Scientia Horticulturae 261 (2020) 108919

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Post-bloom and preharvest treatment of 'Braeburn' apple trees with prohexadione-calcium and GA_{4+7} affects vegetative growth and postharvest incidence of calcium-related physiological disorders and decay in the fruit

Cassandro V.T. do Amarante[a,*], João Paulo G. Silveira[a], Cristiano A. Steffens[a], Sergio T. de Freitas[b], Elizabeth J. Mitcham[c], Aquidauana Miqueloto[a]

[a] Universidade do Estado de Santa Catarina (UDESC), Lages, SC, Brazil
[b] Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Petrolina, PE, Brazil
[c] Department of Plant Sciences, University of California, Davis, CA, USA

ARTICLE INFO

Keywords:
Malus domestica Borkh
Anti-gibberellin
Fruit mineral content
Gene expression of Ca transporters
Water-soluble Ca content
Electrolyte leakage
Bitter pit
Skin cracking

ABSTRACT

Calcium (Ca) deficiency disorders in apple fruit have been associated with high gibberellins (GAs) activity in the tree. This study was carried out to assess the effects of treatments of 'Braeburn' apple trees with prohexadione-calcium (ProCa, an inhibitor of GAs biosynthesis) or gibberellins (GA_{4+7}) on vegetative growth of the trees and postharvest incidence of Ca-related physiological disorders and decay in the fruit. ProCa (300 mg L^{-1}) or GA_{4+7} (300 mg L^{-1}) treatments were applied post-bloom (PB) and preharvest (PH). PB treatments started 15 days after full bloom (DAFB), with one application every week and six applications in total. PH treatments started five weeks before anticipated harvest (125 DAFB), with one application every week and four applications in total. Control trees were left untreated. When applied PB, ProCa reduced and GA_{4+7} promoted vegetative growth of the trees. ProCa PB delayed the impairment of xylem functionality (at the proximal region of the fruit) during fruit growth on the tree. Treatments had no effect on fruit weight, pectinmethylesterase (PME) activity or the expression of *CAX3*, *CAX6* and *V-ATPase* (transporters of Ca into the vacuole) genes assessed in the external cortical tissue at the distal end of the fruit at harvest. When sprayed PB, ProCa increased the total Ca content and reduced K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (K + Mg + N)/Ca ratios in the flesh at the distal portion of the fruit, compared to the treatment with GA_{4+7}, but without differing from the control. In general, ProCa application PB or PH reduced the expression of Ca-*ATPase1*, *Ca-ATPase2*, H^+-*PPase* and *CAX2* (Ca transporters into the vacuole) genes, increased total water-soluble Ca and reduced electrolyte leakage in the fruit at harvest. After two months of cold storage followed by five days of shelf-life, the incidence and severity of bitter pit (BP) was lower on fruit from trees treated with ProCa PH, and higher on fruit from trees treated with GA_{4+7} PB. GA_{4+7} PB also increased the incidence of skin cracking and decay in the fruit. The results showed that ProCa application represents a feasible tool to reduce the incidence of BP. However, ProCa is more effective to reduce BP if applied weekly for five week before harvest.

1. Introduction

The postharvest incidence of physiological disorders in apple fruit, such as bitter pit (BP), has been associated with the low calcium (Ca) content and high content of magnesium (Mg), potassium (K) and nitrogen (N) in the fruit (Ferguson and Watkins, 1989; Amarante et al., 2006; Amarante et al., 2011; De Freitas et al., 2012; De Freitas and Mitcham, 2012; De Freitas et al., 2016). In addition, the development of such disorders has also been associated with a high activity of gibberellins (GAs) in the trees during fruit growth and development (Saure, 2005; Saure, 2014; De Freitas et al., 2016).

GAs treatment close to full bloom promoted fruit growth (Atkinson et al., 2009; Han et al., 2015). However, this can lead to the compression and collapse of xylem vessels, impairing xylem functionality and Ca transport into the fruit (Dražeta et al., 2004; Miqueloto et al., 2014). In addition, GAs promote vegetative growth, increasing the competition between leaves and fruitlets for Ca that might reduce fruit Ca content and increase fruit susceptibility to physiological disorders, such as BP in apples (Saure, 2005) and blossom-end rot in tomatoes (De Freitas et al., 2012).

* Corresponding author.
E-mail address: amarante.cav@gmail.com (C.V.T. do Amarante).

https://doi.org/10.1016/j.scienta.2019.108919
Received 23 July 2019; Received in revised form 2 October 2019; Accepted 4 October 2019
Available online 12 November 2019
0304-4238/

Prohexadione-calcium (ProCa) reduces the biosynthesis of active GAs (GA_1, GA_3, GA_4 and GA_7) in plants by the inhibition of dioxygenase enzymes (GA_{20}-oxidase and GA_3-oxidase) (Davies, 2004). Therefore, ProCa treatment of apple trees can reduce vegetative growth (Cline et al., 2008; Hawerroth et al., 2012; Silveira et al., 2012) that reduces the competition between leaves and fruits for Ca, and reduce fruit susceptibility to Ca deficiency related physiological disorders (Greene, 1991; Silveira et al., 2012; De Freitas et al., 2016).

Fruit susceptibility to Ca deficiency disorders is not always fully explained by total Ca content in the fruit. The abnormal homeostasis of Ca in the cell, associated with depletion of soluble Ca in the apoplast, can predispose fruit to physiological disorders incidence (De Freitas et al., 2010). Pectinmethylesterase (PME) activity can increase Ca binding to the cell wall and, thus, reduce the apoplastic pool of free Ca required to preserve the integrity of the plasma membrane (Ralet et al., 2001), eventually leading to higher fruit susceptibility to BP. In addition, soluble Ca in the apoplast can be reduced by its transport into the vacuole, where Ca can form complexes with phenols, oxalates and phosphates (White and Broadley, 2003). The transport of Ca into the vacuole occurs by the activity of proteins such as Ca-ATPases (at the expense of ATP) and Ca^{2+}/H^{+} antiporters (CAXs, at the expense of electrochemical gradient between vacuole and cytoplasm) (De Freitas et al., 2010). Therefore, the activity of PME in the apoplast and of Ca pumps and transporters in the tonoplast can have a great impact on Ca homeostasis in the cell, determining fruit susceptibility to physiological disorders (De Freitas and Mitcham, 2012; De Freitas et al., 2016).

Tomato plants sprayed with GAs from the early stages of fruit development have higher expression of *Ca-ATPases* and *CAXs* in the fruit, reduced content of apoplastic Ca, and increased incidence of blossom-end rot (De Freitas et al., 2012). Therefore, the treatment of plants with ProCa could reduce the levels of active GAs, and provide a tool to regulate cellular homeostasis in the fruit by maintaining high Ca content in the apoplast thereby preserving the functionality of the plasma membrane and reducing fruit susceptibility to Ca related disorders (De Freitas et al., 2018).

Treatment with GAs have been used commercially at post-bloom to reduce the incidence of russeting (Elfving and Allen, 1987) and promote fruit set or fruit elongation (Ghosh and Halder, 2018), but this can advance fruit maturity and ripening and exacerbate storage breakdown (Sharples and Johnson, 1986; Silveira et al., 2014). GAs are also sprayed preharvest to delay fruit maturation and ripening in apples (Turner, 1972). Also, spraying of GAs have been associated to the developments of calcium deficiency disorders in the fruit (Saure, 2005), and treatment with ProCa reduce the risk of such disorders (De Freitas et al., 2012; Silveira et al., 2012; De Freitas et al., 2016). However, for the best of our knowledge, the effect of application timing of ProCa or GAs (post-bloom or preharvest) on incidence of calcium deficiency disorders in apple fruit has not been studied.

This study was carried out to assess the effects of post-bloom (PB) and preharvest (PH) treatment of 'Braeburn' apple tree with ProCa and GA_{4+7} on vegetative growth, and on mechanisms that regulate Ca homeostasis and the incidence of postharvest physiological disorders in the fruit.

2. Materials and methods

The experiment was carried out in 2013 in a commercial orchard, with 10 year old 'Braeburn' apple trees (*Malus domestica* Borkh) trained to a central leader and planted at medium density with 2.0 m x 6.0 m (in-row x between-row). The orchard was located in Elk Grove, California, USA (38°21'23.27"N, 121°32'13.62"W, altitude of 123 m). The apple trees were sprinkler irrigated between May and August. The trees were fruit thinned leaving two fruits per bunch to keep uniform tree crop load between treatments. The maximum and minimum temperatures and precipitation by month during the experiment are shown in Table 1.

Table 1
Maximum and minimum temperatures and precipitation by month during the period from January to October of 2013.
Souce: U.S. Climate data

Month	Average temperatures (°C)		Precipitation (mm)
	Maximum	Minimum	
January	13.3	0.2	24.4
February	16.8	2.2	9.1
March	20.8	6.9	35.0
April	25.5	9.8	17.6
May	28.1	12.4	7.7
June	31.8	14.2	5.6
July	33.9	15.2	0.0
August	32.5	15.0	0.0
September	29.5	13.9	14.9
October	25.7	8.5	0.0

Trees were sprayed with ProCa (300 mg L^{-1}) or GA_{4+7} (300 mg L^{-1}) PB and preharvest (PH). PB treatments started 15 days after full bloom (DAFB), with one application every week and six applications in total. PH treatments started five weeks before anticipated harvest (125 DAFB), with one application every week and four applications in total. The trees were sprayed with a total volume of 1,000 L ha^{-1} until "run off". The commercial product containing ProCa was Apogee® (with 27.5% ProCa) while for GA_{4+7} was TypRus® (with 2.0% GA_{4+7}). Control trees were left untreated.

In October 2013 (180 DAFB), trees were assessed to determine current season branch growth (in cm) with a tape measure. This was assessed at random on 15 branches on each side of the trees, at the middle third of the canopy. Branches were grouped in four categories in terms of size (< 30 cm; ≥ 30 and < 60 cm; ≥ 60 and < 90 cm; and ≥ 90 cm), followed by the calculation of percentage of branches in each category.

Xylem functionality was assessed according to the method of Dražeta et al. (2004), only for treatments with ProCa and GA_{4+7} sprayed PB and for the control. Fifteen fruit per replicate were harvested at 55, 81, 125 and 149 DAFB. Each fruit had the base of its peduncle removed (approximately 1 mm), followed by immediate immersion of the peduncle into a staining solution (1% acid safranin). The fruits were infiltrated with the stain solution for approximately 8 h, with transpiration under ambient conditions (25 ± 2 °C and 70 ± 10% RH) using a fan to remove the boundary layer. Fruit were cut in half on the equator and transverse 10 mm-thick slices were removed from middle portions of distal (blossom end), and proximal (peduncle end) sections, as described by Miqueloto et al. (2014). Each slice was assessed for the number of stained primary vascular bundles and staining intensity of the secondary cortical vascular system. The counting method through visual analysis was used to determine the number of functional xylem vascular bundles in the primary cortical system. Lightness (L) and hue angle (h°) of the cortex (in proximal and distal regions) were measured to determine the staining intensity of the xylem in the secondary vascular system, using a Minolta model CR 400 colorimeter, according to the methodology described by Miqueloto et al. (2014). It was possible to quantify the red staining intensity of the cortex that resulted from the transport of the stain solution via the xylem vessels by multiplying $L \times h^{\circ}$. High values of $L \times h^{\circ}$ indicate higher lightness (less red staining in the cortex), which indicates lower functionality of the xylem in the secondary vascular system.

Fruit (170 fruit per replicate) were harvested at commercial maturity (160 DAFB). Fifteen fruit per replicate were assessed at harvest for flesh mass, length and diameter, PME activity, electrolyte leakage, and mineral analysis (total contents of N, P, K, Ca, Mg, S and B, and total water-soluble Ca). Five fruit per replicate were assessed at harvest for gene expression of Ca transporters (*Ca-ATPase 1*, *Ca-ATPase 2*, *CAX2*, *CAX3*, and *CAX6*) and electrogenic pumps (H^{+}-*PPase* and *V-*

ATPase) in the tonoplast. PME activity, electrolyte leakage, minerals, and gene expression of Ca transporters and electrogenic pumps were assessed in the flesh tissue sampled immediately below the skin at the distal end of the fruit (after fruit cut in half on the equator). This analysis was performed in the distal end of the fruit, since this is the region where Ca deficiency disorders normally manifest (such as bitter pit in apple and blossom-end rot in tomato) (Dražeta et al., 2004; De Freitas et al., 2012; De Freitas and Mitcham, 2012), and therefore is more suitable for sampling to assesses mineral composition for prediction of Ca deficiency disorders in tomato (De Freitas et al., 2010) and apple (Amarante et al., 2013) fruit.

One hundred and fifty fruit per replicate were held in cold storage (0 ± 0.5 °C / 90-95 % RH) for two months, followed by five days of shelf life (25 ± 2.0 °C / 60-70% RH), and then evaluated visually for incidence (%) of BP, skin cracking and decay. The index of BP was assessed using a scale with six levels of visual damage: none (0), one (1), two (2), three (3), four (4), five (5) and more than five spots (6) of BP per fruit. BP index was calculated according the formula described by Pesis et al. (2009):

$$\text{BP index} = \sum_{0}^{6} \frac{(\text{index level}) \times (\text{fruit at this level})}{\text{total number of fruit}}$$

For PME measurement, the spectrophotometric assay of Hagerman and Austin (1986) was used. The method is based on determination of a pH change due to an increase in unesterified carboxyl groups using the pH indicator bromothymol blue. The measured activity is expressed in absolute values (mol s^{-1} kg^{-1} fw).

Analysis of electrolyte leakage was carried out according to the method described by De Freitas et al. (2011). Briefly, four fruit discs of 1 cm diameter and 0.5 cm thickness were cut from the distal position and sectioned with a double-bladed knife 1 mm under the skin. Each sample of four discs from three fruit represented one replication, which was placed into a 50 mL conical tube on a rotary shaker in a 0.2 M mannitol solution. The conductivity of the mannitol solution was recorded periodically over 6 h. Then, samples were frozen and thawed twice to determine the total conductivity. The results were expressed as the percentage increase in electrolyte leakage (conductivity) per gram of tissue per hour relative to the total tissue conductivity.

The contents of N, P, K, Ca, Mg, S and B (mg kg^{-1} dw) were quantified in the pulp tissue 0.5 cm below the skin at the distal end of the fruit. Nitrogen was quantified according the method described by the AOAC (2006). Phosphorus, K, Ca, Mg, S and B were quantified by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) after sample microwave digestion with nitric acid/hydrogen peroxide (Meyer and Keliher, 1992).

Total content of water-soluble Ca (mg kg^{-1} fw) was assessed by sampling the flesh tissue 0.5 cm below the skin (~70 g) at the distal end of the fruit followed by juice extraction with a food processor, and according to the methodology described by Amarante et al. (2013). The content of water-soluble Ca in the juice was quantified with an ICP-AES.

For gene expression analysis, total RNA was extracted from the outer cortical fruit tissue as described in 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, 5 µg of total RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative, real-time PCR was then performed with the addition of 1 × SYBR green (Applied Biosystem, Foster City, CA, USA) to each sample containing about 100 ng of the synthesized cDNA. The data obtained were normalized based on the expression of the housekeeping apple 18S rRNA. All primers designed were 20 nucleotides long with a melting point temperature of 58 ± 3 ∘C. For *CAX/H*$^+$, *Ca-ATPases*, *V-ATPase* and *H*$^+$*-Pirofosfatase*, the nucleotide sequences were obtained in the UC Davis apple (*Malus domestica*) Expressed Sequence Tags (EST) database (http://cgf.ucdavis.edu/home/). The expressed nucleotides were named *Ca-ATPase1* (CTG1060377) (NCBI: XM 002325215), *Ca-ATPase2* (CTG1063686) (NCBI: AJ310848), *CAX2/H*$^+$ (CTG1073422) (NCBI: NM_112177), *CAX3/H*$^+$ (CTG1076982) (NCBI: XM_002533684), *H*$^+$*-Pirofosfatase* (EB143723) (NCBI: NM 101437) and *V-ATPase* (CTG1058311) (NCBI: NM_001036222), as described by De Freitas et al. (2010).

Table 2
Percentage of current season branches in each class of length of 'Braeburn' apple trees treated with GA_{4+7} and ProCa post-bloom (PB) or preharvest (PH).

Treatments	Percentage of branches in each class of length			
	< 30 cm	≥ 30 cm and < 60 cm	≥ 60 cm and < 90 cm	≥ 90 cm
Control	0.00 b	23.0 b	41.0 ab	35.8 a
GA_{4+7} PB	0.00 b	2.25 c	57.5 a	40.2 a
ProCa PB	22.5 a	72.5 a	5.00 c	0.00 b
GA_{4+7} PH	2.00 b	33.2 b	27.5 b	37.0 a
ProCa PH	0.00 b	41.2 b	24.2 b	34.2 a
CV (%)	91.7	50.9	43.2	49.8

Means (of four replicates) followed by the same letter within the columns are not significantly different by Tukey's test ($p < 0.05$).

The experiment followed a randomized block design, with five treatments and four blocks, each replicate consisting of a plant. Data in percentage were transformed to arc sin $[(x+5)/100]^{1/2}$ before statistical analysis. Treatment means were compared by Tukey's test ($p < 0.05$). Data were analyzed using the statistical program SAS (SAS Institute, 2009).

3. Results and discussion

3.1. Trees vegetative growth

Trees treated with GA_{4+7} PB (starting 15 DAFB) had the highest percentage of branches with length ≥ 60 and < 90 cm, and the lowest percentage of branches with length ≥ 30 and < 60 cm (Table 2). Trees sprayed with ProCa PB had the highest percentage of branches with length < 30 cm and ≥ 30 and < 60 cm (Table 2). Therefore, when treated PB, ProCa reduced while GA_{4+7} promoted vegetative growth. ProCa treatment PB has been shown to inhibit the biosynthesis of active GAs (Davies, 2004) and reduce vegetative growth of apple trees (Cline et al., 2008; Hawerroth et al., 2012; Silveira et al., 2012).

Neither ProCa or GA_{4+7} had an effect on vegetative growth, compared to the control, when sprayed PH (staring 125 DAFB) (Table 2). According to Byers and Yoder (1999), apple trees reduced their vegetative growth 30 DAFB. Treating apple trees PH may have had no effect on vegetative growth because the growth rate had already reduced to lower levels than PB.

3.2. Xylem functionality of the fruit in plants treated with GA_{4+7} and ProCa post-bloom

The number of stained primary cortical vascular bundles decreased from the proximal to the distal end of the fruit, and decreased in both parts from 55 to 149 DAFB (Fig. 1). ProCa PB delayed the loss of functionality of primary cortical vascular bundles in the proximal part of the fruit until 81 DAFB in comparison to the control and GA_{4+7} PB. The functionality of the secondary cortical system (expressed in terms of intensity of cortex staining - $L \times h^{\circ}$) showed no differences between treatments, regardless of fruit part (proximal or distal) or date of fruit sampling (Fig. 1). De Freitas et al. (2012) reported a reduction of xylem functionality in the early stage of tomato fruit development in response to GAs treatments, but this was not observed in apple. Exogenous treatment with GAs causes a more substantial differentiation of phloem than of xylem (De Freitas and Mitcham, 2012). This can reduce the

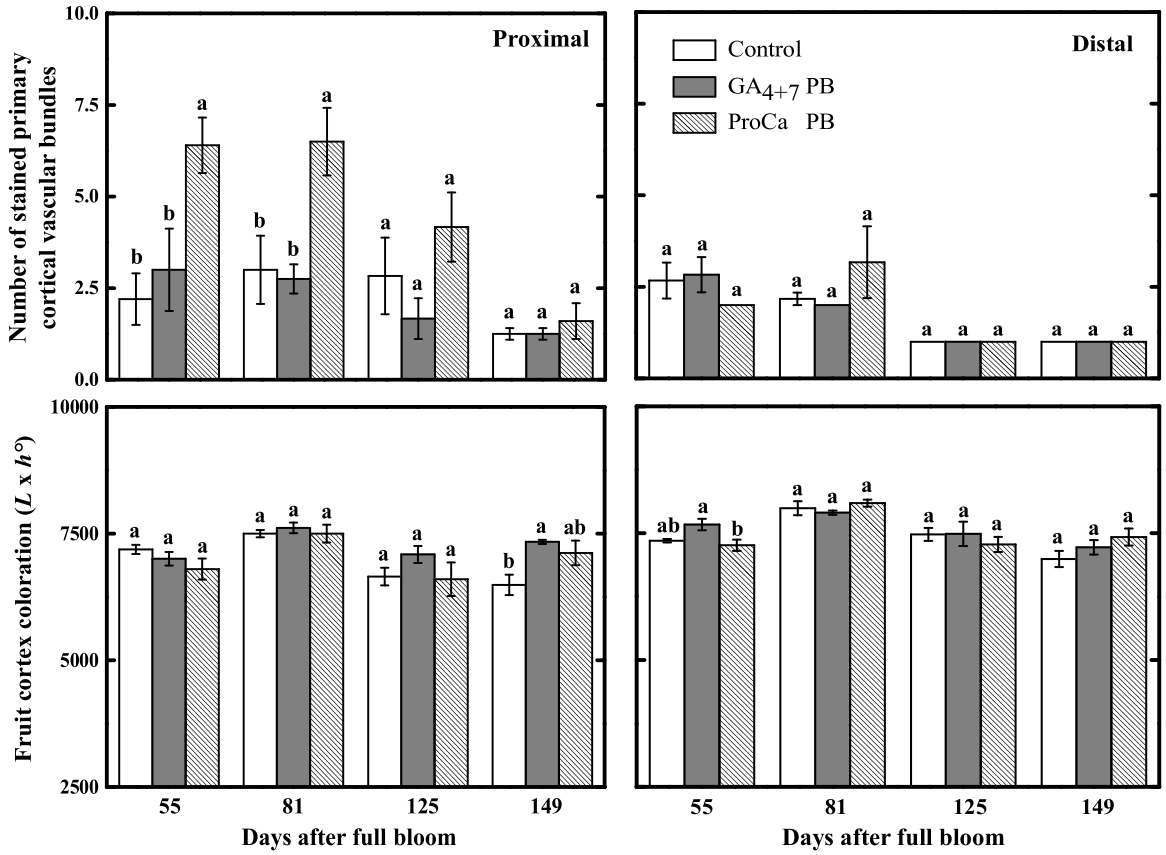

Fig. 1. Number of primary cortical vascular bundles stained (top) and intensity of cortex staining ($L \times h^{\circ}$) (bottom), in the proximal (left) and distal (right) portions of the fruit, harvested from 'Braeburn' apple trees treated with GA_{4+7} and ProCa post-bloom (PB). Increased $L \times h^{\circ}$ values indicate a reduction in the staining of the vascular bundles and loss of xylem function in the secondary cortical system. Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter (comparing treatments within dates) are not significantly different by Tukey's test ($p < 0.05$).

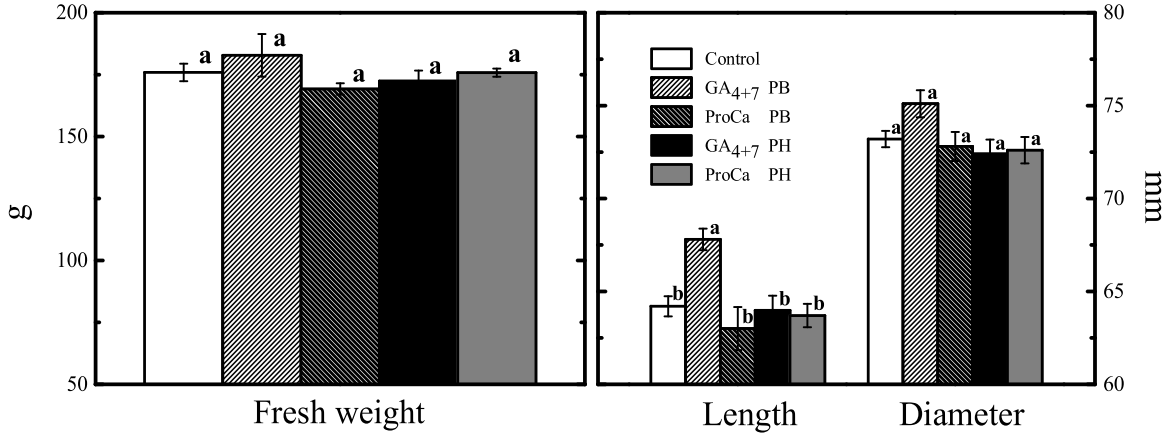

Fig. 2. Fresh weight, length and diameter of the fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} and ProCa post-bloom (PB) and preharvest (PH). Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter are not significantly different by Tukey's test ($p < 0.05$).

transport of Ca (that occurs almost exclusively through the xylem) without reducing the transport to the fruit of N, K and Mg (that occurs also through the phloem), increasing the risk of Ca deficiency disorders in the fruit. The loss of xylem functionality is also associated with increases in fruit mass during development (Dražeta et al., 2004). Since fruit mass was not different between treatments at harvest (Fig. 2), there was no substantial difference between treatments on xylem functionality (Fig. 1).

3.3. Fresh weight, length and diameter of the fruit

Fresh weight (FW) and diameter of the fruit at harvest were not different between treatments, however GA_{4+7} PB increased fruit length (Fig. 2). Treatment with GAs at early stages of apple fruit development results in a higher fruit length/diameter ratio (Nakagawa et al., 1967) and promotes fruit growth (Braidwood et al., 2014). Since GA_{4+7} PB also promoted vegetative growth (Table 2), this might have increased the competition between vegetative growing tissues and fruitlets for the available carbohydrates, resulting in a similar fresh weight of the fruit compared to the other treatments. Treatment with ProCa PB has been

shown to reduce the biosynthesis of GAs and reduce cell expansion in the fruit (Davies, 2004). However, ProCa PB benefits the transport of carbohydrates to the fruitlets as the result of reduced vegetative growth of the trees (Medjdoub and Blanco, 2004). Therefore, fruit from this treatment had similar FW at harvest compared to fruit from the other treatments.

PH treatment with GA_{4+7} and ProCa (starting 125 DAFB) did not affect FW, length or diameter of the fruit at harvest (Fig. 2). Cell expansion and growth of apple fruit slows greatly beyond 120 DAFB (Lakso et al., 1995), and it is not surprising that treatment with GA_{4+7} and ProCa at this stage had no impact on fruit growth.

3.4. Fruit mineral content

The total contents of N, P, K, Mg, S and B of the flesh in the distal portion of the fruit were not different between treatments (data not shown), with average values (± SE) of 2,705 (± 117.7), 952 (± 24.0), 7,830 (± 204.7), 320 (± 5.2), 240 (± 7.2) and 32 (± 0.8) mg kg^{-1} dw, respectively.

ProCa PB increased the total Ca content and reduced K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (K + Mg + N)/Ca ratios in the flesh at the distal portion of the fruit compared to GA_{4+7} PB, but without differing from the control (Table 3). Silveira et al. (2012) also reported reduction in total Ca content and increase in K/Ca and (K + N + Mg)/Ca ratios in the skin tissue of the fruit from 'Fuji' apple trees treated with GA_3 (319 mg L^{-1}) PB compared to fruit from trees treated with ProCa (319 mg L^{-1}) PB and control. The increase of vegetative growth of trees treated with GA_{4+7} PB (~25%), and the reduction in vegetative growth of trees treated with ProCa PB (~50%) compared to the control, might explain these results for fruit mineral content. Greene (1991) reported reduced vegetative growth of apple trees treated with paclobutrazol (an inhibitor of GAs biosynthesis), leading to an increase in Ca content in the fruit and reduction in the incidence of BP. According to the author, the reduction in vegetative growth of apple trees treated with paclobutrazol reduced the competition between leaves and developing fruit for Ca, and increased the content of Ca in the fruit. A similar mechanism might be expected for apples trees treated with ProCa PB. On the other hand, treatment with GAs increases the GAs/auxin ratio in the plant that promotes the differentiation of phloem (Aloni et al., 1990) and increases the transport of N, K and Mg to the fruit. Therefore, ProCa PB can increase Ca content and reduce K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (K + Mg + N)/Ca ratios in the fruit compared to GA_{4+7} PB.

The total Ca content, and K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (K + Mg + N)/Ca ratios in the fruit were not different between treatments with GA_{4+7} and ProCa applied PH and the control (Table 3). GA_{4+7} and ProCa applied PH did not affect vegetative growth (Table 2) or fruit size at harvest (Fig. 2) compared to the control. Xylem functionality (number of primary cortical vascular bundles stained) at the distal region of the fruit was low and did not change from 125 DAFB (Fig. 1). Therefore, it is expected that treatments with GA_{4+7} and ProCa PH (starting 125 DAFB) would have no impact on xylem functionality or on total Ca content and K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (K + Mg + N)/Ca ratios in the fruit at harvest (Table 3).

3.5. PME activity and expression of tonoplast transporters

PME activity and expression of *CAX_3*, *CAX_6* and *V-ATPase* genes in the flesh tissue at the distal end of the fruit was not different between treatments at harvest (data not shown). Fruit from trees treated with GA_{4+7} PB had high expression of *Ca-ATPase1*, *Ca-ATPase2*, *H^+-PPase* and *CAX2* genes. Considering the same treatment time (PB or PH), there was a trend towards higher expression of all these four genes in fruit from trees treated with GA_{4+7} than those treated with ProCa. When treatments were applied PB, fruit from trees treated with GA_{4+7} had higher expression of *Ca-ATPase1*, *Ca-ATPase2* and *CAX2* genes than those from trees treated with ProCa. For treatments applied PH, the expressions of *Ca-ATPase2* and *CAX2* genes were higher in fruit from trees treated with GA_{4+7} than those from trees treated with ProCa (Fig. 3).

De Freitas et al. (2012) previously showed that tomato plants treated with GAs had higher expression of *Ca-ATPase* and *H^+-PPase* in the fruit and higher incidence of blossom-end rot. The higher expression of these transporters can increase Ca content in the vacuole (De Freitas et al., 2010), reducing water-soluble Ca in the apoplast required to preserve the integrity of the plasma membrane (Marschner, 2012). According to De Freitas et al. (2010), the reduced pool of water-soluble Ca in the apoplast increases ion leakage on the plasma membrane and increases the risk of BP in apple fruit.

3.6. Total water-soluble Ca content and electrolyte leakage

For treatments applied PB, total water-soluble Ca content was higher and electrolyte leakage lower in fruit from trees treated with ProCa than those treated with GA_{4+7} (Fig. 4). When treatments were applied PH, values for total water-soluble Ca were higher on fruit from GA_{4+7} treated trees than on fruit from control trees (Fig. 4A), while electrolyte leakage was not different between treatments (GA_{4+7}, ProCa and control) (Fig. 4B).

There was an exponential increase in apple fruit tissue electrolyte leakage with the reduction in total water-soluble Ca content (Fig. 5). Fruit with a total water-soluble Ca content below ~13 mg kg^{-1} fw had a substantial increase in electrolyte leakage, and corresponded to the fruits harvested from trees treated with GA_{4+7} PB. Njoroge et al. (1998) also reported an increase in electrolyte leakage with a reduction in total water-soluble Ca content in tomato fruit.

When treatments were applied PB, GA_{4+7} reduced the total Ca content (Table 3), and this was also reflected in a reduction of total water-soluble Ca content, in comparison to ProCa (Fig. 4A). The lower values of total water-soluble Ca in fruit from trees treated with GA_{4+7} PB likely resulted in a loss of plasma membrane integrity and increased electrolyte leakage compared to ProCa PB (Fig. 4B).

3.7. Assessment of bitter pit, skin cracking and decay after storage

Fruit from trees treated with GA_{4+7} had higher incidence and severity (index) of BP than those from trees treated with ProCa, regardless of the application period (PB or PH) (Fig. 6). For treatments applied PB,

Table 3
Total content of Ca, and K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (Mg + K + N)/Ca ratios in the flesh tissue at the distal end of the fruit, harvested from 'Braeburn' apple trees treated with GA_{4+7} and ProCa post-bloom (PB) or preharvest (PH).

Treatments	Ca (mg kg^{-1} fw)	K/Ca	Mg/Ca	N/Ca	(Mg + K)/Ca	(Mg + K + N)/Ca
Control	180 bc	47.3 ab	1.87 ab	16.18 ab	49.1 ab	65.3 ab
GA_{4+7} PB	155 c	53.3 a	2.18 a	20.6 a	55.5 a	76.1 a
ProCa PB	262 a	29.8 b	1.29 b	11.6 b	31.1 b	42.7 b
GA_{4+7} PH	227 ab	37.4 ab	1.48 b	11.3 b	38.9 ab	50.2 ab
ProCa PH	230 ab	36.9 ab	1.46 b	11.8 b	38.3 ab	50.2 ab
CV (%)	23.3	38.5	31.7	42.8	38.2	39.1

Means (of four replicates) followed by the same letter within the columns are not significantly different by Tukey's test ($p < 0.05$).

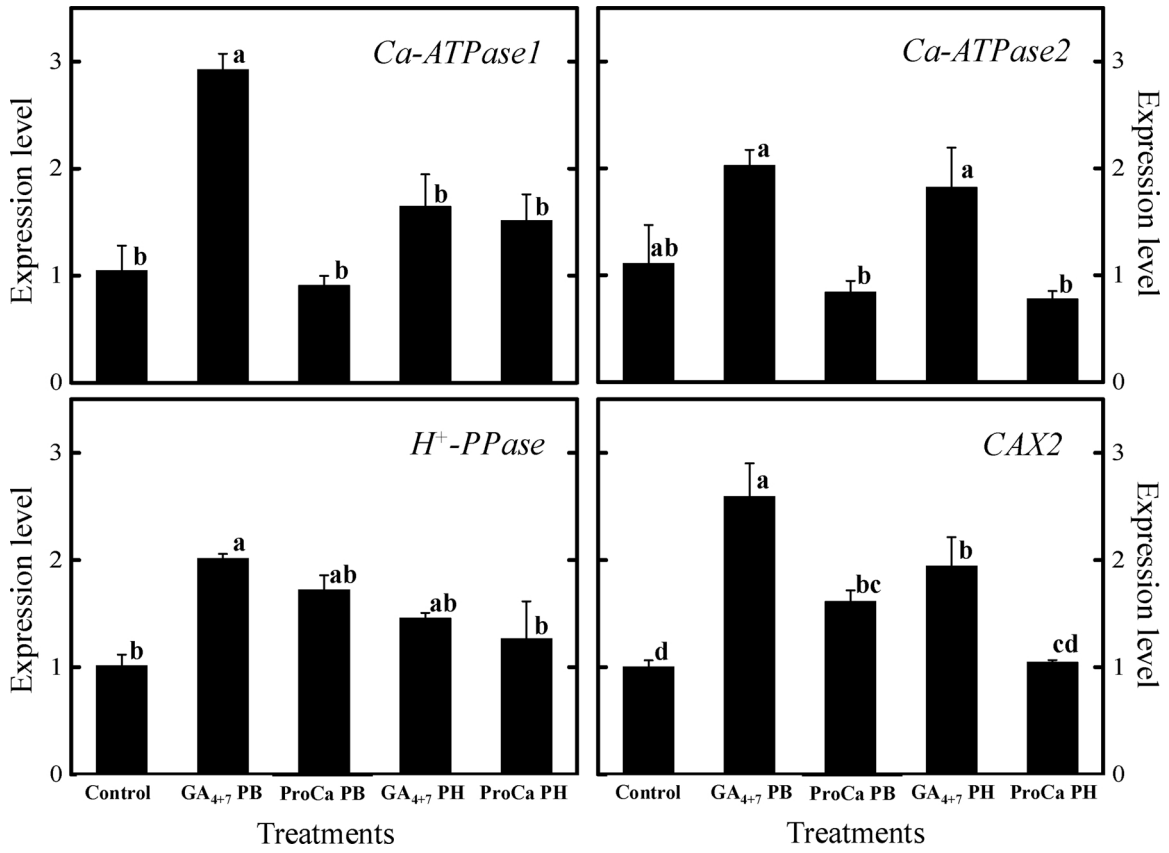

Fig. 3. Expression of genes involved in Ca transport in the outer cortical tissue at the distal end of apple fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} and ProCa post-bloom (PB) or preharvest (PH). *Ca-ATPase1* and *Ca-ATPase2* (putative Ca^{2+} pumps), *H^+-PPase* (pyrophosphatase) (putative H^+ pump) and *CAX2* (putative Ca^{2+}/H^+ exchanger). Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter are not significantly different by Tukey's test ($p < 0.05$).

GA_{4+7} had higher incidence and severity of BP than ProCa as a result of lower total Ca content [and higher K/Ca, Mg/Ca, N/C, (Mg + K)/Ca and (Mg + K + N)/Ca ratios] and lower total water-soluble Ca content in the fruit.

Plasma membrane structure and integrity is believed to be dependent on a free pool of Ca in the apoplast (Hanson, 1960; Plieth, 2001). When the concentration of Ca in the apoplast is below the threshold required to maintain membrane structure and function, membrane leakiness results and BP develops in the tissue (De Freitas et al., 2016). However, other nutrients, such as Mg, K and N, may also be involved in BP development in apples, and the ratios K/Ca, Mg/Ca, N/Ca and (K + Mg)/Ca have been used to predict fruit susceptibility to BP (Dris et al., 1998; Amarante et al., 2006; Lanauskas and Kvikliene, 2006; Amarante et al., 2013). The effects of Mg and K may result from their competition with Ca for binding sites at the plasma membrane surface, but these elements cannot replace Ca's role in membrane structure and stability (Schonherr and Bukovac, 1973; Yermiyahu et al., 1994).

BP incidence is not entirely explained by the total mineral content in

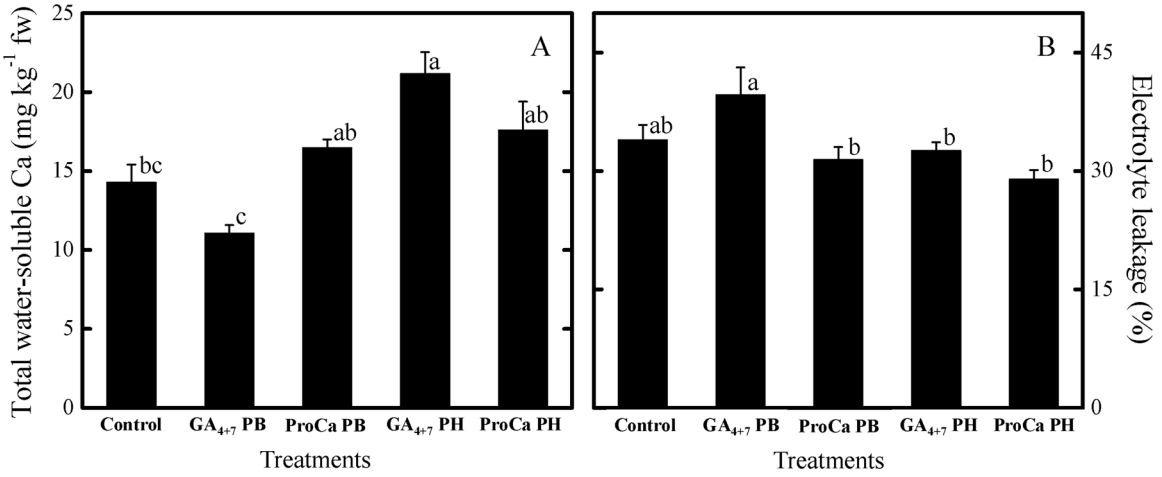

Fig. 4. Total water-soluble Ca content (mg kg^{-1} fw) (A) and electrolyte leakage (B) in the outer cortical tissue at the distal end of the apple fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} or ProCa, post-bloom (PB) or preharvest (PH). Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter are not significantly different by Tukey's test ($p < 0.05$).

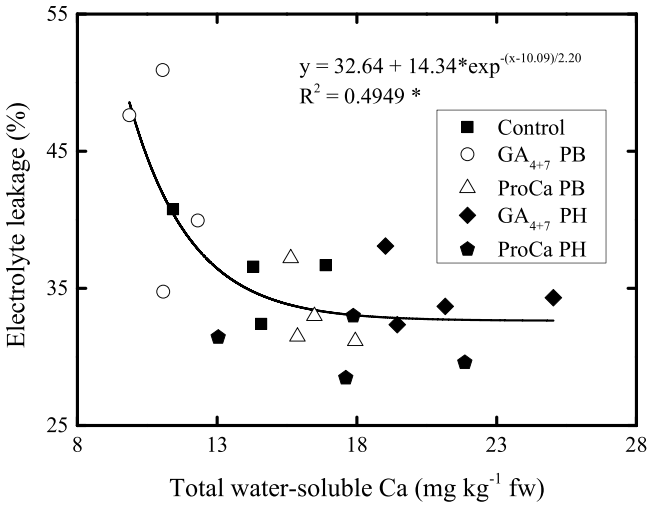

Fig. 5. Relationship between total water-soluble Ca content and electrolyte leakage in the outer cortical tissue at the distal end of apple fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} or ProCa, post-bloom (PB) or preharvest (PH). Each data point represents a replicate of different treatments. *: exponential fitted model is significant ($p < 0.05$).

the fruit, but also by altered Ca homeostasis in the cell. The increasing organellar and decreasing apoplastic Ca pools may result in higher membrane permeability and Ca deficiency disorder development (De Freitas et al., 2010). Fruit from trees sprayed with GA_{4+7} had higher expression of *ATPase 1*, *Ca-ATPase2*, H^+*-PPase* and *CAX2* in the tonoplast and this could increase Ca accumulation inside the vacuole, further depleting the apoplastic pool of free Ca. This might result in localized membrane breakdown and higher incidence of BP in fruit from trees treated with GA_{4+7} than in fruit from trees treated with ProCa, regardless of application time (PB or PH). In tomato plants, treatment with GA_{4+7} increased the expression of *Ca-ATPase* and the incidence of blossom-end rot in the fruit (De Freitas et al., 2012).

According to Saure (2014), the incidence of Ca deficiency disorders in the fruit might be associated with the formation of reactive oxygen species (ROS). GAs might cause cell death and the development of blossom-end rot in tomato fruit by inhibition of the activity of antioxidant enzymes, such as superoxide dismutases, catalases and ascorbate peroxidases, increasing cell susceptibility to oxidative stress and cell damage (Saure, 2014; De Freitas et al., 2018). In addition, GAs promote the degradation in the cell of DELLA proteins. DELLA proteins are nuclear-localized growth repressors, and GAs promote growth by promoting DELLA degradation (Braidwood et al., 2014). DELLA proteins also prevent the formation of ROS under environmental stress, delaying cell damage and death (Saure, 2014). Therefore, treatment with GAs, besides reducing Ca content, can increase the content of ROS in the cells that increases the risk of BP in the fruit.

Fruit from trees treated with ProCa PH had a lower incidence of BP than fruit from control trees, despite the lack of difference between control fruit and fruit from trees treated with ProCa PB (Fig. 6). Therefore, despite having no effect on vegetative growth of the trees, the treatment with ProCa PH might have positively impacted cellular Ca homeostasis, with an increase in total water-soluble Ca (Fig. 4A), preservation of plasma membrane integrity (represented by a lower electrolyte leakage) (Fig. 4B), and therefore reduction in BP incidence (Fig. 5). Bizjak et al. (2012) reported higher content of total phenolic compounds (TPC) in the peel and pulp tissues at harvest in fruit from 'Braeburn' apple trees treated with 250 mg/tree of ProCa three weeks before commercial maturity. Higher content of TPCs could prevent formation of reactive oxygen species (ROS) that might also be associated with the manifestation of Ca deficiency disorders on apples (Saure, 2014). Therefore, ProCa treatment close to fruit maturity may reduce BP by improving the total antioxidant activity in the fruit. Our results show that ProCa PH is more effective to reduce BP than ProCa PB. Only ProCa PH treated fruit had statistically lower incidence and severity of BP than the control fruit (Fig. 6). The treatment of 'Braeburn' apple trees with ProCa PB and PH inhibits ethylene production and delays fruit maturity, with a bigger effect at PH than at PB (Amarante et al., 2019). Less mature fruit is known to be more susceptible to BP than fruit harvested at more advances maturity stages (Ferguson and Watkins, 1989; De Freitas et al., 2016). Therefore, despite of a stronger effect of ProCa PH delaying fruit maturity, which has been shown to increase fruit susceptibility to BP, this treatment resulted in the lowest incidence and severity of BP in the fruit. The fact that ProCa PH delayed fruit development, suggests that it could be possible to delay harvest, which could reduce even further the risk of BP incidence in the fruit.

Fruit harvested from trees treated with GA_{4+7} PB had the highest incidence of skin cracking (18.2%) and decay (6.6%) after storage. Fruit from all the other treatments had < 2.0% incidence of skin cracking and < 3.1% incidence of decay (Fig. 7).

In apple fruit, manifestation of skin cracking was associated with higher expression of expansin genes in the mesocarp than in the pericarp (Kasai et al., 2008). Also, GAs were found to promote the expression of a xyloglucan endotransglycosylase (XET) gene (Atkinson et al., 2009; Han et al., 2015). XET was shown to catalyze the splitting

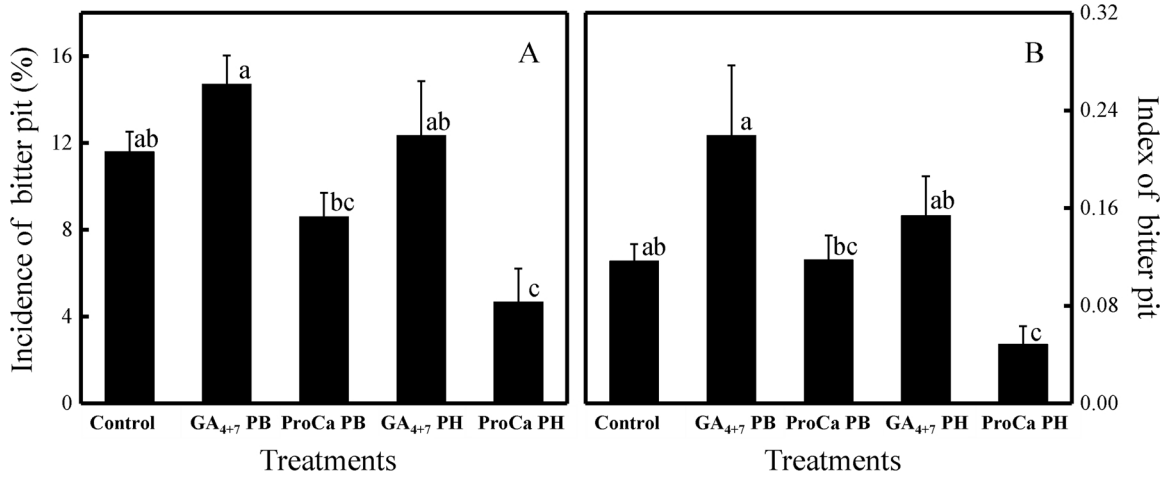

Fig. 6. Incidence (A) and index (B) of bitter pit in apple fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} and ProCa, post-bloom (PB) or preharvest (PH), and held in cold storage for two months (0 ± 0.5 °C / 90-95 % RH), followed by five days of shelf life (25 ± 2.0 °C / 60-70% RH). Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter are not significantly different by Tukey's test ($p < 0.05$).

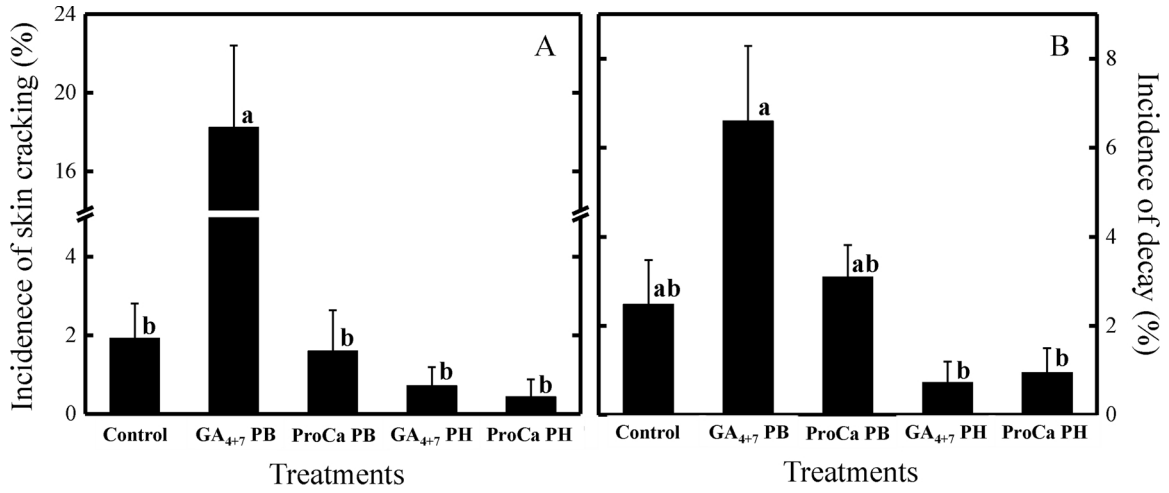

Fig. 7. Incidence of skin cracking (A) and decay (B) in apple fruit harvested from 'Braeburn' apple trees treated with GA_{4+7} or ProCa post-bloom (PB) or preharvest (PH), and held in cold storage for two months (0 ± 0.5 °C / 90-95 % RH), followed by five days of shelf life (25 ± 2.0 °C / 60-70% RH). Values represent the mean of four replicates ± standard error. Vertical bars followed by the same letter are not significantly different by Tukey's test ($p < 0.05$).

of xyloglucan chains, thereby loosening the cell wall and allowing for cell expansion, mainly in the mesocarp, of kiwifruit (Schröder et al., 1998). Therefore, in fruit harvested from trees treated with GA_{4+7} PB, the expansion of pericarp cells might not accommodate the expansion of mesocarp cells, causing increased skin cracking. Also, fruit harvested from trees treated with GA_{4+7} PB had the lowest total Ca content [and highest K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (Mg + K + N)/Ca ratios] (Table 3). Low Ca content has been associated with higher incidence of skin cracking during cold storage in apple fruit (Perring, 1984).

The high incidence of skin cracking may have also increased the incidence of decay during cold storage in fruit harvested from trees treated with GA_{4+7} PB (Fig. 7). For some pathogens that cause postharvest decay in apple fruit, such as *Penicillium expansum* Link, the infection is facilitated by cracks in the epidermal cells (Miedes and Lorences, 2004). Silveira et al. (2014) reported lower Ca content and lower firmness in the skin tissue of apple fruit harvested from trees treated with GA_3 (319 mg L^{-1}) PB, and this might make the fruit more susceptible to skin cracking and decay. A lower Ca content has also been associated with greater susceptibility to postharvest decay in apple fruit (Conway et al., 1994), which might also explain the higher incidence of decay in fruit from trees treated with GA_3 PB.

The results show that treatment with GA_{4+7} PB promoted vegetative growth of the trees and increased the postharvest incidence of BP, skin cracking and decay in the fruit. On the other hand, ProCa reduced vegetative growth when applied PB, and reduced the incidence of BP when applied PB and PH. ProCa application represents a feasible tool to reduce the incidence of BP in apple orchards under climatic and management conditions that increase the risk of development of such physiological disorder. However, our results show that ProCa PH is more effective to reduce BP than ProCa PB.

4. Conclusions

ProCa inhibits and GA_{4+7} promotes vegetative growth of apple trees when applied post-bloom;

When treatments were applied post-bloom, ProCa increased total Ca content and reduced K/Ca, Mg/Ca, N/Ca, (Mg + K)/Ca and (Mg + K + N)/Ca ratios in the fruit compared to GA_{4+7} treatment;

Regardless of application time (post-bloom or preharvest), ProCa reduced the expression of *Ca-ATPase1*, *Ca-ATPase2*, H^+*-PPase* and *CAX2* genes, increased total water-soluble Ca content, and reduced electrolyte leakage in the fruit in comparison to treatment with GA_{4+7};

ProCa is more effective to reduce BP if applied weekly for five week before harvest;

GA_{4+7} applied post-bloom increased the incidence of bitter pit, skin cracking and decay after cold storage.

Acknowledgement

This work was supported by the National Council for Scientific and Technological Development of Brazil (CNPq) (grant numbers 471399/2013-2 and 223522/2012-0).

References

Automated method for microchemical determination of carbon, hydrogen, and nitrogen. In: AOAC (Ed.), Official Methods of Analysis of AOAC International. 18. AOAC International, Gaithersburg, MD.

Aloni, R., Tollier, M.T., Monties, B., 1990. The role of auxin and gibberellin in controlling lignin formation in primary phloem fibers and in xylem of *Coleus blumei* stems. Plant Physiol. 94, 1743–1747. https://doi.org/10.1104/pp.94.4.1743.

Amarante, C.V.T., Chaves, D.V., Ernani, P.R., 2006. Multivariate analysis of nutritional attributes associated with bitter pit in' Gala' apples. Pesq. Agropec. Bras. 41, 841–846. https://doi.org/10.1590/S0100-204X2006000500017.

Amarante, C.V.T., Ernani, P.R., Steffens, C.A., Argenta, L.C., 2011. Skin calcium content is indicative of bitter pit susceptibility in' Fuji' apples. Rev. Bras. Frutic. 33, 180–186. https://doi.org/10.1590/S0100-29452011005000017.

Amarante, C.V.T., Miqueloto, A., De Freitas, S.T., Steffens, C.A., Silveira, J.P.G., Corrêa, T.R., 2013. Fruit sampling methods to quantify calcium and magnesium contents to predict bitter pit development in 'Fuji' apple: A multivariate approach. Sci. Hortic. 157, 19–23. https://doi.org/10.1016/j.scienta.2013.03.021.

Amarante, C.V.T., Silveira, J.P.G., Steffens, C.A., De Freitas, S.T., Mitcham, E.J., 2019. Post-bloom and preharvest treatment of 'Braeburn' apple trees with prohexadione-calcium and GA_{4+7} affect fruit quality. Sci. Hortic (submitted).

Atkinson, R.G., Johnston, S.L., Yauk, Y.-K., Sharma, N.N., 2009. Analysis of xyloglucan endotransglucosylase/hydrolase (XTH) gene families in kiwifruit and apple. Postharvest Biol. Technol. 51, 149–157. https://doi.org/10.1016/j.postharvbio.2008.06.014.

Bizjak, J., Jakopic, J., Slatnar, A., Stampar, F., Stich, K., Halbwirth, H., Zadravec, P., Veberic, R., 2012. Late prohexadione-calcium application on maturing apple cv. 'Braeburn' fruit reduces anthocyanins and alters the phenolic content. Eur. J. Hortic. Sci. 77, 154–162.

Braidwood, L., Breuer, C., Sugimoto, K., 2014. My body is a cage: Mechanisms and modulation of plant cell growth. New Phytol. 201, 388–402. https://doi.org/10.1111/nph.12473.

Byers, R.E., Yoder, K.S., 1999. Prohexadione-calcium inhibits apple, but not peach, tree growth, but has little influence on apple fruit thinning or quality. HortScience 34, 1205–1209. https://doi.org/10.21273/HORTSCI.34.7.1205.

Cline, J.A., Embree, C.G., Hebb, J., Nichols, D.S., 2008. Performance of prohexadione-calcium on shoot growth and fruit quality of apple: Effect of spray surfactants. Can. J. Plant Sci. 88, 165–174. https://doi.org/10.4141/CJPS07087.

Conway, W.S., Sams, C.E., Wang, C.Y., Abbott, J.A., 1994. Additive effects of postharvest calcium and heat treatment on reducing decay and maintaining quality in apples. J. Amer. Soc. Hortic. Sci. 119, 49–53. https://doi.org/10.21273/JASHS.119.1.49.

Davies, P.J., 2004. Plant Hormones: Biosynthesis, Signal Transduction and Action, 3rd edition. Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 775p.

De Freitas, S.T., Amarante, C.V.T., Labavitch, J.M., Mitcham, E.J., 2010. Cellular approach to understand bitter pit development in apple fruit. Postharvest Biol. Technol. 57, 6–13. https://doi.org/10.1016/j.postharvbio.2010.02.006.

De Freitas, S.T., Padda, M., Wu, Q., Park, S., Mitcham, E.J., 2011. 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 Physiol. 156, 844–855. https://doi.org/10.1104/pp.111.175208.

De Freitas, S.T., Mitcham, E.J., 2012. Factors involved in fruit calcium deficiency disorders. Hortic. Rev. 40, 107–146. https://doi.org/10.1002/9781118351871. ch3.

De Freitas, S.T., Jiang, C.Z., Mitcham, E.J., 2012. Mechanisms involved in calcium deficiency development in tomato fruit in response to gibberellins. J. Plant Growth Regul. 31, 221–234. https://doi.org/10.1007/s00344-011-9233-9.

De Freitas, S.T., Amarante, C.V.T., Mitcham, E.J., 2016. Calcium deficiency disorders in plants. In: Pereek, S. (Ed.), Postharvest Ripening Physiology of Crops. CRC Press, Boca Raton, pp. 477–502 Chapter 15.

De Freitas, S.T., Martinelli, F., Feng, B., Reitz, N.F., Mitcham, E.J., 2018. Transcriptome approach to understand the potential mechanisms inhibiting or triggering blossom-end rot development in tomato fruit in response to plant growth regulators. J. Plant Growth Regul. 37, 183–198. https://doi.org/10.1007/s00344-017-9718-2.

Dražeta, L., Lang, A., Cappellini, C., Hall, A.J., Volz, R.K., Jameson, P.E., 2004. Vessel differentiation in the pedicel of apple and the effects of auxin transport inhibition. Physiol. Plant. 120, 162–170. https://doi.org/10.1111/j.0031-9317.2004.0220.x.

Dris, R., Niskanen, R., Fallahi, E., 1998. Nitrogen and calcium nutrition and fruit quality of commercial apple cultivars grown in Finland. J. Plant Nutr. 21, 2389–2402. https://doi.org/10.1080/01904169809365572.

Elfving, D.C., Allen, O.B., 1987. Effects of gibberellin A_{4+7} applications on 'Golden Delicious' fruit russet. Crop Res. 27, 11–18.

Ferguson, I.B., Watkins, C.B., 1989. Bitter-pit in apple fruit. Hortic. Rev. 11, 289–355. https://doi.org/10.1002/9781118060841.ch8.

Ghosh, S., Halder, S., 2018. Effect of different kinds of gibberellin on temperate fruit crops: A review. The Pharma Inn. J. 7, 315–319.

Greene, D.W., 1991. Reduced rates and multiple sprays of paclobutrazol control growth and improve fruit quality of 'Delicious' apples. J. Amer. Soc. Hortic. Sci. 116, 807–812. https://doi.org/10.21273/JASHS.116.5.807.

Hagerman, A.E., Austin, P., 1986. Continuous spectrophotometric assay for plant pectin methyl esterase. J. Agric. Food Chem. 34, 440–444. https://doi.org/10.1021/jf00069a015.

Han, Y., Zhu, Q., Zhang, Z., Meng, K., Hou, Y., Ban, Q., Suo, J., Rao, J., 2015. Analysis of xyloglucan endotransglycosylase/hydrolase (XTH) genes and diverse roles of isoenzymes during persimmon fruit development and postharvest softening. PLOS ONE 10, e0123668. https://doi.org/10.1371/journal.pone.0123668.

Hanson, J.B., 1960. Impairment of respiration, ion accumulation, and ion retention in root tissue treated with ribonuclease and ethylenediamine tetraacetic acid. Plant Physiol. 35, 372–379. https://doi.org/10.1104/pp.35.3.372.

Hawerroth, J.F., Petri, J.L., Leite, G.B., Yoshikawa, E.R., 2012. Application timing of prohexadione calcium on vegetative growth control of' Imperial Gala' apples. Rev. Bras. Frutic. 34, 957–963. https://doi.org/10.1590/S0100-29452012000400001.

Kasai, S., Hayama, H., Kashimura, Y., Kudo, S., Osanai, Y., 2008. Relationship between fruit cracking and expression of the expansin gene MdEXPA3 in 'Fuji' apples (*Malus domestica* Borkh.). Sci. Hortic. 116, 194–198. https://doi.org/10.1016/j.scienta.2007.12.002.

Lakso, A.N., Grapadelli, L.C., Bernard, J., Goffinet, M.C., 1995. An expolinear model of the growth pattern of the apple fruit. J. Hortic. Sci. 70, 389–394. https://doi.org/10.1080/14620316.1995.11515308.

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

Marschner, P., 2012. Mineral Nutrition of Higher Plants, 3rd edition. Oxford, United Kingdom 651p.

Medjdoub, R., Blanco, V.J.A., 2004. Prohexadione-Ca inhibits vegetative growth of 'Smoothee Golden Delicious' apple trees. Sci. Hortic. 101, 243–253. https://doi.org/10.1016/j.scienta.2003.11.005.

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

Miedes, E., Lorences, E.P., 2004. Apple (*Malus domestica*) and tomato (*Lycopersicum esculentum*) fruits cell-wall hemicelluloses and xyloglucan degradation during *Penicillium expansum* infection. J. Agric. Food Chem. 52, 7957–7963. https://doi.org/10.1021/jf048890f.

Miqueloto, A., Amarante, C.V.T., Steffens, C., Santos, A., Mitcham, E., 2014. Relationship between xylem functionality, calcium content and the incidence of bitter pit in apple fruit. Sci. Hortic. 165, 319–323. https://doi.org/10.1016/j.scienta.2013.11.029.

Nakagawa, S., Bukovac, M.J., Hiratai, N., Kurookai, H., 1967. Morphological studies of gibberellin-induced parthenocarpic and asymmetric growth in apple and Japanese pear fruits. J. Jpn. Soc. Hortic. Sci. 37, 9–18. https://doi.org/10.2503/jjshs.37.9.

Njoroge, C.K., Kerbel, E.L., Briskin, D.P., 1998. Effect of calcium and calmodulin antagonists on ethylene biosynthesis in tomato fruits. J. Sci. Food Agric. 76, 209–214. https://doi.org/10.1002/(SICI)1097-0010(199802)76:2<209::AID-JSFA936>3.0.CO;2-J.

Perring, M.A., 1984. Lenticel blotch pit, watercore, splitting and cracking in relation to calcium concentration in the apple fruit. J. Sci. Food Agric. 35, 1165–1173. https://doi.org/10.1002/jsfa.2740351104.

Pesis, E., Ibáñez, A.M., Phu, M.L., Mitcham, E.J., Ebeler, S.E., Dandekar, A.M., 2009. Superficial scald and bitter pit development in cold-stored transgenic apples suppressed for ethylene biosynthesis. J. Agric. Food. Chem. 57, 2786–2792. https://doi.org/10.1021/jf802564z.

Plieth, C., 2001. Plant calcium signaling and monitoring: pros and cons and recent experimental approaches. Protoplasma 218, 1–23. https://doi.org/10.1007/BF01288356.

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. Carbohydr. Res. 336, 117–125. https://doi.org/10.1016/S0008-6215(01)00248-8.

SAS Institute, 2009. Getting Started with the SAS Learning Edition. SAS, Cary 200p.

Saure, M.C., 2005. Calcium translocation to fleshy fruit: its mechanism and endogenous control. Sci. Hortic. 105, 65–89. https://doi.org/10.1016/j.scienta.2004.10.003.

Saure, M.C., 2014. Why calcium deficiency is not the cause of blossom-end rot in tomato and pepper fruit - A reappraisal. Sci. Hortic. 174, 151–154. https://doi.org/10.1016/j.scienta.2014.05.020.

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. https://doi.org/10.1007/BF00385454.

Schröder, R., Atkinson, R.G., Langenkämper, G., Redgwell, R.J., 1998. Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. Planta 204, 242–251. https://doi.org/10.1007/S004250050253.

Sharples, R.O., Johnson, D.S., 1986. Effects of some growth regulators on the ripening and storage quality of apples and pears. Acta Hortic. 179, 721–730. https://doi.org/10.17660/ActaHortic.1986.179.124.

Silveira, J.P.G., Amarante, C.V.T., Steffens, C.A., Miqueloto, A., Katsurayama, J.M., 2012. Inhibition of gibberellin synthesis reduces vegetative growth of apple trees and provides control of bitter pit in the fruit. Rev. Bras. Frutic. 34, 328–335. https://doi.org/10.1590/S0100-29452012000200004.

Silveira, J.P.G., Amarante, C.V.T., Steffens, C.A., Correa, T.R.C., Paes, F.N., 2014. Yield potential and fruit quality of apple trees treated with gibberellin and inhibitor of gibberellins biosynthesis. Rev. Bras. Frutic. 36, 771–779. https://doi.org/10.1590/0100-2945-352/13.

Turner, J.N., 1972. Practical uses of gibberellins in agriculture and horticulture. Outlook on Agr. 7, 14–20.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/aob/mcg164.

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. J. Membr. Biol. 138, 55–63. https://doi.org/10.1007/BF00211069.