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Changes of reactive oxygen species and scavenging enzymes of persimmon fruit treated with CO_2 deastringency and the effect of hydroxyl radicals on breakdown of cell wall polysaccharides *in vitro*

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ABSTRACT

Few studies have examined the non-enzymatic disassembly of cell walls in persimmon fruits. The objective of the study was to investigate the changes in the levels of reactive oxygen species and in the activity of antioxidative enzymes and, to evaluate the effect of •OH on the *in vitro* breakdown of cell wall polysaccharides of persimmon fruit at different ripening stages. After harvest, persimmon (*Diospyros kaki*, 'Mopan') fruits were treated with 95% CO_2 at room temperature, and the results showed that fruit firmness rapidly decreased and the respiratory and ethylene release rates increased. At the same time, hydroxyl radicals (•OH) and O_2^- rapidly accumulated, and the activities of related antioxidant enzymes such as dismutase peroxidase, catalase and peroxidase superoxide, also changed. The effects of •OH induced by the Fenton reaction on the cell wall materials *in vitro* were investigated; the water soluble pectin and CDTA-soluble pectin contents increased, and Na_2CO_3-soluble pectin content decreased. Due to the increasing levels of reactive oxygen species, especially •OH, during persimmon fruit softening, and the *in vitro* effect of •OH on the scission of cell wall polysaccharides, we postulated that •OH may be involved in persimmon fruit softening.

1. Introduction

Persimmon (*Diospyros kaki* L.) originated from China and has a long history of cultivation. All persimmon cultivars are classified as either pollination-constant and non-astringent (PCNA), pollination variant and non-astringent (PVNA), pollination-constant and astringent (PCA) or pollination-variant and astringent (PVA) (Sugiura, 2005). For the PCA and PVA cultivars, artificial deastringency treatments are required for fruit consumption, due to their high contents of soluble tannins. The application of CO_2 at levels higher than 95% for 24 h at room temperature has been established as the optimal treatment conditions to ensure the removal of astringency (Besada et al., 2010a; Ogawa et al., 2011; Yamada et al., 2002). The effectiveness of the CO_2 treatment in removing astringency is based on the insolubilization of soluble tannins by mediating the effects of acetaldehyde generated during anaerobic respiration. However, after exposure to high CO_2 atmosphere, persimmon fruits soften rapidly, reducing their shelf life (Harima et al., 2003; Salvador et al., 2007).

Fruit softening is primarily correlated with cell wall modification, particularly due to solubilization and depolymerization of pectin and hemicellulose. Additionally, due to the accumulation of solutes in the cell wall space, turgor pressure is reduced and leads to fruit softening (Brummell, 2006). Cell wall-degrading enzymes have been considered the important factors involved in the softening mechanisms of fruit. However, other studies have shown that non-enzymatic factors can also influence cell wall components (*e.g.*, pectin and xyloglucan), resulting in fruit ripening and softening (Cheng et al., 2008; Fry and Dumville, 2003; Müller et al., 2009).

Reactive oxygen species (ROS) (*e.g.*, •OH, $^1O_2$, $•O_2^-$ and H_2O_2) are normal products of plant metabolism. Plants produce many reactive oxygen species even under normal physiological conditions and the rate of reactive oxygen species production increases when the plants are stressed. Under normal conditions, the activity of ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), maintain their normal catalytic states.

Abbreviation: •OH, hydroxyl radicals; APX, ascorbate peroxidase; CAT, catalase; CO_3-SP, Na_2CO_3 soluble pectin; CSP, CDTA soluble pectin; CWM, cell wall materials; H_2O_2, hydrogen peroxide; HC, hemicellulose; HC1, easily soluble cellulose; HC2, difficult soluble cellulose; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; WSP, water soluble pectin.

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In studies on maize and soybean, •OH was found to be capable of cleaving cell wall polysaccharides *via* site-specific reaction, which led to the loosening and elongation of cell walls of living coleoptiles and hypocotyls (Schopfer, 2001). Fry et al. (2001) also found that cell wall polysaccharides will be attacked by accumulated •OH in pear fruit softening process, indicating that •OH affected fruit softening by enhancing the non-enzymatic mechanisms of cell wall dissolution. •OH has been shown to increase the water-soluble uronic acid and total sugar contents in cell walls of banana and longan fruits (Cheng et al., 2008; Duan et al., 2011).

Fruit treated with high concentrations of CO_2 are characterized by hypoxic stress that induces the accumulation of ROS (Novillo et al., 2014a,b). However, the relationship between ROS accumulation and fruit softening, and whether •OH has a role in cell wall modification leading to rapid softening in persimmon fruits remains unknown. Therefore, to explore the non-enzymatic mechanism of fruit softening, this work studied the changes in the levels of reactive oxygen species and antioxidases, and the role of •OH on the cell walls of persimmon fruits *in vitro*.

2. Materials and methods

2.1. Plant materials and experimental arrangement

Persimmon fruits (*Diospyros kaki*, 'Mopan') were harvested from an orchard in the Fangshan District of Beijing. The fruit color index was approximately 13.3 and was calculated according to the equation, Color index = 1000 $a/(L \times b)$, where the values of L, a and b were measured from the top of persimmon fruit with a Colorimeter (CR-410, Konica Minolta, Japan). Fruits (approximately 230 g) were selected for shape, color and size uniformity, while any blemished or diseased fruits were discarded. The experimental groups included 2 treatments: (1) Control: fruits were stored at room temperature without any treatment; (2) CO_2-treated: fruits were stored at room temperature in sealed containers with 95% CO_2 for 24 h. In the CO_2-treated group, the fruits were peeled and the pulps were stored in a freezer at −80 °C for the later determination of physiological and biochemical indices. In the control group, the fruits were sampled to measure changes in fruit firmness and other measurements as the CO_2-treated group. At each sampling point, 3 replicate measurements for each of 5 fruits.

2.2. Firmness determination

After cutting off the pericarp sections on the two opposite sides of the equitorial section of each persimmon fruit, a firmness tester (FT327, Breuzzi, Italy) with an 8-mm diameter probe was used to measure the fruit firmness. The data collected were the means of 3 replicates for each of 5 fruits, and are expressed as kg cm^{-2}.

2.3. Respiration rate determination

Respiratory rates of the sample persimmon fruits were measured by a respiratory meter (FQ-3150H, Beijing JunFang Institute, China), and the results were expressed as mg(CO_2) kg^{-1} h^{-1}.

2.4. Ethylene release rate determination

Five fruits were placed into a vacuum drier. After 2 h, 2 mL of the headspace volume were sampled and injected into a gas chromatograph (Shimadzu GC-17A, Japan) to measure the amount of ethylene produced. The chromatography conditions were: nitrogen gas flow rate of 50 mL/min, hydrogen flow rate of 70 mL/min, air flow rate of 500 mL/min, inlet sample temperature of 120 °C, column temperature of 55 °C, and detector temperature of 160 °C. The results were expressed as μL kg^{-1} h^{-1}.

2.5. Determination of •OH, $•O_2^-$ and H_2O_2

The •OH content was estimated according to a method described by Cheng et al. (2008) with some modification. First, 2 g of fruit tissue was homogenized in liquid nitrogen with 10 mL of 0.02 M phosphate buffer (pH 6.0, containing 20 mM 2-deoxy-D-ribose). The solution was then incubated with shaking for 12 h at 25 °C in darkness. The suspension was centrifuged (MIKRO 220R, Hettich, Germany) for 15 min at 12,000 rpm, and the supernatant was collected. A mixture of 1 mL of the supernatant, 1 mL of 1.0% (w/v) 2-thiobarbituric acid (in 0.05 M NaOH) and 1 mL of 2.8% (w/v) trichloroacetic acid was prepared. After heating in boiling water for exactly 15 min, the mixture was cooled in tap water and clarified by centrifugation at 12,000 rpm for 15 min. The reaction product was fluorometrically measured at 532 nm and 353 nm excitation and emission wavelengths, respectively, against reagent blanks with a spectrophotometer (F-4500, Hitachi, Japan). The amount of •OH was expressed in relative fluorescence intensity.

The $•O_2^-$ content was estimated according to a method described by Elstner (1976) with some modification. First, 2 g of fruit tissue was homogenized with 5 mL of 50 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. A reaction mixture consisting of 1 mL of 1 mM hydroxyammonium chloride and 1 mL of supernatant was prepared and incubated at 25 °C for 1 h. A color change was observed after the addition of 1 mL of 17 mM sulfanilic acid and 1 mL of 7 mM 1-naphthylamine for 20 min at 25 °C. The specific absorption at 530 nm was determined with a UV/vis spectrophotometer (UNICO UV-2800AH, Unico Instrument Co., Ltd., Shanghai, China). Sodium nitrite was used as a standard to calculate the $•O_2^-$ content. The results are expressed as nmol g^{-1} FW.

The hydrogen peroxide (H_2O_2) levels were determined according to a method described by Velikova et al. (2000) with some modifications. First, 2 g of fruit tissue was homogenized in liquid nitrogen with 5 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. A mixture, containing 0.5 mL of 100 mM phosphate buffer (pH 7.0), 1 mL of 1 M KI and 0.5 mL of the supernatant, was prepared. The absorbance of the mixture was measured at 390 nm. The H_2O_2 levels were determined using a standard curve and are expressed as nmol g^{-1} FW.

2.6. Measurement of scavenging enzyme activity of reactive oxygen

Two grams of frozen tissue were homogenized with 10 mL of 50 mM cold phosphate buffer (pH 7.8, containing 1 mM dithiothreitol, 1% w/v polyvinyl-pyrrolidone and 1 mM ethylene diamine tetraacetic acid). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected to measure enzyme activity.

For SOD determination, a reaction mixture consisting of 3 mL of 50 mM phosphate buffer (pH 7.8), 0.5 mL of 130 mM methionine, 0.5 mL of 750 μM nitroblue tetrazolium, 0.5 mL of 0.1 mM $EDTANa_2$, 0.5 mL of distilled water, 0.2 mL of crude persimmon extract and 0.5 mL of 20 μM riboflavin were combined. After a 20 min exposure, the absorbance was measured at 560 nm. One SOD unit is defined as the amount of enzyme that inhibited the rate of NBT reduction by 50% of the control.

The POD activity was determined according to a method described by Srivastava and Dwived (2000) with some modifications. A reaction mixture of 1.7 mL of 50 mM phosphate buffer (pH 7.0), 1 mL of 50 mM guaiacol, 0.1 mL of 0.5% H_2O_2 and 0.2 mL of POD