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Enhancing Post-Harvest Quality of Areca Nuts: Using Heat Pre-treatment to Counteract Cold Stress

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ABSTRACT: Fresh areca nut is widely favored by consumers in South and Southeast Asia. However, postharvest areca nut perished quickly and was vulnerable to chilling injury (CI) and lignification during traditional cold storage. In order to alleviate this situation, hot water treatment was applied to investigate its effect on CI and lignification of fresh areca nut during cold storage at 13 °C. Areca nuts were submersed in hot water at 45 °C (HW45) and 50 °C (HW50) for short-term 5 min compared to fruit submersed in water at 20 °C (CT), then stored at 13 °C with 90% humidity for 60 days. CI, malondialdehyde (MDA), elec- trolyte leakage (EL), lignin and total phenolic content, related enzymes including phenylalanine ammonia-lyase (PAL), cinnamyl alcohol dehydrogenase (CAD) and per- oxidase activity (POD) were examined. Results indicated that two HW treatments both induced chilling tolerance and delayed lignification of areca nut to varying degrees during cold storage compared with the CT. Among which, HW45 treated-areca nuts had the lowest CI, MDA content and EL while maintaining the highest total phenolic con- tent. Moreover, no significant effects were found between HW45 and HW50 on tissue lignification, but they both effectively blocked lignin accumulation by inhibiting PAL, CAD and POD activities compared with the CT. The pre- sent study provided a safe physical method to mitigate CI and delay tissue lignification in cold-stored areca nut.
Keywords: Hot water treatment · Areca nut · Chilling injury · Lignification · Cold storage · Total phenolic content

I. INTRODUCTION

Areca (Areca catechu L.) nut is obtained from A. catechu tree which native to West Malaysia, and mainly distributed in South, Southeast Asia and Pacific islands including India, Sri Lanka, Thailand, Bangladesh, Myanmar, China, etc. (Gupta and Warnakulasuriya 2002). Previous resear- ches have reported that areca nut contains two alkaloids owning acetylcholine properties at central ganglionic and peripheral nicotinic and muscarinic receptors. One is a volatile cholinergic alkaloid and central nervous system stimulant called arecoline, the other is its hydrolyzed pro- duct called arecaidine (Nelson and Heischober 1999). Areca nut is known as the fourth worldwide addictive substance after caffeine, nicotine and alcohol (Gupta and Ray 2004). In addition, areca nut is used as an effective medicine to treat various diseases such as gingivitis, con- junctivitis, edema, flatulence, dysuria, etc. due to its anti- inflammatory, bactericidal, detumescent, diuretic and other effects (Peng et al. 2015). Chewing areca nut as a kind of herbal medicine or leisure food is widespread among all levels of society (Alrmalli et al. 2011).

Usually, local people prefer to chew fresh areca nut compared with the processed or dried ones. This is because fresh ones contain high valuable constituents especially the stimulating substances such as alkaloids (Lord and Lim 2002), and they can also reduce the intake of smoke dirt, benzopyrene and other harmful pollutants produced after boiling, baking or roasting. However, fresh areca nut is a highly perishable fruit due to microbial infection and mechanical damage after harvest, it is difficult to store and keep fresh to meet the demand of consumers in non-harvest seasons (Zhang et al. 2017). Cold storage is one of the most common used technologies to delay metabolic processes and prolong postharvest life. As a tropical fruit, areca nut is susceptible to chilling injury (CI) when stored below 14 °C, expressed as external pitting, water ulcerating, nuclear browning and heavy lignification, resulting in a reduction in both internal and external fruit quality. Pre- vious research has reported that the postharvest CI occur- rence is closely linked to membrane malfunction, and malondialdehyde (MDA) and relative electrolyte leakage (EL) are widely used to evaluate the integrity of cell membrane as physiological markers of membrane lipid peroxidation and membrane permeability respectively(Sharom and Thompson 1994). Moreover, lignification is the result of synthesis, accumulation and polymerization of lignin monomers. Phenylalanine ammonia-lyase (PAL), cinnamyl alcohol dehydrogenase (CAD) and peroxidase activity (POD) are involved in the biosynthesis of lignin (Singh et al. 2010). Conclusively, improving the cold resistance and delaying tissue lignification have become urgent problems to be solved in the process of storage, transportation and circulation of fresh areca nuts.

As a feasible non-chemical and environment-friendly technology, heat treatment (HT) has been paid increasing attention to alleviating postharvest physiological disorders, reducing CI and maintaining fruit quality (Lurie 1998). There are several effective combinations of heating med-

ium (hot water, vapour and air), temperature (43–53 °C) and time (from a few minutes to 2 h) depending on cultivar and fruit size (Mirdehghan et al. 2007). HT has been shown to maintain fruit quality and alleviate internal browning in peach (Chen et al. 2017), mitigate CI with the reduction of MDA and EL in banana (He et al. 2012), and HT was also effective in delaying the synthesis of lignin and suppressing lignin-involved synthase activities in bamboo shoot(Luo et al. 2012) and carrot (Howard et al. 2010). In general, HT produces a variety of positive outcomes and occupies a dominant position in postharvest treatment.

So far, no information is available to fresh areca nut on the use of postharvest HT, regarding fruit lignification and CI symptoms. Therefore, the main objective of this study was to investigate CI incidence and lignification of areca nut in response to hot water (HW) treatment at 45 and 50 °C for 5 min. The CI symptoms and images were observed, physiological changes (MDA content and rela- tive EL) in the cell membrane were investigated, as well as total phenolic content, lignin accumulation and related enzymes activities such as PAL, CAD and POD were analyzed during a 2-month cold storage period. A potential strategy to mitigate CI and delay lignification of cold-s- tored areca nut is proposed in this study.

II. MATERIALS AND METHODS

Fruit materials and treatments

Fresh areca nuts were harvested from a commercial orchard located in Tuncang county, Hainan province, China, and transported to the laboratory within the same day. After arrival, fruits were pre-cooled for 12 h at 14 ± 0.5 °C. Areca nuts were selected for uniformity of size, appearance and damaged materials discarded. For short hot water treatments, fruits were randomly divided into three groups of 240 per group (each group consists of three replicates of 80 areca nuts), and then two groups exposed to hot water treatments: one group was submersed in hot water at 45 °C (HW45), another group was sub- mersed in hot water at 50 °C (HW50), while the third group was submersed in water at 20 °C as control (CT), all were submersed for short-term 5 min. Temperature selec- tion for hot water treatment was choose as being optimal basing on the preliminary experiments at 40, 45, 50 and 55 °C. Following treatment, fruits were air-dried at room temperature, then packaged in polyvinyl chloride bags (30 lm thickness) and stored at 13 °C (relative humidity of 90%) for 60 days. CI index and total phenolic content were evaluated at 20 days intervals, and other physiological indicators were measured at 10 day intervals. At each time point, 15 labeled areca nuts from each replicate were taken for the evaluation of CI index and a random set of 5 fruit were taken for the determination of EL. Another samples per storage duration were frozen in liquid nitrogen, then crushed into powder and stored at - 80 °C until used for the measurement of MDA, lignin and total phenolic con- tent, PAL, CAD and POD activities.

Chilling injury assessment

Chilling injury was visually assessed using 15 whole areca nuts from each replicate. A group of five researchers from State Key Laboratory of Food Nutrition and Safety, Ministry of Education of China was professionally trained to recognize and score the CI symptoms.CI in areca nut were characterized by two aspects: external injury was evaluated by the occurrence of dark colored pitting, scald and shrivelling in the skin, internal injury is evaluated by nuclear browning and pulp water soaking. The CI index was performed by percentage of affected area on 0–4 scale, where, 0 = no abnormality, 1 = trace symptoms, small pits, nuclear browning area 1–10%, 2 = moderate symptoms, blotchy appearance, nuclear browning area 11–25%, 3 = moderate to severe symptoms, nuclear browning area 26–50%, 4 = severe symptoms, more than 50% nuclear browning area. CI index = (CI scale 9 fruit number in this level) / (4 9 total fruit number in the treatment).

Malondialdehyde (MDA) content and electrolyte leakage (EL) determination

MDA content was determined with the method employed by Yang et al. (2011) with slight modifications. Five grams of frozen sample were homogenized in 5.0 mL of 100 g L^{-1} trichloroacetic acid and centrifuged at 12,000 9 g for 20 min. The incubation mixture contained 2.0 mL of supernatant and 2.0 mL of 0.67% (w/v) thiobarbituric acid, incubated in boiling water for 20 min. After cooling rapidly on ice and centrifuging at 10,000 9 g for 10 min, absorbance of the supernatant was measured at 450, 532 and 600 nm using an UV-spectrophotometer (Shimadzu UV-1800, Japan). MDA content (mmol g^{-1} FW) = [6.45 9(OD_{532} - OD_{600}) - 0.56 9OD_{450}] 9Vt 9Vr /(Vs 9 m 9 1000), where Vt, Vr and Vs were the total volume of the extract solution, the total volume of the reaction solution and the volume of the extract solution used in the determination, and m was the mass of areca nut samples.

EL of areca nut peel was measured using 10 disks (7 mm diameter and 2 mm thickness) of flesh tissue of 5 fruit. The disks were immersed in 25 mL = deionized water in glass conical flask at 25 °C. After 20 min, the initial conductivity was measured by a digital conductivity meter (DDS-11A, Shanghai Mitsuko Industrial Co. Ltd, China), then the disks were boiled for 10 min to achieve 100% EL and cooled at ambient temperature, and total conductivity was measured. Relative EL (%) was calcu- lated by dividing the initial conductivity by the total conductivity.

Lignin content measurement

Lignin was extracted and measured using the method

described by Luo et al. (2012) with slight modifications. Ten grams of frozen sample were extracted three times with 50 mL methanol (containing 110 mM HCl) at 25 °C for 60 min each time under stirring constantly and cen- trifuged at 5000 g for 25 min. Following this, the residue was added with 10 mL of 72% H_2SO_4 and degraded for 4 h at room temperature so that the cellulose was completely dissolved. The final residue obtained by rinsing with dis- tilled water for twice was used for lignin analysis. The residue was hydrolyzed for 3 h in 12 M H_2SO_4 at 25 °C. Then, diluted the H_2SO_4 up to 1 M and heated for 6 h in a boiling bath in the fume hood, vacuum filtered and rinsed with 100 °C distilled water. Finally, the filter was dried to constant weight and weighed. Lignin content was expres- sed as %.

PAL, CAD and POD activity extraction and assay

PAL activity was measured by a previous method descri- bed by Assis et al. (2001) with slight modifications. Frozen powder samples (10 g) were homogenized in 10 mL of boric acid buffer (pH 8.8) containing 40 g/L PVP, 2 mM EDTA and 5 mM b-mercaptoethanol. The homogenate was centrifuged at 5000 9 g for 25 min at 4 °C and the supernatant collected for enzyme activity determination. PAL activity was measured by incubating 1 mL enzyme extract at 37 °C for 60 min with 5 mL boric acid buffer mentioned above and 1 mL of 20 mM L-phenylalanine solution. The substrate was added after 10 min of prein- cubation and the reaction was ceased with 0.1 mL of 6 N HCl. Then, the increase in absorbance at 290 nm was measured. PAL activity was defined as a change of 0.01 in A_{290} per g per h and expressed as U g^{-1} fresh weight.

CAD activity was analyzed according to the method used by Goffner et al. (1992) with slight modifications. Tengrams of frozen sample were extracted using 8 mL of 200 mM Tris–HCl buffer, pH 7.5, then centrifuged at 5000 9 g for 25 min at 4 °C and the supernatant was used as an extract for enzyme assay. For CAD activity assay, the reaction mixture contained 2.5 mL sodium phosphate buffer (pH 6.5), 2.0 mL of 3 mM NADP?, 2.0 mL of 3.2 mM trans-cinnamic acid and 1.0 mL of extract. The increase in absorbance at 340 nm, due to the formation of coniferyl aldehyde, was recorded every 1 min for at least 6 times. CAD activity was defined as a change of 0.01 in A_{340} per g per min and expressed as U g^{-1} fresh weight.

POD activity was analyzed according to the method employed by Xu et al. (2009) with slight modifications. Frozen samples (10 g) were homogenized in 10 mL of 25 mM sodium phosphate buffer, pH 7.8, containing 0.8 g/ L PVPP and 1 mM EDTA, then centrifuged at 5000 9 g for 25 min at 4 °C. The supernatant was col- lected as the crude enzyme extract for assay. The assay mixture consisted of 6.0 mL above buffer substrate, 2.0 mL of 0.5 M guaiacol, 1.0 mL enzyme extract and 1.0 mL of 2% H_2O_2. Absorbance at 470 nm was recorded every 1 min for at least 6 times. POD activity was defined as a change of 0.01 in A_{470} per g per min and expressed as U g^{-1} fresh weight.

Total phenolic content determination

Total phenolic content was found using the Folin–Ciocal- teu method as described by Liu et al. (2018) with some modifications. Briefly, ten grams of the frozen sample were added with 10.0 mL of 80% (v/v) acetone and centrifuged at 10,000 9 g for 10 min. After that, 1.0 mL supernatant was added with 2.0 mL of folin-phenol reagent and 10.0 mL of 10% (w/w) sodium carbonate solution, shaken well and placed in a dark place for 60 min. The absorbance value at 765 nm was measured and total phenolic content was expressed as gallic acid equivalent values in g/100 g fresh weight.

Statistical analysis

All tests were repeated three times and each repeated test was served as a block in the statistical design. The SPSS (SPSS19.0 for Windows) statistical software was used to calculate the analysis of variance (ANOVA). LSD means comparison test was used in figures. Significance differ- ences were determined at the P \ 0.05 level. The graphic program used to create the figures was Origin 8.

III. RESULTS AND DISCUSSION

CI index in areca nuts

The skin of freshly harvested areca nut was bright green and shiny (Fig. 1b, day 0). After low temperature storage at 13 °C, different degrees of skin pitting and browning occurred in all treatments (Fig. 1b, day 60). Areca nuts from HW45 had the lowest surface pitting and browning areas, whereas fruit from CT had the severe cold damage. HW50 treated- fruit produced yellowing phenomenon in the skin, although the CI was alleviated to some extent. Trace CI symptoms were initially found in fresh areca nut from CT and HW50 after 20 days of storage at 13 °C, and increased rapidly with the prolongation of storage time (Fig. 1a). There was a significant difference between HW treatment and control fruit during the whole storage (p \ 0.05). Compared with CT, HW treatment for 5 min inhibited the skin pitting and browning, thus reduced CI occurrence. The results on heat-treated areca nut were consistent with those for the reduction of CI on tomato (Zhang et al. 2013) and grapefruit (Sapitnitskaya et al. 2006). Among which, HW45 was much more effective than HW50 in reducing CI index and incidence. This may be due to the heat damage caused by the combination of HW50 and cold stress (Schirra et al. 1997), and the result was similar to the report of supra- optimal high temperature effects on the response of CI- sensitive plant exposed to following cold storage (Lurie 1998). On the 60th day, CI index of HW45 was only 0.18, which alleviated 40% and 50% in contrast to HW50 and CT

respectively. Ma et al. (2014) also noted that HW treatment on kiwifruit at 45 °C effectively reduced the CI index and incidence. These findings conclude that short hot water treatment can induce chilling tolerance of areca nut during refrigerated storage, while HW45 could achieve better effects. The beneficial effects to reduce CI may be relevant to: suppressing the reactive oxygen species that cause oxidative damage by enhancing antioxidant enzyme activities (Shadmani et al. 2015); maintaining a high level of phospholipids in mem- branes by stabilizing lipid bilayer structure and inhibiting lipid peroxidation (Wang 1994). Both of the factors may contribute to the fluidity and flexibility of cell membranes and the reduction of CI.

Changes in MDA content and relative EL

Membrane lipid conversion from the liquid-crystalline state to the solid-gel state under low temperature stress leads to an increase in membrane permeability and leakage of ions (Galindo et al. 2004). As the primary response of CI, EL is one of the physiological indicators for assessing fruit tissue integrity (Sharom and Thompson 1994). Meanwhile, MDA content is usually used to reflect the degree of membrane oxidative damage and the chilling tolerance of fruit as one of the main products of membrane lipid peroxidation.

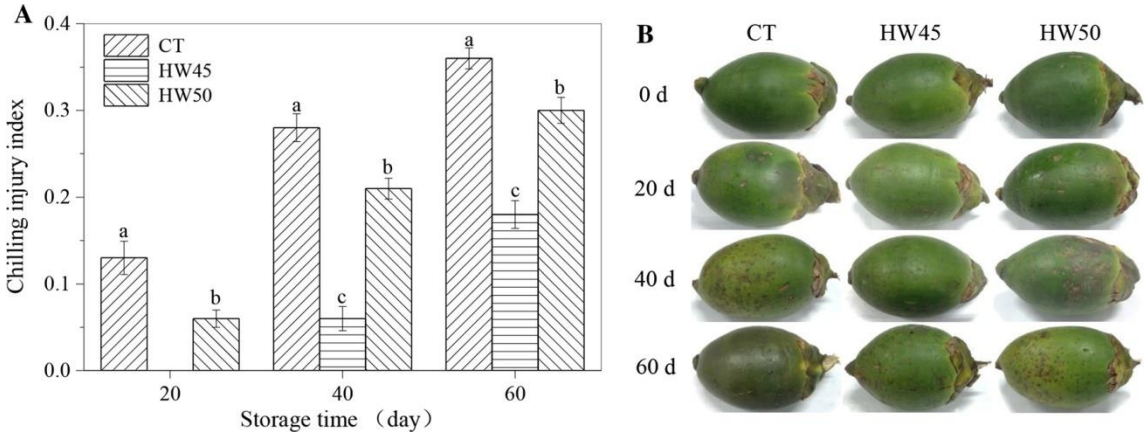

Fig. 1 Effect of short hot water treatment on chilling injury index (a) and image (b) of areca nut during storage at 13 °C for 60 days. Verticalbars represent the standard deviation of the means. Different letters indicate significant differences among treatments (p \ 0.05)

We observed a similar gradually increasing trend with storage time in MDA content and EL of cold-stored areca nut under all treatments (Fig. 2a, b). Campos et al. (2003) reported that the increase of MDA was closed related to CI and EL, which was similar to our results. In the initial 20 days, there was no significant difference between HW treatments and CT. After that, MDA content and EL of HW treated areca nuts were significantly lower than CT fruit (p ＼ 0.05). Moreover, HW45 was more effective in reducing MDA content and EL among three groups. This may have been due to moderate heat treatment (HW45) changed cell membrane permeability by affecting the activity of related membrane proteins, improved the cold resistance and stability of membranes, and thereby inhib- ited the increase of EL and MDA content (Wang et al. 2010). But supra-optimal high temperature (HW50) caused heat stress and damage of areca nut, especially after storage for 20 days (Fig. 2a, b). At the end of storage, MDA content of HW45 was 46.5% and 21.4% lower than CT and HW50 respectively, EL values of HW45 was 10.49% and 2.38% lower than CT and HW50 respectively. As a result, CT treatment resulted in increasing in EL and MDA con- tent much more than HW treatments. In other words, HW treatments exhibited a protective effect on areca nut fruit with less ion leakage across membranes. HW45 had a greater effect on improving the cold resistance of areca nut by reducing lipid peroxidation. Similar effects have been obtained for other cold-sensitive fruit, including tomato, cucumber and papaya (Saltveit 2005; Nasef 2018; Shad- mani et al. 2015).

Lignin content and PAL, POD, CAD activities

Another important symptom of areca nut fruit chilling injury is the aggravation of lignification, manifested asrigid fiber layer texture and less juice etc. PAL is the first marker enzyme that catalyzes the synthesis of lignin monomer, and its activity increases rapidly at the begin- ning of lignin synthesis (Boerjan et al. 2003). CAD cat- alyzes the final step of lignin monomer formation, along with the conversion of aldehydes into corresponding alcohols, while POD catalyzes the oxidation polymeriza- tion of lignin monomers, which is the last step of lignin biosynthesis (Weng and Chapple 2010). As shown in Fig. 3a, lignin content of CT areca nut slowly increased in the initial 20

days of storage at 13 °C, then sharply increased after that, with 22.69% increase within 2 months. Compared with CT, two HW groups significantly inhibited the accumulation of lignin content during the entire storage ($p < 0.05$). Among them, HW45 showed lower lignin content than HW50 (Fig. 3a). At day 60, lignin content of HW45 and HW50 were 13.14% and 14.33%, lowered the values by 15.9% and 14.71% in comparison to CT, respectively.

As illustrated in Fig. 3b–d, PAL, POD and CAD activ- ities in three groups of fresh areca nut all showed a pro- gressive increase within the early 30 days and then decreased until the end of cold storage. They reached a plateau at day 30, indicating that lignin synthesis was faster at this stage. The application of HW treatments signifi- cantly suppressed activities of related enzymes in areca nutrelative to CT ($p < 0.05$), among which, HW45 had the lowest enzyme activities in particular. For PAL, there were no significant differences between HW45 and HW50 throughout entire storage (Fig. 3b). PAL peak levels of HW45 and HW50 were the same 1.93 U/g, reduced about 17.9% compared with CT, while PAL levels at day 60 of HW45 and HW50 were 1.46 U/g and 1.51 U/g, reduced about 15.6% and 12.7% compared with CT, respectively. HW45 showed a significantly beneficial effect in inhibiting CAD activity compared with HW50 after 30 days of storage, while for POD, the inhibitory effect was significant after 40 days of storage. CAD peak level of HW45 was 1.10 U/g, lowered by 0.77 U/g in comparison to CT and by 0.22 U/g to HW50 (Fig. 3c). POD peak level of HW45 was 1.85 U/g, lowered by 0.87 U/g in comparison to CT and by 0.26 U/g to HW50 (Fig. 3d). At the end of storage phase, HW45 had the lowest PAL, CAD and POD activities, which were positively correlated with lignin content.

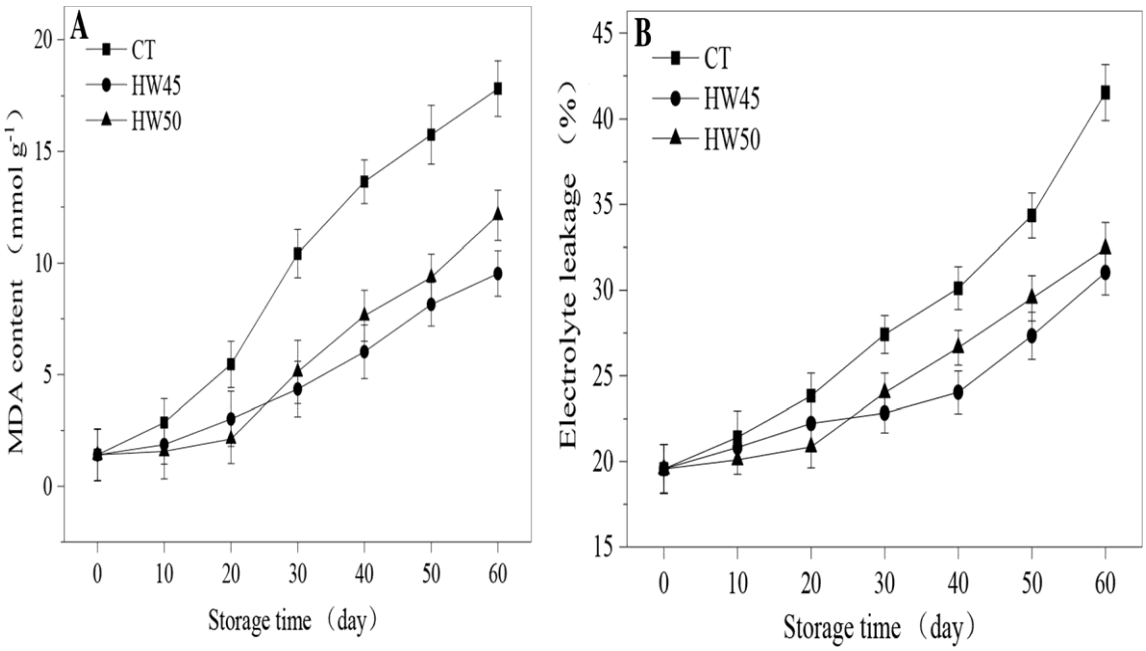

Fig. 2 Effect of short hot water treatment on MDA content (a) and electrolyte leakage (b) of areca nut during storage at 13 °C for 60 days. Vertical bars represent the standard deviation of the means

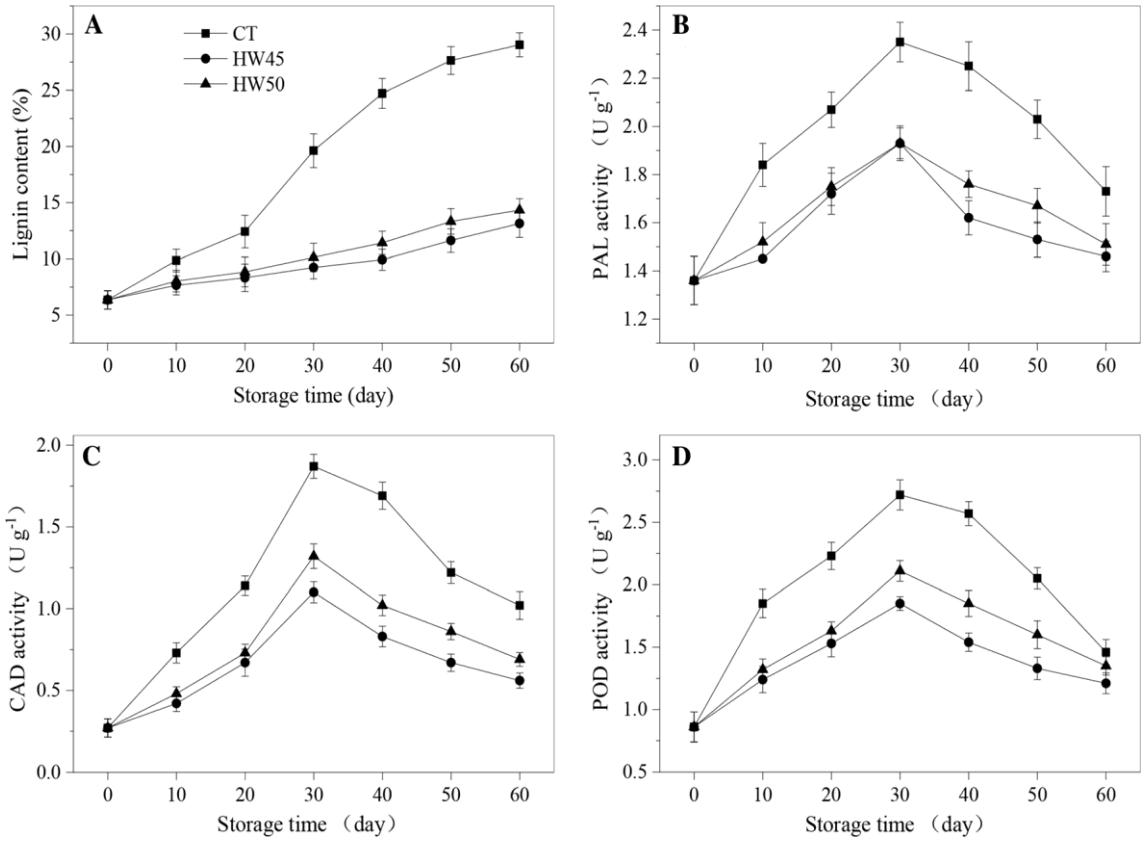

Fig. 3 Effect of short hot water treatment on lignin content (a), PAL (b), CAD (c) and POD (d) activities of areca nut during storage at 13 °C for60 days. Vertical bars represent the standard deviation of the means

PAL, CAD and POD are involved in physiological process of lignification and played important roles in cold- damaged tissues by participating in the synthesis, accu- mulation and oxidation cross-linking of lignin monomers, thus lower enzyme activities mean less tissue lignification and tissue damage. Heat treatment resulted in suppressing PAL activity in mango (Dang et al. 2008), suppressing CAD activity in mangosteen (Dangcham et al. 2008) and suppressing POD activity in water chestnut (Peng and Jiang 2010). The study of Cai et al. (2006) indicated that above three enzymes were positively associated with accumulation of lignin in loquat fruit. The study of Luo et al. (2012) indicated that 45 °C heat-treated bamboo shoots delayed the rise in PAL, CAD and POD activities involving in tissue lignification. In the current study, lignin content and related enzyme activities were suppressed in heat-treated areca nut. HW45 exhibited a beneficial effect at any time of cold storage on delaying the lignification process and further reducing the chilling injury symptoms of areca nut.

Total phenolic content

Oxidation of phenolic substances can promote the synthesis of lignin. The accumulation of lignin and the disruption of vacuoles by chilling injury in areca nut were associated with the loss of phenolics (Toor and Savage 2006). As the extension of storage time, we observed a reduction in total phenolic content under all treatments (Fig. 4), from 5.23 g/ 100 g (freshly harvested) to 2.86 g/100 g (CT, on the 60th day). In the first 20 days, there was insignificant difference among three groups. After that, HW45 exhibited a significantly higher content than CT (p \ 0.05). Meanwhile, HW50 had no significant effect at day 40 (p [0.05) but had significant effect at day 60 compared with CT (p \ 0.05). Till the end, total phenolic content in areca nut from high to low was HW45 (4.16 g/100 g) [HW50 (3.51 g/100 g) [CT (2.86 g/100 g), reduced by 20.5%, 32.9% and 45.3% compared with the initial value, respectively. Thus, the better treatment for maintaining total phenolics was HW45 in comparison, this may be due to the reduction of oxidase activity and the integrity of membrane structure by hot water dipping at optimal tem- perature (Nasef 2018).

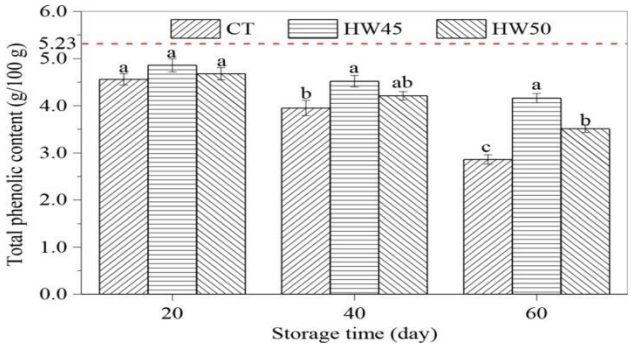

Fig. 4 Effect of short hot water treatment on total phenolic content of areca nut during storage at 13 °C for 60 days. Dotted line in the figure indicates the initial arecoline content of fresh areca nut (0.46%). Vertical bars represent the standard deviation of the means. Different letters indicate significant differences among treatments($p < 0.05$)

IV. CONCLUSION

As far as we know, this is the first report on the effect of HW treatment on CI and lignification of fresh areca nut during refrigerated storage. Application of HW dipping alleviated chilling injury of areca nut stored at 13 °C, maintained membrane integrity by inhibiting the increase of MDA content and cell membrane permeability. Although HW45 and HW50 could both improve chilling tolerance in areca nut compare with CT, HW45 showed more effective than HW50 not only in reducing CI index and membrane damage, but also in suppressing PAL, CAD and POD activities, preventing the synthesis of lignin and maintaining the total phenolic content. These results provide a safe and effective method for delay chilling injury and lignification of cold-stored fresh areca nut.

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