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

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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.

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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

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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