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Changes in enzymatic activity, accumulation of proteins and softening of persimmon (*Diospyros kaki* Thunb.) flesh as a function of pre-cooling acclimatization

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ABSTRACT

One of the major causes of 'Fuyu' persimmon loss after cold storage (CS) is the breakdown of its flesh, which results in the production of a translucent fruit (a *water-soaked* fruit). It is believed that the cause of this disturbance is linked to disorganization of the cytoskelet and endomembrane system, which changes the synthesis and transport of proteins and metabolites, resulting in incomplete ripening. To test this hypothesis, 'Fuyu' persimmon was subjected to three different postharvest treatments (T): Control – harvested and kept at 23 ± 3 °C and relative humidity (RH) of 85 ± 5% (room temperature, RT) for 12 days, T1 – harvested and kept under cold storage (CS) (1 ± 1 °C and RH of 85 ± 5%) for 30 days followed by RT storage for 2 days, T2 – kept under RT for 2 days (acclimatization) followed by CS for 30 days. Control and T2 resulted in fruit with decreased flesh firmness (FF), and increased soluble solids (SS) and ascorbic acid (AA) contents. In these fruit the activity of endo-1,4-ß-glucanase (endo-1,4-ß-gluc), pectin methylesterase (PME), polygalacturonase (PG) and ß-galactosidase (ß-gal) increased. T1 resulted in translucent fruit with decreased FF, without any enzymatic activity changes, probably due to the physical disruption of the cytoskeleton. Further, there was an increased content of proteins corresponding to expansins in fruit kept under Control and T2 conditions, which suggests that these conditions do contribute to the synthesis and/or transport of proteins involved in the process of solubilization of the cell wall. In these fruit, there was also a major accumulation of gene transcripts corresponding to heat shock proteins (HSPs) of organelles related to endomembrane, which suggests participation of these genes in the prevention of damage caused by cold conditions. These data proved the hypotheses that acclimatization contributes to the expression of HSPs, and synthesis and transportat of proteins involved in the solubilization of the cell wall. The expression of these genes results in the normal ripening of the persimmon, as confirmed by the evolution of ethylene production.

1. Introduction

Among the methods used to maximize the storage period of 'Fuyu' persimmon, cold storage (CS), storage in a modified atmosphere (MA) and storage under a controlled atmosphere (CA) have been suggested. With CS at 0–1 °C, damage caused by cold is often observed and is characterized by the breakdown of flesh 1–2 days after its removal from the cold chamber (George et al., 1997; Krammes et al., 2006).

During persimmon ripening, softening is caused by the activity of the hydrolytic enzymes of the cell wall and by proteins that facilitate these activities such as expansins (Nakano et al., 2003). However, there is little information regarding this process or regarding the enzymatic behavior after CS or after acclimatization followed by CS. In other species, enzymes that are more involved with the metabolism of the cell wall, and thus responsible for the solubility of its macromolecules, include endo-1,4-ß-glucanase (endo-1,4-ß-gluc), pectin methylesterase (PME), polygalacturonase (PG) and ß-galactosidase (ß-gal) (Brummell, 2006; Nakamura et al., 2003). Moreover, it is known that adequate ripening is the result of the evolution of ethylene production and the maintenance of the endomembrane system, which is responsible for the transportation of proteins including ones involved in

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cell wall solubilization (Rose et al., 1998). Several reports describe that pre-storage treatments such as the use of growth regulators (Krammes et al., 2006; Pegoraro et al., 2010) or physical treatments (Boston et al., 1996; Sabehat et al., 1998; Sun et al., 2010) can contribute to the preservation of cellular homeostasis and prevent damage by cold storage.

The goal of this study was to identify the mechanisms involved in the sudden loss of flesh firmness (FF) soon after removing the fruit from the cold and to verify the effect of acclimatization prior to CS in the prevention of chilling injury. Acclimatization is the condition that fruit are conditioned at temperature lower or higher than room temperature (RT), before CS. From exploratory studies in with several temperature combinations (15–50 °C in warmed water or air) and multiple time periods (from 2 min to 4 days) were tested we found that the optimal acclimatization for prevention of chilling injury in persimmon fruit 'Fuyu' was 23 ± 3 °C for 2 days. In this case the acclimatization corresponded to fruit conditioning at RT.

To achieve this, we both related enzymatic activities with FF loss and evaluated the content of ascorbic acid (AA), which are indicators of the evolution of the ripening processes of fruit that contain significant concentrations of pectins (Gomez and Lajolo, 2008). During normal ripening of fruit, it is expected that solubilization of pectins will cause an increase in AA (Barata-Soares et al., 2004; Gomez and Lajolo, 2008). Moreover, it is believed that delaying CS of persimmon could stimulate activity of HSPs genes (Sabehat et al., 1998; Sun et al., 2010), thus protecting the synthesis and transport of proteins and organelle homeostasis, which would result in adequate ripening.

2. Materials and methods

2.1. Plant material and experimental design

Persimmon (*Diospyros kaki*, cv. Fuyu) fruit from a commercial orchard located in the municipality of Canguçu/RS, South of Brazil, were harvested in 2010 when the epicarp were orange in color. The average values for L*, a* and b* were 56.3, 34.7 and 49.1, respectively. The Hue angle was 54.7, and the SS content was 15.2%. The FF average was 82.6 Newton (N), and the average fruit weight was 256.5 g. Prior to treatment application, the fruit were randomly divided into 42 experimental units with 10 persimmons in each group. Then, each treatment was applied to a random fruit, and all experiments were repeated in triplicate. Persimmons were stored under the following conditions:

Control: 23 ± 3 °C and RH of 85 ± 5% (room temperature, RT) for 12 days;
T1: 1 ± 1 °C and RH of 85 ± 5% (cold storage, CS) for 30 days, followed by storage at 23 ± 3 °C and RH of 85 ± 5% for 2 days;
T2: 23 ± 3 °C and RH of 85 ± 5% for 2 days (acclimatization) followed by the same conditions described for T1 for 30 days.

For storage under Control, the first sample collection was performed soon after harvest, and thereafter at intervals of 2 days until the twelfth day of storage. A total of seven additional sample collections were made. On the other hand, T1 and T2 treated fruit were analyzed after 30 days under CS and then at 12 h interval for 2 days. The FF, SS, AA content, enzymatic activities of endo-1,4-ß-gluc, PME, PG and ß-gal and the immunodetection of expansins were measured. The accumulation of transcripts of the genes corresponding to expansins (Exp3) and HSPs were also evaluated, via RT-qPCR. There were multiple HSP genes examined, associated with different regions of the cell: cytosol (Ps-CI sHSP1 and Ps-CI sHSP2), lumen of the endoplasmic reticulum (HSP40-1er), chloroplasts (HSP70 chl and HSP17.8 chl) and mitochondria (HSP60 mit and HSP26.5 mit). The RT-qPCR of the Control was analyzed at 0, 24, 48 and 72 h after harvesting. The Exp3 was also evaluated. The RT-qPCR results of the T1 and T2 samples were analyzed 0, 12, 24, 36 and 48 h after removal from the cold chamber. Variance analysis was performed on the data, and the least significant difference (LSD) and Tukey test ($p < 0.05$) among treatments were determined using the Winstat program (Machado and Conceição, 2003).

2.2. Physico-chemical analysis

The flesh firmness (FF) was measured using a manual penetrometer, with a tip of 11 mm, and the results were reported in Newtons (N). The content of soluble solids (SS) was determined by refractometry, with results expressed as percentages (m/m). The content of ascorbic acid (AA) was determined using the extraction method of Vinci et al. (1995) in which 3 g of fruit is added to 5 mL of metaphosphoric acid at 4.5% (v/v) for 1 h in a light-proofed flask. Then, the samples were filled to a volume of 10 mL with ultra-pure water and homogenized. After homogenization, two filtrations with Whatman no. 3 paper were performed. The filtrate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was used for analysis in a Shimadzu chromatograph equipped with the following modules: solvent mixer LC-10AT$_{VP}$, degasser FCV-10A$_{VP}$, pump reodine DGU-14$_{A}$, control system SCL-10$^{A}_{VP}$, column oven CTO-10AS$_{VP}$ and autosampler SIL-10AF. A reverse phase analytical separation column, Nova-Pak C_{18} (3.9 cm × 150 mm × 4 μm), was used. The mobile phase consisted of two solvents: (A) 0.1% acetic acid solution and (B) methanol. A total of 10 μL of sample was injected at a rate of 0.8 mL min^{-1} at a column temperature that was maintained between 25 and 40 °C. The elution time of the sample was 30 min. Compound identification was obtained from the absorption spectrum in the UV–visible using a model SPD-10AV$_{VP}$ at the wavelength of 254 nm. The recovery rate of the method was determined to be 93%, and the data were acquired and processed using the software Class-VP. Results were expressed as mg of AA per 100 g of fresh material.

2.3. Enzymatic activities

To determine enzymatic activities, methods based on the procedures previously calibrated for strawberries (Martínez and Civello, 2008) and peaches (Girardi et al., 2005) were used with slight modifications. In these studies, the flesh was frozen in liquid nitrogen and stored at either −85 °C or −20 °C prior to analysis. For persimmon, it was found that using the exact procedure of those previous studies led to poor reproducibility in the enzymatic activities measurements. Better results were obtained by adding the extraction buffers (3 mL g^{-1} of flesh) prior to storage of samples at −85 °C. Good separation of the supernatant and high reproducibility were obtained using this modification.

The determination of the enzymatic activities of endo-1,4-ß-gluc was performed with a sample of frozen persimmons and its extraction buffer containing 50 mM sodium acetate and 10 g L^{-1} polyvinylpolypyrrolidone (PVPP) at pH 6.0 (buffer A). After disintegration of the frozen flesh in the presence of the buffer at a volume ratio of 1:3, the suspension was centrifuged at 20,000 × *g* at 4 °C for 30 min and the supernatant was discarded. The precipitate was resuspended in 15 mL of buffer A, homogenized for 30 min and centrifuged again. The precipitate from this stage was resuspended in 15 mL of buffer containing 50 mM sodium acetate, 1 M of NaCl and 10 g L^{-1} PVPP at pH 6.0 (buffer B) and kept under agitation at 4 °C for 1 h. After this period, the material was centrifuged at 20,000 × *g* at 4 °C for 1 h. For each analysis repetition of the enzyme assay, 1.5 mL of supernatant was added to 0.5 mL of buffer B with 0.5% (m/v) of carboxymethylcellulose. The mixture was then incubated at 30 °C,