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Phytochemical content, antioxidants and cell wall metabolism of two loquat (*Eriobotrya japonica*) cultivars under different storage regimes

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ABSTRACT

Changes in quality, phytochemical content and cell wall metabolism of two loquat cultivars (*Eriobotrya japonica* cvs. 'Morphitiki', 'Karantoki') under different storage regimes were studied. The fruit were harvested at commercial maturity stage and analyzed after 1, 3, 5, 7, and 11 days maintenance at room temperature (RT, ~20 °C) or after cold storage (14 days at 4 °C) and additional ripening at RT for 1, 3 and 5 days, respectively. Compositional analysis revealed substantial cultivar differences; the 'Morphitiki' fruit was more acidic and showed higher contents of total phenolics, flavonoids and hydroxycinnamic acid-derivatives as well as greater antioxidant potency. Although firmness did not change markedly during storage, the cell wall exhibited extensive remodeling. Greater changes were observed in the pectin backbones than in polyuronide side chains and cross-linking glycans. Polygalacturonase (PG) showed better association with cell wall solubilization at RT than the enzymes involved in arabinan or galactan disassembly. During postharvest ripening after harvest, 'Karantoki' showed more extensive pectin solubilization than 'Morphitiki'. Interestingly, cold storage inhibited the cell wall disassembly in 'Karantoki' but not in 'Morphitiki', suggesting that the cultivars may differ in their susceptibility to chilling-related wall disorders. Low temperature-induced alterations in wall disassembly may impact juice and phytochemical release upon consumption.

1. Introduction

Loquat (*Eriobotrya japonica*) is a subtropical, evergreen fruit tree that blooms in fall and early winter, and ripens in the spring (Badenes et al., 2013), a period that few fleshy fruits are offered in the fresh market. Due to its unusual phenology, reverse to that of the well-known temperate fruit crops, growers of early-harvested loquats can obtain high prices in the market (Pinillos, Hueso, Marcon, Jose, & Cuevas, 2011). Loquat can be sorted into yellow-, orange-, red- and white-fleshed cultivars, mainly related to the capacity of fruit to accumulate carotenoids. It is regarded as a non-climacteric fruit type regarding its ripening pattern; however a climacteric-type increase in ethylene production and respiration rate during loquat developmental stages has been observed (Jiang et al., 2011).

Once regarded as a low value crop, indigenous in China, the interest in loquat commercial production has risen. The fruit is characterized by a refreshing flavor (Xu & Chen, 2011) and worldwide interest has fostered development of new breeding programs with the aim to deliver loquat cultivars of premium quality (Badenes et al., 2013). Only recently, a metabolomic approach was employed, offering new clues about on-tree ripening-related changes in loquat cultivars with special reference to aroma (Besada, Salvador, Sdiri, Gil, & Granell, 2013).

Different to other fruit species that may be destined for the industry, loquats are almost exclusively consumed fresh (Ding, Chachin, Ueda, Imahori, & Wang, 2001). Fresh loquat is highly perishable and low temperature storage is recommended to prevent deterioration. However, loquat is considered as a sensitive commodity to low-temperature storage (Cai et al., 2006a,b), thus incidence of chilling injury (CI) symptoms after extended cold storage periods reduce fruit market life and consumers' acceptance (Cai, Cao, Yang, & Zheng, 2011). CI symptoms have been monitored as tissue leatherness, loss of flavor and taste, browning, reduced juiciness and increased ion leakage from the skin tissue, as well as hardening of fruit flesh (Cai et al., 2006b; Cao, Zheng, Wang, Jin, & Rui, 2009a; Cao, Zheng, Wang, Rui, & Tang, 2009b; Cao, Zheng, Yang, Wang, & Rui, 2009c).

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Compositional changes and postharvest properties of some loquat cultivars have been characterized over the recent years (Cai, Xu, Li, Ferguson, & Chen, 2006c; Cai et al., 2006a,b; Cao et al., 2009a,b). However, most commercial loquat cultivars remain unexplored in major quality attributes, phytochemical composition, ripening behavior and storage response. Therefore, it is not clear the extent of varietal differences in terms of organoleptic and nutritional quality as well as in the susceptibility to physiological disorders after prolonged exposure at chilling temperatures. In this comparative study, the changes in quality attributes, phytochemical and cell wall compositional properties of two loquat cultivars after storage at chilling and non-chilling temperatures were investigated.

2. Materials and methods

2.1. Fruit material and storage conditions

The fruit from two loquat cultivars (*Eriobotrya japonica*, cvs. 'Morphitiki', 'Karantoki') were harvested from a commercial orchard (Episkopi, Lemesos, Cyprus) based on fruit size and external color, typical for each cultivar. Management system included the covering of the orchard with plastic trays after pollination until harvest. After elimination of defective samples, the fruit were separated into 8 lots of 30 fruits of uniform weight and size per cultivar. The fruit were either maintained at room temperature (RT, ~20 °C) for 1, 3, 5, 7, and 11 days, respectively or cold stored (14 days at 4 °C) and subsequently transferred at RT for 1, 3 and 5 days, respectively.

At each storage time, the fruit were separated into three 10-fruit sub-lots and initially were used to determine weight loss, tissue firmness, soluble solids content (SSC) and titratable acidity (TA). Subsequently, the fruit material was cut into wedge-shaped slices and flesh tissue was immediately frozen in liquid nitrogen and stored at −20 °C until analysis. Such material was used for the phytochemical analysis, preparation of cell wall material and determination of cell wall modifying enzymes, as described in the following sections.

2.2. Quality attributes

The weight loss (%) was calculated on 30 individual fruits per cultivar and storage time considered. Tissue firmness was measured as described elsewhere (Cao, Zheng, Wang, Rui, & Tang, 2010), at two points of the equatorial region of 30 fruit from each replicate (skin removed) with a texture analyzer (TA.XT *plus*, Stable Micro Systems, Surrey, U.K.), using a 5 mm diameter probe at a speed of 1 mm s^{-1}, the penetration depth was 5 mm.

Flesh tissue, held at −20 °C until needed, was ground and the homogenate centrifuged at 10,000×*g* for 10 min. The supernatant from 3 replicates of 10 fruit each was used for the determination of SSC and TA. Soluble solids content was measured in a portable digital refractometer (DR103L, Sun Instruments Corp. USA) and TA was determined by potentiometric titration with 0.1 mol L^{-1} NaOH up to pH 8.1, using 5 mL juice aliquots taken to a final volume of 50 mL with deionized water. The measurements were carried out using a DL22 Mettler Toledo titrator (Mettler-Toledo, Inc., Columbus, Ohio, USA) and results expressed as percentage of malic acid. The SSC/TA ratio was also calculated.

2.3. Phytochemical analysis

Five grams of flesh tissue from each replicate was homogenized with 15 mL of 950 mL L^{-1} cold ethanol and centrifuged at 10,000×*g* for 10 min. The pellet was then re-extracted with 10 mL of 800 mL L^{-1} cold ethanol. The supernatants were combined to make a final volume of 25 mL (Cao et al., 2009b).

The determination and classification of phenolic content was performed according to Obied, Allen, Bedgood, Prenzler, and Robards (2005), this protocol allows the simultaneous determination of total phenolics, hydroxycinnamic acid derivatives and flavonols. Briefly, 1 mL of each fruit extract was mixed with 1 mL 0.1% (v/v) HCl–ethanol solution and 8 mL 2% (v/v) HCl–ethanol solution into a 10 mL volumetric flask. The absorbance was measured at 280 nm to determine total phenolics using gallic acid as standard, at 320 nm to determine hydroxycinnamic acid derivatives using caffeic acid as standard, and at 360 nm to estimate flavonols using rutin as standard.

The carotenoid content was monitored as elsewhere described (Cao et al., 2009c). The absorbance of ethanolic extracts was read at 665, 649 and 470 nm and the carotenoid content was calculated according to the equations for ethanol solvent and expressed as mg 100 g^{-1} fresh weight (FW).

2.4. Determination of antioxidant activity by ferric reducing antioxidant power (FRAP)

A sample containing 3 mL of freshly prepared FRAP solution (0.3 mol L^{-1} acetate buffer (pH 3.6) containing 10 mmol L^{-1} 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) and 40 mmol L^{-1} $FeCl_3.10H_2O$) and 100 μL of fruit extract was incubated at 37 °C for 4 min and the absorbance was measured at 593 nm. A standard curve of L-ascorbic acid (AsA) was prepared and results were expressed as μmol AsA 100 g^{-1} FW (Goulas & Manganaris, 2011).

2.5. Determination of DPPH· scavenging activity

Two mL of each fruit extract were mixed with 1 mL of 0.3 mmol L^{-1} solution of 2,2-diphenyl-2-picrylhydrazyl (DPPH·) in methanol, incubated in the dark for 30 min and the absorbance of the mixture was monitored at 517 nm. Different concentrations of each sample were tested and the % of free radical scavenging activity was determined by the following equation: % scavenging activity = 100 – [(Abs of sample – Abs of blank) 100/Abs of control]. EC_{50} values are referred to the extract concentration (mg mL^{-1}) required for the 50% of antioxidant activity (Goulas & Manganaris, 2011).

2.6. Determination of antioxidant activity by phosphomolybdenum assay

One mL of fruit extract was combined with 1 mL of reagent solution (0.6 mol L^{-1} H_2SO_4, 28 mmol L^{-1} sodium phosphate, and 4 mmol L^{-1} ammonium molybdate). The test tubes were incubated at 95 °C for 90 min. The absorbance of the solution was measured at 695 nm against a blank sample, and total antioxidant capacity was expressed as μmol AsA 100 g^{-1} FW (Goulas & Manganaris, 2011).

2.7. Cell wall isolation

For cell wall isolation, 20 g of mesocarp tissue were placed in 100 mL absolute ethanol, homogenized in an Ultraturrax (IKA® – Werke GmbH & Co. KG, Germany) and boiled for 30 min to ensure the inactivation of enzymes and the extraction of low molecular weight solutes. The insoluble material was vacuum filtered and sequentially washed with 40 mL of ethanol, 40 mL of chloroform:methanol (1:1, v/v), and 40 mL of acetone. The insoluble material was dried overnight at 37 °C, yielding the alcohol insoluble residue (AIR). The dried residue was weighed. Two independent

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extractions were made for each treatment and storage time analyzed (Manganaris, Vicente, Crisosto, & Labavitch, 2008).

2.8. Cell wall fractionation

Fractions enriched in cell wall components were obtained by sequential extraction in different solvents. Approximately 40 mg of AIR residue from each sample were suspended into 10 mL of water and stirred at room temperature for 3 h under constant shaking and subsequently centrifuged at 6000×*g* and vacuum filtered. The filtrate was taken to 14 mL with water and designated as water soluble fraction (WSF). The residue was then extracted with 10 mL of 50 mM Na_2CO_3 for 1 h with shaking. The slurry was centrifuged and the supernatant was saved, taken to 14 mL with deionized water and designated as Na_2CO_3 soluble fraction (NSF). The Na_2CO_3 insoluble pellet was then extracted with 10 mL of 4 M KOH for 1 h, with shaking, and the extracted solution was designated as the 4 M KOH-soluble fraction (4KSF). Two cell wall samples were analyzed for each cultivar and storage time analyzed and each sample was extracted in duplicate. Samples of the different fractions obtained were assayed for uronic acid (UA) and neutral sugar (NS) contents as described below. Cell wall fractionation results expressed as relative values of UA and NS of the different fractions.

2.9. Uronic acids

Uronic acids (UA) were measured according to Blumenkrantz and Asboe-Hansen (1973). Aliquots (50–200 μL) of the different cell wall fractions were pipetted into test tubes and taken to 200 μL with deionized water. After that, 1 mL of 75 mM sodium borate in 98% (w/w) H_2SO_4 was added in and placed in ice water bath. Samples were gently agitated and incubated at 100 °C for 10 min. After boiling the reaction mixtures were cooled in a water ice bath and 20 μL of 0.15% w/v *m*-phenylphenol in 0.5% w/v NaOH were added. After vortexing, 300 μL of each sample were loaded in 96-well plates and the absorbance at 520 nm was measured in a plate reader (model Infinite 200 PRO, Tecan GmpH, Austria). The calibration curve was prepared with galacturonic acid (0–50 μg mL^{-1}).

2.10. Neutral sugars (NS)

Neutral sugars (NS) were measured by the anthrone method (Yemm & Willis, 1954). Aliquots (100–300 μL) from the different cell wall fractions were pipetted into test tubes and taken to 500 μL with water. After that 1 mL of 2 g L^{-1} anthrone (in 98% w/w H_2SO_4) was added in a water–ice bath. After vortexing, the samples were incubated for 10 min at 100 °C. The reaction mixtures were cooled in a water–ice bath, agitated and 300 μL were loaded in 96-well plates. The absorbance at 620 nm was measured in a plate reader, as previously mentioned. The calibration curve was done with glucose (0–30 μg mL^{-1}).

2.11. Cell wall degrading enzymes

The cell wall degrading enzymes were determined according to Vicente, Powell, Greve, and Labavitch (2007a). Approximately 5 g of fruit were homogenized in an T-25 digital Ultra-Turrax with 15 mL of 50 mM NaAc–HAc pH 5.0 containing 10 g L^{-1} polyvinylpolypyrrolidone (PVPP), and 1 M NaCl. The homogenates were then stirred at 4 °C for 1 h and centrifuged at 5000*g* for 20 min. The supernatant was filtrated and dialyzed against 10 mM NaAc pH 5.0 and used for assays of enzymatic activities. Two independent extracts were prepared for each treatment and storage time and each extract was measured in duplicate.

For β-galactosidase (β-gal) and α-arabinofuranosidase (α-araf) reaction mixtures containing 500 μL of enzyme extract, 1000 μL of 50 mM NaAc–HAc buffer pH 5.0 and 200 μL of 3 mM *p*-nitrophenyl-galactopyranoside or *p*-nitrophenyl-arabinofuranoside, respectively were incubated at 37 °C. 200 μL aliquots were taken at intervals, 1000 μL of 0.4 M Na_2CO_3 were added and the absorbance at 410 was measured.

Polygalacturonase (PG) activity was measured in reaction mixtures containing 800 μL of 50 mM NaAc–HAc buffer pH 5.0, 400 μL of 0.15% w/v polygalacturonic acid and 800 μL of enzymatic extract. The mixtures were incubated at 37 °C. At different times, 200 μL aliquots were taken and 1 mL of 1 M sodium borate was added. Reducing sugars liberated were measured with 2-cyanoacetamide according to Gross (1982).

For endo-1,4-β-D-glucanase/β-glucosidase (EGase) activity the reaction mixtures contained 800 μL of 50 mM NaAc–HAc buffer pH 5.5, 400 μL of 0.2% (w/v) carboxymethyl-cellulose (CMC) and 800 μL of enzymatic extract. The mixtures were incubated at 37 °C. At different times, 200 μL aliquots were taken and assayed for reducing sugars as described for PG activity (Bach & Schollmeyer, 1992).

2.12. Statistical analysis

Data were subjected to analysis of variance (One-Way ANOVA) and least significant differences (LSD) at the 5% level ($P \leqslant 0.05$) were used for comparing means using the software package SPSS v20 (SPSS Inc., Chicago, USA). Correlation analysis was carried out and R-square values are reported. Graphs were created using Prism v5.01 (Graph Pad Inc., San Diego, USA) and results were presented as means ± standard error.

3. Results and discussion

3.1. Quality attributes

Both cultivars showed substantial weight loss during the shelf life period both after harvest and after removal from cold storage. Quantitative losses were particularly excessive after 7 days at 20 °C and after 2 weeks at 4 °C + 5 d at 20 °C and were accompanied by qualitative losses (wrinkled epidermis), thus rendering the fruit commercially unacceptable (Fig. 1A). Dehydration, due to substantial weight loss, may partially explain the slight differences detected in tissue firmness among fleshly harvested and fruit subjected to cold storage prior to ripening at RT (Fig. 1B). Intriguingly, a significant increase of firmness of 'Luoyangqing' loquat fruits during extended cold storage, attributed to lignin accumulation has been reported (Cai et al., 2006c), indicating that loquat firmness properties are largely dependent on the cultivar considered.

Although loquat is harvested based on skin color, a minimum soluble solids content of 10% is often required for commercialization (Pinillos et al., 2011). SSC content measured in this work was well above the preferable quality threshold for the consumers for both cultivars (Fig. 2A). Previous studies reported that great genotypic differences exist regarding SSC content in loquats grown in Mediterranean countries (Ercisli, Gozlekci, Sengul, Hegedus, & Tepe, 2012) and China (Xu & Chen, 2011), with reported values in the range between <10% and >20%. A gradual reduction in SSC with the progress of cold storage concomitant with severe TA decrease has been reported (Cao, Zheng, & Yang, 2011), leading to fresh loquat flavor deterioration. TA markedly decreased throughout the shelf life period (Fig. 2B) in accordance with previous reports (Cao et al., 2009c, 2011). TA was always higher in 'Morphitiki' fruit for all storage regimes analyzed resulting in a

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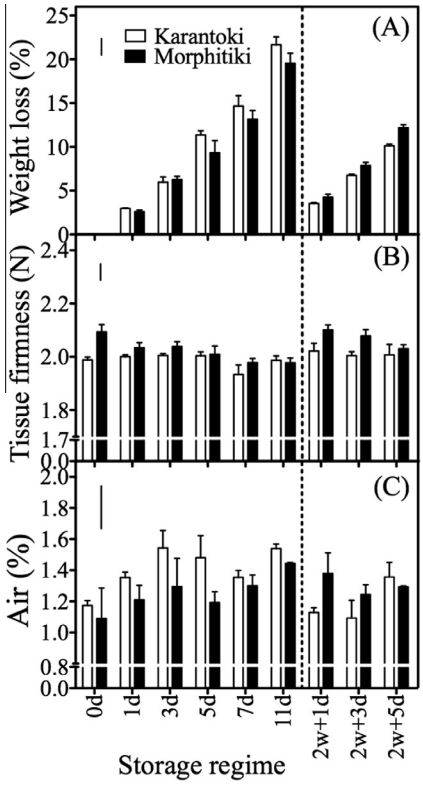

Fig. 1. Weight loss (%, A), tissue firmness (Newtons, B) and alcohol insoluble residue (AIR) (C) of loquat fruit (*Eriobotrya japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0 d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period for 1, 3 or 5 d, respectively. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

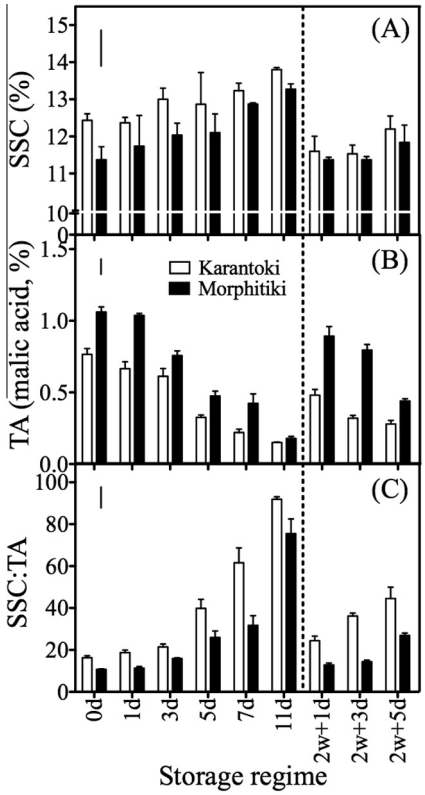

Fig. 2. Soluble solids content (%, A), titratable acidity (% Malic Acid, B) and SSC/TA ratio (C) of loquat fruit (*E. japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0 d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period for 1, 3 or 5 d, respectively. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

lower SSC/TA ratio compared to 'Karantoki' fruit for all storage regimes applied (Fig. 2C). It should be noted that 'Morphitiki' is highly appreciated by the consumers due to superior taste (Dr. G. Chatzipieris, personal communication); therefore a central role of TA in loquat fruit flavor can be attributed. While SSC could be used as harvest maturity index as elsewhere proposed (Pinillos et al., 2011), TA seems in this commodity highly relevant to fulfill consumer's expectations.

3.2. Phytochemical composition

At harvest, the phenolic content of 'Morphitiki' loquat (39.0 ± 0.5 mg gallic acid equivalents (GAE) 100 g^{-1} FW) was significantly higher than the corresponding content of 'Karantoki' fruit (31.6 ± 1.9 mg GAE 100 g^{-1} FW) and remained comparatively higher regardless of storage regime (Fig. 3A). The phenolic content has been reported to vary greatly among loquat cultivars (Xu & Chen, 2011). During the first 3 d at 20 °C total phenolics increased in 'Morphitiki' as opposed to 'Karantoki' which showed no changes. The phenolic content of both cultivars decreased after 5 days at RT, indicating that fruit deterioration is accompanied by a marked reduction of phenolic compounds. Hence low-temperature storage has been generally regarded to reduce loss of phenolics; 2-week cold storage did not inhibit the loss of phenolic compounds, in line with a previous study (Cao et al., 2011). A significant decrease in the total phenolic content of 'Luoyangqing' fruit during cold storage (0 and 5 °C) has been also reported (Cao et al., 2009c).

Hydroxycinnamic acid derivatives followed a similar pattern with total phenolics (Fig. 3B). Chlorogenic acid, neochlorogenic acid, and 5-feruloylquinic are usually the main hydroxycinammic acids found in loquat fruits (Ding et al., 2001; Ferreres et al., 2009). As for total phenolics, long storage times led to a significant loss of hydroxycinnamic acid-derivatives (Fig. 3A, B). Flavonol content also followed a similar pattern with total phenolics (Fig. 3C) but they were present at relatively low contents in both cultivars in accordance with a previous report (Xu & Chen, 2011).

Loquat is considered as a source of carotenoids which affect both fruit color and health-promoting properties (Azqueta & Collins, 2012; Zhou, Li, Xu, Sun, & Chen, 2011a). Carotenoid content is highly variable depending on the cultivar considered (Zhou, Xu, Sun, Li, & Chem, 2011b). In the current study, 'Morphitiki' demonstrated higher carotenoid content than 'Karantoki' (Fig. 3D). Despite its non-climacteric type, carotenoid biosynthesis increased as shelf life increased markedly during storage; pigment concentration during postharvest storage has been also reported (Ding, Chachin, Hamauzu, Ueda, & Imahori, 1998). Extended shelf life periods are accompanied with visible dehydration symptoms thus contributing to the increase in carotenoid content measurement on a fresh weight basis. It is worth noting that after 2 weeks of low-temperature storage and subsequent transfer at RT, carotenoids reached comparable levels with fruit continuously held at 20 °C. This differs from what has been found in some chilling sensitive fruit species, in which long term storage at low temperature reduced final pigment concentration upon attaining full ripening (Lurie, 1998). Even some cold tolerant commodities such as strawberries have been shown to accumulate less anthocyanin at the red ripe stage, if subjected to a cold storage period (Vicente, Martínez, Civello, & Chaves, 2002).

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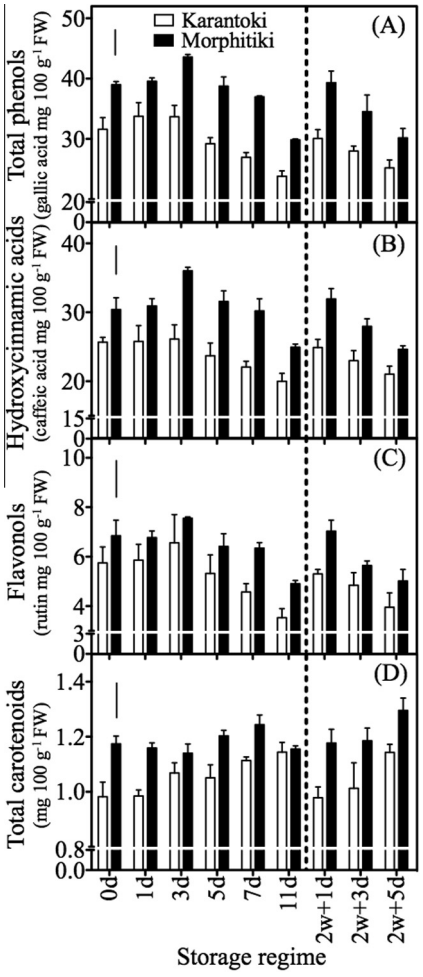

Fig. 3. Total phenols (gallic acid equivalents, (A) hydroxycinnamic acids (caffeic acid equivalents, (B) total flavanols (rutin mg 100 g^{-1} FW, (C) and total carotenoids (D) of loquat fruit (*E. japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0 d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period for 1, 3 or 5 d, respectively. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

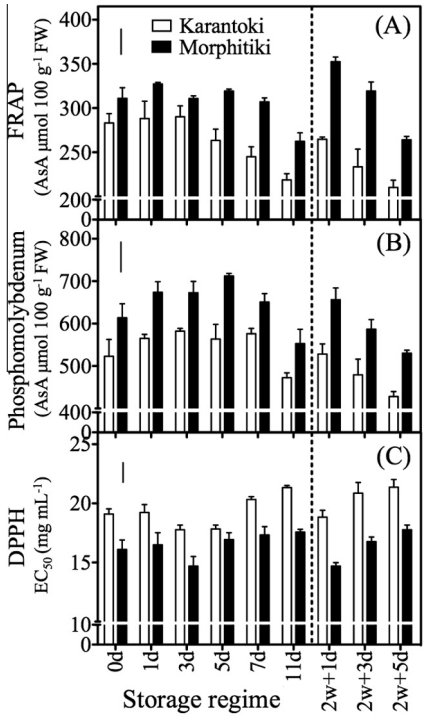

Fig. 4. Total antioxidant capacity of loquat fruit (*E. japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0 d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period at 20 °C for 1, 3 or 5 d, respectively, evaluated with three *in vitro* assays: (a) ferric reducing antioxidant power (FRAP), (B) phosphomolybdenum assay and (C) 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

3.3. Antioxidant properties

The antioxidant (AOX) capacity of loquat fruits, evaluated with the FRAP and phosphomolybdenum assays, followed a similar pattern, demonstrating a higher antioxidant capacity in 'Morphtiki' fruits at all storage regimes and time points tested (Fig. 4A, B). As found for phenolic antioxidants, AOX activity decreased in association with fruit deterioration. In 'Karantoki' fruit, AOX activity dropped, starting from day 5 at RT, while 'Morphitiki' showed no variations until day 7. After cold storage and subsequent transfer to 20 °C, the AOX capacity was progressively decreased for both cultivars. However, 'Morphitiki' still maintained higher AOX capacity than 'Karantoki'. The DPPH· scavenging activity assay also indicated low antioxidant capacity (higher EC_{50} values) in both cultivars after extended shelf life period (7 and 11 days after harvest and 3 and 5 days after 2-week cold storage) when fruit deterioration was noticeable (Fig. 4C).

With a view to rationalize the antioxidant properties of loquat fruits, the correlation coefficients for total phenolics, hydroxycinnammic acid-derivatives, total flavonols, total carotenoids and antioxidant potency were calculated (data not shown). Relatively high positive correlation ($r \geqslant 0.892$) was found between phenolic content and antioxidant potential. Furthermore, data showed that hydroxycinammic acid-derivatives and flavonols are the major groups contributing to the ethanol soluble AOX activity. Our findings are comparable to that reported for a list of twelve selected loquat cultivars (Xu & Chen, 2011).

3.4. Cell wall yield and solubilization

The alcohol insoluble residue at harvest was 1.08% and 1.17% in 'Morphitiki' and 'Karantoki' fruit, respectively (Fig. 1C), representing mainly the cell wall material, since no starch in loquat pulp has been detected (Femenia, Garcia-Conesa, Simal, & Rossel, 1998). Intriguingly, wall content increased during storage. Since the increase of AIR (%) was higher to what would be expected due to weight loss, the *de novo* deposition or the cross link of pre-existing polymers might have occurred during storage at higher rates than disassembly. This is uncommon, as AIR usually decreases during ripening, as wall disassembly proceeds. In many products such as kiwifruit, blueberry and raspberry the cell wall content diminishes dramatically as ripening progresses (Redgwell, Melton, & Brasch, 1992; Vicente, Ortugno, Powell, Greve, & Labavitch, 2007b,c). Other loquat studies have also reported an increase in cell wall contents during storage at chilling temperatures (Cao et al., 2009b). In our case, results indicated that this increase can also occur during normal ripening at RT suggesting that it is more probable to be considered as a developmental rather than a chilling stress response.

To evaluate possible differences in the modifications of wall components of both cultivars, we fractionated AIR to yield the water soluble fraction (WSF) enriched in loosely bound pectin, a

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Na_2CO_3 soluble fraction (NSF) mainly composed of tightly-bound pectin and a 4KSF fraction accounting predominantly with wall cross-linking glycans (Brummell, 2006). Marked changes were detected in pectin fractions, indicating extensive cell wall remodeling in spite of the low firmness modifications detected. At harvest, uronic acids were mainly associated with the NSF, while the WSF and KSF only accounted for *ca.* 20% and 5% of the total UAs, respectively (Fig. 5A). The fact that loquat AIR contained high levels of pectins has been also reported: Femenia et al. (1998) showed that polyuronides contributed up to 70% of total cell wall polysaccharides in the flesh of loquat fruit. Results reported herein suggest that pectic polymers are also the main wall constituents being modified during postharvest storage. During maintenance at 20 °C after harvest, a large increase in water soluble UAs was found in 'Karantoki' fruit, particularly after 11 days shelf life, while no major modifications in pectin solubility were detected in 'Morphitiki'. Interestingly enough, after cold storage and subsequent transfer at RT, pectin solubility showed no changes in 'Karantoki', indicating that 2 weeks maintenance at low temperature resulted in some inhibition of normal wall disassembly. In contrast, substantially higher water soluble pectins were observed in cold-stored 'Morphitiki', reflecting a frequent pattern found in fruits during normal ripening. It has been reported that cold storage (at 0–1 °C) may affect pectin and hemicellulose turnover and induce lignin accumulation in loquat (Cao et al., 2009b), and our results suggest varietal-dependent responses which may reflect cultivar differences in chilling tolerance.

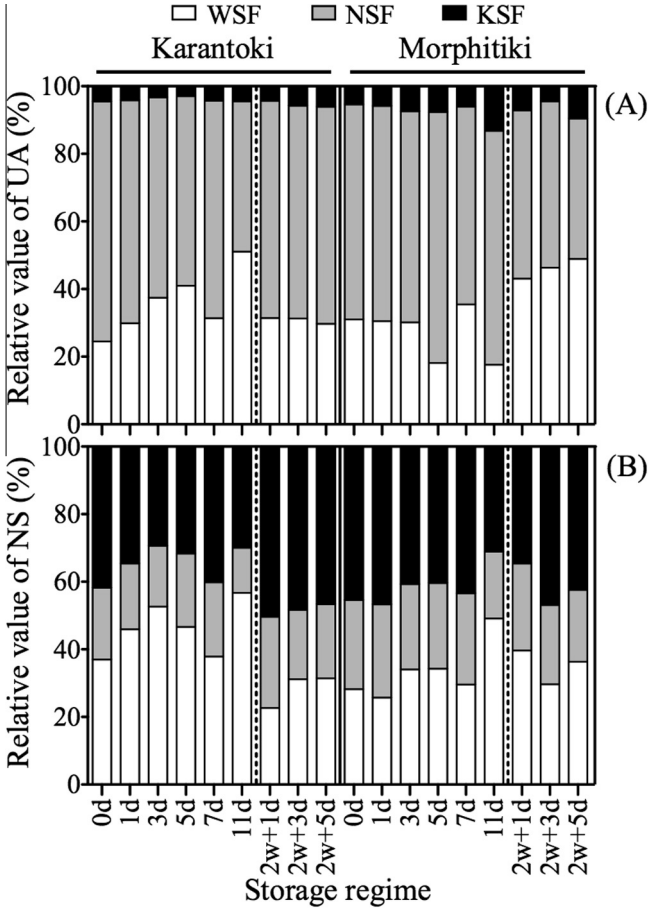

Fig. 5. Relative distribution of uronic acids (UA, A) and neutral sugars (NS, B) in the water- (WSF), Na_2CO_3– (NSF) and 4 M KOH-Soluble (KSF) fractions of loquat fruit (*E. japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period for 1, 3 or 5 d, respectively. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

Approximately 60% of the wall NS content was associated with the two pectin-rich fractions (WSF and NSF) (Fig. 5B). The most abundant neutral sugars commonly present in water or Na_2CO_3 fruits extracts are arabinose (ara) and/or galactose (gal) (Gross & Sams, 1984). Loquat fruit, in particular, is highly abundant in arabinose (Femenia et al., 1998), thus suggesting a high degree of pectin branching. Studies in other fruit species indicated that pectin side chain removal is an event occurring during early or mid-ripening (Brummell & Harpster, 2001). However, in loquat fruit the shift of NS from the NSF to the WSF occurred after 11 d shelf life in both cultivars. Unexpectedly, a marked increase in KOH soluble neutral sugars was found in Karantoki fruit after two weeks of cold storage. This seemingly resulted from the insolubilization of pre-existing wall polysaccharides and/or form de novo biosynthesis of alkali soluble wall material. Chilling injury in loquat fruit has been related to modification of cell wall polysaccharides (Cai et al., 2006c), such as increased hemicellulose content (Cao et al., 2009b). In addition, chilling-induced lignin biosynthesis may increase the cross-linking of cell wall components (Carpita & McCann, 2000). Results of the current study suggest the higher chilling sensitivity in 'Karantoki' fruit is due to anomalous wall disassembly that could be detected even after relatively short (2 week) cold storage regimes (4 °C). Future experiments with extended storage periods remain as a means to support the anomalous wall disassembly theory. The differences observed in pectin disassembly among cultivars did not result in modifications in tissue firmness. However, the solubility of cell wall polymers was certainly altered and this may result in marked differences in tissue hydration, viscosity and solubility during ingestion (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011).

Turnover of both pectin and cellulose-hemicellulose matrices during ripening may be important not only in terms of sensory quality (e.g., to reach desirable textural properties) but also to facilitate the release of health promoting phytochemicals (Palafox-Carlos et al., 2011). There is evidence indicating that cell wall polysaccharides may directly interact with the food antioxidants and interfere with their assimilation. Besides its lower contents of phenolics and carotenoids the disruption of cell wall disassembly in 'Karantoki' when this cultivar is subjected to a cold storage, may arrest phytochemical release during digestion.

It should be also noted that reduction and/or alleviation in CI symptoms has been correlated with enhanced antioxidant enzyme activity (Cao et al., 2009c). Since accumulating evidence exists regarding the role of oxidative stress in CI symptoms, the selection of loquat cultivars destined for extended cold storage may be determined based also on their antioxidant status.

3.5. Cell wall degrading enzymes

β-gal activity increased during ripening in both cultivars, but more markedly in 'Morphitiki' fruit after 7 and 11 days shelf life (Fig. 6A). Low temperature storage seemed not to affect this enzymatic activity. Refrigerated fruit recovered similar enzymatic activity than non-chilled loquats after an additional shelf life period at RT for 3 and 5 days.

α-Araf also increased during storage at RT in both cultivars, however, differently to what was found in β-gal, α-araf activity was not recovered in any cultivar after cold storage (Fig. 6B). In peach, α-araf, activity was reduced by half in chilling-injured fruit, evident as mealy, relative to juicy fruit even after only 1 week of cold storage (Brummell, Dal Cin, Lurie, Crisosto, & Labavitch, 2004). However, it is worth noting that the changes in the *in vitro* activity of these enzymes in loquat did not correlate with

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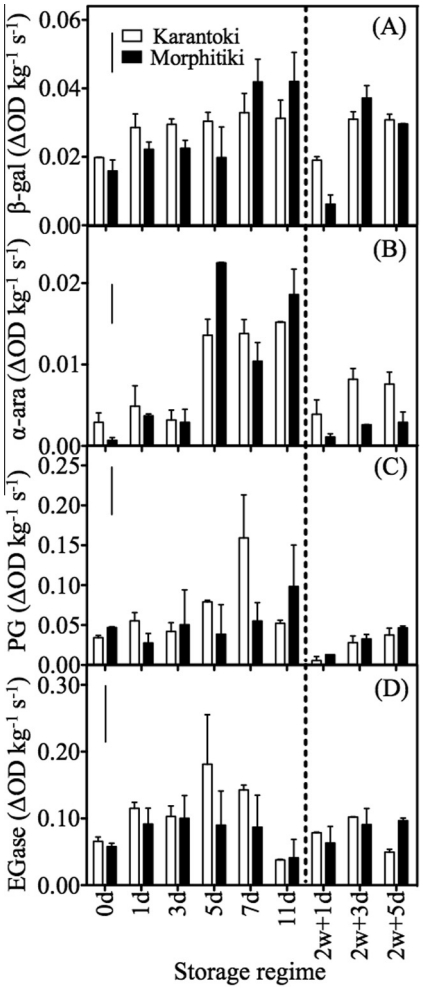

Fig. 6. β -galactosidase (β-gal, A), α-arabinofuranosidase (α-araf, B), polygalacturonase (PG, C) and endo-1,4-β-D-glucanase/glucosidase (EGase, D) activities in loquat fruit (*E. japonica*, cvs. 'Karantoki', 'Morphitiki') at harvest (0 d), during maintenance at 20 °C (shelf life) for 1, 3, 5, 7 or 11 d and after 2 weeks (w) cold storage (4 °C) and an additional shelf life period for 1, 3 or 5 d, respectively. Results are the means ± standard error. The vertical bar represents the least significant difference (LSD, $P \leqslant 0.05$).

the modifications occurring in cell wall neutral sugars (Figs. 5B & 6A, B).

PG increased markedly during maintenance at RT after harvest, particularly in 'Karantoki' fruit (Fig. 6C). However, after 2 weeks at 4 °C and subsequent transfer to 20 °C much lower PG activity was reached; this decrease was more evident in the 'Karantoki' fruit. A marked decrease in the PG levels for both cultivars was monitored 1 d after removal from cold storage. Furthermore, the PG activity levels detected during room temperature storage in both cultivars could be related with the decreasing and unchanging UA content in NSF observed in cv. 'Karantoki' and cv. 'Morphitiki', respectively.

Finally, EGase in fruit ripened continuously at 20 °C showed generally higher activity in 'Karantoki' fruit (Fig. 6D). However, EGase did not reach the same levels when the fruit was held at RT after a 2 week cold storage. 'Morphitiki' showed lower EGase activity than 'Karantoki' when ripened continuously at 20 °C, but interestingly no alterations were observed after storage at low temperature. In this case, the enzyme increased earlier than that of PG, peaking at day 5 (instead of day 7) and decreased thereafter. Intriguingly and opposite to what occurred in 'Karantoki' fruit, EGase was not altered by chilling in 'Morphitiki'. Overall, the inhibition of pectin solubilization as well as the decreased activity of wall degrading enzymes (EGase and PG) in ripening 'Karantoki' fruit after removal from cold storage compared to fruit maintained at non-chilling temperatures, suggests that this cultivar might be less tolerant to chilling and more prone to show dysfunctional cell wall turnover. Our findings are in line with studies outlining the alleviation of CI symptoms through enhanced cell wall polysaccharide solubilization (Cao et al., 2010).

The CI syndrome in loquat has been associated with multiple effects, including increased production of reactive oxygen species (ROS), tissue browning, lignification, alterations of membrane composition and function and cell wall turnover (Cai et al., 2006b; Cao et al., 2009a; Xu, Dong, Zhang, Xu, & Sun, 2012). The nature of the alterations in wall disassembly is significantly different from that reported in stone fruits. This is supported by the facts that ethylene ameliorates CI in climacteric peach and plum and 1-methylcyclopropene (1-MCP) exacerbates the problem (Lurie & Crisosto, 2005) while the opposite is true in non-climacteric loquat (Cao et al., 2009b). A major difference is the accumulation of lignin in chilling-injured loquat (Cai et al., 2006c). Another important distinction is that in loquat the changes are not associated with modifications of pectin side chain-removing enzymes (β-gal and α-araf) as in peach (Brummell et al., 2004) and plum (Manganaris et al., 2008), respectively. The polygalacturonase:pectin methyl esterase ratio is a criterion that has been suggested as indicator of CI in Chinese loquat cultivars (Cao et al., 2010). The current study outlined that the softening related PG and EGase are the enzymes inhibited by cold storage in chilling sensitive 'Karantoki' loquat fruit. The changes in polymer cross-linkage and wall pore sizes, by deposition of lignin or hemicelluloses may limit *in vivo* activities of wall loosening agents as well. Other cell wall proteins unexplored herein such as expansins may be involved in the differences observed (Yang et al., 2008). Although further work is needed to determine the biological basis of cold induced alterations in normal cell wall disassembly in loquat fruit, the present study showed that genotypic differences in the susceptibility to CI disorders exist.

4. Conclusions

Results indicated genotypic variations in quality attributes, phytochemical composition and responses to postharvest management in loquat. Identifying and delimiting the extent of such intercultivar variations is necessary to provide the fruit industry more appropriate recommendations for postharvest management, which would ultimately maximize both sensory and nutritional quality maintenance of fresh loquat. Although 'Karantoki' fruits are highly appreciated by the growers and consumers due to productivity and fruit size characteristics, the current study demonstrates 'Morphitiki' as a superior cultivar in terms of antioxidant capacity, phenolic and carotenoid contents, being at the same time less prone to cold-induced alterations in cell wall disassembly. While such disruption in the "organized disorganization" of fruit cell wall did not directly impact firmness it may reduce juice and phytochemical release upon consumption.

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