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Precooling and ozone treatments affects postharvest quality of black mulberry (*Morus nigra*) fruits

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ABSTRACT

Mulberry (*Morus* spp.) fruits are delicious and nutritious, but they are highly perishable and have a very short shelf-life for sale in the market. This study investigated the effect and mechanisms of 2 ppm ozone and precooling treatments on the postharvest quality of mulberry fruit during refrigerated storage. The results revealed that mulberry fruit subjected to ozone and precooling treatment had higher levels of titratable acidity and total soluble solids content, better retention in firmness and color, and lower decay rate, respiratory intensity, and polyphenol oxidase activity compared to the control. From the analysis of cell ultrastructure and cell wall components of fruit, ozone and precooling treatments also induced shrinkage of the stomata in the epidermis, inhibited bacteria invasion, reduced water transpiration, and delayed the decomposition of the cell walls and the degradation of epidermal tissues.

1. Introduction

Horticultural plants are very important for human diet as sources of vitamins, minerals and dietary fiber and they become a significant part of human life moreover due to their medicinal and environmental uses as well as aesthetics and economic values. The stem, leaf, flowers, roots and the fruits of vegetables and fruit crops have the highest potential of export (İpek et al., 2016; Kaczmarska et al., 2015; Mlcek et al., 2015; Tsou, Li, & Vijayan, 2016).

The mulberry fruit belongs to the genus *Morus* of the family *Moraceae* (Chen, You, Abbasi, Fu, & Liu, 2015). The fruits are usually eaten either fresh (Ercisli & Orhan, 2007) or processed into wine (Wang et al., 2015), juice (Zou et al., 2016) and jam (Kim, Kim, Seok, & Seo, 2015). Previous studies revealed that mulberry fruit contain high amount of phenolics (Yu et al., 2014), flavonoids (Ercisli & Orhan, 2007), polysaccharides (Ying, Han, & Li, 2011) and ascorbic acid (Ercisli & Orhan, 2008), and possess multiple biological activities, such as hepatoprotective (Tang, Huang, Lee, Tang, & Wang, 2013), antioxidative (Du, Zheng, & Xu, 2008), antibacterial (Yang & Lee, 2012), and hypolipidemic (Yang, Yang, & Zheng, 2010), to immunomodulating (Lee et al., 2013), anti-inflammatory (Liu & Lin, 2013) and anti-apoptotic (Kim et al., 2010) activities. Mulberry fruit are also used as traditional folk medicines for treating fever, anemia, sore throat, and hypertension in China and Korea (Lee et al., 2013; Ma et al., 2015). According to traditional Chinese medicine, mulberry fruit can protect against kidney and liver damage, improve eyesight, strengthen the joints and have radio protective and anti-aging effects (Wattanathorn et al., 2012). Due to their characteristics in quality, distinctive taste, and nutritional value, the production and consumption of mulberry fruit has increased rapidly in recent years.

Mulberry fruit are highly perishable due to high water content (more than 70%) and soft texture (Chen, Zhu, & Han, 2011). This, along with its short harvesting period (usually early May for 3 weeks) (Doymaz, 2004), makes it difficult to sell them as fresh fruits in the market. Therefore, it is important to develop economically feasible, easily adaptable, and highly efficiency strategies to improve the shelf-life of fresh mulberry fruit.

Ozone is known to be a good alternative sanitizer for fresh fruit and vegetables because it can decompose ethylene and destroy microorganisms by progressive oxidation of the phospholipid and protein molecules in the cell membrane (Ji, Pang, & Li, 2014), which are vital for functioning of the membrane. This increase the permeability of cell membranes, allowing for the leakage of cellular contents and eventually leading to the death of microorganisms (Segat et al., 2014). Ozone treatment is highly effective against a wide range of bacteria, fungi, yeasts, and viruses. This, along with its high reactivity, lack of residue and high permeability, is why there is a great deal of interest in using ozone as a sanitizer on the surfaces of foods and to increase the shelf-life of fruits and vegetables

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(Guzel-Seydim, Greene, & Seydim, 2004). Recent studies have indicated that ozone is an effective postharvest treatment for extending shelf-life and decreasing fungal deterioration in fresh fruit, such as grapes (Sarig et al., 1996), longans (Whangchai, Saengnil, Singkamanee, & Uthaibutra, 2010) and strawberries (Pérez, Sanz, Ríos, Olias, & Olías, 1999). Sarig et al. (1996) revealed that ozone may induce resistance of plants to pathogens at appropriate concentrations. Possible biochemical reactions of plants to ozone are the production of pathogenesis-related proteins, induction of ethylene, or elicitation of phytoalexins, such as the stilbenes. Since the phytoalexins found in grapes are the stilbenes resveratrol, pterostilbene and several viniferins, it was considered possible that ozone could be used to induce resistance to postharvest decay in fruit.

Postharvest mulberry fruits usually contain a large amount of field heat due to the fact that they are harvested during the hot and rainy season (Chen et al., 2015). It resulted in high respiration rate, and rapid growth and reproduction of microorganisms on the fruit surface. Precooling (PC) is a cooling process in which the field heat is removed from fruit rapidly after harvest. This treatment reduces the rate of biochemical and microbiological changes, thus extending the storage period. Therefore, PC plays an extremely important role in the maintenance of the postharvest quality of fresh fruit throughout the whole cold chain (Pathare, Opara, Vigneault, Delele, & Al-Said, 2012).

Although both PC and ozone (OZ) treatments have been extensively studied in a variety of fruit and vegetables, little has been done in mulberry fruit. By considering the different physiological and biochemical characteristics and growth environment and conditions, it is necessary to identify the appropriate PC and OZ preservation conditions that are specific for mulberry fruit. Therefore, the main purpose of this study was to investigate 1) the senescence inhibition efficacy of PC and OZ treatments on the physicochemical properties of mulberry fruit, and 2) the preservation mechanism and pathway by analyzing the cell ultrastructure and cell wall components of postharvest mulberry fruit during refrigerated storage.

2. Materials and methods

2.1. Materials

Fresh mulberry fruit were hand-harvested at commercially mature stage from a farm located in Wuyi, Zhejiang Providence, China on May 19, 2015. Undamaged 'Dashi' mulberry fruit at eight to nine maturity, which were of red to black color and of uniform size, full juice, and firm were selected. Fresh mulberry fruit was transported to the laboratory by a refrigerated car within 2 h.

2.2. Per-cooling and ozone treatments

PC and non-cooling mulberry samples were randomly divided into portions of 100 g and packed in polyethylene terephthalate (PET) plastic transparency box (150 × 200 mm). PC samples were treated in a forced-air precooling unit (cooler info) to reach core temperature below 5 °C within 30 min after harvest.

Followed the PC treatment, the mulberry fruit was treated with OZ, which was considered as PC + OZ groups. OZ gas was generated from a laboratory corona discharge OZ generator using oxygen, with a working voltage of 220 V, 50 Hz (Model L-1000, Tianjin, China). OZ treatment was carried out in a chamber (2 m^3) with the gaseous OZ concentration at 2 ppm (based on our previous optimization studies (data not shown)). The temperature and relatively humidity of the chamber for OZ treatment was 5 ± 1 °C and 80 ± 10%, respectively.

All sample groups were placed in a plastic bag (thickness at 0.25 mm) at 0 ± 1 °C and 80 ± 10% RH. Different quality analyses were performed over 15 consecutive days, at 0, 3, 6, 9, 12 and 15 days during storage. At each sampling date, 100 samples of fruit were taken from each chamber.

2.3. Decay rate

During storage, the number of fruit showing decay which was visually evaluated. Fruit with visible mold growth was considered rotten, and the percentage of decay fruit was used for expressing decay rate. Five replicates were performed for this analysis, in which twenty fruit were used for each replicate.

2.4. Physicochemical properties

2.4.1. Fruit firmness

Fruit firmness was assessed using an Instron Universal Testing Machine (Model 5540, USA). Ten fruit in each replicate were penetrated using the 50 mm diameter probe, at a speed of 50 mm/min on three points in the equatorial region of the whole fruit. The compression force measured at the maximum peak of the recorded force on the chart was expressed in Newton (N). Five replicates were performed for this analysis.

2.4.2. Respiratory intensity

Fruit respiration intensity was measured by placing 100 g of fruit inside a 200 mL can after which the can was sealed. After 2 h, headspace CO_2 content was measured using a gas chromatograph with a flame ionization detector. The analytical conditions were: column temperature at 60 °C, carrier gas flow at 40 mL/min, air flow rate at 450 mL/min, detection chamber temperature at 120 °C, and the injection volume at 1 mL. CO_2 content was expressed at mL CO_2/(kg h). Three replicates were performed for this analysis.

2.4.3. Color measurement

Fruit surface color was determined on three different locations of each individual fruit using the Minolta CR-300 Chroma Meter (Minolta Corp., Japan). Lightness (L^*) was reported. Five replicates were performed for this analysis, in which ten fruit were used for each replicate.

2.4.4. Titratable acidity (TA)

TA was titrated by NaOH with Automatic pH titrator (Mettler Toledo L-100 type, USA). Citric acid was used as reference and result was expressed as%. Three replicates were performed for this analysis, in which five fruit were used for each replicate.

2.4.5. Total soluble solids (TSS)

TSS were expressed as % using a hand-held sugar measuring instrument (Atago PAL-1, Japan). Three replicates were performed for this analysis, in which five fruit were used for each replicate.

2.5. Polyphenol oxidase (PPO) activity analysis

PPO was extracted from mulberry fruit according to the method of Li, Rojas, and Hinestroza (2012). Briefly, 2 g of mulberry fruit was incubated in 10 mL of extraction buffer (0.1 M phosphate–citric acid buffer, pH 6.0) in a 15 mL centrifuge tube and shaken at 4 °C for 12 h, followed by centrifuging at 10,000 rpm for 15 min and the supernatant was used as the crude extracts of PPO. PPO activity was determined by measuring the absorbance at 420 nm (A 420) using a spectrophotometer (Unocal UV-2800, USA). The enzyme activity was expressed as unit per ml (U ml^{-1}) enzyme. One unit of enzyme activity was defined as an increase

in absorbance of 0.001/min at 420 nm. Three replicates were performed for this analysis, in which five fruit were used for each replicate.

2.6. Observation of ultrastructure with Scanning Electron Microscope (SEM)

To investigate the ultrastructure of epicuticle waxes, the mulberry skin samples (approx. 5.0 mm × 5.0 mm sizes) were cut from the fruit using a blade. Each sample was delicately placed on aluminum stubs covered with bi-adhesive tape, freeze-dried for 3–5 h, and then coated with gold (25 nm thick) in a Balzers Union SCD 040 Sputter Coater (Balzers, Wiesbaden, France). Representative areas were examined with a Scanning Electron Microscope (Philips XL 20) at 20 kV.

2.7. Cell wall substance content

The method from Jung, Samac, and Sarath (2012) was used with slightly modification. Ten fresh mulberry fruit were milled with deionized water at 0 °C, centrifuged at 1000*g* for 10 min, and the supernatant was discarded. The precipitate was then washed successively using ice cold deionized water, acetone and methanol: chloroform (1:1, v/v). Starch in cell wall was removed by α-amylase treatment at 37 °C for 30 min. Crude extract of the cell wall material was obtained after freeze drying, and weighed. Three replicates were performed for this analysis.

2.8. Experimental design and data analysis

Treatments were arranged in a completely randomized design. Experimental data were analyzed using analysis of variance (ANOVA) via SAS 9.1 software. Mean differences at $P < 0.05$ were considered to be significant using Duncan's Multiple Range Test (DMRT).

3. Results and discussion

3.1. Decay rate

PC + OZ treatments employed in this study resulted in a significant effect on the decay rate of mulberry fruit as shown in Fig. 1. Based on prior preliminary screening tests, we identified the optimal duration (30 min) and OZ concentration (2 ppm) exposure to control postharvest diseases of mulberry fruit. No decay symptom observed in treated groups during the first 3d of storage (Fig. 1). Nevertheless, fruits in the control group decayed during the first 3d, and the degree of decay was significantly higher than in fruits in the PC group. The results in Fig. 1 showed that decay symptoms were observed after 9d in fruits in PC + OZ group, and the degree of decay was significantly lower. Whangchai, Saengnil, and Uthaibutra (2006) found that OZ destroyed microorganisms by oxidation of cellular components, such as sulfhydryl groups of amino acids in enzymes leading to cell membrane damage. Our decay rate results also suggested that longer exposure to OZ damaged the cellular membrane, and that PC + OZ treatments could reduce the rate of decay in mulberry fruit.

3.2. Physicochemical properties

3.2.1. Firmness

The firmness of mulberry fruit in control group continuously declining during storage, however, the firmness of mulberry fruit in treated group increased slightly during the first 3d and then decreased after 6 days (Fig. 2). Throughout the storage period, the firmness of fruits in treated group was significantly higher than the control fruit ($P < 0.05$), while there was no significant difference between fruits in the PC + OZ group and PC group ($P > 0.05$). These results indicated that PC treatment significant impacts fruit firmness, which might be related to decreased respiration rate in early stage of storage. However, possible pathways need to be explored further.

3.2.2. Respiratory intensity

Respiration rate is an important indicator for reflecting the effect of storage. In general, the higher the intensity of respiration, the more the vigorous of respiration, and the sooner the nutrients are consumed (Navarro, Flores, Garrido, & Martinez, 2006). After 15 d of storage, respiration rate of control, PC and PC + OZ groups were 11.21, 9.58 and 9.73 mg/kg·h, respectively (Fig. 3). PC and PC + OZ treatments significantly reduced respiration intensity, but no difference ($P > 0.05$) between fruits in PC and PC + OZ group were found. It was indicated that PC treatment could help maintain respiration rate at a low level, thus delaying the physiological activity of fruit during storage.

3.2.3. Color

The L^* value of mulberry significantly ($P < 0.05$) increased after 6d of storage, and thereafter decreased gradually thoughout the ramaining 9d of storage (Table 1). The initial L^* values of treated groups were significantly higher than those of the control group,

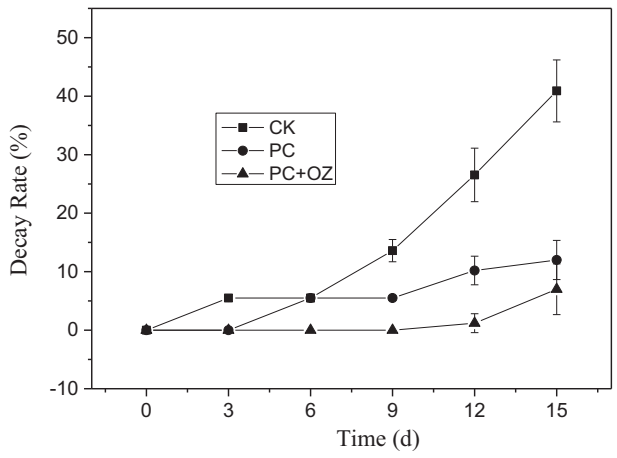

Fig. 1. Effect of precooling (PC), ozone (OZ) and PC + OZ treatments on the decay rate of mulberry fruit during postharvest storage at 0 ± 1 °C and 80 ± 10% RH.

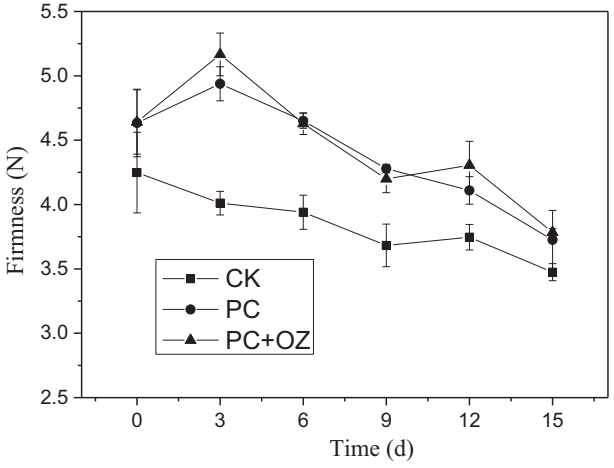

Fig. 2. Effect of precooling (PC), ozone (OZ) and PC + OZ treatments on the firmness of mulberry fruit during postharvest storage at 0 ± 1 °C and 80 ± 10% RH.

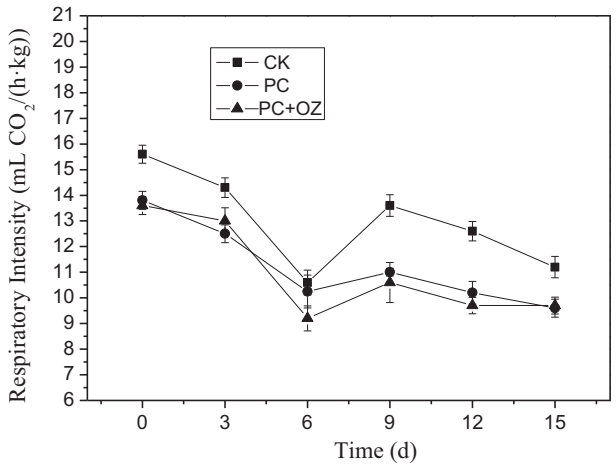

Fig. 3. Effect of precooling (PC), ozone (OZ) and PC + OZ treatments on the respiratory intensity of mulberry fruit during postharvest storage at 0 ± 1 °C and 80 ± 10% RH.

Table 1
Effect of PC and PC + OZ treatments on color, TA, TSS, and PPO activity in post-harvest mulberry fruits.

Physicochemical properties	Storage period (days)	Treatments*		
		CK	PC	PC + OZ
Color (L* value)	0	16.22^{b}	17.34^{a}	17.24^{a}
	3	17.27^{a}	17.95^{a}	17.91^{a}
	6	20.12^{b}	20.34^{b}	21.73^{a}
	9	18.58^{b}	19.46^{a}	19.31^{ab}
	12	17.52^{b}	18.75^{ab}	19.02^{a}
	15	17.25^{b}	18.46^{a}	18.96^{a}
Titratable acidity (%)	0	20.27^{a}	20.45^{a}	20.66^{a}
	3	20.01^{a}	20.28^{a}	20.32^{a}
	6	18.64^{c}	19.86^{b}	20.38^{a}
	9	14.35^{c}	17.68^{b}	18.35^{a}
	12	13.56^{c}	16.65^{b}	17.32^{a}
	15	13.24^{c}	15.35^{b}	16.53^{a}
Total soluble solids (%)	0	11.30^{a}	11.28^{a}	11.29^{a}
	3	10.34^{b}	11.36^{a}	11.46^{a}
	6	10.51^{b}	11.68^{a}	11.58^{a}
	9	10.15^{c}	11.61^{b}	12.14^{a}
	12	9.29^{c}	10.39^{b}	11.24^{a}
	15	7.80^{c}	9.98^{b}	10.98^{a}
Polyphenol oxidase activity (U ml^{-1} enzyme)	0	20.01^{a}	16.87^{a}	23.53^{a}
	3	35.65^{ab}	29.65^{b}	43.86^{a}
	6	108.30^{a}	84.23^{b}	93.25^{b}
	9	99.37^{a}	94.56^{a}	95.36^{a}
	12	82.50^{a}	65.36^{b}	72.30^{b}
	15	72.35^{a}	68.68^{ab}	60.32^{b}

* Means within each row with the different letters indicate significant difference ($p < 0.05$) between treatments.

indicating that PC treatment could effectively remove field heat and maintain the color of mulberry fruit. The maximum L* value of PC + OZ group was significantly ($P < 0.05$) higher than the peak values of other groups. As compared to the control group, fruits of treated groups showed a distinctly higher L* value during the longer storage time (9–15d). The results indicated that PC treatment, especially in conjunction with OZ treatment, could maintain the brightness of mulberry fruit during storage.

3.2.4. Titratable acidity

TA represents the content of organic acid in fruit (Melgarejo, Salazar, & Artes, 2000). TA values declined throughout the entire storage period for all treatments. However, both PC and PC + OZ treatments delayed the loss of TA, which might be associated with the metabolic activity and respiratory rates of fruit. Similar results were demonstrated by Chen et al. (2015) that fresh mulberry fruit showed a decreasing tendency in TA value during storage at low temperature.

3.2.5. Total soluble solids content

TSS content is an important indicator of fruit maturity and intrinsic quality. There were no significant ($P > 0.05$) differences among the individual groups initially (Table 1), but TSS content in control group declined continuously during storage, except for the slight increase at 3d. The TSS content increased in the PC + OZ group from an initial value of 11.29 to a peak value of 12.14 at 9d of storage. At the end of storage (15d), there was a significant difference among the TSS contents of the control, PC, and PC + OZ groups. These results were in accordance with the finding of Leccese, Bartolini, and Viti (2012). PC and OZ treatment could effectively maintain TSS of mulberry fruit during the early days of storage.

3.3. Effect of OZ and PC treatment on PPO activity

The mechanism by which OZ limits decay development in mulberry fruit was investigated by analyzing the enzyme activity related to senescence and defense response. PPO has long been considered as a predominant factor leading to the discoloration of fruit (Li et al., 2012). As shown in Table 1, the activity of PPO in OZ and PC treated (43.86 U mL^{-1}) enzyme fruit was higher than that in the untreated fruit during the initial days of storage (day 3). The induction of PPO activity suggest that OZ and PC treatment can induce resistance of mulberry fruit against pathogen and thus reduce the decay rate. However, PPO activity declined rapidly in both OZ treated and untreated fruit starting at Day 9, which might be because the fruit became senescent and over-ripe. Similar result was reported by Whangchai et al. (2006) that PPO activity of fruit was significantly inhibited after exposed to OZ, and it further decreased slightly after 9 d of storage. Furthermore, at the end of storage, reduction of PPO activity can be achieved by the combination of OZ and PC treatment, compared with OZ treatment alone. These results implied complicated changes in the mulberry as affected by OZ treatment.

3.4. Changes in ultrastructure of fruit

3.4.1. Scanning electron microscopy (SEM) observation

SEM images of control fruit showed the presence of seriously deformed stomata, thinned guard cells, and external projections on the surface, indicating the loss of moisture control functions in the epidermal cells (Fig. 4A). Mycelium also appeared on the surface of control fruit, which cracked the skin and destroyed the epidermal tissue (Fig. 4B). Conversely, the stomata were regular on the fruit surface of PC + OZ group, morphology of guard cells was complete, the closed status could be effectively suppressed water loss (Fig. 4C). At the same time, there were no obvious cracks on the surface and cellular tissue of PC and PC + OZ treated fruit (Fig. 4D). PC + OZ treatment also significantly suppressed the degradation of epidermal tissue of fruit, allowing for maintenance of their morphological features. Ozone can reduce the size of pores on fruit skin (Keutgen & Pawelzik, 2008), allowing for the simultaneous inhibition of bacterial infection and water transpiration. Based on SEM observation and variations of firmness, OZ effectively inhibited the decomposition of mulberry fruit skin and suppressed fruit softening.

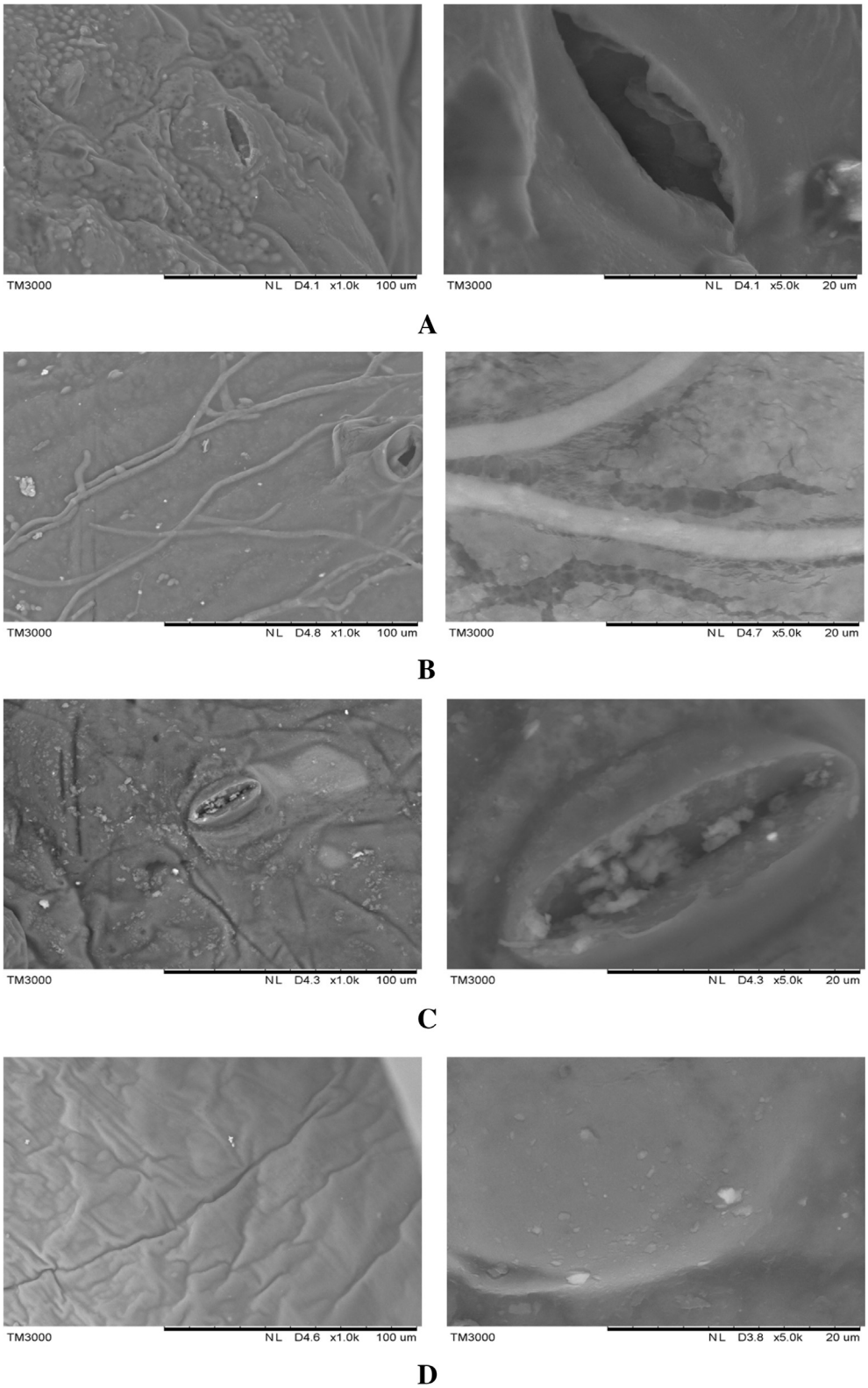

Fig. 4. Observation of ultrastructure of mulberry fruit by scanning electron microscope. A: Mulberry stomata of control group; B: Mulberry skin of control group; C: Mulberry stomata treated group; D: Mulberry skin of treated group.

3.4.2. Cell wall substance content

The content of cell wall substance increased slightly initially, and then rapidly declined after 3 d, especially in the control group (Fig. 5). It was found that cell wall substance of per unit area epidermal tissue generally declined during storage. The degradation rate in treated group was significantly lower than the control group, consistent with the variation of fruit firmness and decay rate. These results were consistent with those from a previous study which reported that the inhibition of cell wall substance decomposition helps to maintain cell morphology, thus

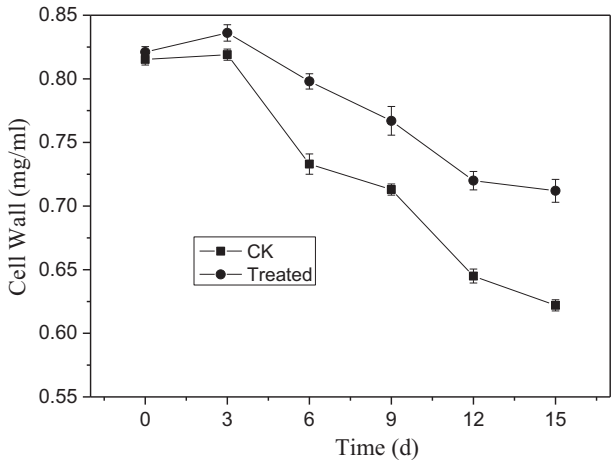

Fig. 5. Effect of treatments (OZ + PC) on cell wall substances of mulberry fruit during postharvest storage at 0 ± 1 °C and 80 ± 10% RH.

maintaining the firmness of fruit and protecting it from spoilage (Bu, Yu, Aisikaer, & Ying, 2013).

4. Conclusion

In the present study, OZ and PC treatments could reduce the decay rate and respiratory intensity, maintain color, and delay softening of mulberry fruit, thus extending their shelf-life during refrigerated storage. The OZ induced disease resistance of mulberry may be related to the enzyme activity of PPO. In addition, the delayed fruit senescence by OZ may be related to its impact on the degradation of cell wall substance. However, the molecular mechanism of the response of mulberries to OZ treatment remains unknown. Our results suggested that OZ in combination with PC treatments can provide a feasible technique for prolonging the postharvest life and ensuring the quality of mulberry fruit during refrigerated storage. It was also found that OZ at appropriate concentrations may induce resistance of mulberries to pathogens by induction of the phytoalexins resveratrol and pterostilbene. Future studies need to focus on the molecular mechanism of mulberry response to ozone treatment.

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