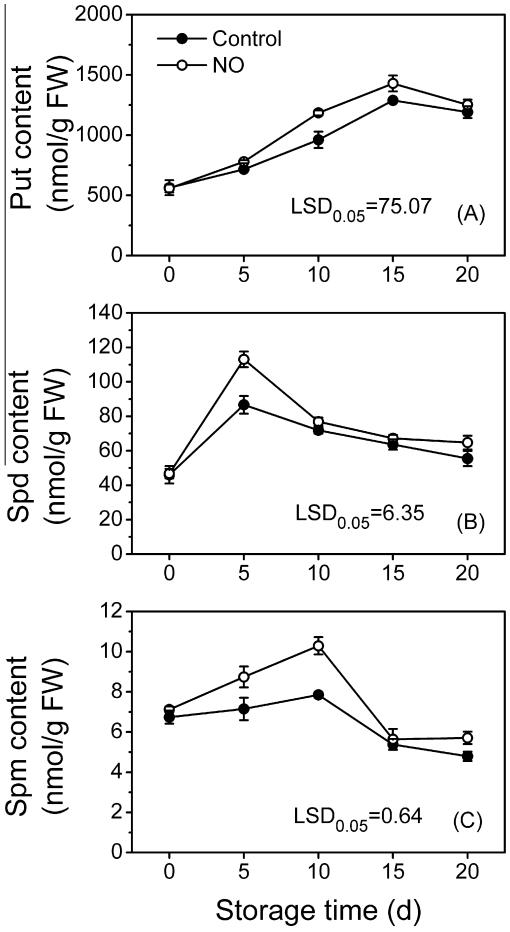

Fig. 2. Effect of NO treatment on contents of Put (A), Spd (B) and Spm (C) of banana fruit during cold storage. Values are the means ± SD of triplicate assays.

NO-treated banana fruit displayed significantly higher levels of Put content than control on day 10 and day 15 ($P < 0.05$). Maximum value of Spd content in NO-treated fruit was recorded on day 5, with 1.3-fold compared to control (Fig. 2B). No significant difference was observed after 10 days of cold storage ($P < 0.05$). Spm content slowly increase in the initial 10 days, following a sharp decrease during the rest of cold storage (Fig. 2C). Spm content in treated fruit was 22.2% and 31.1% higher than control fruit on day 5 and day 10, respectively.

3.3. ADC, ODC, DAO and PAO activities

In the NO-treated fruit, the ADC activity increased rapidly in the first 5 days of storage, and then decreased gradually during the remainder of storage (Fig. 3A). Compare to control, significantly higher level of ADC activity in treated fruit was recorded on day 5. ODC activity in both control and treated fruit increased during the first 10 days of storage, and then slowly decreased (Fig. 3B). NO treatment promoted the ODC activity during the whole storage. DAO activity showed a sharp increase during the first 10 days of storage in view of the slight decrease for the rest of storage period (Fig. 3C). DAO activity in treated fruit on day 10 was 1.4-fold compare to control. PAO activity was also promoted by NO treatment (Fig. 3D). At the end of storage, PAO activity in NO-treated fruit was 15.6% higher than control fruit.

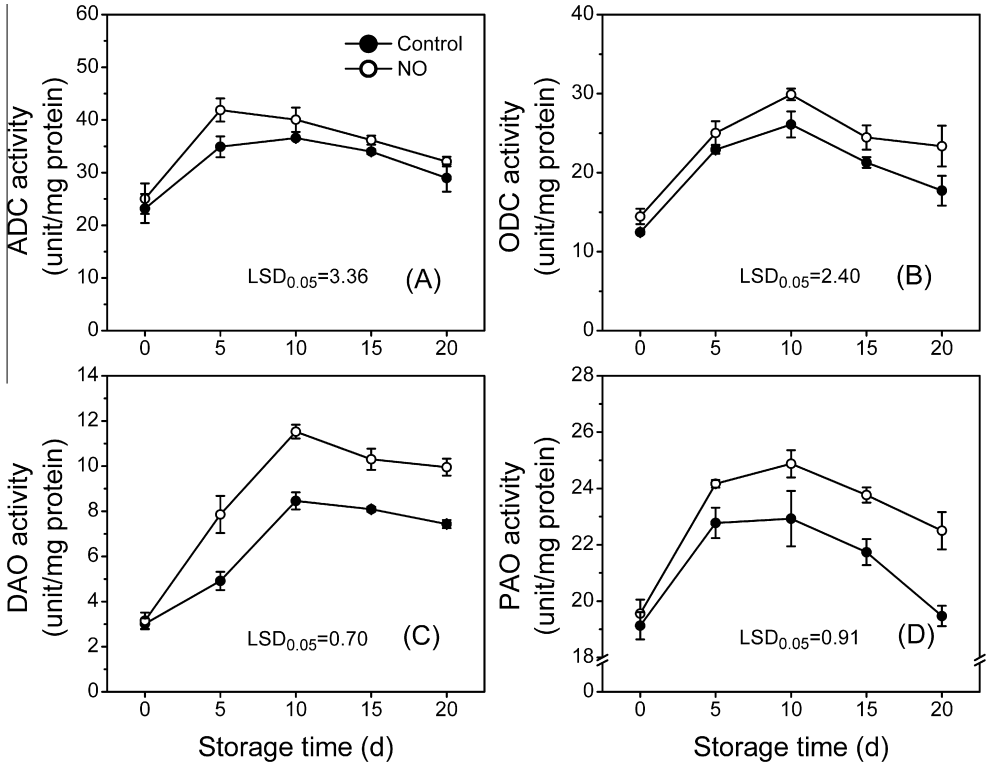

Fig. 3. Effect of NO treatment on activities of ADC (A), ODC (B), DAO (C) and PAO (D) of banana fruit during cold storage. Values are the means ± SD of triplicate assays.

3.4. GABA content, GAD and GABA-T activities

GABA content increased during the whole storage period and the increase was promoted by NO treatment (Fig. 4A). On day 20, GABA content in treated fruit is 41.1% higher than control. Compared to control, NO-treated fruit displayed remarkable higher GAD activity throughout the cold storage (Fig. 4B). A minor shift was observed on day 5 in fruit with NO treatment. GABA-T activity in control fruit reached the peak value on day 5, while GABA-T activity in NO-treated fruit gradually increased (Fig. 4C). NO treatment retarded the variation of GABA-T activity.

3.5. Proline content and OAT activity

Proline content increased in the first 10 days and then declined (Fig. 5A). NO-treated fruit exhibited a higher level of proline content compared to control. On day 5, proline content in NO-treated fruit was 21.4% higher than the control. OAT activity showed a slight increase in the first 5 days and then a sharp decrease for the rest of cold storage (Fig. 5B). NO treatment promoted the OAT activity during the whole storage.

4. Discussion

Previous studies suggested that NO could regulate key protective metabolite levels in plants via enzymatic and transcriptional modulation of proline and polyamine biosynthetic pathways (Filippou, Antoniou, & Fotopoulos, 2013). PAs had also been suggested to be involved in multiple physiological processes and stress responses (Gupta et al., 2013). It is claimed that NO may act downstream of PAs in plant under abiotic stress (Arasimowicz-Jelonek et al., 2009). Due to their polycationic nature at physiologic pH, PAs can bind strongly to negatively charged groups in cellular components, which is crucial for maintaining cellular homeostasis under stress. Moreover, PAs can function as ROS scavengers and protect the activity of antioxidant enzymes (Gupta et al., 2013). Exogenous PAs treatment can significantly alleviate the chilling symptoms of many chilling-sensitive fruits, such as cucumber (Zhang et al., 2009), apricot (Saba, Arzani, & Barzegar, 2012) and zucchini fruit (Palma, Carvajal, Ramos, Jamilena, & Garrido, 2015). As depicted in Fig. 2, NO-treated banana fruit displayed a significantly higher level of PAs than control fruit. Similar results were observed in MeSA-treated tomato fruit, in which the reduction of CI symptoms was associated with up-regulation of PAs and NO levels (Zhang et al., 2011). Our results indicated that PAs may get involved in the development of chilling tolerance induced by NO in cold-stored banana fruit, but the concrete mechanism is still unclear.

The biosynthesis of PAs is initiated with the formation of Put, with arginine and ornithine as the two main amino acid sources respectively. One involves the transformation from ornithine to Put, catalyzed by ODC. The other route involves decarboxylation of arginine regulated by ADC, via agmatine and N-carbamoylputrescine intermediates which are finally converted to Put (Alcazar et al., 2010). Compared to the control, NO treatment promoted the activities of ADC and ODC in cold-stored banana fruit (Fig. 3A and B). In this study, NO may get involved in the accumulation of PAs through promoting ADC and ODC activities, thus contributing to enhanced chilling tolerance in banana fruit. Polyamine degradation is catalyzed by DAO and PAO (Gupta et al., 2013). As depicted in Fig. 3C and D, NO treatment promoted the activities of DAO and PAO, Based on the results, it can be inferred that the key biosynthesis enzymes ADC and ODC, rather than degradation enzymes DAO and PAO, play dominant roles in the accumulation of PAs in NO-treated banana fruit, thus resulting in an enhanced chilling tolerance.

Contribution of PAs and other related metabolites, such as GABA and proline, has been suggested to be involved in the development of chilling tolerance of postharvest fruits (Palma, Carvajal, Jamilena, & Garrido, 2014). GABA shunt, rather than polyamine

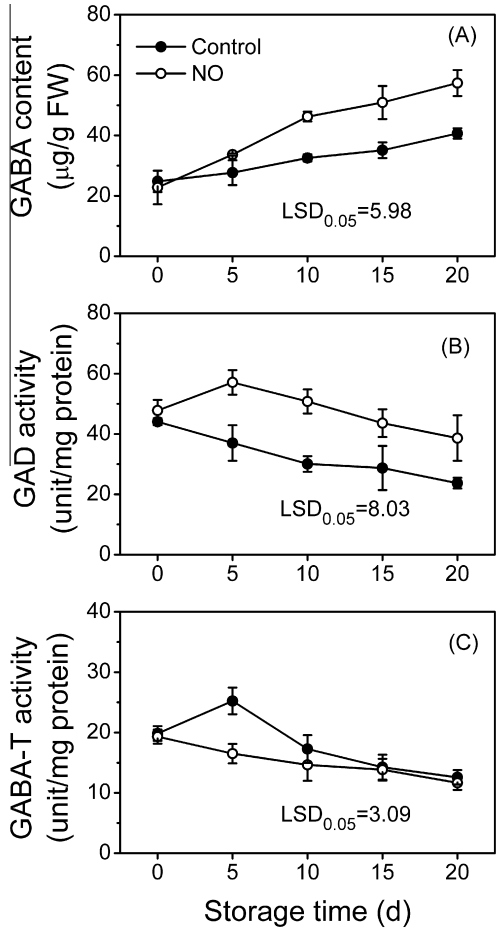

Fig. 4. Effect of NO treatment on GABA content (A), activities of GAD (B) and GABA-T (C) of banana fruit during cold storage. Values are the means ± SD of triplicate assays.

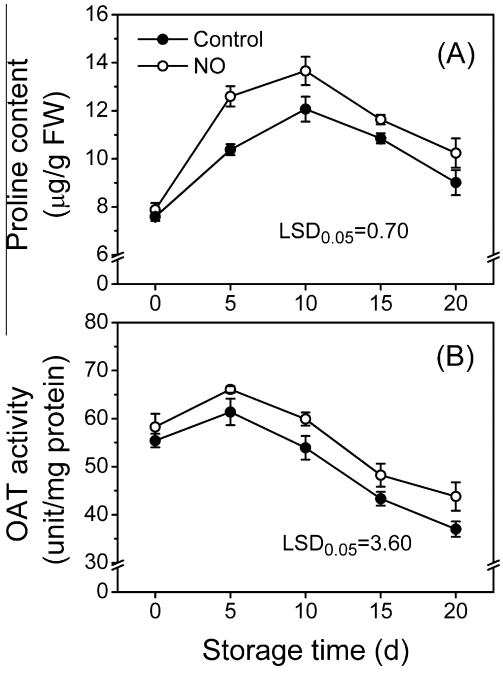

Fig. 5. Effect of NO treatment on proline content (A) and OAT activity (B) of banana fruit during cold storage. Values are the means ± SD of triplicate assays.

degradation pathway, is the main pathway for GABA biosynthesis in plants (Hyun et al., 2013). Some researchers suggested that GAD activity played a dominant role in the accumulation of GABA, thus involved in the enhancement of chilling tolerance in postharvest fruits under cold storage (Cao et al., 2012; Shang, Cao, Yang, Cai, & Zheng, 2011). However, some researchers believed that it was the activity of GABA-T rather than GAD contributes to GABA synthesis (Li et al., 2013). Higher GABA production together with lower GABA-T activity was observed in tomatoes with CO_2 treatment, suggesting that GABA accumulation might be attributed to a retarded GABA degradation (Deewatthanawong et al., 2010b). Except for the regulation of GAD and GABA-T enzymes, GABA accumulation may also be regulated at transcriptional/translational and post-translational levels (Deewatthanawong et al., 2010a). GABA was over accumulated in tomato plants through the suppression of GABA-T genes (Koike, Matsukura, Takayama, Asamizu, & Ezura, 2013). Our results showed that NO treatment promoted GABA levels with a high GAD activity compared to control fruit during cold storage; by contrast, lower GABA-T activity was observed in NO-treated banana (Fig. 4). Based on these results, we suggest that the NO treatment could mediate the GABA shunt pathway during cold storage via an induction of GAD activity and inhibition of GABA-T activity, leading to accumulation of GABA, which may be an adaptive mechanism to protect against chilling stress.

In addition, Put can be converted into GABA through the activity of DAO (Gupta et al., 2013; Shelp et al., 2012; Yang et al., 2011). Previous studies reported that in germinating fava bean under hypoxia, 30% of GABA formed was supplied by polyamine degradation pathway (Yang, Guo, & Gu, 2013). Put treatment induced different pathways including GABA shunt and other related nitrogen metabolites as stress defense mechanisms, which could contribute to enhanced chilling tolerance of zucchini fruit (Palma et al., 2015). However, the role of NO signaling pathway in PAs metabolism, as well as its relationship with GABA needs to be further explored.

Just like GABA, some amino acids, such as proline, play an important role in plant responses to stress (Rai, 2002). It is well known that proline accumulates in plants in response to various stress (Verbruggen & Hermans, 2008). The contribution of proline to alleviating chilling injury has been reported in peaches and loquat fruit during cold storage (Cao et al., 2012; Shang et al., 2011). The elevated levels of proline, together with accumulation of PAs, were determined in heat-treated fruits, which displayed an enhanced chilling tolerance (Zhang et al. (2013a, 2013b)). In higher plants, proline can be synthesized from either glutamate or ornithine, catalyzed by Δ1-pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT), respectively. As a key metabolism intermediate, ornithine played an important role in the biosynthesis of Put and proline (Hussain et al., 2011). OAT may act as determinant in the accumulation of proline under cold-shock (Ruiz et al., 2002). According to our previous study, the higher activities of P5CS, together with lower proline dehydrogenase (PDH) activity in NO-treated banana fruit, may account for the accumulation of proline in NO-treated fruit (Wang et al., 2013). In the present study, NO treatment retarded the decrease of OAT activity in banana fruit, associated with a higher level of proline (Fig. 5A and B). The results indicated that NO could promote proline accumulation through the upregulated ornithine pathway. In agreement with our research, Shang et al. (2011) demonstrated that exogenous GABA treatment enhanced accumulation of endogenous GABA and proline in peach fruit, which resulted from the increased GAD, P5CS, and OAT activities and decreased PDH activity (Shang et al., 2011). Similar results were observed in MeJA-treated loquat fruit (Cao et al., 2012). Our study indicated that interplay between proline and GABA metabolism may take an important role in the NO-induced chilling tolerance of postharvest banana fruit under cold storage.

In conclusion, exogenous NO treatment notably reduced CI in banana fruit during cold storage. The elevated chilling tolerance in NO-treated banana fruit may be attributed to enhanced accumulation of PAs and GABA in fruit, as a result of the increased activities of ADC, ODC, GAD, DAO and PAO. Besides, NO treatment increased the activity of GAD but repressed activity of GABA-T, which led to the accumulation of GABA. Accumulation of proline may also get involved in the enhanced chilling tolerance of cold-stored banana fruit. The accumulation of PAs, GABA and proline increased the chilling resistance of NO-treated banana fruit, which can be attributed to their active catabolism. However, further studies are required to clarify the complex molecular networks regulated by NO, especially its signal pathway in response to chilling stress.

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