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Effects of Wax Treatment on the Physiology and Cellular Structure of Harvested Pineapple during Cold Storage

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ABSTRACT: Pineapple (*Ananas comosus* (L.) Merr. cv. 'Paris') is sensitive to low temperature and highly susceptible to blackheart during cold storage, which causes serious fruit decay. This work investigated the effect of wax treatment (Sta-Fresh 2952, 60 g/L) on blackheart of pineapple under chilling stress. Wax treatment significantly reduced blackheart symptoms after 14 days of storage and markedly delayed changes in firmness and flesh color during the whole period of storage. The weight loss of wax-treated fruit (2.6%) was less than the control (3.1%) at the 24th day of storage. The treatment decreased the activities of PG and EGase for maintaining cell wall stability during the later period of storage. In the control fruit, the structure of flesh cells was significantly damaged under chilling stress, with looser cell wall, absence of middle lamella, loss of membrane integrity, and many cells near the vascular tissue collapsed. The subcellular elements could be barely observed in the control after storage. These destructive symptoms were significantly alleviated in the wax-treated fruit. The results suggest that wax treatment could reduce blackheart of pineapple under chilling stress via maintenance of cell integrity.

KEYWORDS: *pineapple, blackheart, wax, cell structure*

INTRODUCTION

Pineapple [*Ananas comosus* (L.) Merr.], an important fruit in many tropical and subtropical countries,[1] has good export potential because of its exceptional flavor when consumed as a fresh fruit.[2] Low-temperature storage is recommended to extend its postharvest life for export via sea shipment.[3,4] However, pineapple is sensitive to low temperature and susceptible to blackheart, which is detected only at the consumer level, creating further problems with product reputation and waste at the cannery.[5] Previous studies showed that a variety of chilling injury symptoms at least partly result from a loss of cell integrity due to damage to membranes.[6] It is accepted that pulp browning is usually caused by the oxidation of phenolic compounds.[7,8] Loss of cellular compartmentalization has been shown to accelerate browning by releasing phenolics stored in the vacuoles.[9] Polygalacturonase (PG, EC 3.2.1.15) and pectin methylesterase (PME, EC 3.1.1.11) are the two important enzymes associated with pectin degradation of the cell wall,[10] and endo-1,4-glucanase (EGase, EC 3.2.1.4) is the enzyme that hydrolyzes internal 1,4-β-glucan bonds of the cell wall, which is considered to be related to the development of chilling symptoms.[11] These findings have encouraged the investigation of the relationship between cell wall-related enzymes and blackheart of pineapple during cold storage. The rapid development of blackheart following low-temperature storage restricts refrigerated seafreight export. Therefore, blackheart has become a major postharvest limitation for the pineapple industry.[6] Also, it is necessary to search for an effective approach to protect pineapple fruit from blackheart during cold storage.

Recently, waxing has been applied to reduce postharvest diseases and stress damage in many horticultural crops, including nectarine and strawberry.[12,13] Many studies have been done on chilling injury symptoms and corresponding responses in fruit physiology, which have been frequently attributed to changes of cell wall metabolism-associated enzymes.[11,14] However, there is little information about the effects of wax treatments on cellular structure in chilling injury fruits, and less information is available about the cell structure related to blackheart in pineapple caused by chilling.[15] To the best of our knowledge, there is no report so far on the application of scanning electron microscopy (SEM) to analyze the cell structure of pineapple flesh.

The present work explores the effect of wax treatment on blackheart of pineapple and analyzes changes of flesh composition and cell structure related to blackheart symptoms in pineapple with two different imaging technologies, SEM and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Plant Material and Experimental Design. Pineapple fruits (*A. comosus* cv. 'Paris') were selected on the basis of fruit color and size uniformity, from a commercial grower in Zhanjiang, Guangdong, China. All fruits were cleaned and soaked in 0.05% (w/v) Iprodione solution (Kuaida, Jiangsu, China) for 2 min to eliminate potential microbes. Fifty fruits (without crown) were dipped in Sta-Fresh 2952 (FMC) wax solution at 60 g/L for 1 min, and another group of 50 fruits were dipped in water for 1 min, as control. Fruits were then allowed to dry for 2 h at 25 °C and subsequently stored at 7 °C. Cell structure of pineapple was investigated upon harvest and after 21 days of storage at 7 °C plus 3 days at 25 °C, and five fruits were taken from each group. Flesh tissue was collected for cellular structure assays in

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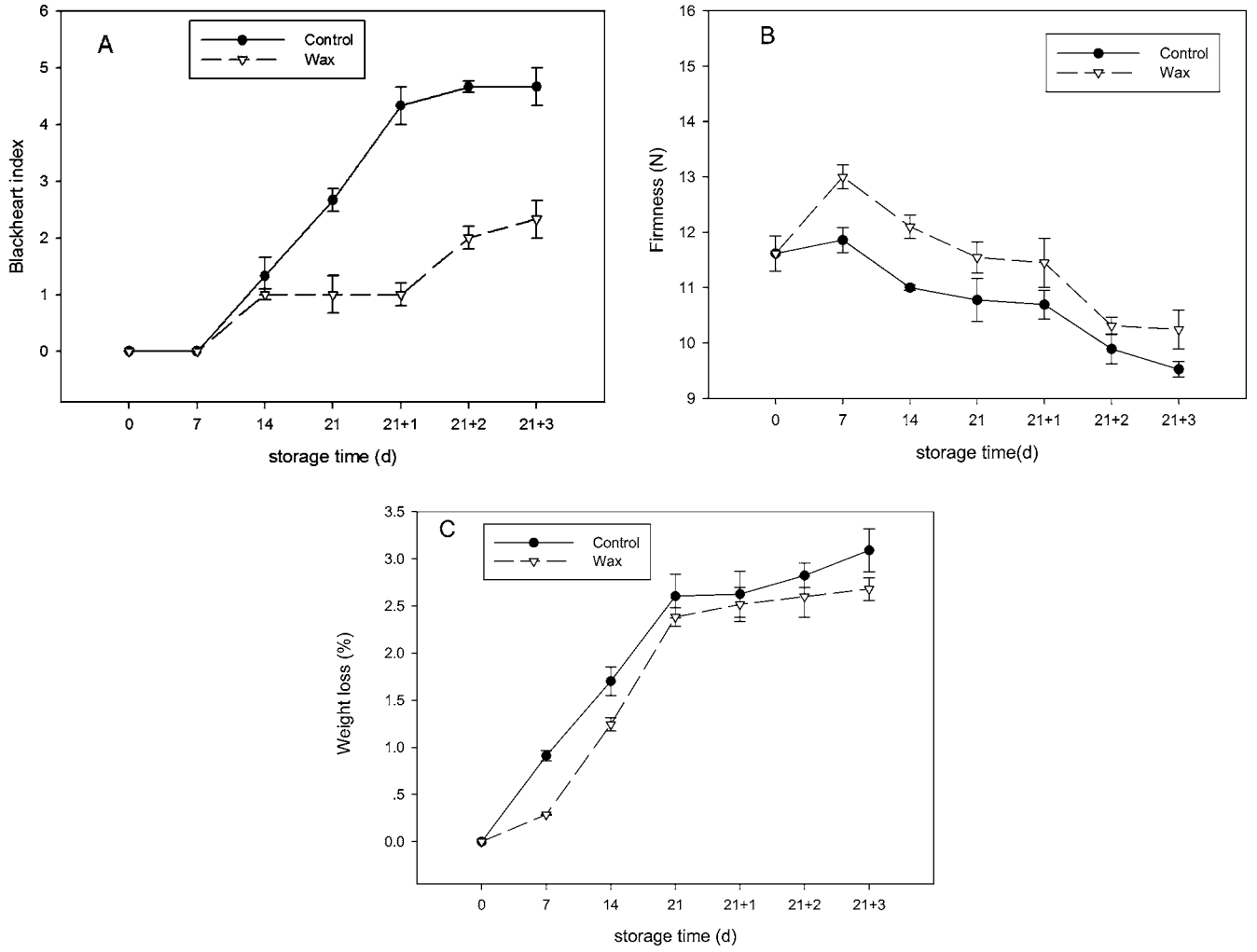

Figure 1. Effects of wax treatment on blackheart index (A), firmness (B), and weight loss (C) of pineapple fruit during storage at 7 °C for 21 days and then transferred to 25 °C for 3 days. 0−21 represent 21 days of storage at 7 °C, and 21 + n (n = 1, 2, 3) indicates 21 days of storage at 7 °C followed by n days of storage at 25 °C. Values are the mean of five replicates, and error bars represent the standard deviation.

the same area (disease-affected tissue adjacent to the core) of each fruit. Five fruits (each fruit is a replicate) were randomly sampled every 7 days during cold storage (21 days at 7 °C) and every day during room temperature storage (3 days at 25 °C) to determine physical and biochemical changes. The flesh adjacent to the core from each fruit was cut, frozen in liquid nitrogen, and stored at −40 °C for enzyme assays.

Blackheart Index (BI) Determination. Fruits were cut longitudinally in halves to determine the BI. For each fruit, BI was scored from 0 to 5 according to the percentage of flesh affected (0, free from blackheart; 1−5, 10, 10−25, 25−50, 50−75, and >75% of flesh blackheart, respectively).[6] The average BI was calculated from each group of fruit.

Firmness and Weight Loss. Fruit firmness (flesh) was measured at six equational regions of each fruit using a penetrometer (Instron5542) fitted with an 8 mm diameter flat probe, and results were expressed as newtons (N). Fruit weight was measured just after harvest and at different sampling dates. The weight loss was expressed as percentage of fresh weight loss relative to the initial value (taken as 100%).[16]

Color Assessment. Readings with a colorimeter were randomly taken at six different locations on each pineapple fruit (flesh), using a total of five fruits from each group. Color saturation (C) and hue angle (H) values of each fruit were recorded with a CR-300 colorimeter (Minolta, Ramsey, NJ). [17]

Assay of PG, PME, and EGase Activities. PG activity was determined according to the method of Pressey and Avants.[18] Briefly, 1 g of frozen flesh was ground with 5 mL of 0.15 mol L^{-1} NaCl. The homogenate was extracted for 20 min and centrifuged at 15000g for 45 min. The supernatant was collected as enzyme extract, which was mixed with an equal volume of 0.5% polygalacturonic acid in 50 mmol L^{-1} sodium acetate buffer (pH 5.5) and incubated at 37 °C for 6 h. The release of reductive group was measured by 3,5-dinitrosalicylic acid reagent at 540 nm. Galacturonic acid was used as a standard of the reductive group, and the controls without incubation were set.

PME activity was determined according to the method of Hagerman and Austin.[19] Briefly, 2 g of frozen flesh was ground with 6 mL of 1 mol L^{-1} NaCl containing 10 g L^{-1} PVPP. The homogenate was stirred for 3 h at 4 °C and centrifuged (10000g, 30 min, 4 °C). The supernatant was collected and extensively dialyzed against water adjusted to pH 7.5 with NaOH. The activity was determined in a mixture containing 2 mL of 0.5% (w/v) pectin (72% methoxyl content), 150 μL of 0.01% bromothymol blue in 3 mmol L^{-1} phosphate buffer (pH 7.5), 0.65 mL of distilled water, and 200 μL of enzymatic extract. The mixture was incubated at 40 °C, and the change in absorbance at 620 nm was measured.

Finally, EGase activity was measured according to the method of Li et al.[20] Briefly, 1 g of frozen flesh was ground with 4 mL of 0.1 mol L^{-1} sodium phosphate buffer (pH 7.8) containing 1.8 mM cyclohexanediaminetetraacetic acid (CDTA) and 1.2 mol L^{-1} NaCl, then extracted for 1 h, and centrifuged for 15 min at 10000g at 4 °C. The

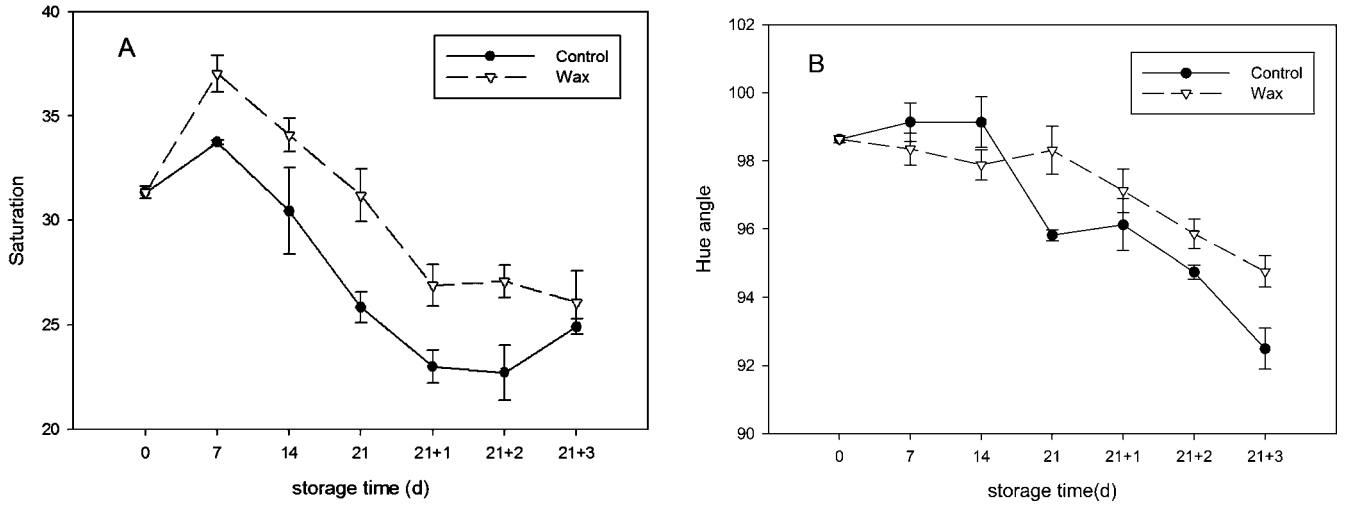

Figure 2. Effects of wax treatment on saturation (A) and hue angle (B) of pineapple fruit during storage at 7 °C for 21 days and then transferred to 25 °C for 3 days. 0−21 represent 21 days of storage at 7 °C, and 21 + *n* (*n* = 1, 2, 3) indicates 21 days of storage at 7 °C followed by *n* days of storage at 25 °C. Values are the mean of five replicates, and error bars represent the standard deviation.

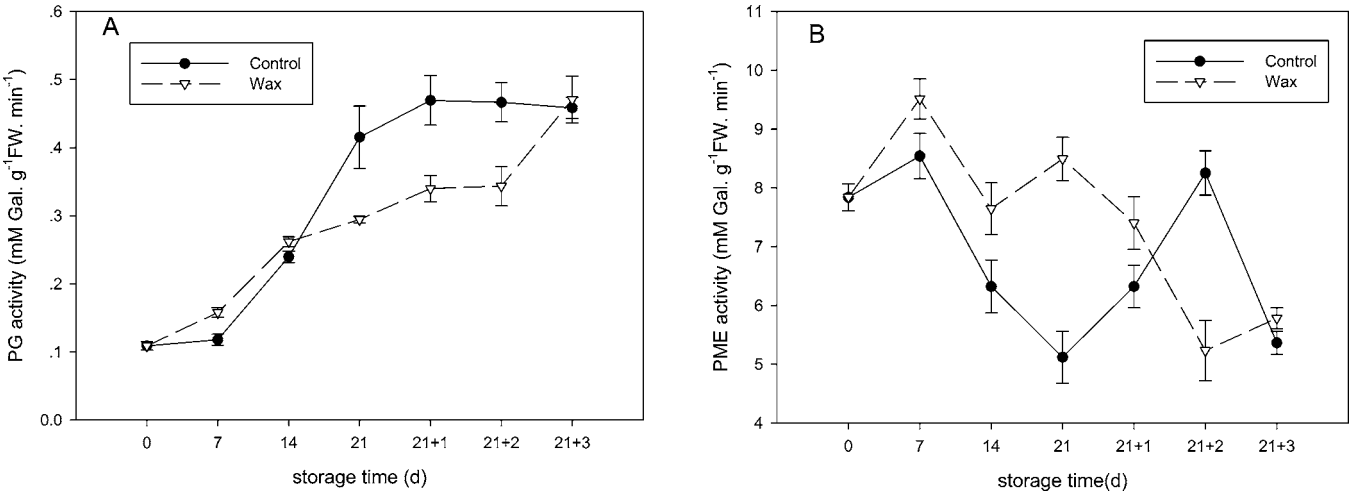

Figure 3. Effects of wax treatment on PG activity (A) and PME activity (B) of pineapple fruit during storage at 7 °C for 21 days and then transferred to 25 °C for 3 days. 0−21 represent 21 days of storage at 7 °C, and 21 + *n* (*n* = 1, 2, 3) indicates 21 days of storage at 7 °C followed by *n* days of storage at 25 °C. Values are the mean of five replicates, and error bars represent the standard deviation.

supernatant was collected and dialyzed against 40 mM sodium acetate buffer (pH 4.6) overnight. The assay mixture containing 0.5 mL of enzymatic extract and 0.5 mL of 1% (w/v) carboxymethylcellulose (CMC) prepared previously in 0.02 M Tris-HCl (pH 8.0) was incubated at 40 °C for 60 min. The reaction was terminated by adding 2.5 mL of 3,5-dinitrosalicylic acid and then boiled for 5 min. After cooling, the absorbance at 540 nm was measured. One unit of enzymatic activity was defined as 1 mmol of glucose (Glu) produced per minute. The enzymatic activity was presented on the basis of the fresh weight.

SEM. A small piece of tissue (5 × 5 × 2 mm^3) was torn with a tweezer. The samples were fixed and preserved in a fixative solution (90 mL of 50% ethanol, 5 mL of formalin, and 5 mL of acetic acid) until further processing. Before scanning, the slices were dehydrated in a series of ethanol solutions and dried at a critical point of liquid CO_2 in a desiccator. The specimens were mounted onto aluminum specimen stubs using conductive silver glue and sputter coated with gold. SEM was carried out with an XL-30 scanning electron microscope (Philips, The Netherlands) at 20 kV.

TEM. Flesh tissue (2 × 2 × 5 mm^3) was taken by a scalpel. Samples were processed according to the methods of Han et al.[21] and fixed in 2% glutaraldehyde (v/v) in 50 mM phosphate buffer (pH 7.2) at room temperature (25 °C) for 1 h and stored overnight in the fixative solution at 4 °C. Then the samples were washed three times with the same buffer and postfixed in 1% OsO_4 (w/v) (Spectrum, USA) for 3 h. After three washings with this buffer, the samples were dehydrated in a series of ethanol solutions, placed in propylene oxide, and embedded in Epon resin 812 (Shell Chemical, USA). The embedded samples were cut with an ultramicrotome (Ultracut R; Leica, Germany). Ultrathin sections (70 nm thick) were mounted on Formvar-coated grids, stained with 2% aqueous uranyl acetate and lead citrate, and observed with an electron microscope (TECNAI 12, Philips) at 80 kV.

Statistical Analysis. Data were analyzed statistically by ANOVA and mean differences were estimated by Duncan's new multiple-range test (DMRT) ($P < 0.05$) using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

RESULTS

Blackheart Index (BI), Firmness, and Weight Loss. Symptoms of blackheart, including watery spot and internal browning, were assessed visually. The BI in the control fruit increased more rapidly than that in the wax-treated fruit. The

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control fruit displayed more severe blackheart symptoms than the wax-treated fruit after 14 days of storage (Figure 1A).

Throughout the storage, the firmness loss of the control fruit was significantly greater than that of the wax-treated fruit ($P < 0.05$), indicating that waxing effectively delayed fruit softening (Figure 1B).

Although the weight loss of both fruits increased steadily during the storage, the wax-treated fruit had less weight drop on the 7th and 14th days compared to the control fruit ($P < 0.05$). At the end of the storage, the control showed 3.1% fresh weight loss, which was higher ($P < 0.05$) than the wax treatment (2.6%) (Figure 1C).

Color. During storage, the degree of color saturation peaked on the seventh day and decreased rapidly afterward in both types of fruits (Figure 2A). The wax treatment substantially reduced the decrease of the color saturation compared to the control fruit ($P < 0.05$).

Figure 2B shows that the hue angle was higher in the wax-treated fruit than in the control fruit after the cold storage, although no significant difference was observed during the cold storage, suggesting that the wax-treated fruit had less browning and better quality after cold storage.

PG Activity and PME Activity. For PG activity, a steady increase was observed in both types of fruits, but the wax-treated fruit showed a higher PG activity before the 14th day of storage, whereas the control fruit displayed a greater increase thereafter, and consequently the control fruit had a higher PG activity than the wax-treated fruit during the later storage period (Figure 3A).

Wax treatment greatly inhibited the decline of PME activity during cold storage and thus maintained a significantly ($P < 0.05$) higher activity of PME during the first 21 days compared to the control. The PME activity of the control increased strongly after the 21st day, whereas that of the wax-treated fruit decreased gradually within the same period (Figure 3B).

EGase Activity. The EGase activity increased continuously during the whole storage in both the control and treated fruits. The control fruit displayed lower EGase activity before day 14 of storage, after which the EGase activity of the wax-treated fruit showed a significantly ($P < 0.05$) lower level than that of the control fruit (Figure 4).

Effect of Wax Treatment on the Cell Structure of Pineapple Flesh. Before cold storage, the fresh fruit view under SEM showed individual cells (arrow) of normal morphology, with a clear cellular framework (Figure 5A); the flesh tissue was largely attached to the vascular bundles (Figure 5D). After 21 days of storage at 7 °C plus 3 days at 25 °C, however, the integrity of the flesh cells in the control fruit was damaged, and most cells displayed an undulated shape; intact cells (arrow) were not identifiable (Figure 5B). In addition, little flesh was attached to the vascular bundles in the control (Figure 5E). In contrast, there were no significant changes in the flesh cells of the wax-treated fruit after the whole period of storage. Here, each individual cell was regular in shape (arrow), having some flocculent substance (cell catabolite) (Figure 5C), and most of the flesh was attached to the vascular bundles (Figure 5F).

Effect of the Wax Treatment on the Subcellular Structure of Pineapple Flesh. At the ultrastructural level, regular thickening of the cell walls and the middle lamellae was observed clearly in the flesh cells of the fresh fruit. The plasma membrane was located tightly against the cell wall, and the cytoplasm was homogeneous with abundant organelles (Figure 6A,D). After 21 days of storage at 7 °C and a further 3 days at 25 °C, none of the organelles in control cells was identifiable, and the cell walls became much looser and swollen. The microfibril in the primary cell walls loosened and the middle lamella of the cell wall disappeared (Figure 6B,E). In contrast, the cell walls of the wax-treated fruit remained intact. Even after 24 days of storage, some cell organelles appeared to be functional (Figure 6C). Meanwhile, the cell walls maintained normal structure, and the middle lamella was clearly observable in the wax-treated fruit, although it had partially been dissolved in some locations (Figure 6F).

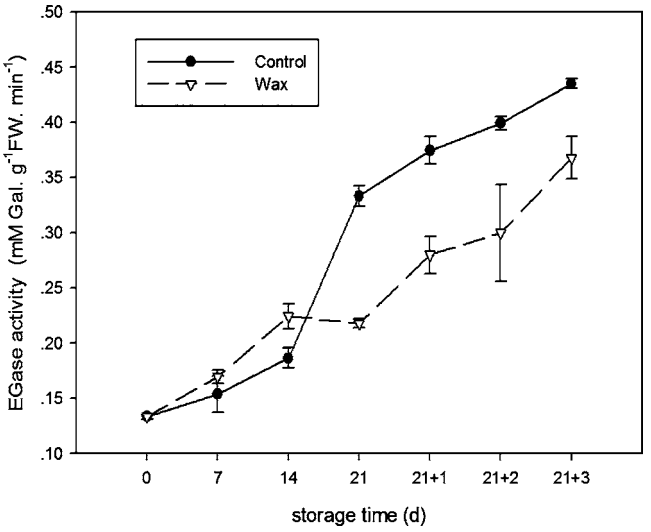

Figure 4. Effects of wax treatment on EGase activity of pineapple fruit during storage at 7 °C for 21 days and then transferred to 25 °C for 3 days. 0−21 represent 21 days of storage at 7 °C, and 21 + n (n = 1, 2, 3) indicates 21 days of storage at 7 °C followed by n days of storage at 25 °C. Values are the mean of five replicates, and error bars represent the standard deviation.

DISCUSSION

Effects of Wax Treatment on the Physiological Responses of Pineapple to Cold Storage. Cell wall degradation has been considered as the main factor involved in fruit softening. Fruit firmness decline during prolonged cold storage occurs in parallel with pectin modification.[22] Our study showed that the wax-treated fruit maintained a higher level of fruit firmness than the control fruit, which is consistent with the findings of Martinezromero et al.[23] The degradation of the cell wall is related to the action of several cell wall proteins and enzymes.[24] Rapid softening is associated with PG activity, which degrades the pectin in the cell wall matrix, causing a decrease in cell wall coherence and leading to softening.[25] In some cases, PG needs de-esterified homogalacturonic acid as its substrate, and PME can catalyze the demethylation of the C6 carboxylic acid group in galacturonosyl residues.[26] The occurrence of blackheart in some fruits during cold storage has been found to be associated with changes in PG and PME activities.[10,26] The activity of PME declined in both the control and waxed fruits during cold storage as has also been reported previously in loquat fruit,[14] probably because low temperature could suppress the activities of PME.[22] The activity of PG in the wax-treated fruit was higher than that in the control fruit during the first 14 days of the cold-storage period, which indicated that wax treatment increased the breakdown of cell walls during the period. However, the cells of both the control

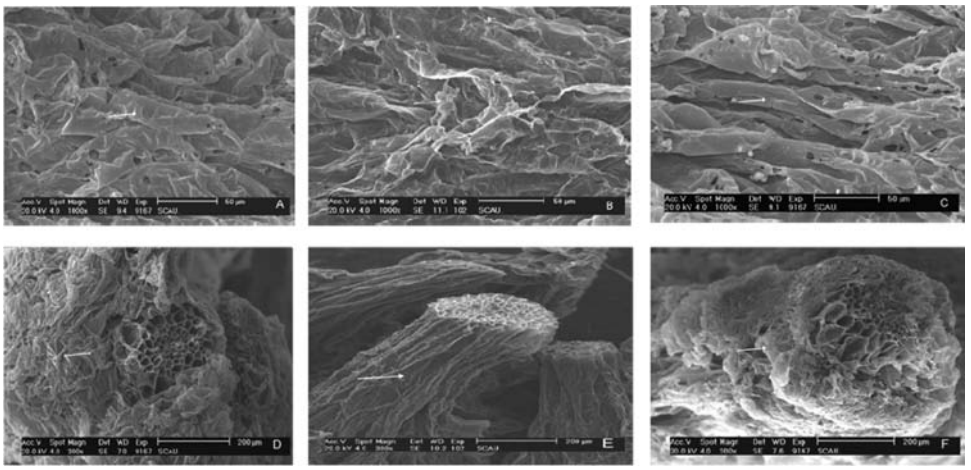

Figure 5. Effects of wax treatment on postharvest pineapple flesh (A, B, and C) and core (D, E, and F) as detected by SEM (A); (D) tissue taken from fruit right after harvest containing normal single cell (arrow) and intact flesh texture; (B, E) tissue taken from the control after storage containing invisible single cell (arrow) and destructive flesh texture; (C, F) tissue taken from the wax-treated fruit after storage containing visible single cell (arrow) and relatively intact flesh texture.

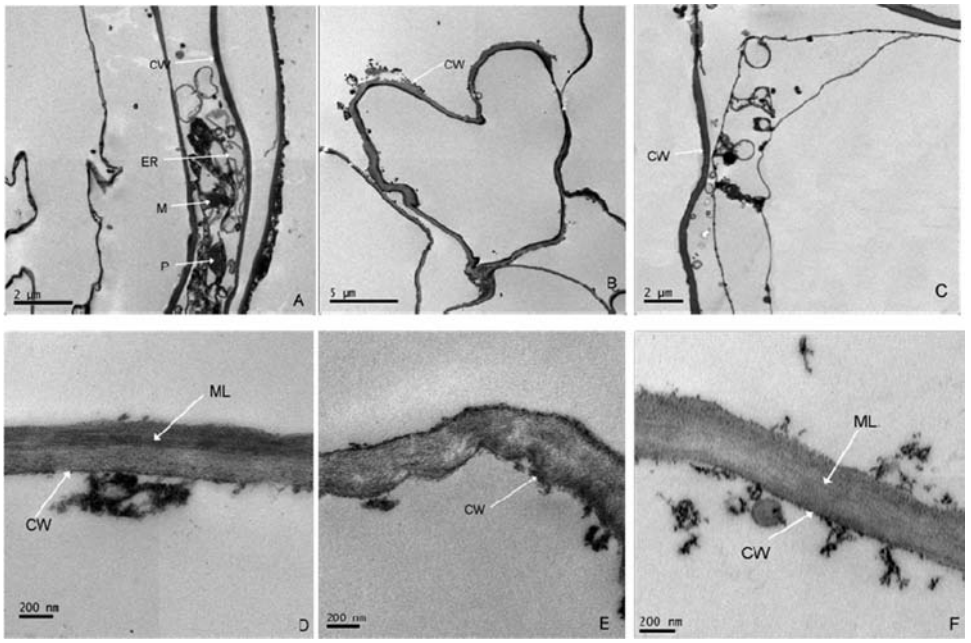

Figure 6. Effects of wax treatment on cell ultrastructure of postharvest pineapple flesh as detected by TEM: (A, D) tissues taken after harvest; (B, E) tissues taken after storage at 7 °C for 21 days and transferred to 25 °C for 3 days in the control; (C, F) tissues taken after storage at 7 °C for 21 days and transferred to 25 °C for 3 days in the wax-treated fruit. CW, cell wall; ER, endoplasmic reticulum; M, mitochondria; ML, middle lamella; P, plastid.

fruit and the wax-treated fruit were relatively intact, and the breakdown of cell walls rarely led to chilling injury during the early cold-storage period.[27] Therefore, the changes of PG activity could not result in the difference of BI between the control and treatment during the first 14 days of cold storage (Figure 1A). The activity of PG in the wax-treated fruit was significantly lower than that in the control during the later storage period. The cells of pineapple have been seriously injured during the later storage period; thus, the cellular structure changes occurring were associated with chilling injury, generally leading to blackheart during the period. The reduced blackheart of wax-treated fruit might result from the decrease of PG activity that was favorable to keep the cell wall integrity during the later storage period as Zhu et al. reported for pears.[11]

Cellulose is one of the major components of the cell wall, and EGase is likely to contribute to cellulose breakdown. The decomposition of cellulose is correlated with cell wall collapse.[24] Cell wall collapse, thus weakening its effectiveness as a barrier, could explain why fruit with chilling injury was more susceptible to diseases.[21] Lower EGase activity during the later period of storage was observed in the wax-treated fruit. The reduced activities of both PG and EGase in the wax-treated fruit might be correlated with a delayed senescence, because an increase in these enzyme activities occurs along with senescence.[27] Consistent with the result reported in tomato,[28] better cellular integrity and higher firmness were maintained in

the wax-treated fruit compared to the control fruit. Moreover, wax treatment was effective in retarding the change in color saturation and water loss during cold storage. These results suggest that the alleviation of blackheart by wax treatment is mainly due to delayed cell wall degradation and retarded moisture loss, which leads to maintenance of fruit quality.

Effect of Wax Treatment on the Cellular Structure of Pineapple. Cell membrane is first targeted for damage when plants suffer from chilling injury.[29] The damage to the cell membrane initiates a cascade of reactions, including disruption of cellular and subcellular structures.[30] In this study, the cell wall was destroyed in the control fruit, resulting in the disappearance of cell framework, as shown by SEM (Figure 5B). Moreover, most flesh in the control fruit was separated from the vascular bundle after storage (Figure 5E), suggesting that the chilling stress led to cell collapse, which was similar to the results obtained by Bauchot et al.,[31] who showed that chilling injury of kiwifruit was associated with the presence of cell collapse. The significant differences found in cell wall structure and flesh composition after storage between the wax-treated and control fruit could be attributed to the difference in water loss as wax treatment gave a physical barrier against evaporation. This result was consistent with the previous study in pear fruit.[32] The presence of the holes in Figure 5A,C images might be the pores of the membrane, which occurs with the onset of senescence in ripe fruit; further studies are required.

Ultrastructural changes due to chilling injury are usually observed in the membrane systems of such organelles as chloroplasts, mitochondria, nuclei, and tonoplast as well as plasma membranes. These changes in membrane system together with disorganization of the cell wall are characteristics of senescence in fruit.[33] In this study, changes in the cell walls, such as swelling of the cell wall, which could be regarded as a symptom of chilling injury,[21,34] degradation of the middle lamella, and organelles disappearance were observed in the control cells after cold storage (Figure 6B,E). The wax treatment significantly retarded these changes (Figure 6C,F) and reduced the blackheart of pineapple as Han et al.[21] showed that methyl salicylate could maintain cellular structure and composition to alleviate flesh browning symptoms in mango fruit. The results suggested that waxing might inhibit the damage to the cellular network caused by low temperatures and protect cell wall integrity.

In summary, wax treatment delayed the changes in firmness and flesh color and decreased weight loss during storage. The activities of PG and EGase during the later period of storage were decreased, and the biomembrane and the cellular integrity were protected by waxing, which thus alleviated blackheart of pineapple under chilling stress. Further studies are needed to investigate the sequence of changes during the development of blackheart in terms of the distribution of polyphenol oxidase and phenolics in pineapple fruit.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BI, blackheart index; EGase, endo-1,4-glucanase; PG, polygalacturonase; PME, pectin methylesterase; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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