Research Article

The Influence of a Novel Chitosan-Based Coating with Natural Antimicrobial Agents on the Storage Properties and Reactive Oxygen Species Metabolism of Harvested Tangelo Fruit

Ying Ji [iD],[1] **Jieqiong Wang** [iD],[2] **Ye Liu,**[1] **Shaoyan Liu,**[1] **Xuanjing Jiang,**[3] **Huaming Huang,**[1] **and Ling Li**[1]

[1]*Fujian Forestry Vocational Technical College, Nanping 353000, China*
[2]*Tea Research Institute Chinese Academy of Agricultural Sciences, Key Laboratory of Biology, Genetics and Breeding of Special Economic Animals and Plants, Ministry of Agriculture and Rural Affairs, 9 South Meiling Road, Hangzhou 310008, China*
[3]*College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou 362000, China*

Correspondence should be addressed to Ying Ji; jiying0599@163.com

This study investigated the effects of a novel antibacterial film based on chitosan, carboxymethyl cellulose, sodium alginate, tea polyphenols, ascorbic acid, and tangelo peel extract on the postharvest quality and reactive oxygen species metabolism of tangelo fruit during storage. The composite film significantly reduced the fruit decay rate and weight loss, delayed the reduction in total soluble solids and titratable acidity, and retained fruit firmness and the appearance of tangelo fruit during storage. Furthermore, the composite film effectively reduced the fruit respiration rate, inhibited the increase in cell-membrane permeability, markedly reduced the generation of superoxide anion, hydrogen peroxide, and malondialdehyde, and enhanced the activity of the antioxidant enzymes superoxide dismutase, catalase, and ascorbate peroxidase. The composite film also reduced losses of the nonenzymatic antioxidants ascorbic acid and glutathione. Overall, the chitosan-based composite antibacterial film effectively maintained the quality of tangelo fruit during storage, enhanced ROS scavenging capacity and antioxidant properties, and then reduced the rot rate of postharvest tangelo.

1. Introduction

Tangelo is a hybrid citrus cultivar, bred in Japan from the *Citrus unshiu* mandarin as the female parent and the *Hassaku* orange as the male parent. Tangelos are orange-yellow, with rough skin and oblate shape. They have the aroma of orange and pomelo, the flesh is golden yellow, and the juice is sweet and refreshing [1, 2]. Tangelo is a good source of vitamins and minerals [3] and has beneficial antioxidant and health functions [4, 5]. Tangelos are grown in China, Iran, Italy, Mauritius, and the United States and have Chinese geographical indication product origin protection; the industry is mature and of great significance to the Chinese economy. However, the metabolism of the tangelo fruit is vigorous; it dehydrates rapidly in storage and

is easily infected by pathogenic fungi, such as *Colletotrichum gloeosporioides*, *Phoma citricarpa*, *Capnodium citri*, and *Botrytis cinerea* [6–8]. *Alternaria alternata* and *Colletotrichum gloeosporioides* were identified as major pathogens on tangelos [9, 10]. These storage problems result in reduced nutritional value and fruit quality, as well as wastage of commercially unacceptable fruit, which restricts the development of the tangelo industry.

Plant senescence is a degenerative process, in which one of the first cellular responses is increased generation of reactive oxygen species (ROS), including superoxide anion $(O_2^{-\bullet})$, hydrogen peroxide (H_2O_2), and hydroxyl radical. Plants have evolved an efficient antioxidant defense system that can prevent the excessive accumulation of ROS and repair oxidative damage, consisting of radical-scavenging

enzymes (e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX)), and nonenzymatic antioxidants (e.g., glutathione (GSH) and ascorbic acid (AsA)). The rate of ROS metabolism appears to be an important factor in determining the storability of fruits and vegetables, and ROS are involved in regulating fruit senescence [11]. ROS accumulation results in juice sac granulation in pummelo [12], and disordered ROS metabolism causes oxidative damage to the cellular membrane and affects the storage quality of postharvest fruits, such as litchi [13], navel orange [14], melon [15], blueberry [16], longan [17], and guava [18].

Coating fruit with a polysaccharide polymer film slows respiration and transpiration and inhibits microbial action, thereby maintaining the fruit's quality in storage for longer periods, for example, orange [19], loquat [20], banana [21], blueberry [22], and avocado [23]. Tangelo has a thick peel, the pores in the pericarp are large and numerous, the white cortex is spongy, and the composition and structure of the cuticle are complex, so any coating has to adhere strongly and have excellent mechanical properties; a single-component coating cannot achieve adequate protection of tangelos. Given the vigorous metabolism and high susceptibility to fungal infection of the tangelo fruit, there is an urgent need for a novel coating that reduces respiration and transpiration and has antioxidant and antifungal activities.

We previously reported that tangelo peel extract (TPE) is a rich source of phenolics and flavonoids, and that TPE is an effective growth inhibitor of *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Penicillium* mold [24]. We also previously reported on the development of a coating film composition for tangelo fruit [25] to optimize factors such as inhibition of respiration, dehydration, and fungal growth. However, there has been no report on the use of compound antibacterial film treatment to enhance the storage properties of tangelos by modulating ROS metabolism.

The aim of this investigation was to evaluate comprehensively the effectiveness of a six-component, chitosan-based composite fruit-coating films as an effective, practical postharvest treatment to increase the practical storage time of tangelos. The incorporation of ascorbic acid (AsA) and tea polyphenols (TPs) to increase the antioxidant and antimicrobial activity of the films was evaluated. Fruit color, firmness, total soluble solids (TSS), titratable acidity (TA), weight loss, percentage of commercially acceptable fruit, respiration rate, cell membrane permeability, $O_2^{-\bullet}$, H_2O_2, malondialdehyde (MDA) levels, antioxidant enzyme activities, and antioxidant content were monitored during 120 days of storage.

2. Materials and Methods

2.1. Materials.
Tangelos were picked at maturity in Nanping, Fujian Province, China, and transported to the laboratory of Food Analysis at Fujian Forestry Vocational Technical College (Nanping, China) on the day of collection. Tangelos for the storage study were selected for uniform size and color and the absence of disease and

mechanical damage. Tangelos were rinsed immediately with sterile water and air-dried before further treatment.

2.2. Preparation of Chitosan-Based Compound Treatment Solution.
Tangelo peel was crushed with a crusher and sieved; the peel powder was mixed with 70% ethanol (solid-liquid ratio 1 : 30). The mixture was extracted ultrasonically for 25 min at 40°C. Then the homogenate was filtered, concentrated, and fixed. After that the extract was stored at 4°C for standby.

Based on our previous research, chitosan-based compound antibacterial film treatment solution in distilled water was prepared as described previously, with the composition (% w/v): chitosan (CH) (1.0%), carboxymethyl cellulose (CMC) (0.5%), sodium alginate (SA) (0.5%), TPs (2.0%), AsA (1.0%), and TPE (2.5%).

The preparation steps are as follows: CMC and SA were mixed with distilled water, and then the mixture was stirred with a magnetic mixer at 60°C until the solid was completely dissolved, and it was kept for 6–8 h in order to defoam. After CH, TPs, AsA, and TPE were added to the film-forming solution, then the composite coating was mixed with ultrasonic for 15 min.

2.3. Postharvest Treatments.
Twenty of the harvested, washed, and dried tangelos were randomly selected and used to characterize the fresh (day 0) fruit using the tests in Sections 2.4–2.13. The remaining tangelos were randomly divided into two batches of 200 fruits. One batch (treatment) was soaked in treatment solution (Section 2.2) for 20 min, and the other batch (control) was soaked in distilled water for 20 min. The fruits were air-dried after treatment, packed in perforated polyethylene storage bags (22 cm × 20 cm, 0.01 mm thickness, five fruits per bag), and stored at 10 ± 1°C with 70% relative humidity for 120 d. During storage, every 30 days, 20 fruits from the control and treatment batches were randomly selected to determine the physiological, biochemical, and ROS metabolism-related indices.

2.4. Determination of Fruit Respiration Rate.
According to the methodology of Chen et al. [26], one tangelo was placed in a 2 L glass jar, tightly sealed and left for 10 min, then the respiration rate was measured using an infrared CO_2 fruit breathing apparatus (3051H, Zhejiang, China) and expressed as mg CO_2 kg$^{-1}\cdot$h^{-1}. The average value was calculated from 10 fruits.

2.5. Determination of Cell Membrane Permeability.
Cell membrane permeability was determined as described previously and expressed as relative electrical conductivity (%) [27]. Electrical conductivity was measured with a DDS-11A digital conductivity meter (Ningbo Hinotek Technology Co., Ltd., Zhejiang, China).

2.6. Determination of Percentage of Commercially Acceptable Fruit.
According to the methodology of Lin et al. [27], the

percentage of commercially acceptable fruit remaining was determined by visually monitoring the fruits every 30 days for signs of decay and designating any fruit with visible decay as not acceptable. The percentage of undecayed fruit was then calculated.

2.7. Determination of Fruit Weight Loss. According to the methodology of Zhou et al. [16], weight loss was determined by weighing the same 20 tangelos every 30 days. The mean of 20 samples was taken as the result.

2.8. Evaluation of Hue Angle in Tangelo Fruit. The altered methodologies of Nie et al. [12] to determine the average color of the tangelo skin were followed; the hue angle was determined for 10 tangelos every 30 days, and the mean of 10 samples was taken as the result. The hue angle was measured from six different places on the equatorial part of the peel on each fruit with an ADCI-60-C Color Analyzer (Chincan Ltd., Zhejiang, China). The hue angle was calculated as follows: $h^{\circ} = 180^{\circ} + \arctan(b/a)$, where h° is the hue angle, a is the red/green reading, and b is the yellow/blue reading.

2.9. Determination of TSS and TA of Tangelo Pulp. After mixing the pulp and juice from 20 tangelos, the contents of TSS and TA in the mixture were determined as described previously [27]. TSS was measured with a digital pocket refractometer (WZ-214/ATC, Beijing, China). TA was determined by titration with 0.1 M NaOH to an end point of pH 8.2 and calculated as citric acid equivalents. Both TSS and TA contents are expressed as percentages.

2.10. Determination of Fruit Flesh Firmness. According to the methodology of Zhang et al. [28], the firmness of the tangelo flesh was evaluated using a fruit penetrometer (GY-15, Kern & Sohn, Balingen, Germany). A section of peel was sliced off to expose the flesh at four equally spaced positions around the equator of the fruit, and then the probe was pushed into a depth of 10 mm. The penetrometer readings (newtons, N) were averaged for 10 fruits.

2.11. Determination of Superoxide Anion Production Rate and Contents of H_2O_2 and MDA. The production rate (expressed as $mmol \cdot kg^{-1} \cdot min^{-1}$) of $O_2^{-\bullet}$ and the contents (expressed as $mol \cdot kg^{-1}$) of H_2O_2 and MDA were determined in samples (2.0 g) from 20 tangelo fruits, as described previously [29, 30].

2.12. Determination of SOD, CAT, and APX Activities. The activities of SOD, CAT, and APX were determined in samples (2.0 g) from 20 tangelo fruits, as described previously [29, 30]. The enzyme activities were expressed as $U \cdot kg^{-1} \cdot protein$.

2.13. Determination of AsA and GSH Content. The contents of AsA and GSH were determined in samples (2.0 g) from 20 tangelo fruits, as described previously [31]. The results were expressed as $mg \cdot kg^{-1}$.

2.14. Statistical Analyses. All experiments, except for the determination of the percentage of commercially acceptable fruit, were carried out in triplicate and repeated in three independent sets of experiments. Data are presented as means ± standard errors. The results were analyzed with analysis of variance, using SPSS statistics version 20.0, with $p < 0.05$ considered significant and $p < 0.01$ very significant. The WPS software was used for graph plotting.

3. Results

3.1. Effects of Composite Coating on Fruit-Storage Properties. The respiration rate of postharvest fruit is an accurate indicator of its physiological metabolic rate, which determines the speed of both ripening and senescence [14]. The respiration rate of postharvest tangelo fruit increased steadily during storage (Figure 1(a)) but coated fruit had a significantly lower rate ($p < 0.05$) than the control at 60 days and a very significantly lower rate ($p < 0.01$) at 90 and 120 days. The respiration rate of the control fruit increased by 36.0% at 120 days, whereas that of the coated fruit increased by only 15.5%.

When fruit is stressed, the integrity of the cell membrane is disrupted, which increases its permeability and results in leakage of intracellular substances [13]. Electrolyte leakage increases electrical conductivity and is a measure of membrane permeability and, indirectly, of membrane damage [16]. The relative electrical conductivity of control fruit increased rapidly up to 30 days, then more slowly (Figure 1(b)), whereas that of coated fruit was very significantly lower ($p < 0.01$) than that of control fruit from 30 to 120 days of storage. Therefore, the composite coating was markedly able to inhibit the loss of cell membrane integrity.

The percentage of commercially acceptable fruit is the most important indicator of the storage stability of fruit [27]; for coated fruit it was constant at 100% until 60 days of storage, whereas for control fruit it decreased very significantly to 83.3% (Figure 1(c)). After 120 days of storage, 96.3% of coated fruit was still commercially acceptable, whereas control fruit was very significantly lower ($p < 0.01$) at 63.3%.

The weight loss of stored fruit because of water loss by transpiration is also an important indicator of quality and freshness [16]. After 120 days of storage, the coated fruit lost significantly less water (7.64%; $p < 0.01$) than the control fruit (11.95%; Figure 1(d)).

3.2. Effects of Composite Coating on Fruit-Storage Quality. Fruit color is an important index of postharvest quality. The hue angle can be used as a comprehensive index for evaluating color change of fruit pericarp; the higher hue angle indicates smaller color change in pericarp of fruit [12]. The skin of newly picked tangelos is yellow/green (hue angle 91.63°) and then becomes completely yellow as the fruit
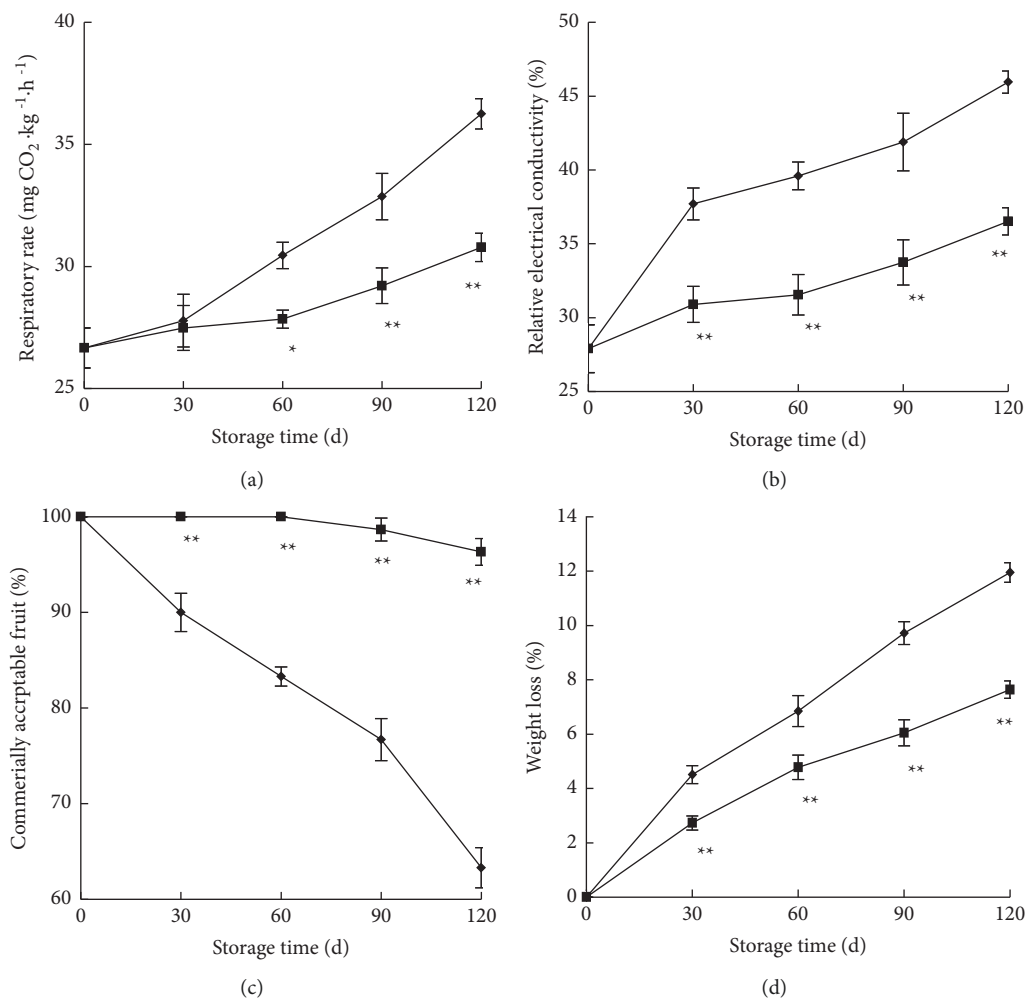
FIGURE 1: Effects of composite film coating on respiration rate (a), relative electrical conductivity (b), percentage of commercially acceptable fruit (c), and weight loss (d) of tangelo fruit during storage. The symbols * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between coated (■) and control (♦) fruit.

ripens. The hue angle decreased during storage, initially rapidly, then much more slowly; the hue angle of coated fruit was consistently and significantly higher ($p < 0.05$) than that of the control (Figure 2(a)). The hue angles of tangelo fruit in the treatment and control were, respectively, 63.24° and 55.53° on storage day 120. The treatment descended more slowly, compared with storage day 0.

TSS refers to soluble compounds, including sugars, acids, minerals, and vitamins, of which sugars and acids are the main components; sugars and acids are substrates for respiration [14]. The TSS increased gradually, by 5.8% in control fruit and by 13% in coated fruit (significantly higher than control, $p < 0.05$), until day 60, then decreased until day 120 (Figure 2(b)); the TSS of coated fruit was 12.96% and that of the control was 12.27% on day 120 of storage. These changes may be explained by the degradation of insoluble starch into soluble sugars during the first half of the storage period and consumption of sugars and acids by respiration during the second half. The slower consumption of sugars and acids in coated fruit appears to be related to its slower respiration rate (Figure 1(a)).

The distinctive, desirable taste of citrus and other fruits is a balance between sweetness from sugars and sourness from acids. TA is a good indicator of sourness [14]. TA decreased during the whole storage period, by about half in control fruit and by a third in coated fruit (Figure 2(c)). TA was very significantly higher ($p < 0.01$) in coated fruit during the whole storage period.

Flesh firmness is an important indicator of fruit maturity and quality, being too high in unripe fruit but too low in over-ripe fruit [28]. The tangelos softened, and their firmness decreased gradually during storage (Figure 2(d)). During the first 30 days of storage, the firmness of coated and control fruit was not significantly different, but the differences were significant ($p < 0.05$) at 60 and 90 days. At storage day 120, the firmness of the control fruit (0.23 N) was very significantly lower ($p < 0.01$) than that of coated fruit (0.29 N).

3.3. Effects of the Composite Coating on $O_2^{-\bullet}$, H_2O_2 and, MDA Content. ROS, such as $O_2^{-\bullet}$ and H_2O_2, can damage membrane lipids by peroxidation of unsaturated fatty acids,
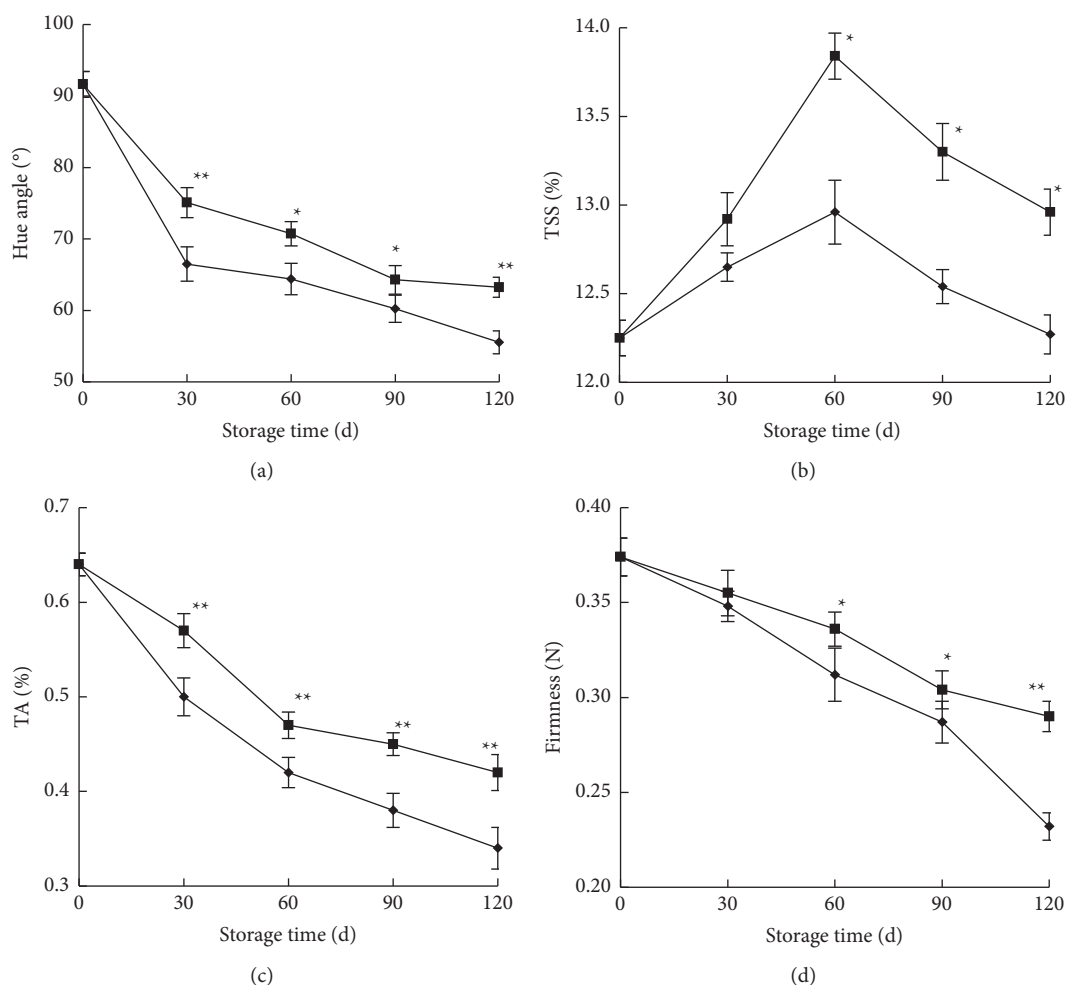
FIGURE 2: Effects of composite film coating on hue angle (a), total soluble solids (TSS) (b), titratable acidity (TA) (c), and flesh firmness (d) in tangelo fruit during storage. The symbols * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between coated (■) and control (◆) fruit.

which disrupts membrane structure and integrity [15]. The production rate of $O_2^{-\bullet}$ was not significantly different between control and coated fruit until day 60 of storage (Figure 3(a)), whereas the control increased markedly at day 90 and was very significantly higher ($p < 0.01$) from day 90 to day 120.

The H_2O_2 content of the stored fruit increased relatively evenly, but that of the control fruit increased faster and was very significantly higher ($p < 0.01$) than that of the coated fruit from day 30 to day 120 (Figure 3(b)).

MDA is one of the end products of lipid peroxidation and a marker of membrane oxidative damage [17]. The MDA content of the stored fruit increased evenly, but that of the control fruit increased faster and was very significantly higher ($p < 0.01$) than that of the coated fruit (Figure 3(c)). The MDA content was 0.16 mol·kg^{-1} at day 0 and the control fruit increased to 0.62 mol·kg^{-1} on day 120 of storage, whereas the coated fruit increased to only 0.44 mol·kg^{-1}. The composite coating very significantly inhibited the production of $O_2^{-\bullet}$ and decreased the H_2O_2 content of the stored fruit, thereby reducing oxidative stress. The lower MDA

content indicated that the reduction in oxidative stress reduced lipid peroxidation, helped to maintain the structural integrity of the cell membrane, and minimized its permeability, which is consistent with the observed inhibition of increased electrical conductivity (Figure 1(b)).

3.4. Effects of Composite Coating on SOD, CAT, and APX Activities.
SOD, CAT, and APX are the main antioxidant enzymes that control the accumulation of ROS in plant cells and protect them from oxidative stress [11]. The SOD activity in the stored fruit increased slowly until day 30 then decreased more rapidly until day 120, indicating that the antioxidant defenses in the fruit were activated (Figure 4(a)). The SOD activity in the coated fruit was very significantly higher ($p < 0.01$) than in the control at days 90 and 120 of storage. The CAT activity in the stored fruit increased during storage, more rapidly in the first 30 days, then more slowly and the activity in the coated fruit was significantly higher ($p < 0.05$) than in the control fruit (Figure 4(b)). The APX activity in the stored fruit decreased rapidly throughout the
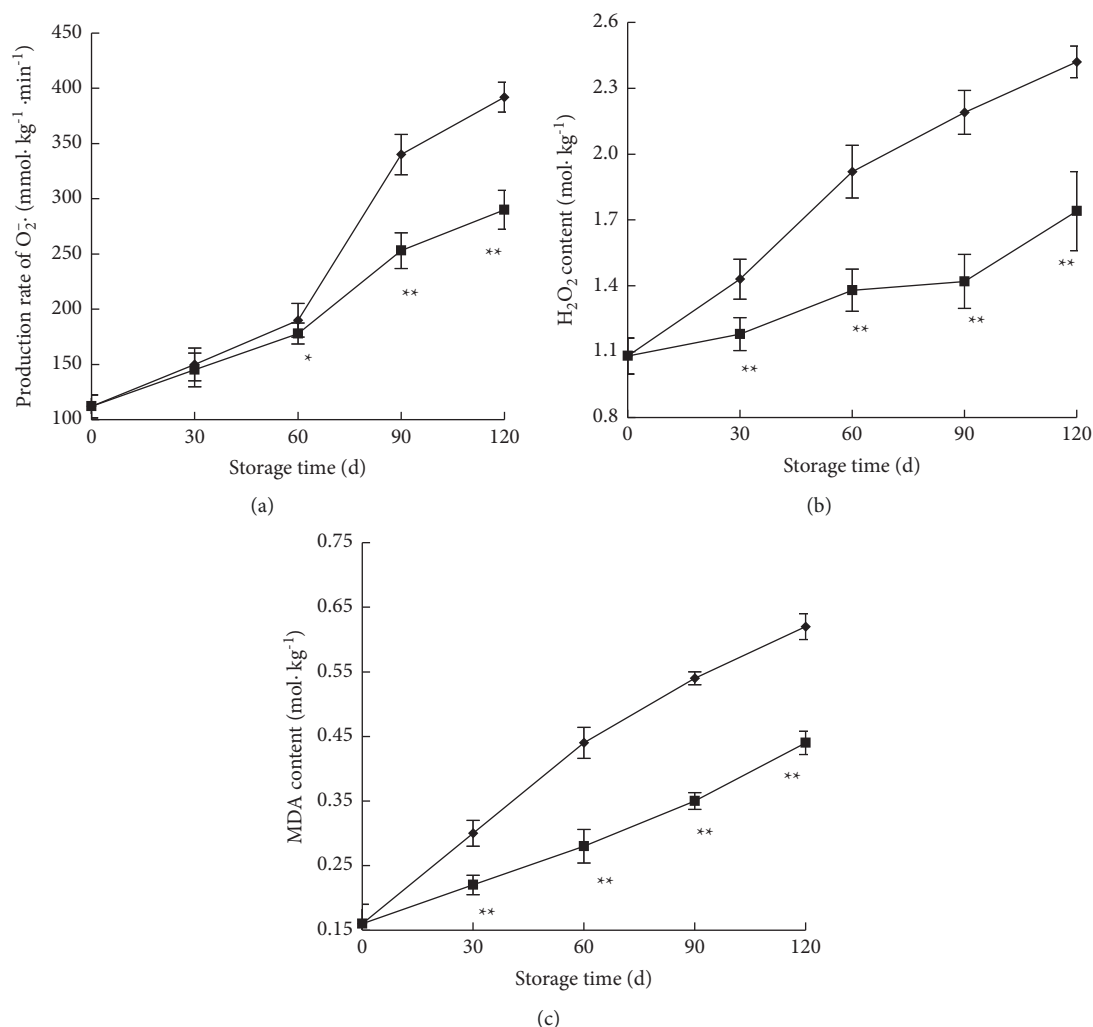
(a)

(b)

(c)

FIGURE 3: Effects of composite film coating on superoxide anion ($O_2^{-\bullet}$) production rate (a), hydrogen peroxide (H_2O_2) content (b), and malondialdehyde (MDA) content (c) in tangelo fruit during storage. The symbols * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between coated (■) and control (◆) fruit.

storage period, but the activity in the coated fruit was very significantly higher ($p < 0.01$) than in the control fruit during the whole storage period (Figure 4(c)).

3.5. Effects of Composite Coating on the Contents of AsA and GSH. The endogenous antioxidants, AsA and GSH, are part of the cellular defenses against oxidative stress [12]. The AsA content of the control fruit decreased rapidly until day 60 of storage, after which the decrease slowed, with a 70% decrease by day 120. The AsA content of the coated fruit decreased slowly until day 60, after which the rate of decrease sped up, being very significantly higher ($p < 0.01$) than the control all through the storage period (Figure 5(a)). The GSH content of the control fruit decreased rapidly until day 30 and then decreased more slowly. The GSH content of the coated fruit decreased slowly until day 60, after which it decreased more rapidly, remaining significantly higher ($p < 0.05$) than the control until day 120, when it was only slightly higher than the control (Figure 5(b)).

4. Discussion

Pathogen infections and vigorous physiological metabolisms, which generates ROS, are the main factors that reduce the quality of stored tangelo fruit. A coating can maintain the quality of the fruit if it reduces respiration and minimizes the growth of microorganisms that cause decay. The composite coating used here combined the film-forming functions of three polysaccharides with the antimicrobial and antioxidant effects of AsA and polyphenols. The individual polysaccharides (CH, CMC, and SA) could not form a uniform film, but the three-polysaccharides-based compound film led to uniform coverage of the fruit and high tensile strength and good gas/water vapor-barrier properties. That is because hydroxyl groups on CH molecules interact with the carbonyl, hydroxyl, and acetyl groups on CMC and SA through hydrogen bonds and ionic bonds.

The composite coating created a microenvironment in tangelos fruit, with high CO_2 and low O_2 concentrations, which inhibited gas exchange through the fruit skin,
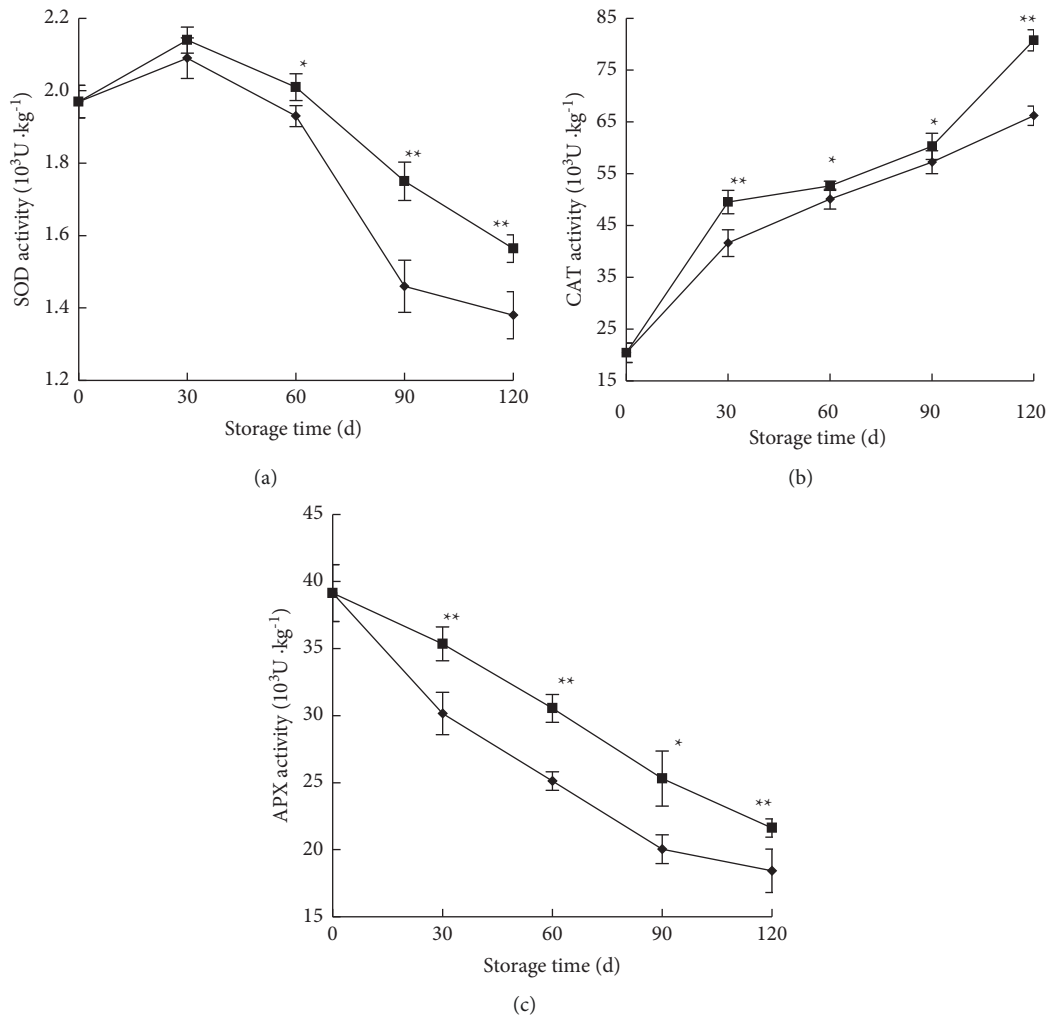
(a)

(b)

(c)

FIGURE 4: Effects of composite film coating on superoxide dismutase (SOD) (a), catalase (CAT) (b), and ascorbate peroxidase (APX) (c) activities in tangelo fruit during storage. The symbols * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between coated (■) and control (◆) fruit.
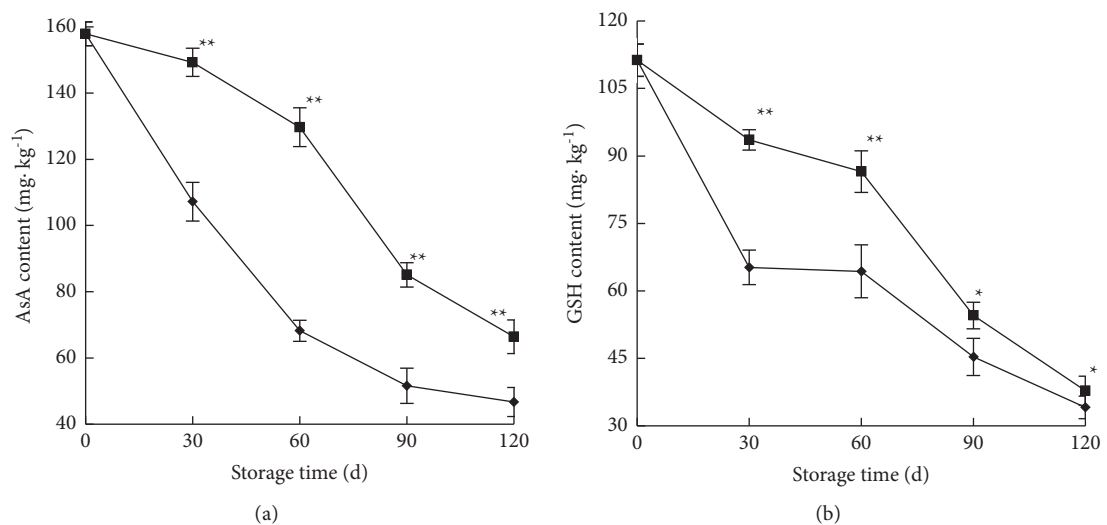

(a)

(b)

FIGURE 5: Effects of composite film coating on the contents of ascorbic acid (AsA) (a) and glutathione (GSH) (b) in tangelo fruit during storage. The symbols * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between coated (■) and control (◆) fruit.
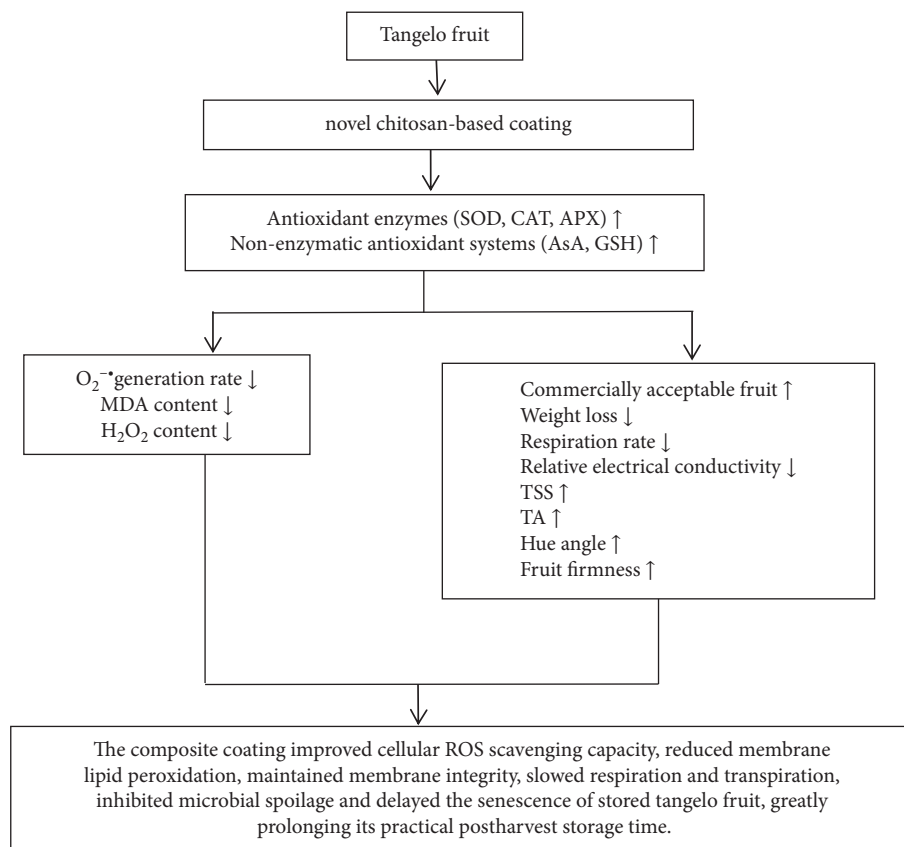
Figure 6: Summary of the mechanism of action of the chitosan-based composite coating.

effectively creating a modified-atmosphere environment inside the fruit and, consequently, inhibiting respiration and decreasing transpiration [27]. Organic acids are consumed as substrates for respiration and are precursors in many biochemical pathways [12], so the reduced respiration rate and consequent reduced metabolic rate in coated fruit would have limited the consumption of acids, which, combined with reduced consumption of sugar, would result in coated fruit having a stronger flavor than control fruit.

The addition of TPs, AsA, and TPE to the formulation strongly inhibited microbial growth and decay [3], which eventually maintained better storage behaviors of tangelos fruit. In addition, the antioxidant activity of the TPs, AsA, and TPE would be expected to protect the cellular membranes from oxidative damage by ROS, thereby maintaining membrane integrity, reducing water leakage from cells, and reducing excessive softening of stored tangelos. The skin of the coated fruit was orange and that of the control was yellow, which showed that the composite coating could accelerate the loss of green color in tangelo fruit, to produce a more attractive color. The coating made from polysaccharides could reduce ROS accumulation, which reduces membrane lipid peroxidation and MDA production, which results in the disruption of cell membrane structure and the loss of membrane integrity [32]. Cellular defenses against oxidative stress caused by ROS include enzymatic and nonenzymatic free-radical-scavenging systems [33, 34]. The role of SOD is to scavenge superoxide anions and protect

cells from damage by its reaction products, such as lipid peroxides and aldehydes [35, 36]. Both CAT and APX catalyze the degradation of peroxide to water and oxygen and are important peroxide-scavenging enzymes [37, 38]. AsA and GSH can scavenge hydroxyl radicals and hydrogen peroxide. Disease development in postharvest fruits is related to excessive accumulation of ROS and the associated oxidative damage to cellular components and membranes [39, 40]. Therefore, efficient scavenging of ROS in plants reduces oxidative damage to cells, enhances stress resistance, and delays senescence. Control of excessive ROS generation is critical to extending the storage time and shelf life of fruit.

In this study, superoxide anion production (Figure 3(a)) and H_2O_2 content (Figure 3(b)) in the control tangelos increased during the storage period, which accelerated the peroxidation of membrane lipids and increased the accumulation of MDA (Figure 3(c)). The accumulation of ROS caused lipid peroxidation in the cellular membrane and increased its permeability, as indicated by the increase in relative electrical conductivity (Figure 1(b)). However, the composite coating markedly reduced ROS and MDA accumulation and the increase in membrane permeability by strengthening cellular enzymatic and nonenzymatic antioxidant defenses.

Based on the above findings, the possible mechanism of action by which the composite coating greatly extends the tangelo storage time can be summarized (Figure 6).

5. Conclusions

The composite coating created a microenvironment in tangelos fruits, which inhibited respiration, delayed the increase in pericarp cell membrane permeability, retarded fruit disease, maintained a higher commercially acceptable fruit rate, lowered weight loss percentage, and maintained the better apparent quality in pericarp of harvested tangelos. Besides, coating treatment could suppress the decline of contents of TSS, while delay the increases in contents of pulp TA in harvested tangelos fruit, which retain a better pulp quality and nutritive properties of tangelos fruit during storage. The coating film combined multiple beneficial effects on the stored tangelos, i.e., strong antimicrobial activity, protection of the fruit cells from oxidative stress, and membrane lipid peroxidation, by stimulating cellular enzymatic and nonenzymatic antioxidant defenses. Our results indicated that the composite coating could provide an alternative approach for keeping the storability and quality properties and prolonging the storage-life of tangelos fruit during postharvest storage.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Y.J was involved in conceptualization, methodology, formal analysis, writing and original draft preparation, supervision, and project administration. X.J.J was involved in conceptualization, writing, reviewing and editing, and supervision. Y.L was responsible for methodology, formal analysis, data curation, and visualization. H.M.H was responsible for data curation. J.Q.W was responsible for data curation and supervision. S.Y.L was responsible for data curation and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

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