Scientia Horticulturae 161 (2013) 266–272

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Shading affects flesh calcium uptake and concentration, bitter pit incidence and other fruit traits in "Greensleeves" apple

Sergio T. de Freitas[a,*], Cassandro V.T. do Amarante[b], Abhaya M. Dandekar[c], Elizabeth J. Mitcham[c]

[a] Brazilian Agricultural Research Corporation, Embrapa Semiárido, Petrolina, PE 56302-970, Brazil
[b] Department of Agronomy, Santa Catarina State University, Lages, SC 88520000, Brazil
[c] Department of Plant Sciences, University of California, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 26 May 2013
Received in revised form 15 July 2013
Accepted 16 July 2013

Keywords:
Calcium deficiency
Cell wall
Nutrients
PAR
Photosynthetically active radiation
Physiological disorder

ABSTRACT

Bitter pit (BP) is a physiological disorder in apple (*Malus* × *domestica* Borkh) believed to be triggered by low Ca^{2+} concentrations in the fruit, and which may be influenced by environmental conditions. The objectives of this study were to explore the effects of tree shading on total and cell wall bound fruit Ca^{2+} content and fruit susceptibility to BP. 'Greensleeves' (GS) apple trees were cultivated under shaded and non-shaded conditions. The shading condition was applied 70 days after full bloom by reducing about 50% of the sunlight reaching the tree canopies. Shading increased stem water potential and leaf stomatal conductance. Bitter pit was observed only in shaded fruit. Cortical Ca^{2+} was most abundant in shaded fruit without BP symptoms, intermediary in shaded fruit with BP, and lowest in non-shaded fruit. The cell wall Ca^{2+} concentration was higher in shaded than non-shaded fruit, but shaded fruit with and without BP had similar cell wall Ca^{2+} concentrations. The degree of pectin deesterification and the expression of two pectin methylesterases (*PME1* and *PME2*) were higher in shaded fruit than in non-shaded fruit. The percentage of total cortical Ca^{2+} bound to the cell wall was highest in shaded fruit with BP. Shaded fruit without BP had lower Mg^{2+} content and Mg^{2+}/Ca^{2+} ratio in cortical tissue than shaded fruit with BP. The results indicate that although shade increases fruit Ca^{2+} uptake, it also enhances fruit susceptibility to BP by increasing Mg^{2+} uptake and Ca^{2+} binding to the cell wall in fruit cortical tissue.

Published by Elsevier B.V.

1. Introduction

Calcium (Ca^{2+}) is required in the fruit for cellular signaling responses, cell wall structure and strength, as a counter-ion inside storage organelles, and for plasma membrane structure and integrity (Kendal, 2004; White and Broadley, 2003). Bitter pit (BP) is a Ca^{2+} deficiency disorder believed to be triggered by low total fruit Ca^{2+} concentrations (Ferguson and Watkins, 1989) and abnormal regulation of cellular Ca^{2+} partitioning (De Freitas et al., 2010).

At the whole plant level, limiting the light intensity that reaches the canopy can potentially affect fruit susceptibility to physiological disorders by changing fruit uptake of water, photosynthates, and nutrients (Ferguson et al., 1999; Woolf and Ferguson, 2000). High light intensity increases leaf and fruit temperature and transpiration rates (Brough et al., 1986; Ho and White, 2005). Since Ca^{2+} moves in the plant exclusively through xylem vessels (Ho and White, 2005), higher leaf and fruit transpiration rates could favor higher leaf and fruit Ca^{2+} uptake. However, high light intensity increases vapor pressure deficit (VPD) that is known to enhance leaf transpiration at higher rates than fruit transpiration, decreasing plant water potential and xylemic fruit water and therefore Ca^{2+} uptake (Adams and Ho, 1993, 1992; Araki et al., 2000; De Freitas et al., 2011a; Guichard et al., 2005; Ho, 1989; Tadesse et al., 2001; Taylor and Locascio, 2004). Accordingly, approaches that increase fruit transpiration are more effective in increasing fruit Ca^{2+} uptake than increasing Ca^{2+} concentrations available in the root system (Paiva et al., 1998). The negative effect of high light intensity and temperature on fruit Ca^{2+} uptake could be overcome by shading techniques to reduce light intensity reaching the canopy (Chen et al., 1998; Dussi et al., 2005; Ma and Cheng, 2004; Montanaro et al., 2006). In this context, whole plant shading techniques could help reducing apple fruit susceptibility to BP, especially in growing regions that have abundant sunny days.

Although total fruit Ca^{2+} uptake has been shown to influence fruit susceptibility to Ca^{2+} deficiency disorders, previous studies have shown that Ca^{2+} binding to the cell wall can be the final mechanism regulating BP incidence (De Freitas et al., 2010). Approximately 60% of Ca^{2+} in apple cortical tissue is bound to the cell wall (De Freitas et al., 2010; Harker and Venis, 1991; White and Broadley, 2003). Therefore, an increase in cell wall Ca^{2+} binding

* Corresponding author. Tel.: +55 87 3866 3710; fax: +55 87 3866 3815.
E-mail address: sergio.freitas@embrapa.br (S.T. de Freitas).

0304-4238/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.scienta.2013.07.019

sites could trigger a cell-localized Ca^{2+} deficiency and BP symptoms in fruit tissue. Increased Ca^{2+} binding to the cell wall could be the result of increased synthesis of deesterified pectins and/or increased expression and activity of enzymes that create Ca^{2+} binding sites in the cell wall matrix, such as pectin methylesterases (PMEs) (Ralet et al., 2001). Increased Ca^{2+} binding to the cell wall of isolated cortical cells is consistent with the finding that damaged tissues usually have more Ca^{2+} than the surrounding healthy tissues, most in a water-insoluble form (Gracia et al., 2008; Steenkamp and Villiers, 1983). In addition, cortical water-soluble Ca^{2+} decreases during ripening and pitted fruit have less water-soluble Ca^{2+} than sound fruit (Pavicic et al., 2004; Steenkamp and Villiers, 1983). Environmental growing conditions are known to affect cell wall composition and metabolism (Goulao and Oliveira, 2008), which can potentially affect the dynamics of Ca^{2+} binding to the cell wall and fruit susceptibility to BP.

The objectives of this study were to explore the effects of tree shading on total and cell wall bound fruit Ca^{2+} content and fruit susceptibility to BP.

2. Materials and methods

'Greensleeves' (GS) apples (*Malus* × *domestica* Borkh) grafted onto M9 rootstocks were cultivated in an orchard located in Davis, California, USA. The 14-years-old trees did not receive any foliar Ca^{2+} sprays in the field during fruit growth. During the entire growing period, the orchard was regularly irrigated to keep the soil moisture near field capacity. The trees were left non-shaded or shaded with black net suspended above the trees at 70 days after full bloom (DAFB). The net was made of high density polyethylene with 50% shading mesh and 100% UV stabilization. The study followed a complete randomized block design with four blocks per treatment and one tree per block. At harvest, comparisons were made between non-shaded and shaded conditions. After three months of storage at 0 °C, the treatments followed a factorial design with combinations between shading conditions (non-shaded or shaded) and BP incidence (with or without BP).

Non-shaded and shaded environmental conditions were analyzed for photosynthetically active radiation (PAR; λ = 400–700 nm), relative humidity (RH), and air temperature at the top of the canopy. The trees were evaluated for leaf stomatal conductance with a steady-state porometer (LI-1600; LI-COR Biotechnology, Lincoln, NE, USA) and stem water potential quantified with a pressure chamber (PMS Instrument Company, Albany, OR) as described by McCutchan and Shackel (1992). Monthly measurements were made between 12 pm and 1 pm, from the onset of shading.

Two hundred fruit from each block were harvested from the middle region of the canopy on each tree at 120 DAFB. Fruit fresh weight was determined by dividing total fruit weight on each block by the total number of fruit. Physico-chemical analyses were accomplished on 10 randomly selected fruit per block. Starch content was estimated by cutting the fruit in half, then dipping the cut end in a solution containing iodine:potassium iodide (QA Supplies, LLC, Norfolk, VA) for 1 min for starch staining. The degree of flesh staining was then evaluated according to the California Granny Smith Starch Index where 0 = 100% starch and 6 = 0% starch (Mitcham, 1993). Fruit firmness was measured as resistance to penetration with an 11 mm probe on opposite sides at the equator of the fruit after removal of a small area of peel using a Fruit Texture Analyzer (Güss, Strand, South Africa). Juice samples were extracted by squeezing two cortical wedges cut from both sides of the fruit in two layers of cheese cloth. Total soluble solids were determined with a digital refractometer (Abbe 10450, American Optical, Buffalo, NY, USA). The acidity, determined as the percentage of malic acid equivalents, was measured with an automatic titrator (Radiometer, Copenhagen, Denmark) by titrating 4 mL of juice with 0.1 N NaOH to pH 8.2. Skin color was determined on opposite sides of each fruit with a Chroma Meter CR-310 (Minolta, Osaka, Japan). The results were expressed as hue angle (h°, where 90° = full yellow and 180° = full green), lightness (L, where 0 = black and 100 = white) and chroma (C, representing color saturation).

After harvest, fruit were stored at 0 ± 0.5 °C and 90–95% RH for three months. At the end of storage, all fruit were analyzed for BP incidence (%) and severity (BP index). BP index was assessed according to a five-point visual scale (0 = no pit, 1 = 1 to 5 pits, 2 = 6 to 10 pits, 3 = 11 to 15 pits, 4 = 16–20 pits, 5 = >20 pits per fruit) and calculated using the formula described by Pesis et al. (2010):

$$\text{BP index} = \sum_{0}^{5} \frac{(\text{index level}) \times (\text{fruit at this level})}{\text{total number of fruit}}$$

Fruit with and without BP were segregated and outer cortical tissue was manually excised from the calyx end, just beneath the skin up to a depth of 5 mm, frozen in liquid N_2 and stored at −80 °C for later analysis. Frozen samples were analyzed for cell wall content, soluble and insoluble pectin content, degree of pectin deesterification, expression level of two *PME* genes, concentration of total calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}) and nitrogen (N), as well as the concentration of Ca^{2+} in the cell wall and in soluble and insoluble pectins. We later calculated the percentage of cortical Ca^{2+} bound to the cell wall and to soluble and insoluble pectins.

Cell walls were extracted as described previously (Campbell et al., 1990). Frozen samples were boiled in 95% ethanol (4 mL g^{-1} fresh weight) for 20 min, homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY, USA) at top speed for 60 s, then centrifuged at 1500 × *g* for 10 min and the supernatant decanted. The pellet was suspended by homogenization in 80% ethanol in the Polytron, centrifuged, and the supernatant decanted. This procedure was repeated until the supernatant was colorless. The crude cell wall pellet was dried at room temperature, then suspended in DMSO:water (9:1, v/v; 20 mL g^{-1} dry weight) and stirred 24 h to remove starch (Selvendran and O'Neill, 1987). The slurry was centrifuged at 1500 × *g* for 10 min, DMSO was decanted, and the pellet was washed repeatedly in 95% ethanol to remove all traces of DMSO. The pellet was dried and suspended once in acetone. The acetone-washed, starch-free cell wall material was air-dried. Water soluble and insoluble pectin fractions were isolated as described by De Freitas et al. (2010).

Nutrient concentrations were determined in the previously extracted, frozen and freeze dried outer cortical tissue at the calyx end of the fruit. Nitrogen was analyzed using a combustion method (AOAC, 2006). Potassium was extracted with 2% acetic acid and quantitatively assessed by atomic emission spectrometry (Johnson and Ulrich, 1959). Calcium and Mg^{2+} were determined by subjecting tissue to microwave acid digestion/dissolution and subsequent analysis by inductively coupled plasma atomic emission spectrometry (Meyer and Keliher, 1992). Cell walls and water soluble and insoluble pectins were also analyzed for Ca^{2+} in a similar fashion and the results presented on a fresh weight basis of cortical tissue and as a percentage of total cortical tissue Ca^{2+} concentration.

The degree of pectin deesterification was determined by the reductive method. Samples were incubated overnight in 1 mL 10 mg mL^{-1} $NaBH_4$ in 50% ethanol. The samples were then neutralized with acetic acid and dried. Incubation with $NaBH_4$ converts esterified uronosyl residues to their respective neutral sugars. Later, reduced and the respective unreduced samples were dissolved in 67% H_2SO_4 and the total uronic acid determined as described by

Table 1
Environmental and plant attributes of 'Greensleeves' apple trees grown in non-shaded and shaded conditions.

Attribute	Non-shaded	Shaded
Photosynthetically active radiation ($\mu E\,m^{-2}\,s^{-1}$)	1740.0 ± 34.6 a[a]	861.3 ± 8.2 b
Relative humidity (%)	35.2 ± 2.3 b	41.2 ± 0.7 a
Air temperature (°C)	36.4 ± 0.6 a	35.2 ± 0.6 b
Leaf stomatal conductance ($\mu mol\,m^{-2}\,s^{-1}$)	266.6 ± 9.4 b	473.8 ± 15.3 a
Plant water potential (MPa)	−2.08 ± 0.13 b	−1.46 ± 0.06 a

[a] Mean values (±SE) within each row followed by different letters were significantly different ($p < 0.05$).

Ahmed and Labavitch (1977). The total uronic acid concentration determined in the reduced samples represents deesterified uronic acid, which is presented as a percentage of the total uronic acid in the respective unreduced sample (Klein et al., 1995).

Pectin methylesterase expression was determined by extracting total RNA from the frozen outer cortical fruit tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The RNA concentration and purity were determined at 260 nm and 260 nm/280 nm, respectively, using a UV spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA). For all samples, 3 μg total RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative, real-time PCR was performed by adding 1xSYBR green (Applied Biosystem, Foster City, CA, USA) to ~100 ng synthesized cDNA from each sample. The data were normalized based on expression of the housekeeping apple 18S rRNA gene (Defilippi et al., 2005). All primers were 20 bp long with a melting point temperature of 58 ± 3 °C. All *PME* nucleotides represent part of Unigene sequences obtained from the apple (*Malus domestica* Borkh) EST data base in the NCBI gene bank (http://www.ncbi.nlm.nih.gov/Genbank/). The putative PMEs were named *PME1* (NCBI: CO168183), with 98% identity to *pectin methylesterase* (NCBI: AB067684.1), and *PME2* (NCBI: CO415488), with 83% identity to *pectin methylesterase* (NCBI: XP 002278061.1).

Statistical analysis was performed on each variable by means of analysis of variance (ANOVA) using the SAS statistical package (SAS Institute, 2002). The mean values of four replicates (±standard error) were compared using Tukey's test ($p < 0.05$).

3. Results

The shade provided by netting reduced the PAR by 50.5% and air temperature by 1.2 °C, and increased RH by 6%, leaf stomatal conductance by 45%, and plant water potential by 0.62 MPa (Table 1). Shading reduced fruit fresh weight and TSS content, but had no effect on fruit starch content, flesh firmness or malic acid content (Table 2). Skin color was greener (higher h° and lower L) in shaded than in non-shaded fruit at harvest (Table 2). The chroma value of skin tissue was similar between non-shaded and shaded apples (Table 2).

There was no BP at the time of harvest. After three months storage at 0 °C, BP was only observed in fruit from shaded trees, which presented 42 (±7.14 SE) % BP incidence and 1.6 (±0.14 SE) BP severity (BP index).

Shading increased cortical Ca^{2+} concentration (Table 3). The highest cortical tissue Ca^{2+} concentration was observed in shaded fruit without BP symptoms (Table 3). Pitted fruit grown in the shade had more cortical tissue Ca^{2+} than non-pitted fruit grown under non-shaded conditions (Table 3). The magnesium concentration of cortical tissues was not significantly different between shaded fruit damaged by BP and non-shaded fruit. The lowest Mg^{2+} concentration was observed in cortical tissue of shaded fruit without BP

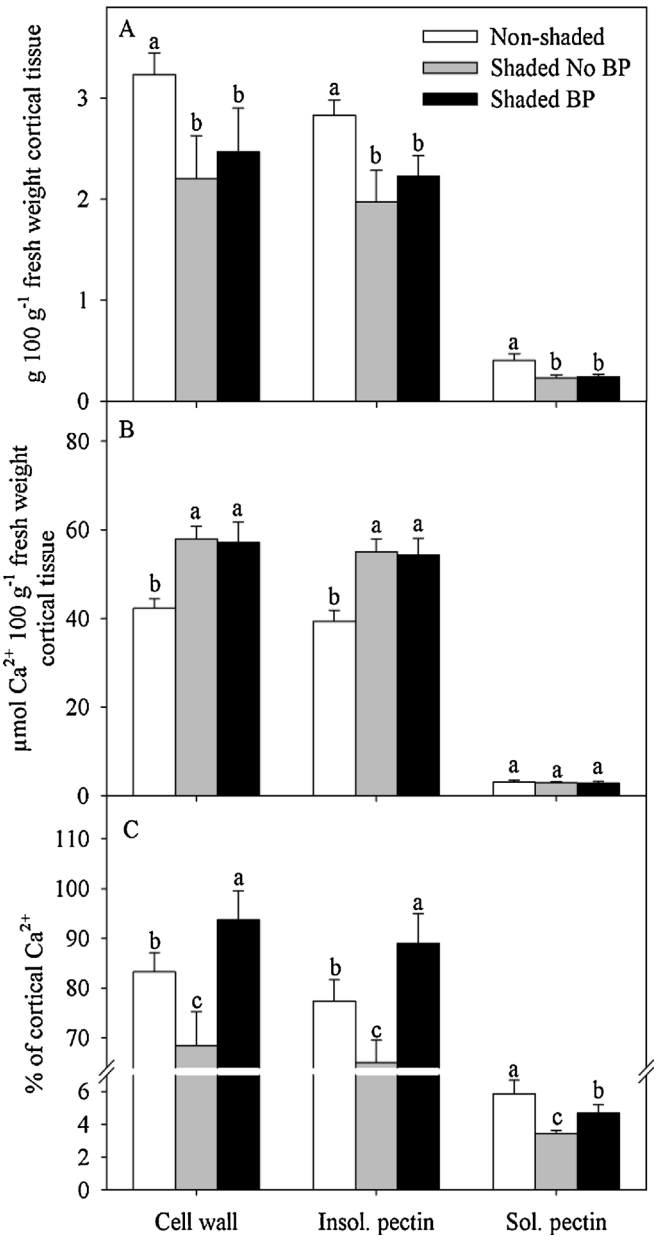

Fig. 1. Concentration of cell wall, water-insoluble pectins and soluble pectins (A) and Ca^{2+} concentration (B) in cortical tissue, and percentage of cortical Ca^{2+} bound to the cell wall, water-insoluble pectins and soluble pectins (C) in 'Greensleeves' apple fruit grown under shaded and non-shaded conditions and stored for three months at 0 °C. Mean values with different letters for the same cell wall fraction were significantly different according to Tukey's test ($p < 0.05$). Values represent the mean of four replicates ± SE.

(Table 3). The concentrations of K^+ and N were similar among non-shaded and shaded fruit with and without BP symptoms (Table 3). The lowest Mg^{2+}/Ca^{2+} and K^+/Ca^{2+} ratios were observed in cortical tissue of shaded fruit without BP symptoms (Table 3). The lowest N/Ca^{2+} ratio was observed in cortical tissue of shaded fruit with BP symptoms (Table 3).

The total cell wall and water-soluble and insoluble pectin content in cortical tissue were higher in fruit grown in non-shaded than in shaded conditions, but there was no difference between shaded fruit with and without BP (Fig. 1A).

Calcium concentrations in the cell wall and water-insoluble pectin were lower in fruit grown under non-shaded than in shaded conditions (Fig. 1B). There was no difference in cell wall and water-insoluble pectin Ca^{2+} concentrations between shaded fruit with

Table 2
Fruit fresh weight (FFW), starch index, flesh firmness, total soluble solids (TSS), malic acid equivalents and skin color of non-shaded and shaded 'Greensleeves' apples at harvest.

Shading	FFW (g)	Starch (1–6)	Firmness (N)	TSS (%)	Malic acid (%)	Skin color		
						h°	C	L
No	134.0 a[a]	2.95 a	66.9 a	15.0 a	0.67 a	105.6 b	48.6 a	77.7 a
Yes	119.7 b	3.12 a	62.2 a	11.5 b	0.60 a	114.3 a	48.1 a	71.8 b
CV (%)	0.66	9.92	8.63	3.05	11.3	1.16	1.25	0.32

[a] Mean values within each column followed by different letters were significantly different ($p < 0.05$).

Table 3
Concentration of Ca^{2+}, Mg^{2+}, K^+ and N, and ratios of Mg^{2+}/Ca^{2+}, K^+/Ca^{2+} and N/Ca^{2+} in the outer cortical tissue of 'Greensleeves' apples grown under non-shaded (without BP) and shaded (with and without BP) conditions after three months of storage at 0 °C.

Shading	BP	Ca^{2+}	Mg^{2+}	K^+	N	Mg^{2+}/Ca^{2+}	K^+/Ca^{2+}	N/Ca^{2+}
		μmol 100 g fw^{-1}		mmol 100 g fw^{-1}				
No	No	50.7 c[a]	208.3 ab	2.58 a	4.24 a	4.12 a	50.9 a	83.5 a
Yes	No	84.5 a	191.6 b	2.55 a	4.40 a	2.27 c	30.1 b	52.1 b
Yes	Yes	61.0 b	213.8 a	2.77 a	4.22 a	3.51 b	45.4 a	45.4 c
CV (%)		1.47	4.86	6.02	7.78	7.77	8.65	3.81

[a] Mean values within each column followed by different letters were significantly different according to Tukey's test ($p < 0.05$).

or without BP (Fig. 1B). There was no difference in water-soluble pectin Ca^{2+} concentration between non-shaded (without BP) and shaded (with and without BP) fruit (Fig. 1B).

The percentage of cortical Ca^{2+} bound to the cell wall and water-insoluble pectin was higher in shaded fruit with BP than in shaded fruit without BP and non-shaded fruit (Fig. 1C). The lowest percentage of cortical Ca^{2+} bound to soluble pectin was observed in shaded fruit without BP (Fig. 1C).

The highest degree of pectin deesterification and expression of *PME1* and *PME2* were observed in cortical tissue of fruit grown in shaded conditions (Fig. 2). The highest degree of pectin deesterification was observed in shaded fruit with BP (Fig. 2). The expression levels of *PME1* and *PME2* were similar between shaded fruit with and without BP (Fig. 2).

4. Discussion

Our study showed that BP incidence and severity in apple fruit was increased by tree shading. Our data suggest that fruit susceptibility to BP may be enhanced by Ca^{2+} binding to the cell wall in fruit cortical tissue. In addition, a higher Mg^{2+}/Ca^{2+} ratio was also associated with higher fruit susceptibility to BP. Shading reduced fruit weight and TSS content, but had no effect on starch content, flesh firmness or

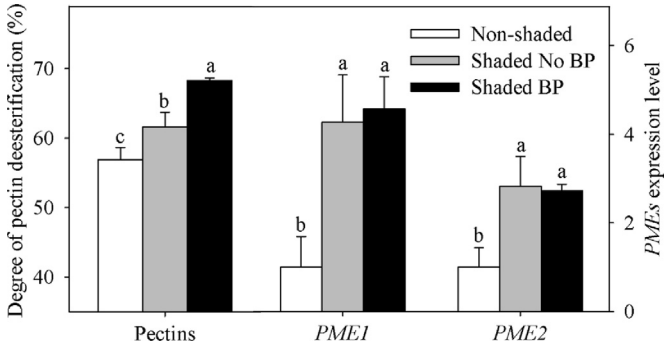

Fig. 2. Degree of pectin deesterification and expression of *PME1* and *PME2* genes in cortical tissue of 'Greensleeves' apple fruit grown under shaded and non-shaded conditions and stored for three months at 0 °C. Mean values with different letters for the same attribute were significantly different according to Tukey's test ($p < 0.05$). Values represent the mean of four replicates ± SE.

malic acid content. Therefore, the shading treatment did not substantially affect fruit development and maturity at harvest that otherwise might have impacted fruit susceptibility to BP.

4.1. Shading decreased fruit growth and dilution of fruit Ca^{2+} concentration

Shaded fruit were smaller and had lower TSS and cell wall content than non-shaded fruit. Studies have shown that much of the carbohydrate output of photosynthesis is channeled into sugars and cell wall polysaccharide biosynthesis in the fruit (Chen et al., 1997; Dussi et al., 2005; Grappadelli et al., 1994; Sorensen et al., 2010). Under low light intensity, apple trees possibly had lower photosynthetic rates, resulting in the lower accumulation of sugars and cell wall polysaccharides and reduced growth rates of the fruit (Amarante et al., 2011; Chen et al., 1997; Dussi et al., 2005; Grappadelli et al., 1994; Palmer, 1989). This suggests that the higher Ca^{2+} concentration observed in shaded fruit tissue could be attributed to lower dilution of the Ca^{2+} accumulated in the fruit due to lower fruit growth rates. However, shading reduced fruit weight by 10.7% and increased Ca^{2+} concentration by 66.6% in fruit without BP and by 20.3% in fruit with BP. Therefore, other factors were also responsible for the higher Ca^{2+} concentration observed in the cortical tissue of shaded fruit.

4.2. Shading increased fruit Ca^{2+} uptake

Although others have shown that shading apple and kiwifruit plants by reducing sunlight incidence by up to 40% decreased fruit Ca^{2+} uptake (Chen et al., 1997; Montanaro et al., 2006), our apple trees showed increased fruit Ca^{2+} uptake with a 50% reduction in PAR. Additionally, this shading maintained a slightly lower air temperature and higher relative humidity in the canopy, along with higher plant water potential. Under conditions of lower plant water potential and high evaporative demand, similar to our unshaded conditions, higher transpiration rates in leaves than in fruit could limit fruit Ca^{2+} uptake or even trigger a back flow of xylemic sap and Ca^{2+} from the fruit back to the plant (Adams and Ho, 1993, 1992; Araki et al., 2004, 2000; Eguchi et al., 2003; Guichard et al., 2005; Ho, 1989; Tadesse et al., 2001; Taylor and Locascio, 2004). In our

study, shading maintained higher plant water potential, indicating lower tension in the xylem vessels connecting plant to fruit, which potentially favored water and Ca^{2+} movement into the low-transpiring fruit, contributing to the observed higher Ca^{2+} content in fruit cortical tissue.

4.3. Shading increased Ca^{2+} binding to the cell wall and fruit susceptibility to BP

Shaded fruit had more total tissue Ca^{2+} and were also more susceptible to BP than non-shaded fruit, suggesting that other factors besides total tissue Ca^{2+} determined BP susceptibility. Our data indicate that susceptibility of shaded fruit to BP was affected by the capacity of the cell wall to bind Ca^{2+}. Although shaded fruit had less total cell wall and water-insoluble and soluble pectins in the cortical tissue, these fruit had higher expression of *PME1* and *PME2*, which resulted in more pectin deesterification, more Ca^{2+} bound to the cell wall, and greater fruit susceptibility to BP than non-shaded fruit. The activity of PMEs creates carboxylate groups on pectin chains that strongly bind Ca^{2+}, forming a structure with high affinity for this ion known as an 'egg-box' structure (Caffall and Mohnen, 2009; Micheli, 2001; Ralet et al., 2001). The strongly bound Ca^{2+} is present in the water-insoluble pectin fraction, which is an important structural component of the cell wall matrix (Caffall and Mohnen, 2009). Accordingly, our results show that most of the cell wall bound Ca^{2+} was in the water-insoluble pectin fraction, suggesting its interaction with the cell wall matrix through the high affinity 'egg-box' structures. Other studies have also suggested that PME activity and Ca^{2+} binding to the cell wall can affect cellular Ca^{2+} partitioning and fruit susceptibility to BP (De Freitas et al., 2010).

Shaded fruit with and without BP had similar cell wall and water-insoluble and soluble pectin content in the cortical tissue, as well as similar Ca^{2+} concentration in all cell wall fractions. However, shaded fruit with BP had a higher percentage of cortical Ca^{2+} bound to the cell wall and water-insoluble and soluble pectins than shaded fruit without BP. Therefore, in shaded fruit, susceptibility to BP was determined by total cortical tissue Ca^{2+}. Shaded fruit without BP had more Ca^{2+} in the cortical tissue, resulting in a greater concentration of cortical Ca^{2+} not bound to the cell wall and available for other cellular functions, reducing fruit susceptibility to BP compared to shaded fruit with BP. The potential reasons for such difference in Ca^{2+} content for shaded fruit could include fruit position in the tree, individual fruit transpiration rates, the number of functional xylem vessels in the fruit and different auxin/gibberellin ratio in the fruit that is believed to determine xylem vessel development and function in fruit tissue (Dražeta et al., 2004; Ferguson and Watkins, 1989; Paiva et al., 1998; Saure, 1996, 2005).

Shading can be viewed as an abiotic stress, known to stimulate auxin biosynthesis and transport in the plant that leads to cell expansion, most of these responses taking place through cytosolic Ca^{2+} oscillations (White and Broadley, 2003; Hornitschek et al., 2012). Studies suggest that during the process of auxin-induced cell growth (Perrot-Rechenmann, 2010), low levels of cytosolic Ca^{2+} in the tissue could result in abnormal auxin-induced signaling responses, and insufficient apoplastic Ca^{2+} concentrations could lead to excessive cell enlargement, both cases leading to cell death and Ca^{2+} deficiency symptom development in the fruit (Ho and White, 2005). Therefore, the higher percentage of cortical Ca^{2+} bound to the cell wall of shaded fruit with BP could result in lower levels of cytosolic and/or free apoplastic Ca^{2+} concentrations, which may explain the higher fruit susceptibility to BP under shade conditions.

4.4. Non-shaded conditions decreased fruit Ca^{2+} content and fruit susceptibility to BP

Apple fruit grown under high light intensity were less susceptible to BP and had lower cortical tissue Ca^{2+} content than shaded fruit. Our data suggest that the lower susceptibility of non-shaded fruit to BP may be due to reduced *PME1* and *PME2* expression, resulting in less pectin deesterification, less Ca^{2+} bound to the cell wall, and therefore a higher percentage of cortical Ca^{2+} not bound to the cell wall and available for other cellular functions than in shaded fruit. It is possible that cortical tissue Ca^{2+} concentration was also more homogeneous among non-shaded fruit than among shaded fruit, resulting in uniformly lower susceptibility of non-shaded fruit to BP.

Fruit flesh temperatures well above 40 °C have been recorded frequently in direct sunlight (Ferguson et al., 1998; Woolf and Ferguson, 2000). Such high temperatures can increase synthesis of heat-shock proteins (HSPs), up-regulate the xanthophyll cycle, increase the activity of antioxidant enzymes, and increase soluble antioxidants of the ascorbate-glutathione cycle in fruit tissue (Ferguson et al., 1998; Ma and Cheng, 2004). HSPs are responsible for protein folding, assembly, and translocation in many cellular processes, stabilizing proteins and membranes and assisting in protein refolding, which is particularly important for cell survival under stress conditions (Wang et al., 2004). HSPs are part of the mechanism that attempts to maintain protein and membrane stability, inhibiting Ca^{2+} deficiency symptom development in fruit tissue (De Freitas et al., 2011b). Similarly, antioxidant mechanisms may also reduce Ca^{2+} deficiency symptom development, possibly due to detoxification of reactive oxygen species in fruit tissue (De Freitas et al., 2011b; Sabban-Amin et al., 2011). Although HSPs and antioxidants potentially reduce fruit susceptibility to Ca^{2+} deficiency disorders, the mechanisms involved are not well understood.

4.5. Suggested role of other cortical nutrients on fruit susceptibility to BP

High BP incidence is usually positively correlated with high Mg^{2+}/Ca^{2+}, K^+/Ca^{2+}, and N/Ca^{2+} ratios in fruit tissue (Amarante et al., 2006; Bramlage et al., 1980; Lanauskas and Kvikliene, 2006). However, the mechanisms through which Mg^{2+}, K^+, and N affect fruit susceptibility to BP are still poorly understood. Our results showed that shaded fruit without BP had the lowest Mg^{2+}/Ca^{2+} ratio due to the combined effect of high Ca^{2+} and low Mg^{2+} content in the cortical tissue. The Mg^{2+} concentration in the cortical tissue of shaded fruit with BP (213.8 μmol 100 g fw^{-1}) was higher than in shaded fruit without BP (191.6 μmol 100 g fw^{-1}). Since cortical Ca^{2+} was higher in shaded fruit without BP (84.5 μmol 100 g fw^{-1}) than shaded fruit with BP (61.0 μmol 100 g fw^{-1}), the high Mg^{2+} uptake may enhance the effect of low Ca^{2+} uptake in increasing fruit susceptibility to BP in shaded fruit without BP. Magnesium at high levels could compete with Ca^{2+} for binding sites at the plasma membrane surface. Magnesium binding at the plasma membrane could then replace Ca^{2+}, but not the role of Ca^{2+} in maintaining proper plasma membrane structure and integrity, which could lead to leaky plasma membranes, the initial symptom of Ca^{2+} deficiency disorders in fruit (De Freitas et al., 2010; Schonherr and Bukovac, 1973; Yermiyahu et al., 1994). Although other studies have suggested that K^+/Ca^{2+} and N/Ca^{2+} ratios are related to fruit susceptibility to BP (Bramlage et al., 1980; Lanauskas and Kvikliene, 2006), our study showed a poor relationship. There were no differences in cortical fruit K^+ and N content among non-shaded and shaded fruit with and without BP. Therefore, the observed differences in the K^+/Ca^{2+} and N/Ca^{2+} ratios were

mostly due to differences in Ca^{2+} concentrations in fruit cortical tissue.

4.6. Suggested mechanisms involved in BP development in apple fruit in response to shade

Shading apple trees not only increased fruit Ca^{2+} content, but also surprisingly increased fruit susceptibility to BP. Under shaded conditions, fruit cortical tissue had a higher capacity to strongly bind Ca^{2+} ions in the water-insoluble pectin network in the cell wall matrix, reducing the levels of Ca^{2+} available for other cellular functions. Under shaded conditions, fruit with lower total Ca^{2+} uptake have a lower concentration of non-cell wall bound Ca^{2+}, thereby enhancing fruit susceptibility to BP. The combined effect of low Ca^{2+} and high Mg^{2+} concentrations in the cortical tissue further contributes to higher fruit susceptibility to BP possibly due to the competition between these cations at the membrane.

References

Adams, P., Ho, L.C., 1993. Effects of environment on the uptake and distribution of calcium in tomato and on the incidence of blossom-end rot. Plant Soil 154, 127–132.

Adams, P., Ho, L.C., 1992. The susceptibility of modern tomato cultivars to blossom-end rot in relation to salinity. Journal of Horticultural Science 67, 827–839.

Ahmed, E.R.A., Labavitch, J.M., 1977. A simplified method for accurate determination of cell wall uronide content. Journal of Food Biochemistry 1, 361–365.

Amarante, C.V.T. do, Steffens, C.A., Argenta, L.C., 2011. Yield and fruit quality of 'Gala' and 'Fuji' apple trees protected by white anti-hail net. Scientia Horticulturae 129, 79–85.

Amarante, C.V.T. do, Chaves, D.V., Ernani, P.R., 2006. Multivariate analysis of nutritional attributes associated with bitter pit in 'Gala' apples. Pesquisa Agropecuária Brasileira 41, 841–846.

AOAC, 2006. Official Methods of Analysis of AOAC International, 8th ed. AOAC International, Gaithersburg, MD.

Araki, T., Eguchi, T., Wajima, T., Yoshida, S., Kitano, M., 2004. Dynamic analysis of growth, water balance and sap fluxes through phloem and xylem in tomato fruit: short-term effect of water stress. Environmental Control in Biology 42, 225–240.

Araki, T., Kitano, M., Eguchi, H., 2000. Dynamics of fruit growth and photoassimilate translocation in tomato plants (*Lycopersicon esculentum* Mill.) under controlled environment. Acta Horticulturae 534, 85–92.

Bramlage, W.J., Drake, M., Lord, W.J., 1980. The influence of mineral nutrition on the quality and storage performance of pome fruit grown in North America. Acta Horticulturae 92, 29–40.

Brough, D.W., Jones, H.G., Grace, J., 1986. Diurnal changes in water content of the stems of apple trees, as influenced by irrigation. Plant, Cell & Environment 9, 1–7.

Caffall, K.H., Mohnen, D., 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydrate Research 344, 1879–1900.

Campbell, A.D., Huysamer, M., Stotz, H.U., Greve, L.C., Labavitch, J.M., 1990. Comparison of ripening processes in intact tomato fruit and excised pericarp discs. Plant Physiology 94, 1582–1589.

Chen, K., Hu, G., Lenz, F., 1998. Apple yield and quality as affected by training and shading. Acta Horticulturae 466, 53–58.

Chen, K., Hu, G.Q., Lenz, F., 1997. Training and shading effects on vegetative and reproductive growth and fruit quality of apple. Gartenbauwissenschaft 62, 207–213.

Defilippi, B., Kader, A.A., Dandekar, A.M., 2005. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. Plant Science 168, 1199–1210.

De Freitas, S.T., Shackel, K.A., Mitcham, E.J., 2011a. Abscisic acid triggers whole-plant and fruit-specific mechanisms to increase fruit calcium uptake and prevent blossom end rot development in tomato fruit. Journal of Experimental Botany 62, 2645–2656.

De Freitas, S.T., Padda, M., Wu, Q., Park, S., Mitcham, E., 2011b. Dynamic alterations in cellular and molecular components during blossom-end rot development in tomatoes expressing *sCAX1*, a constitutively active Ca^{2+}/H^{+} antiporter from *Arabidopsis*. Plant Physiology 156, 844–855.

De Freitas, S.T., Amarante, C.V.T. do, Labavitch, J.M., Mitcham, E.J., 2010. Cellular approach to understand bitter pit development in apple fruit. Postharvest Biology and Technology 57, 6–13.

Dražeta, L., Lang, A., Hall, A.J., Volz, R.K., Jameson, P.E., 2004. Causes and effects of changes in xylem functionality in apple fruit. Annals of Botany 93, 275–282.

Dussi, M.C., Giardina, G., Sosa, D., Junyent, R.G., Zecca, A., Reeb, P., 2005. Shade nets effect on canopy light distribution and quality of fruit and spur leaf on apple cv. Fuji. Spanish Journal of Agricultural Research 3, 253–260.

Eguchi, T., Araki, T., Yoshida, S., Kitano, M., 2003. Xylem sap backflow from tomato fruit under water deficit condition. Acta Horticulturae 618, 347–351.

Ferguson, I.B., Volz, R., Woolf, A., 1999. Preharvest factors affecting physiological disorders of fruit. Postharvest Biology and Technology 15, 255–262.

Ferguson, I.B., Snelgar, W., Lay-Yee, M., Watkins, C.B., Bowen, J.H., 1998. Heat shock response in apple fruit in the field. Australian Journal of Plant Physiology 25, 155–163.

Ferguson, I.B., Watkins, C.B., 1989. Bitter pit in apple fruit. Horticultural Reviews 11, 289–355.

Goulao, L.F., Oliveira, C.M., 2008. Cell wall modifications during fruit ripening: when a fruit is not the fruit. Trends in Food Science & Technology 19, 4–25.

Gracia, J.V.M.A., Monge, E., Blanco, A., 2008. Visual detection of calcium by GBHA staining in bitter pit-affected apples. Food Science and Technology International 14, 315–319.

Grappadelli, L.C., Lakso, A.N., Flore, J.A., 1994. Early season patterns of carbohydrate partitioning in exposed and shaded apple branches. Journal of the American Society for Horticultural Science 119, 596–603.

Guichard, S., Gary, C., Leonardi, C., Bertin, N., 2005. Analysis of growth and water relations of tomato fruit in relation to air vapor pressure deficit and plant fruit load. Journal of Plant Growth Regulation 24, 201–213.

Harker, F.R., Venis, M.A., 1991. Measurement of intracellular and extracellular free calcium in apple fruit cells using calcium-selective microelectrodes. Plant, Cell & Environment 14, 525–530.

Ho, L.C., White, P.J., 2005. A cellular hypothesis for the induction of blossom-end rot in tomato fruit. Annals of Botany 95, 571–587.

Ho, L.C., 1989. Environmental effects on the diurnal accumulation of ^{45}Ca by young fruit and leaves of tomato plants. Annals of Botany 63, 281–288.

Hornitschek, P., Kohnen, M.V., Lorrain, S., Rougemont, J., Ljung, K., López-Vidriero, I., Franco-Zorrilla, M., Solano, R., Trevisan, M., Pradervand, S., Xenarios, I., Fankhauser, C., 2012. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant Journal 71, 699–711.

Johnson, C.M., Ulrich, A., 1959. Analytical Methods for Use in Plant Analysis. Bulletin 766. Agricultural Experiment Station, University of California, Berkeley, CA.

Kendal, D.H., 2004. The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiology 136, 2438–2442.

Klein, J.D., Hanzon, J., Irwin, P.L., Ben-Shalom, N., Lurie, S., 1995. Pectin esterase activity and pectin methyl esterification in heated 'Golden Delicious' apples. Phytochemistry 39, 491–494.

Lanauskas, J., Kvikliene, N., 2006. Effect of calcium foliar application on some fruit quality characteristics of 'Sinap Orlovskij' apple. Agronomy Journal 4, 31–36.

Ma, F., Cheng, L., 2004. Exposure of the shaded side of apple fruit to full sun leads to up-regulation of both the xanthophylls cycle and the ascorbate-glutathione cycle. Plant Science 166, 1479–1486.

McCutchan, H., Shackel, K.A., 1992. Stem water potential as a sensitive indicator of water stress in prune trees (*Prunus domestica* L. cv. French). Journal of the American Society for Horticultural Science 117, 607–611.

Meyer, G.A., Keliher, P.N., 1992. An overview of analysis by inductively coupled plasma-atomic emission spectrometry. In: Montaser, A., Golightly, D.W. (Eds.), Inductively Coupled Plasmas in Analytical Atomic Spectrometry, vol. 2. VCH Publishers, New York, NY, pp. 473–516.

Micheli, F., 2001. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. Trends in Plant Science 6, 414–419.

Mitcham, E.J., 1993. Maturity standards for California-grown 'Granny Smith' apples. In: Proceedings of the 9th Annual Washington Tree Fruit Postharvest Conference, Wenatchee Convention Center, Wenatchee, WA, pp. 21–22.

Montanaro, G., Dichio, B., Xiloyannis, C., Celano, G., 2006. Light influences transpiration and calcium accumulation in fruit of kiwifruit plants (*Actinidia deliciosa* var. deliciosa). Plant Science 170, 520–527.

Paiva, E.A.S., Martinez, H.E.P., Casali, V.W.D., Padilha, L., 1998. Occurrence of blossom-end rot in tomato as a function of calcium dose in the nutrient solution and air relative humidity. Journal of Plant Nutrition 21, 2663–2670.

Palmer, J.W., 1989. Canopy manipulation for optimizing utilization of light. In: Wright, C. (Ed.), Manipulation of Fruiting, vol. 1. Butterworths, London, pp. 245–262.

Pavicic, N., Jemric, T., Kurtanjek, Z., Cosic, T., Pavlovic, I., Blaskovic, D., 2004. Relationship between water-soluble Ca and other elements and bitter pit occurrence in 'Idared' apples: a multivariate approach. Annals of Applied Biology 145, 193–196.

Perrot-Rechenmann, C., 2010. Cellular responses to auxin: division versus expansion. Cold Spring Harbour Perspectives on Biology 2, 1–15.

Pesis, E., Ebeler, S.E., de Freitas, S.T., Padda, M., Mitcham, E.J., 2010. Short anaerobiosis period prior to cold storage alleviates bitter pit and superficial scald in 'Granny Smith' apples. Journal of the Science of Food and Agriculture 90, 2114–2123.

Ralet, M.C., Dronnet, V., Buchholt, H.C., Thibaulta, J.F., 2001. Enzymatically and chemically de-esterified lime pectins: characterisation, polyelectrolyte behaviour and calcium binding properties. Carbohydrate Research 336, 117–125.

Sabban-Amin, R., Feygenberg, O., Belausov, E., Pesis, E., 2011. Low-oxygen and 1-MCP pretreatments delay superficial scald development by reducing reactive oxygen species (ROS) accumulation in stored 'Granny Smith' apples. Postharvest Biology and Technology 62, 295–304.

SAS Institute, 2002. Statistical Analysis System, v.8.2. SAS Institute, Cary, NC.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: its mechanism and endogenous control. Scientia Horticulturae 105, 65–89.

Saure, M.C., 1996. Reassessment of the role of calcium in development of bitter pit in apple. Australian Journal of Plant Physiology 23, 237–243.

Schonherr, J., Bukovac, M.J., 1973. Ion exchange properties of isolated tomato fruit cuticular membrane: exchange capacity, nature of fixed charges and cation selectivity. Planta 109, 73–93.
Selvendran, R.R., O'Neill, M.A., 1987. Isolation and analysis of cell walls from plant material. In: Glick, D. (Ed.), Methods of Biochemical Analysis, vol. 32. Wiley Interscience, New York, pp. 25–153.
Sorensen, I., Domozych, D., Willats, G.T., 2010. How have plant cell walls evolved? Plant Physiology 153, 366–372.
Steenkamp, J., Villiers, O.T., 1983. The role of organic acids and nutrient elements in relation to bitter pit in 'Golden Delicious' apples. Postharvest Physiology and Storage 138, 35–42.
Tadesse, T., Nichols, M.A., Hewett, E.W., Fisher, K.J., 2001. Relative humidity around the fruit influence the mineral composition and incidence of blossom-end rot in sweet pepper. Journal of Horticultural Science and Biotechnology 76, 9–16.
Taylor, M.D., Locascio, S.J., 2004. Blossom-end rot: a calcium deficiency. Journal of Plant Nutrition 27, 123–139.
Wang, W., Vinocur, B., Shoseyov, O., Altman, A., 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science 9, 244–252.
White, P.J., Broadley, M.R., 2003. Calcium in plants. Annals of Botany 92, 487–511.
Woolf, A.B., Ferguson, I.B., 2000. Postharvest responses to high fruit temperatures in the field. Postharvest Biology and Technology 21, 7–20.
Yermiyahu, U., Nir, S., Ben-Hayyim, G., Kafkafi, U., 1994. Quantitative competition of calcium with sodium or magnesium for sorption sites on plasma membrane vesicles of melon (*Cucumis melo* L.) root cells. Journal of Membrane Biology 138, 55–63.