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Postharvest control of litchi (*Litchi chinensis* Sonn.) pericarp browning by cold storage at high relative humidity after enzyme-inhibiting treatments

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ABSTRACT

Enzyme inhibitors were studied as adjuvant treatments in the control of litchi pericarp browning by protection against desiccation during reefer transport. Various organic acids (acetic, malic, citric, and oxalic acid) and inorganic salts (NaCl, $CaCl_2$) were investigated for *in vitro* inhibitory effects on the peroxidases (POD) and phenol oxidases (including laccase) extracted from litchi pericarp. Promising inhibitors were tested on 'Hong Huey' litchi fruit for their capability to prevent pericarp browning during cold storage (21 d, 5 °C, 90% relative humidity) with and without foil wrapping. An enzyme assay had been optimized for measuring the *in vitro* activities of phenol oxidases toward (−)-epicatechin, being the natural phenolic key substrate in litchi. Phenol oxidase activities were chiefly pH-dependent and completely inactivated at pH ≤ 3.5 by the organic acids used for buffering, whereby chelating agents performed best, especially oxalic acid. POD activity was stable over wider pH and ionic strength ranges, with inhibition being maximal (84%) in 0.25 M oxalic acid buffer (pH 3.5). $CaCl_2$ (0.25 mol L^{-1}) decreased POD activity by 68%, while rising doses increased the initial lag phase up to 2.5 min. In contrast to these *in vitro* enzyme-inhibiting effects, postharvest fruit treatments with these phenol oxidase and peroxidase inhibitors did not improve color retention during cold fruit storage, but proved ineffective or even favored pericarp browning compared to the control fruit. Pericarp color retention was maximal (96–97%) throughout cold storage of fruit in gas-permeable but moisture-retaining foil bags for at least two weeks, whether the fruit had been dipped into cold water (control) or into citrate (25 mmol L^{-1}) solution. Consequently, pericarp color retention only required an intact pericarp at harvest and postharvest protection against desiccation. Preventing water loss through preservation of cell compartmentation thus proved to be crucial and sufficient for the control of enzymatic browning under reefer conditions.
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1. Introduction

Enzymatic browning of the pericarp is one of the key factors limiting the marketability of fresh litchi (*Litchi chinensis* Sonn.) fruit, because the rapid loss of its attractive red color often creates a false impression of spoilage (Reichel et al., 2010, 2013). Due to enzyme inhibition and other protective effects, sulfur dioxide fumigation is still the current practice to prevent litchi pericarp browning (Liang et al., 2012), but undesirable effects on fruit quality and health concerns have led to consumer rejection and legal restriction (Sivakumar et al., 2010). This has necessitated the search for alternative processes (Bhushan et al., 2015). Prevention of pericarp browning has been understood to require measures that reduce the pericarp pH, but chiefly slow pericarp dehydration and weight loss (Joas et al., 2005). The aim is to improve pericarp appearance and freshness during distribution in reefer containers (Ducamp-Collin et al., 2008; Wang et al., 2010; Shafique et al., 2016) used for long distance transport. However, according to our previous study (Reichel et al., 2013), the rather high relative humidity (RH) of 90% could not prevent the loss of pericarp

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moisture and thickness upon cold storage (5 °C) without additional measures. Consequently, slow desiccation of fruits lying close together resulted in dark brownish-red pericarp due to prolonged oxidation of (−)-epicatechin and co-oxidation of anthocyanins. More rapid water loss of exposed pericarp favored surface scurf formation, yielding unattractive light brown fruit that showed much better anthocyanin retention but greater losses of flavanol oligomers (Reichel et al., 2013). Notable residual activities of polyphenol oxidases (PPO) and peroxidases (POD), which were found in the pericarp of those fruit despite declining enzyme activities with ongoing desiccation, suggested the additional application of enzyme inhibitors and/or further measures to improve moisture retention.

Pericarp browning in litchi has mainly been ascribed to PPO (Jiang et al., 1997, 1999), showing the highest activity in the exocarp (Underhill and Critchley, 1995). Directly after harvest, PPO gene expression was induced by pericarp desiccation and rapidly upregulated until reaching an early maximum, in parallel to PPO activity (Wang et al., 2014). The greater the water loss or the storage temperature, the higher these maxima were found to be. High affinity to (−)-epicatechin, being the natural substrate due to its prevalence in the pericarp, was shown for litchi PPO (Liu et al., 2007; Reichel et al., 2011) and a recently described laccase (LAC) that was mainly found in vacuoles of mesocarp cells in intact litchi pericarp (Fang et al., 2015). While LAC gene expression was always notably high, LAC was secreted to the extracellular space after beginning pericarp browning and significantly contributed to the overall polyphenol oxidizing activity in litchi pericarp (Fang et al., 2015). Litchi POD, being specific for H_2O_2, accept a wide range of co-substrates and are thus nonspecifically involved in postharvest browning, chiefly through stress-induced surface scurf formation (Reichel et al., 2011, 2013). Litchi exocarp has been shown to have a dense palisade-like layer of elongated suberized cells directly below the epidermis (Riederer et al., 2015).

Dipping into organic-acid solutions is common to prevent enzymatic browning of fresh or minimally processed fruits and vegetables (Buta and Moline, 2001; Son et al., 2001). While acids inhibit enzyme proteins nonspecifically due to pH effects, chelating agents among the carboxylic acids additionally block central or stabilizing ions of their active sites (Yoruk and Marshall, 2003; Jiang et al., 2008). Likewise, effects of buffer concentration on conformation of the enzyme protein due to the resultant ionic strengths have to be considered (Laurenti et al., 2000). The latter also plays a role in the application of salt solutions. Halides being known to inhibit LAC (Morozova et al., 2007) have also been discussed as PPO inhibitors (Son et al., 2001; Liu et al., 2007). Their cations, such as Na^+ or Ca^{2+}, may also have an effect on the structural stability and activity of both PPO and POD (Rasmussen et al., 1998; Sun et al., 2008). The inhibitory effects of organic acids and halide salts described for isolated litchi pericarp PPO and POD (Gong and Tian, 2002; Jiang et al., 1997, 1999; Sun et al., 2008) should still be verified *in vitro* for crude enzyme extracts in the presence of the natural substrate for a deeper understanding of the potential that these inhibitors can have in view of the coacting browning enzymes *in vivo*.

High doses of organic acids in combination with chitosan coating (Joas et al., 2005) or Ca^{2+} application (Wang et al., 2010) were shown to support color retention efficiently, but they could only slow browning during cold storage for 8–10 d, which might be too short for long-distance transport. Concurrently, pericarp integrity was impaired, because acid impregnation required wetting (Wang et al., 2010) or pretreatments enhancing pericarp porosity (Caro and Joas, 2005). Inconsistent responses to treatments, including varietal differences (Ducamp-Collin et al., 2008), and the viscosity of coating solutions (Caro and Joas, 2005) have turned out to be other limiting factors. However, for acidic calcium sulfate (Wang et al., 2010) and $CaCl_2$ dipping (Kou et al., 2015), the slower activity decline of various oxygen-scavenging enzymes was found to contribute to browning prevention, thus suggesting more complex protective mechanisms.

Thus, the aim of this follow-up study of our work (Reichel et al., 2013) was to improve pericarp color retention upon cold and humid storage (5 °C, 90% RH), exploring adjuvant fruit treatments with moderate doses of organic acids and chloride salts alone and in combination with fruit packaging in gas-permeable but moisture-retaining foil bags. The inhibitor doses should preferably not enhance pericarp porosity, but become effective locally, where cell compartmentation was lost. Concurrent application of surfactants was thus to be avoided. The *in vitro* inhibitory effects of the organic acids and chloride salts on POD and the litchi phenol oxidases (PPO+LAC) were to be verified for crude enzyme extracts in the presence of the natural substrate (−)-epicatechin, considering the impact of pH, inhibitor concentration, and ionic strength.

2. Materials and methods

2.1. Chemicals used

Unless otherwise stated, reagents were from VWR International (Darmstadt, Germany). (−)-Epicatechin, polyvinylpolypyrrolidone (PVPP), and tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the *in vivo* trials, tripotassium citrate monohydrate and oxalic acid dihydrate were supplied by Ajax Finechem (Auckland, New Zealand) and sodium chloride by Lab-Scan (Bangkok, Thailand). Deionized water was used throughout.

2.2. Fruit material under study

The *in vitro* tests (*cf.* 2.3) required a large amount of homogenous *Litchi chinensis* Sonn. pericarp powder having high activities of browning enzymes. It was obtained from 'Chacapat' fruit, because this cultivar is known for its large fruit, which can yield a considerable amount of pericarp per unit due to notable pericarp thickness, while the pericarp is rather unsusceptible to browning directly after harvest despite sufficiently high enzyme activities (Reichel et al., 2010, 2013). Fully ripe 'Chacapat' fruit, which had a mean size of 37.7 ± 0.4 g, pericarp color values of L^* = 38.5 ± 0.5, a^* = 33.0 ± 0.6, and b^* = 26.2 ± 0.4, and a litchi maturity index of 6.5 according to Reichel et al. (2010) (*LMI*, *cf.* Eq. (3) in 2.4.2), were bought on the local fresh-fruit market in Chiang Mai, northern Thailand, (02 June 2008) maximally 10 h after they had been harvested in Fang (~125 km north of Chiang Mai) and subsequently distributed in traditional baskets, which were lined with paper to protect the fruit against desiccation. The fruit were directly brought to the laboratory at Chiang Mai University in Chiang Mai, debranched, and shock-frozen in liquid nitrogen. The pericarp removed from 40 fruits was kept at −80 °C until lyophilization. Afterwards, it was vacuum-packed and sent deep-frozen (−40 °C) to Hohenheim University, Stuttgart, Germany. There, it was finely ground, vacuum-packed, and stored at −80 °C until use.

For the *in vivo* trials (*cf.* 2.4) of this follow-up study, fruit of the commercially most important Thai litchi cultivar 'Hong Huey' were used exemplarily, as before (Reichel et al., 2013). 'Hong Huey' and 'Chacapat' fruit were shown to be comparable in terms of the PPO and POD activities directly after harvest, but fruit of the former cultivar are more prone to rapid browning and surface scurf formation due to their specific pericarp morphology and polyphenol composition (Reichel et al., 2013). To ensure homogenous fruit quality, the 'Hong Huey' fruit were picked from two adjacent trees in a research orchard in Mae Sa Mai, northern Thailand (18.8° North

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