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Chilling injury is associated with changes in microsomal membrane lipids in guava fruit (*Psidium guajava* L.) and the use of controlled atmospheres reduce these effects

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ABSTRACT

Storage of guava fruit cv 'Media china' at temperatures below 8 °C causes chilling injury associated with changes in cell membrane lipids. The aim of the present study was to evaluate the effects of two temperatures (4 and 10 °C) and three controlled atmospheres (air; 10 kPa O_2; 5 kPa CO_2 and 10 + 5 kPa of O_2 and CO_2) on microsomal lipid profiles in ripened fruit or fruit storage during three weeks. The study revealed that chilling injury is associated with a decrease in microsomal membrane volume and microsomal protein and phospholipids content (5%, 47% and 22%; respectively). Also, it was observed a decrease in Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), Phosphatidylinositol (PI), phosphatidic acid (PA), cardiolipin (CL) contents and a substitution of saturated fatty acids (FA) for unsaturated FA in the microsomal membrane. Importantly, it was found that storing guava fruit 'Media China' at 10 °C under 5 kPa CO_2 reduced chilling injury prevalence and increased shelf life, suggesting that these storage conditions maintained membrane functionality. Overall, these results indicate that CI is associated to deep alterations in membrane lipids pathway.

1. Introduction

Guava is a sub-tropical fruit native from South East Mexico and Central America. Worldwide, the main producers of this fruit are India ('Allahabad Safeda'), Brazil, and Mexico (Lopes Da Cunha et al. 2012), where varieties 'Beaumount', 'Pedro Sato', 'XXI Century' and 'Media China' are cultivated. 'Media China' is an aromatic variety with high vitamin C content. It is mostly consumed as fresh fruit due to its short shelf life. To increase shelf life, it is recommended to storage guava fruit at low temperatures; however, temperatures below 8 °C cause chilling injury (CI) (Mercado-Silva et al. 1998). For example, variety 'Media China' can be stored during one week at 5–8 °C in mature stage or two weeks at 8–10 °C in mature green stage before CI symptoms are apparent (Kader, 1999). CI symptoms in 'Media China' variety include water soaking, failure to ripen, and surface and flesh discoloration (Benito-Bautista and Mercado-Silva, 1997).

Modified or controlled atmospheres (CA) are used to extend shelf life and reduce CI symptoms (Wang and Wang, 1993; Whitaker and Wang et al., 1987). Kader (2001), recommended a CA containing 2–5% O_2 and 0–1% CO_2 for guava fruit stored at 5–15 °C. Also, Singh and Pal (2008) reported that storing guava during 30 days at 8 °C under 5% O_2 + 2.5% CO_2, 5% O_2 + 5% CO_2, and 8% O_2 + 5% CO_2 delayed the appearance of CI symptoms in varieties 'Lucknow-49′, 'Allahabad Safeda', and 'Apple Color'; respectively. Teixeira and Durigan (2010), stored 'Pedro Sato' guava during 28 days at 12.5 °C under 1–5% O_2, without symptoms of damage. Benito-Bautista and Mercado-Silva (1997) observed that the use of 5% CO_2 at 4 °C during two or three weeks decreased CI incidence in guava fruit 'Media China'. These studies provide evidence supporting the use of CA to reduce CI symptoms in guava; however, our knowledge about the mechanisms mediating these effects remains to be elucidated.

Four decades ago, it was proposed that the primary cause of CI in fruits and vegetables was the thermotropic phase transition of membrane lipids (Lyons, 1973; Raison and Orr, 1990). Later, it was suggested that plants with a high ratio of saturated/unsaturated fatty acids could be more sensitive to CI (Whitaker, 1994). In the 90 s, Nishida and Murata (1996) reinforced this hypothesis by overexpressing desaturase genes in plants and reducing CI symptoms in tobacco and Arabidopsis.

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This observation was confirmed by Changfeng and Shiping (2009) showing that high levels of unsaturation FA reduce CI symptoms in peach fruit.

Recently, Li et al. (2015) proposed that cold stress in plants (*Arabidopsis thaliana*, *Atriplex lentiformis,* and *Triticum aestivum*) generates a dynamic response in membrane lipid metabolism to overcome this stress. Based on this premise, we hypothesized that storage of guava at low temperatures causes changes in membrane lipid metabolism which provoke the CI symptoms. To address this idea we analyzed the effects of storing guava at different temperatures on membrane lipid metabolism and the potential role of CA to reduce CI symptoms.

2. Materials and methods

2.1. Biological material and experimental set up

Guava fruit (*Psidium guajava* L.) cv 'Media China' changes colour from green to yellow during the ripening process; thus, guava fruit was harvested at mature-green stage (green yellowish, 12.0% solids soluble content and 0.6% titratable acidity) in a commercial orchard located in Calvillo, Aguascalientes; Mexico. Fruit (n = 1200) were pre-cooled at 10–12 °C during 18 h to remove field heat; then, fruits were divided randomly into ten groups (n = 40) to assess the effects of two different temperatures and four CA conditions. These experimental groups were compared against two additional groups, fruit without CI symptoms (guava stored 7 days at 20 °C) and fruit with severe CI symptoms (guava stored 4 weeks at 4 °C). The study was performed in triplicate (Table 1).

For CA treatments, fruit were placed into glass containers (volume: 20 L) under continuous air flushing (90% HR) and mixed with CO_2 and N_2 gas using a static tube mixer. Atmosphere composition was monitored by collecting 20 μL of atmosphere samples from each container and analyzed by gas chromatography (Perkin Elmer Autosystem) using a capillary column Chromapac 7537, (10 m in length and 0.7 mm in diameter) and Helium as gas carrier at 60 mL min^{-1}. A TCD detector was used to register CO_2 signals in the sample. Temperatures of injector, oven, and detector were 150, 80, and 175 °C; respectively. Quantification of CO_2 was performed by injecting 20 μL of an external standard of 0.5% CO_2 in N_2.

2.2. Lipids analysis in microsomal membranes

2.2.1. Microsomal membranes extraction

Microsomal membranes were obtained by phase partitioning as described by González de la Vara and Medina (1988). Briefly, tissue (120 g) was homogenized at 4 °C during 30 s using 180 mL of extraction solution (250 mM sucrose, 4 mM DTT, 0.5 mM EDTA, 0.1% (w/v) bovine albumin, 0.5% (w/v) insoluble PVP, and 70 mM Tris-HCl, and pH 8.0). The homogenate was filtered and centrifuged at 10,000 *g* for 15 min; then, supernatant was collected and centrifuged at 12,000 g for 2.5 h to separate microsomal membranes. Isolated membranes were suspended in 1.6 mL of a storage solution at pH 6.5 [sucrose 250 mM, 20 mM of DTT, 50 mM of hydroxyl methyl amino methane, and morpholino ethane sulfonic acid (Tris/MES)], and stored at -80 °C.

Table 1
Experimental design used to evaluate the effects of temperature and controlled atmospheres (CA) storage.

Treatment	Temperature (°C)	Storage (weeks)	Transference at 20 °C after treatments (days)
Fruit without CI	20	1	0
Fruit with severe CI	4	4	3
Air	4	3	3
Air	10	3	3
10 kPa O_2	4	3	3
5 kPa CO_2	4	3	3
10 kPa O_2 + 5 kPa CO_2	4	3	3
10 kPa O_2	10	3	3
5 kPa CO_2	10	3	3
10 kPa O_2 + 5 kPa CO_2	10	3	3

Table 2
Protein content, microsomal membranes volume and phospholipids contents in guava fruit stored at different controlled atmospheres (CA) during three weeks at 4 and 10 °C.

Treatment	Protein content (μg g^{-1})$^{\Delta}$	Microsomal membranes volume (μL g^{-1})$^{\Delta}$	Phospholipids content (mg mL^{-1}) †
Fruit without CI	7.60 a	2.03 b	19.21 a
Fruit with severe CI	4.04 e	1.93 c	15.06 e
4 °C			
Air	4.83 d	1.98 c	16.73 dc
10 kPa O_2	5.47 c	2.06 b	17.23 b
5 kPa CO_2	7.42 a	2.12 b	19,01 a
10 O_2 + 5 CO_2	5.32 c	1.98 c	17.54 b
10 °C			
Air	4.74 d	2.03 b	16.51 d
10 kPa O_2	5.27 c	2.09 b	17.09 cb
5 kPa CO_2	7.18 b	2.21 a	18.81 a
10 O_2 + 5 CO_2	5.20 c	2.04 b	17.31 d

Fruit without CI was ripening for 1 week at 20 °C and fruit with severe CI was maintained 4 weeks at 4 °C. Different letters in the same column represent statistical differences *P ≤ 0.05. $^{\Delta}$content expressed for g of fresh tissue †content expressed per mL of microsomal membranes recovered.

2.2.2. Total phospholipids content

Total phospholipid content was measured by the Ames (1966) method; briefly, 250 μL of perchloric acid was added to 50 μL of microsomal membranes and heated to complete digestion. The residue was cooled and suspended in 5 mL of distilled water, filtered and brought to 10 mL. One mL of this solution was mixed with 2 mL of Ames reagent (1.2 mL ammonium molybdate, 0.42% of sulfuric acid 1 N, 0.2 mL of ascorbic acid 10%, and 0.48 mL water) for 45 min at 30 °C; and then, the absorbency at 820 nm was measured. Quantification was performed using a standard curve of KH_2PO_4. Results were expressed as PC equivalents with a molecular weight of 760 g mol^{-1}.

2.2.3. Phospholipids and galactolipids separation and fatty acids (FA) analysis

Phospholipids and galactolipids from microsomal membranes were separated by thin layer chromatography (TLC) using the method described by Bligh and Dyer (1959) and Whitaker (1988). Briefly, 2.5 mL of isopropanol were added to 200 μL of microsomal membranes, heated 15 min at 70 °C, and cooled to room temperature. Then, 5 mL of chloroform:methanol (2:1 v/v) were added, heated at 70 °C, and filtered. After filtration, 0.5 mL of 0.88% KCI were added. Polar and non-polar phases were separated using a separation funnel. Non-polar phase was re-extracted twice with 3 mL of methanol-water (1:1 v/v), and the mixed with the polar phase. The extracted phospholipids and galactolipids were dried under N_2 flow at 60 °C and stored at -20 °C until TLC separation.

The extracted phospholipids and galactolipids were diluted in 500 μL of chloroform and aliquots of 50 μL were placed and separated on a silica gel 60 TLC plate (Merck®). Pure standards of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglyceroll (PG), cardiolipin (CL), phosphatidylinositol (PI), monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG) were also included in the analysis. The mobile phase consisted of a mixture of chloroform:methanol:acetic acid:water (160:40:8:4). After running time, the silica gel plates were dried in a N_2 flow and