Food Research International 44 (2011) 1905–1911

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effect of pure oxygen atmosphere on antioxidant enzyme and antioxidant activity of harvested litchi fruit during storage

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ARTICLE INFO

Article history:
Received 11 September 2010
Accepted 17 October 2010

Keywords:
High oxygen
Litchi fruit
Pericarp browning
Antioxidant

ABSTRACT

The effects of pure oxygen on pericarp browning, reactive oxygen species (ROS) metabolism, antioxidant enzyme and antioxidant activity of harvested litchi fruit were investigated. Application of pure oxygen significantly prevented pericarp browning and delayed the increase in membrane permeability of litchi fruit during storage. Litchi fruit exposed to pure oxygen showed a lower level of lipid peroxides, compared to control fruit, with the delay in the increases of both H_2O_2 content and superoxide production rate. Furthermore, it was found that the treatment with pure oxygen induced the activities of superoxide dismutase (SOD), ascorbated peroxidase (APX) and catalase (CAT), which could be beneficial in scavenging of H_2O_2 and superoxide and alleviating lipid peroxidation. In addition, antioxidant ability (reducing power and free-radical scavenging activity against DPPH radical, superoxide anions and hydroxyl radical) of methanol extracts from litchi fruit pericarp declined gradually, with decreasing contents of anthocyanins and phenolic compounds, as storage time of the fruit progressed. There was a linear relationship between the contents of either anthocyanins or phenolic compounds and antioxidant ability or free radical scavenging activity. Treatment with pure oxygen markedly increased antioxidant ability, which was related to higher levels of anthocyanins and phenolic compounds, compared with those of control fruit. It is suggested that enhanced antioxidant activity and antioxidant enzyme induced by pure oxygen may contribute to alleviating lipid peroxidation and maintenance of membrane integrity, which reduced decompartmentation of enzymes and substrates, resulting in enzymatic browning.

1. Introduction

Litchi (*Litchi chinensis* Sonn.) is a non-climacteric subtropical fruit with high commercial value for its white translucent aril and attractive red peel color. However, harvested litchi fruit deteriorates rapidly due to pericarp browning, which leads to reduced market value (Jiang, Duan, Joyce, Zhang, & Li, 2004). Pericarp browning has mainly been attributed to degradation of red pigments and oxidation of phenolics by PPO (Jiang, Zauberman, & Fuchs, 1997; Lin et al., 1988; Zauberman et al., 1991; Zhang, Pang, Ji, & Jiang, 2001). Some reports have shown that lipid peroxidation, which reduced membrane fluidity and increased membrane permeability (Jiang et al., 2004; Lin et al., 1988; Liu, Jiang, Chen, Zhang, & Yi, 1991), might result in loss of compartmentalization between enzymes and their substrates and, thereby, may be responsible for enzymatic browning. Therefore, inhibiting lipid peroxidation could be an important method to delay pericarp browning and extend the storage life of litchi fruit.

Controlled atmosphere (CA) with low oxygen concentration is effective in maintaining quality and extending shelf life of horticultural products, including inhibition of tissue browning (Kader, 1993; Mahajan & Goswami, 2004). However, the accumulation of ethanol and acetaldehyde in litchi fruit under modified atmosphere packaging can influence the storage life, due to anaerobic respiration. Recent researches have shown that high O_2 concentration effectively inhibited enzymatic browning. It was found that high oxygen treatment inhibited the browning of some horticultural products, such as iceberg lettuce (Heimdal, Kuhn, Poll, & Larsen, 1995), apples (Lu & Toivonen, 2000), litchi (Duan, Jiang, Su, & Zhang, 2004), grape (Deng, Wu, & Li, 2005), and potato slice (Limbo & Piergiovanni, 2006). Nevertheless, sensitivity to O_2 toxicity varies among species. Increased O_2 concentrations around and within the commodity may also result in higher levels of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical, that can damage plant tissues (Fridovich, 1986). Unfortunately, little information is available on the effect of elevated O_2 on browning of fruit in relation to antioxidant activity, ROS metabolism and lipid peroxidation.

The objective of this study was to investigate the effects of pure oxygen on ROS metabolism, lipid peroxidation, antioxidative enzymes and antioxidant activity of harvested litchi fruit associated with

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doi:10.1016/j.foodres.2010.10.027

membrane integrity and then to understand the role of pure oxygen in litchi peel browning.

2. Materials and methods

2.1. Plant materials and treatment

Litchi fruit (*Litchi chinensis* Sonn.) cv. Huaizhi at 80% maturation was harvested from a commercial orchard in Guangzhou, China. Fruit were selected for uniformity of shape and color and freedom from blemished or disease fruits. Fruit was dipped for 3 min in 0.1% Sportak fungicide solution and air-dried for 2 h at 28 °C. Fifty fruit were put into a 6-litre plastic jar, and nine jars were used for each treatment. The jars were connected to a continuous flow (30 ml/min) of humidified air (control) and 100% O_2. Oxygen concentration was checked regularly with O_2/CO_2 analyzer (Model CYES-II, Shanghai Scientific Instruments, China) and maintained at ±0.5% during storage. Samples were taken at 2-day intervals during storage, frozen in liquid nitrogen and stored at −80 °C for analysis of lipid peroxidation, ROS level, contents of anthocyanins and total phenol, enzyme activities and antioxidant activity. Browning index and relative leakage rate were evaluated immediately after removing the samples from the plastic containers.

2.2. Assesment of pericarp browning

Litchi pericarp browning was assessed by evaluating the browned area on each pericarp of 30 fruit on the following scale: 1 = no browning (excellent quality); 2 = slight browning; 3 = <1/4 browning; 4 = 1/4–1/2 browning; 5 = >1/2 browning (poor quality). The browning index was calculated as $\sum$ (browning scale × proportion of corresponding fruit within each class).

2.3. Measurement of membrane permeability

Membrane permeability was expressed by relative leakage rate. Pericarp discs were taken from the equatorial region with a cork borer (10 mm in diameter). Thirty discs (about 2 g) were rinsed twice and then incubated in 25 ml of 0.3 M mannitol at 25 °C for 30 min. Electrolyte leakage was determined with a conductivity meter (Model DDS-11A, Shanghai Scientific Instruments). A matching batch of discs was boiled for 15 min in 25 ml of 0.3 M mannitol, then cooled to 25 °C to assess total electrolyte levels. Relative electrolyte leakage was expressed as a proportion (%) of total electrolytes.

2.4. Determination of lipid peroxidation

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content in pericarp according to the method of Guidi, Tonni, and Soldatini (2000) with minor modifications. Pericarp tissue (5 g) was homogenized in 25 ml of 50 g/l trichloroacetic acid (TCA) and centrifuged for 10 min at 4000×*g*. The supernatant was collected to determine MDA concentration. A 1-ml aliquot of supernatant was mixed with 3 ml of 0.5% TBA in 10% TCA and incubated for 20 min in boiling water, then cooled quickly and finally centrifuged for 10 min at 4000×*g*. Absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. Since anthocyanins also have absorption at 532 nm, the tube containing TCA instead of TBA was used for adjustment (control). MDA concentration was calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Determination of superoxide generation rate

Superoxide generation rate was measured according to the method of Yan, Dai, Liu, Huang, and Wang (1996), with some modifications. Litchi pericarp (5 g) were homogenized with 20 ml of 50 mM Na-phosphate buffer (pH 7.8, containing 0.5 g polyvinylpolypyrrolidone and 0.3% Triton X-100). The homogenate was centrifuged at 5000×*g* for 10 min. The supernatant was used for measuring the superoxide production rate. A 1-ml aliquot of the supernatant was mixed with 0.9 ml of 50 mM Na-phosphate buffer, pH 7.8 and 0.1 ml of 10 mM hydroxylammonium chloride. After incubation for 20 min at 25 °C, 1 ml of the above reaction mixture was added to 1 ml of 17 mM 4-aminobenzene sulphonic acid and 1 ml of 7 mM α-naphthylamine and mixed, then separated into two layers using ether to remove the interference caused by pigments. The absorbance of the lower pink water-phase was measured at 530 nm.

2.6. Measurement of H_2O_2 content

The levels of H_2O_2 in pericarp were measured by monitoring the absorbance of the titanium-peroxide complex at 415 nm, following the method of Patterson, Macrae, and Ferguson (1984). Absorbance values were calibrated to a standard curve generated using known concentrations of H_2O_2.

2.7. Extraction and assays of SOD, APX and CAT activities

Litchi pericarp tissues (5 g) were homogenized with 20 ml of 0.05 M Na-phosphate buffer (pH 7.0) containing 0.5 g insoluble polyvinylpolypyrrolidone (PVPP), 0.1 mM EDTA and 0.2 mM ascorbic acid (AsA) at 4 °C. The homogenate was filtered through four layers of gauze, and then centrifuged for 20 min at 12,000×*g* and 4 °C. The supernatant was used as the crude extract for assays of SOD, CAT and APX activities.

SOD activity was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Rao, Paliyath, and Ormrod (1996). The photo-induced reactions were performed in an aluminium foil–lined box with two 30-W fluorescent lamps. The distance between the reaction solution and the lamp was adjusted until the intensity of illumination reached about 5,856 mW m^{-2}. Enzyme extract (50 µl) was mixed with 3 ml of reaction buffer solution consisting of 1.3 µM riboflavin, 13 mM methionine, 63 µM nitro blue tetrazolium and 100 µM EDTA (pH 7.8). The reaction solution was illuminated for 15 min at 25 °C. A non-irradiated reaction mixture that did not develop color was used as a control. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT activity was determined as the decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Beers & Sizer, 1952). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2. CAT activity, expressed as µmol H_2O_2 degraded per mg protein per minute, was calculated using an extinction coefficient of 39.4 mM^{-1} cm^{-1}.

APX activity was determined in a 3 ml reaction mixture containing 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0). The decrease of the absorbance at 290 nm was recorded for 2 min (Nakano & Asada, 1989). Enzyme activity, expressed as µmol of ascorbate oxidised per mg protein per minute, was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Protein contents were determined according to the method of Bradford (1976), with bovine serum albumin as the standard.

2.8. Preparation of litchi pericarp extract and analysis of antioxidant activity

Litchi pericarp (10 g) was ground with liquid nitrogen in a pestle and a bowl, and extracted in 60 ml of methanol containing sodium metabisulphite (0.5%) for 30 min. The homogenate was filtered through four layers of cheesecloth and the residue was added, with

20 ml of the same extraction solvent for two successive re-extractions. The collected filtrate was centrifuged at 20,000×*g* and 4 °C for 20 min. The supernatants were transferred to vials, stored at −80 °C, and then used for analysis of antioxidant activity, including reducing power, scavenging activities of DPPH radical, superoxide radical and hydroxyl radical.

The reducing power was determined according to the method of Oyaizu (1986) with some modification. A 0.25-ml aliquot of methanol extract was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated for 20 min at 50 °C. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged for 10 min at 650×*g*. A 5-ml aliquot of the upper layer was mixed with 5 ml of distilled water and 1 ml 0.1% ferric chloride, and the absorbance at 700 nm was measured. A higher absorbance indicated a higher reducing power.

DPPH radical scavenging activity was assessed according to the method of Larrauri, Sanchez-Moreno, and Saura-Calixto (1998) with some modifications. Briefly, 10 μl of methanol extract was mixed with 3 ml of 0.1 mM DPPH-methanol solution. After the solution was incubated for 30 min at 25 °C in dark, the decrease of the absorbance at 517 nm was measured. Control contained methanol instead of the methanol extract while blanks contained methanol instead of DPPH solution. The scavenging of DPPH radicals by the samples was calculated according to the following equation: DPPH scavenging activity (%) = [1−(absorbance of sample−absorbance of blank) / absorbance of control] × 100.

Superoxide radical scavenging activity was evaluated according to the method of determining SOD activity above mentioned.

Hydroxyl radicals scavenging activity was evaluated by determining the inhibitory effect of hydroxyl radical on deoxyribose degradation. The inhibitory effect of litchi pericarp extract on deoxyribose degradation by hydroxyl radicals was determined by measuring the reaction activity between either antioxidants and hydroxyl radicals (referred as non–site-specific scavenging assay) or antioxidants and iron ions (referred as site-specific scavenging assay), described by Lee, Kim, Kim, and Jang (2002). For the non–site-specific scavenging assay, a 0.1-ml aliquot of methanolic extracts from litchi pericarp was mixed with 1 ml of reaction buffer (100 μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM L-ascorbic acid, pH 7.4) and incubated at 37 °C for 1 h. One milliliter of 0.5% 2-thiobarbituric acid in 0.025 M NaOH and 1 ml of 2.8% trichloroacetic acid were added to the mixture and heated at 80 °C for 30 min. Finally, the mixture was cooled on ice and the absorbance was measured at 532 nm. Site-specific scavenging activity, which represented the ability of samples to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. Percent inhibition of deoxyribose degradation was calculated as (1−absorbance of sample / absorbance of control) × 100.

2.9. Determination of total phenol and anthocyanin contents

Total phenol contents in litchi pericarp were determined by the method of Folin-Ciocalteu reaction (Singleton & Rossi, 1965), using gallic acid as standard. The phenolic contents were expressed as gallic acid equivalents (GAE) on fresh weight (FW).

Anthocyanin contents in litchi pericarp were determined using the pH differential method (Zhang, Pang, Yang, Ji, & Jiang, 2004). The contents were expressed as milligrams of cyanidin-3-glucoside equivalents on fresh weight.

2.10. Data handling

The experiments were arranged in completely randomized design, and each was comprised of three replicates. Data were tested by analysis of variance using SPSS version 7.5. Least significant differences (LSD) were calculated to compare significant effects at the 5% level.

3. Results

3.1. Peel browning and relative leakage rate

As shown in Fig. 1A, browning index and relative leakage rate of litchi fruit pericarp increased rapidly as storage time progressed. Exposure of pure O_2 atmosphere delayed the increases of browning index and relative leakage rate in litchi pericarp. After 6 days of storage, the browning index for control (air stored) fruit was 4.6, while fruit kept in pure O_2 had a browning index of 3.6. At the same time, the relative leakage rates of control and pure oxygen–exposed fruit were 61% and 38.9%, respectively.

3.2. Lipid peroxidation

Malondialdehyde (MDA) is one of the products of membrane lipid peroxidation. As shown in Fig. 2A, MDA contents increased sharply in control fruits until 4 days, and then declined slightly. However, MDA contents in litchi fruit exposed to pure oxygen increased in a very slow rate until 4 days, and then significantly increased. During all storage, MDA contents of litchi fruit exposed to pure oxygen was significantly lower than those of the control fruit.

3.3. H_2O_2 content and superoxide production rate

Effects of pure oxygen exposure on superoxide production rate and H_2O_2 content in litchi pericarp were shown in Fig. 2A. Application of pure oxygen resulted in a significantly lower superoxide production rate and (Fig. 2B) and H_2O_2 content (Fig. 2C) until 4 days, compared with the control. At 4 days, superoxide production rate were increased by 88.0% and 28.8%, and H_2O_2 content increased by 25.5% and 10.0% for control

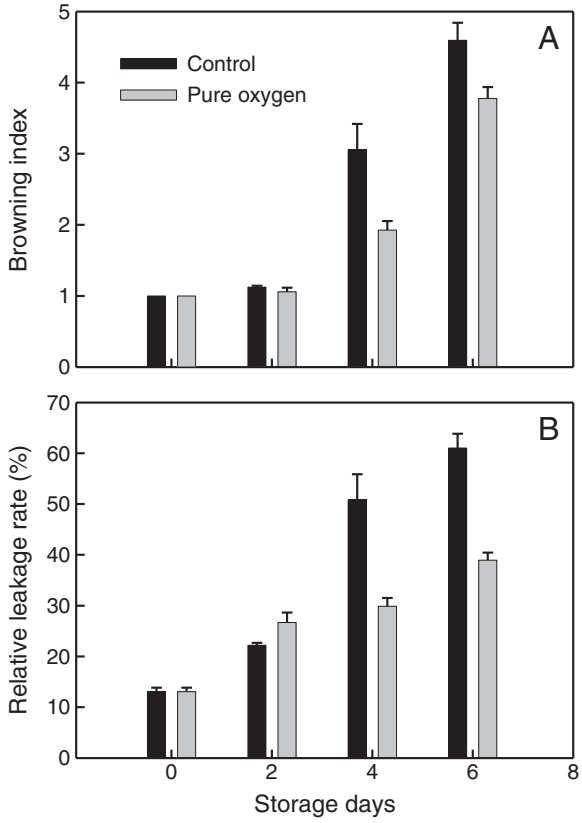

Fig. 1. Effect of pure oxygen on pericarp browning (A) and relative leakage rate (B) of litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

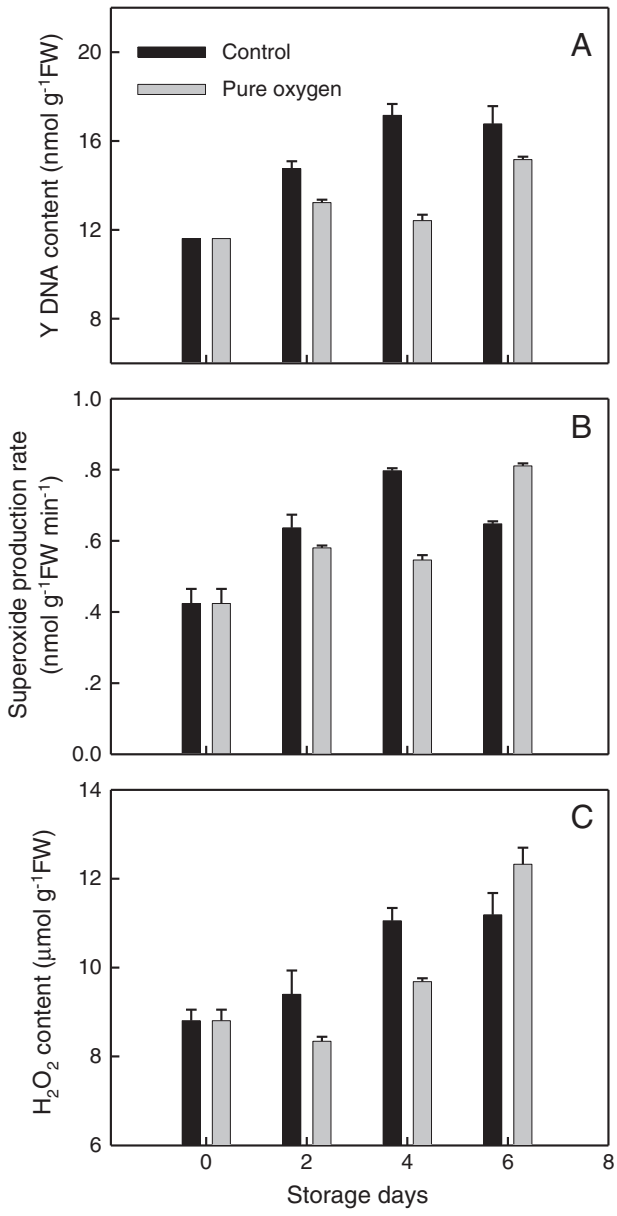

Fig. 2. Effect of pure oxygen on MDA content (A), superoxide production rate (B) and H_2O_2 level (C) of litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

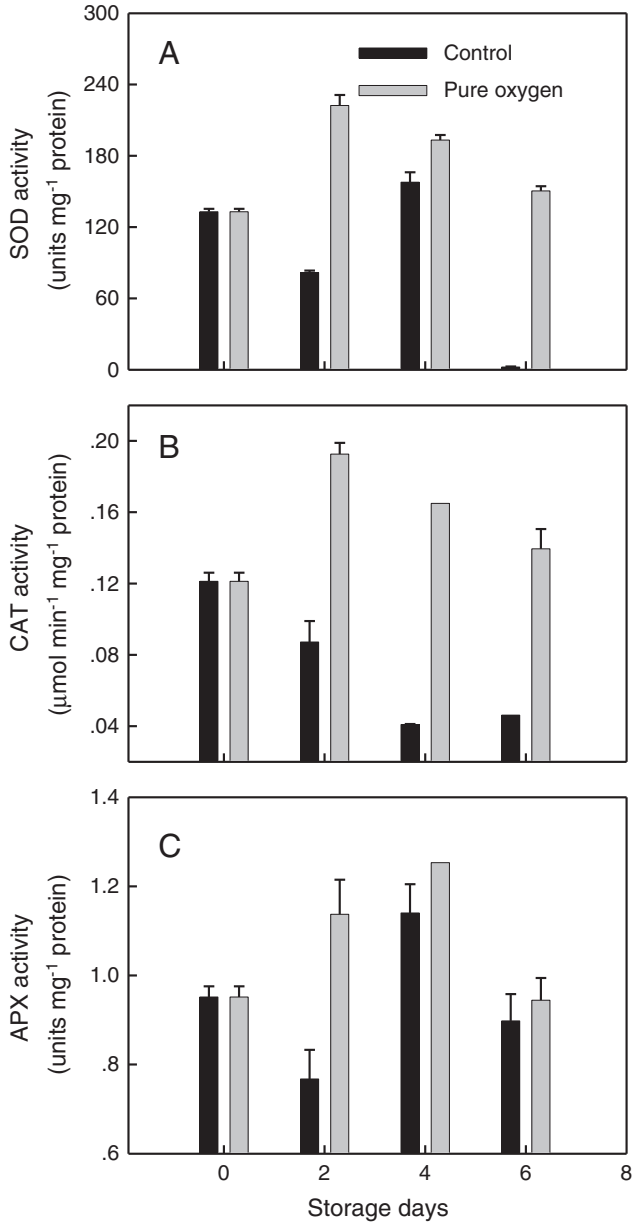

Fig. 3. Effect of pure oxygen on activities of SOD (A), CAT (B) and APX (C) of litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

and treatment, respectively. However, superoxide production rate and H_2O_2 content was higher in pure oxygen–exposed fruit than in control fruit after 6 days of storage.

3.4. Antioxidant enzyme and contents of phenolic compounds and anthocyanins

Fig. 3 shows the effects of pure oxygen exposure on several antioxidant enzymes such as SOD (Fig 3A), the enzyme for catalyzing the dismutation of superoxide radical to O_2 and H_2O_2, CAT (Fig. 3B), the enzyme mainly responsible for eliminating H_2O_2, and APX (Fig. 3C), the key enzyme of the Halliwe-Asada pathway for the removal of H_2O_2. In general, the time course of the activities of SOD and CAT in control litchi was characterized by a decreasing trend during storage, except for an increase of SOD at 4 days. APX activity of control fruit decreased within 2 days, then increased rapidly, and finally decreased after 4 days of storage. Exposure of pure oxygen caused increases in the activities of the three antioxidant enzymes. At 2 days, litchi fruit exposed to pure oxygen enhanced the activity of SOD by 20%, and CAT by 40%, compared with that of 0 days. Thereafter, the activities decreased steadily, but were still maintained at high levels. Different from the activities of SOD and CAT, the highest activity of APX occurred at 4 days of storage. As pericarp browning index increased, the contents of total phenol compounds and anthocyanins decreased rapidly (Fig. 4). Pure oxygen exposure delayed the reduction of total phenols and anthocyanins in litchi fruit pericarp (Fig. 4). There was no significant difference for the contents of total phenol and anthocyanins between control and pure oxygen–exposed fruit at 2 days. After 4 and 6 days of storage, the contents of total phenol compounds and anthocyanins in pure oxygen–exposed fruits were much higher than in control fruits.

3.5. Antioxidant activity

Figs. 5 and 6 show the changes of reducing power and free radical scavenging activity of methanol extracts from litchi fruit exposed to

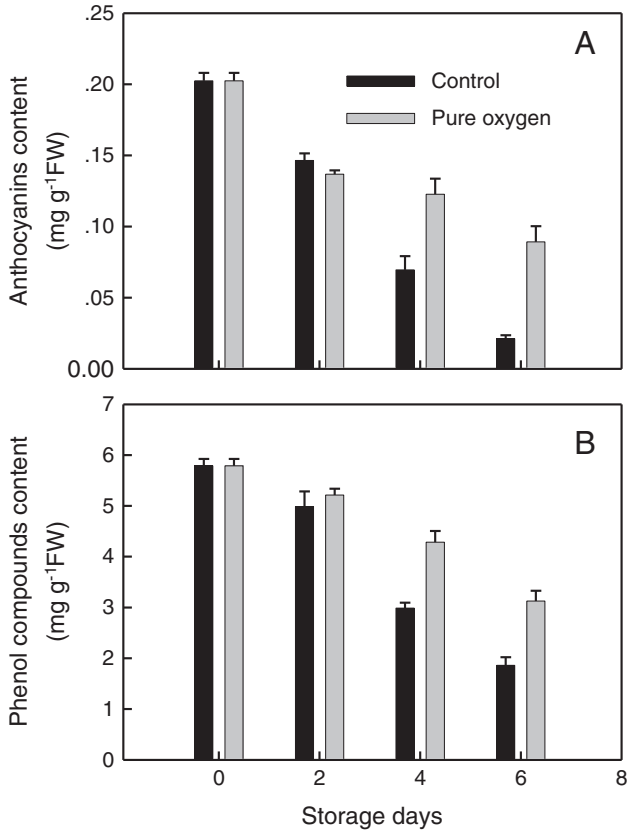

Fig. 4. Effect of pure oxygen on content of anthocyanins (A) and phenol compounds (B) of litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

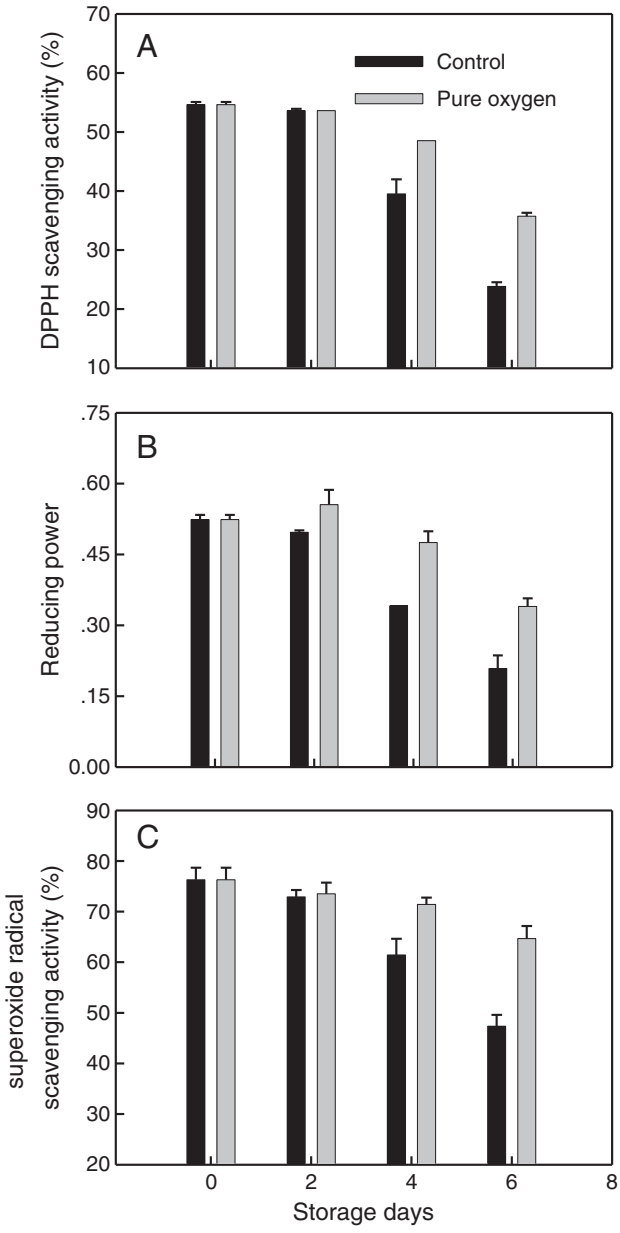

Fig. 5. Effect of pure oxygen on DPPH scavenging activity (A), reducing power (B) and superoxide radical scavenging activity (C) of harvested litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

air or pure oxygen. Reducing power and scavenging activities of DPPH radicals, superoxide anions and hydroxyl radical of methanol extracts from litchi fruit pericarp declined gradually as storage time of the fruit progressed. Application of pure oxygen delayed the decrease of reducing power and free radical scavenging activity of harvested litchi fruit. After 4 and 6 days of storage, reducing power and scavenging activities of DPPH radicals, superoxide radical anions and hydroxyl radical of fruits treated with pure oxygen were significantly higher than those of control fruits. In addition, non–site-specific scavenging of hydroxyl radical was much higher than site-specific scavenging activity in litchi pericarp, which indicated that the enhanced hydroxyl radical scavenging activity by pure oxygen was mainly by means of chelating Fe^{3+}.

4. Discussion

It has been well established that litchi pericarp browning is generally attributed to the oxidation of phenolic compounds (Duan et al., 2004; Jiang et al., 2004). Initiation of enzymatic browning is related to a loss of membrane integrity, which resulted in the decompartmention of enzymes and substrates (Jiang et al., 2004; Liu et al., 1991). Membrane permeability, expressed as relative leakage rate, is an important index of membrane integrity. In the present study, pure oxygen exposure effectively reduced pericarp browning in litchi fruit (Fig. 1A) and alleviated relative electrolyte leakage (Fig. 1B), suggesting that pure oxygen concentration helps to maintain the compartmention of enzymes and substrate.

The accumulation of ROS due to the altered balance between ROS production and scavenging capacities will result in lipid peroxidation, which is the main cause of the membrane deterioration, and reduce the storage quality and marketability of horticultural products (Marangoni, Paima, & Stanley, 1996). Malondialdehyde (MDA), a lipid peroxidized product, can reflect the extent of membrane lipid peroxidation induced by oxidative stress. In this experiment, pure oxygen exposure alleviated lipid peroxidation in litchi fruit (Fig. 2A), coinciding with lower superoxide radical production rate and H_2O_2. It is suggested that pure oxygen exposure could reduce the accumulation of ROS, thus, preventing the membrane from peroxidation.

To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes scavenging ROS, such SOD, CAT and peroxidases. SOD catalyzes the dismutation of superoxide radical to H_2O_2, while CAT and APX are the enzymes mainly responsible for eliminating H_2O_2 (Bloknina, Virolainen, & Fagerstedt, 2003). Some reports have shown that high oxygen concentration induced the activities of SOD and CAT and maintained membrane integrity in peach (Wang, Tian, & Xu, 2005), loquat (Ding et al., 2006) and strawberry (Chen et al., 2005). Yang, Zheng, and Cao

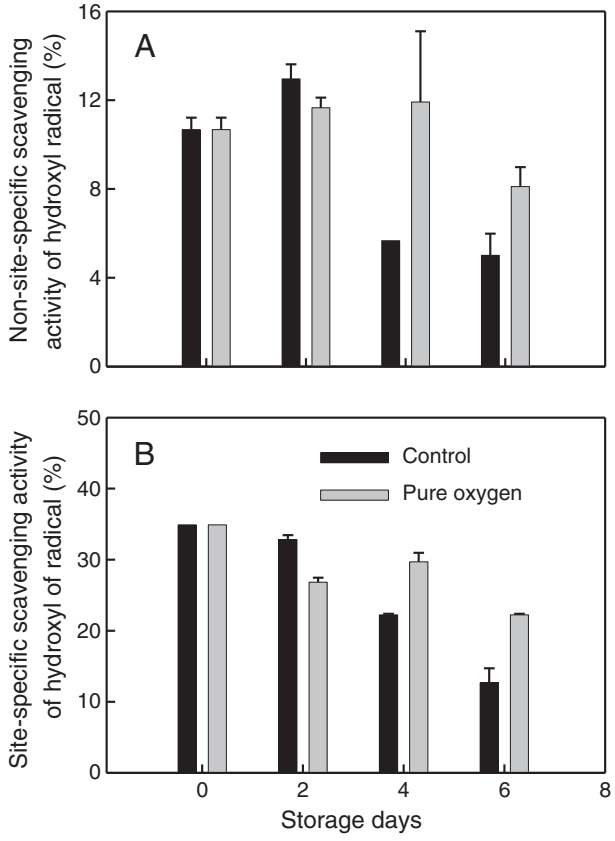

Fig. 6. Effect of pure oxygen on non–site-specific (A) and site-specific (B) scavenging activity of hydroxyl radical of harvested litchi fruit during storage at 28 °C and 90–95% relative humidity. Each value was presented as a mean ± standard error ($n = 3$).

(2009) also found that application of high oxygen maintained higher activities of CAT, APX and POD and alleviated lipid peroxidation and, thus, delayed the decay of Chinese bayberry. In the present study, the activities of SOD, CAT and APX were induced in litchi fruit by exposure of pure oxygen, accompanied by a lower level of superoxide production rate and H_2O_2 content and decreased lipid peroxidation compared to the control fruits. These data suggest that exposure of pure oxygen could be beneficial in maintaining the balance of ROS metabolism by the induction of antioxidant enzymes activities in litchi fruit, thus, alleviating the lipid peroxidation and stabilizing membrane integrity.

In addition to antioxidant enzyme system, plants possess a network of low molecular mass antioxidants, including ascorbate, glutathione, phenolic compounds, anthocyanins and tocopherols (Faller & Fialho, 2009; Ferreira, Barros, & Abreu, 2009). Reducing power and free radical scavenging activity of plant extract, including DPPH radical, superoxide radical, and hydroxyl radical, are measures of such non-enzymatic antioxidant activity (Kang & Saltveit, 2002; Yang et al., 2009). In this study, the non-enzymatic antioxidant activity (reducing power and free radical scavenging activity) decreased gradually as storage or senescence progressed. Exposure of pure oxygen inhibited the decrease in reducing power and all three type of free radical scavenging activity of methanol extract in litchi fruit pericarp tissue during storage. Furthermore, it was found that there was a linear relationship between the contents of either anthocyanins or phenolic compounds and antioxidant ability or free radical scavenging activity in control and pure oxygen–exposed litchi fruit (data not shown). Our previous studies also indicated that exogenous treatments with anthocyanins or phenolic compounds reduced browning and delayed increase in relative leakage rate (Duan, Jiang, Su, Zhang, & Shi, 2007). Addition of gallic acid, catechin and quercetin efficiently delayed lipid oxidation of olive oil-in-water emulsions (Di Mattia, Sacchetti, Mastrocola, & Pittia, 2009). These results suggest that the role of the anthocyanins and phenolic compounds in enhancing non-enzymatic antioxidant activity and alleviating lipid peroxidation in pure oxygen–exposed litchi fruit.

In conclusion, high activities of SOD, CAT and APX and high non-enzymatic antioxidant activity in pure oxygen–exposed litchi fruit corresponded to low levels of ROS accumulation, lipid peroxidation and relative electrolyte leakage rate. These effects were evidently responsible for reduced pericarp browning in litchi fruit during storage.

Acknowledgement

This work was supported by National Natural Science Foundation of China (grant no. 30972071 and U0631004), Guangdong Provincial Science Foundation of China (grant no. 06200670), and Science and Technology Planning Project of Guangdong Province, China (grant no. 2010B090300060).

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