Postharvest Biology and Technology 108 (2015) 68–77

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Cell wall metabolism and chilling injury during postharvest cold storage in zucchini fruit

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ARTICLE INFO

Article history:
Received 25 February 2015
Received in revised form 27 May 2015
Accepted 28 May 2015
Available online 6 June 2015

Keywords:
Zucchini
Chilling injury
Pectin
Cellulose
Lignin
Cell wall-modifying proteins

ABSTRACT

Postharvest cold storage in zucchini fruit extends the commercial life but causes the appearance of chilling injury (CI), characterized by the development of pits and damaged areas at the surface of the fruit. This physiological disorder has been related to alterations of the cell wall metabolism in several fruit. We have analyzed the relationship between the development of CI and the changes that take place at the cell wall due to cold storage in zucchini fruit, as well as the effect of a preconditioning treatment on these changes. Microscopical observations have shown that the surface depressions detected in chilling injured fruit were caused by cell death and cell collapse. Low temperature induced the solubilization of the more soluble pectins, as evidenced by the highest levels of neutral sugars and uronic acids found in control fruit after the cold storage in water-soluble fraction (WSF) and CDTA-soluble fraction (CSF). The results obtained in Na_2CO_3-soluble fraction (NSF) for both parameters was opposite; higher levels were detected in preconditioned fruit and lower in more damaged fruit. The same behaviour was found in the base soluble hemicelluloses, 1 M KOH- and 4 M KOH-soluble fractions (1KSF and 4KSF), and in the insoluble cellulose content. Zucchini fruit stored at low temperature showed an increase of the enzymatic activities pectin methylesterase (PME), polygalacturonase (PG), and cellulase (CEL), and an accumulation of mRNA corresponding to a expansin (EXP) gene. Preconditioned fruit showed the lowest levels of these enzymatic activities. Microscopic analysis of CI fruit correlated with the biochemical changes observed in cell wall. Lignin content was higher in control than preconditioned fruit, suggesting a possible role of the lignification process in CI development in zucchini.

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1. Introduction

Chilling injury (CI) is a physiological disorder that takes place when fruits from tropical or subtropical origin are exposed to low but not freezing temperatures. The macroscopic symptoms of this disorder include alterations of the metabolism, that lead to an abnormal ripening, and the appearance of damaged areas at the surface of the fruit (Sevillano et al., 2009). Several of these modifications can be related to cell wall integrity and metabolism, which is affected in many chilling susceptible fruit and vegetables. In many fruit the softening associated with ripening is altered by low temperature that induces changes in the cell wall turnover (Almeida and Huber, 2008; Cao et al., 2009, 2010). Cold storage also affects cell-wall enzyme activities required for normal ripening, leading to different symptoms such as mealiness, leatheriness, and woolliness (Brummell et al., 2004; Fruk et al., 2014). The relationship between the cell wall modifications and the development of damage at the fruit surface has been less studied. Mercer and Smittle (1992) described that tissue breakdown in cucumber results in increased concentrations of water soluble pectins, the more soluble components of cell wall. Other storage disorders that affect to citrus fruit, such as non-chilling peel pitting, lead to the appearance of collapsed areas in the flavedo, the outer part of the peel (Cajuste and Lafuente, 2007); these lesions have been associated with structural and compositional changes in cell wall (Cajuste et al., 2011; Vicente et al., 2013).

All the symptoms described above are partly due to changes in the solubilization and depolymerization of pectin. Solubilization process increases the capacity of pectin to be extracted from the cell wall by aqueous solvents whereas depolymerization represents a lowering of the molecular mass due to the activity of cell wall-modifying enzymes (Redgwell et al., 1992, 1997). The major enzymes involved in depolymerization of pectin are pectin methylesterase (PME) and polygalacturonase (PG), along with pectate lyase, galactosidase, and arabinosidase. PME removes methyl side group of cell wall galacturonans, converting methyl-

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http://dx.doi.org/10.1016/j.postharvbio.2015.05.013
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esterified pectin into de-esterified pectin, which are substrates that PG can act upon. PG cleaves the α-1,4-linkages between the galacturonic acid residues of galacturonans. Cellulase (CEL) is another important cell wall-modifying enzyme that hydrolyzes a number of β-1,4-linked glucans and are likely to target the glucan backbone of xyloglucan, the hemicellulose that bridges between cellulose microfibrils. The non-enzymatic proteins expansins (EXP) also have an important role in cell wall modification. EXP acts disrupting hydrogen bonds between cell wall polymers and the cellulose microfibril surface. These cell wall-modifying enzymes and proteins have been associated with the rearrangement that takes place during the postharvest cold storage in different fruit (Brummell et al., 2004; Figueroa et al., 2012; Khademi et al., 2014). Moreover, in some species stresses such us chilling induce lignification processes associated with the development of injuries (Cai et al., 2006; Dangcham et al., 2008). Lignin is a generic name for a large group of aromatic polymers derived from the phenylpropanoid pathway that is deposited in the cell wall making the cells rigid and impervious (Vanholme et al., 2010).

Zucchini (*Cucurbita pepo* L. morphotype *Zucchini*) is a non-climacteric fruit harvested at an immature stage. Since zucchini is of subtropical origin, it belongs to the group of fruit that suffer CI when stored at low temperatures. CI symptoms in zucchini fruit are mainly characterized by the appearance of pits and damaged areas on the exocarp tissue. The pits may be the result of a cellular loss of integrity caused by damages to cell membrane or to cell wall (Balandrán-Quintana et al., 2002). The alterations suffered by the zucchini fruit membranes and their relationship with the CI have been studied, and a positive correlation with lipid peroxidation and electrolyte leakage have been reported (Carvajal et al., 2011, 2015; Palma et al., 2015). However, little information is available about changes induced by storage at low temperature on cell wall metabolism. With respect to cell wall-modifying enzymes, an increase in PG activity has been reported in zucchini fruit stored at 2.5 °C after 9 days, as compared to fruit stored at 12 °C (Balandrán-Quintana et al., 2007). Some postharvest treatments have been proven to be effective in reducing CI in zucchini fruit. Among them are the exogenous application of polyamines such us putrescine, or the preconditioning of the fruit before cold storage (Carvajal et al., 2015; Palma et al., 2015). The effect of the treatments with polyamines has been associated with a reduction in PG activity (Martínez-Téllez et al., 2002) but no data about changes in cell wall have been reported in fruit that have been preconditioned.

Therefore, in this study we have examined the temporal pattern of changes in cell wall composition and in the activity of a range of enzymes associated with these changes during postharvest cold storage in zucchini fruit. Moreover, we also show the effect that a preconditioning treatment that improves chilling tolerance has on the prevention of cell wall modifications. The main objective has been to elucidate the relationship between cell wall deterioration and the development of CI symptoms.

2. Materials and methods

2.1. Plant material and treatments

Zucchini fruit (*C. pepo* L. morphotype *Zucchini*) of the commercial hybrid Sinatra (Clause–Tezier) were provided by E.H. FEMAGO S.A. After harvest, fruit were divided in three groups for the following treatments: group 1 (fruit at harvest); group 2 (control fruit) fruit stored at 4 °C; and group 3 (preconditioned fruit) fruit preconditioned during 48 h at 15 °C and, after that, stored at 4 °C. Three replicates per treatment and storage period were prepared, each consisting in 6 fruits of similar size. After 7 and 14 days of storage at 4 °C, total exocarp of each fruit was separated (1–1.5 mm of thickness), mixed per replicate, homogenized in liquid nitrogen, and stored at −80 °C.

2.2. Estimation of chilling injury index

Chilling injury index was evaluated using a subjective scale of visual symptoms described by Martínez-Téllez et al. (2002). Fruits were rated according to the following scale: 0, no pitting; 1, slight (10% or less of the evaluated fruit); 2, medium (10–20% of the evaluated fruit); and 3, severe pitting (>20% of the evaluated fruit). CI index was determined using the formula: Σ (pitting scale (0–3) *x* number of fruits in each class)/total number of fruits estimated.

2.3. Preparation of tissue for light microscopy

Healthy and injured areas from exocarp tissue were excised (2 mm × 2 mm) with a razor blade and fixed in 4% (v/v) formaldehyde and 2% glutaraldehyde (v/v) in phosphate-buffered saline (PBS) pH 7.4, rinsed four times in PBS and dehydrated in a graded ethanol series. Tissue was then embedded in Embed 812 resin, and sections of 1.0–1.5 μm were cut with a Reichert microtome (Leica, Germany) and stained with 1% (w/v) toluidine blue in borax. Light micrographs were taken using an Olympus BX41 microscope with an Olympus DP70 digital camera (Olympus, Tokyo, Japan).

2.4. Cellular integrity and cell death assay

To evaluate cellular integrity, electrolyte leakage was determined in the exocarp of freshly-harvested fruit and in healthy and injured areas from this tissue after 14 days of cold storage. For that, we followed the method described by Mao et al. (2007). Briefly, exocarp was separated with a vegetable peeler and 10 discs were taken from each replicate with an 11 mm diameter stainless-steel cork borer. Four replicates from each treatment were measured. Each replicate was rinsed with 50 mL of deionized water three times for 3 min. After being incubated for 30 min and shaken at 100 rpm in 50 mL of deionized water, this solution was measured for conductivity at room temperature using a conductimeter. Total conductivity was determined after boiling the flasks for 10 min and cooling at room temperature. The electrolyte leakage was expressed as percentage of total conductivity.

Cell death assay was carried out by estimation of trypan blue uptake according to Qu et al. (2009) with some modifications. Exocarp was separated with a vegetable peeler and 5 discs were taken from each replicate with an 11 mm diameter stainless-steel cork borer. Four replicates from each treatment were measured. Exocarp discs were submerged in 0.25% (w/v) trypan blue in Petri dishes an incubated on a platform shaker for 10 min. After that, the discs were rinsed with deionized water until no more blue stain was eluted and then dried by filter paper. The dry discs were

Table 1
PCR primers used in gene expression analysis.

Gene symbol	Name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Accession no.
CpEXP1	Expansin 1	TCGTGAGGGTGAGTGTGAAA	CACGTTCCATGATGTTGAGG	KP792597
EF-1α	Elongation factor-1α	GCTTGGGTGCTCGACAAACT	TCCACAGAGCAATGTCAATGG	HO702383

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