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Mechanisms regulating bitter pit development in 'Greensleeves' apples with suppression of ethylene biosynthesis

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ABSTRACT

The objectives of this study were to understand the role of ethylene and nutrients (Ca^{2+}, Mg^{2+}, K^{+} and N) on bitter pit (BP) development in wild type (GS) and ethylene suppressed (68G and 103Y) 'Greensleeves' apples. The transgenic line 68G is suppressed for 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) and line 103Y is suppressed for ACC synthase (ACS). Suppression of ethylene biosynthesis reduced BP incidence and severity. Lower ethylene biosynthesis, in ethylene-suppressed genotypes, had no effect on Ca^{2+}, Mg^{2+}, K^{+} and N concentrations in fruit cortical tissue. In all genotypes, fruit with BP had lower Ca^{2+} and higher Mg^{2+} concentrations and higher Mg^{2+}/Ca^{2+} ratio in cortical tissue. The results indicate that high levels of ethylene biosynthesis and Mg^{2+} in cortical tissue can enhance fruit susceptibility to BP incidence.

Keywords: *Malus domestica*, calcium, ACCO, ACCS, physiological disorder.

RESUMO

Mecanismos envolvidos no desenvolvimento de "bitter pit" em maçãs 'Greensleeves' silenciadas para enzimas da síntese de etileno

Os objetivos deste trabalho foram avaliar o efeito do etileno e nutrientes (Ca^{2+}, Mg^{2+}, K^{+} e N) sobre o desenvolvimento de "bitter pit" (BP) em maçãs 'Greensleeves' tipo selvagem e silenciadas para enzimas da síntese de etileno (68G a 103Y). A linhagem transgênica 68G é silenciada para ácido 1-carboxílico-1-aminociclopropano (ACC) oxidase (ACO) e a linhagem 103Y é silenciada para ACC sintase (ACCS). O silenciamento das enzimas ACCO e ACCS diminuiu a incidência e a severidade de BP, mas não teve efeito sobre as concentrações de Ca^{2+}, Mg^{2+}, K^{+} e N no tecido cortical dos frutos. Em todos genótipos, frutos com BP apresentaram baixas concentrações de Ca^{2+} e

altas de Mg^{2+}, resultando em alta razão Mg^{2+}/Ca^{2+} no tecido cortical. Estes resultados indicam que alta síntese de etileno e alta concentração de Mg^{2+} no tecido cortical dos frutos pode aumentar a susceptibilidade dos mesmos a incidência de BP.

Palavras-chave: *Malus domestica*, cálcio, ACCO, ACCS, desordem fisiológica.

Although bitter pit (BP) is believed to be a calcium (Ca^{2+}) deficiency disorder, it may also be regulated by ethylene and other nutrients in fruit tissue (AMARANTE; CHAVES; ERNANI, 2006; LÖTZE; THERON; JOUBERT, 2010). Ethylene is key regulator of many metabolic processes controlling fruit ripening that can affect fruit susceptibility to BP. Plant nutrients such as K^+ and Mg^{2+} may enhance fruit susceptibility to BP by competing with Ca^{2+} for binding sites in the cell and inhibiting Ca^{2+}-binding dependent cellular processes (HO; WHITE, 2005; SAURE, 2005). High N content is usually related to high shoot growth, which may enhance Ca^{2+} movement towards the leaves and decrease Ca^{2+} in the fruit (HO; WHITE, 2005). N and K^+ also trigger cell expansion (HO; WHITE, 2005; SAURE, 2005), suggesting that high levels of these nutrients could favor rapid plant and fruit growth leading to a reduction in fruit Ca^{2+} uptake and dilution of fruit Ca^{2+} content. The objectives of this study were to understand the role of ethylene and other nutrients (Mg^{2+}, K^+ and N) on BP development in wild type and ethylene suppressed 'Greensleeves' apples.

MATERIALS AND METHODS

Wild type 'Greensleeves' (GS) apple trees (*Malus domestica*) and trees from two ethylene biosynthesis-suppressed lines developed at the University of California-Davis - 68G (*1-aminocyclopropane-1-carboxylate oxidase* (*ACO*) suppressed) and 103Y (*1-aminocyclopropane-1-carboxylate synthase* (*ACS*) suppressed) - were cultivated in an orchard located in Davis, California. The trees were 14 years old and did not receive any Ca^{2+} supplement in the field during fruit growth or after harvest. A factorial design was used, with combinations between apple genotypes (GS, 68G, 103Y) and BP incidence (with or without BP). There were four blocks per treatment and one tree per block. All plants were shaded at 70 days after full bloom (DAFB) by covering the plants with a black net suspended above the trees that reduced the light intensity reaching the canopy of the plants by ~50%. Shading was used to avoid fruit damage by sunlight. Two hundred preclimacteric fruit from each block were harvested at 120 DAFB. After harvest, fruit were stored at 0 (±0.5) ºC and 90 to 95% RH for three months. The ethylene concentration in the storage environment was minimized by constantly circulating the air through a potassium permanganate filter.

At harvest, each genotype was analyzed for starch content, flesh firmness, soluble solids content (SSC) and titratable acidity (TA). These analyses were accomplished using four replications with 10 fruit for each genotype. Starch clearing was estimated by cutting the fruit in half, then dipping the fruit in a solution containing iodine:potassium iodide (1:4) (QA Supplies, LLC, Norfolk, VA) for 1 min for 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. Fruit flesh 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). Soluble solids content (SSC) and titratable acidity (TA) were determined in juice sample extracted by squeezing two cortical wedges cut from both sides of the fruit in two layers of cheese cloth. Soluble solids were determined with an Abbe 10450 digital refractometer (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 4mL of juice with 0.1N NaOH to pH 8.2.

At three months of storage, all fruit were analyzed for BP incidence and severity. Fruit with and without BP were then segregated and outer cortical tissue was manually cut from the calyx end right underneath the skin up to a depth of 5 mm, frozen in liquid N_2 and stored at -80ºC for later analysis. Frozen samples were analyzed for total nitrogen (N), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) concentrations. Bitter pit was assessed by 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 to 20 pits, 5 = >20 pits per fruit) and calculated with the formula described by Pesis et al. (2010):

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

Nitrogen concentration was analyzed by a combustion method. Potassium was extracted with 2% acetic acid and quantitatively assessed by atomic emission spectrometry. Calcium and Mg^{2+} were determined by subjecting tissue to microwave acid digestion/dissolution and subsequent analysis by inductively coupled plasma atomic emission spectrometry.

Statistical analysis was performed for each variable by means of analysis of variance (ANOVA) using the SAS statistical package. The mean values (of four replicates ± standard error) were compared using Tukey's test ($p = 0.05$). Canonical discriminant analysis (CDA) was performed to identify the best mineral variable (Ca^{2+} concentration and nutrient concentration ratios Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+}, and N/Ca^{2+} in cortical tissue) to discriminate between fruit with and without visual symptoms of BP by using the PROC CANDISC procedure of SAS. The power of each variable to discriminate between fruit with and without BP was investigated by calculating the standardized canonical coefficients (SCC), canonical correlation (r) between canonical discriminant function 1 (CDF_1) and the mineral variables, and the parallel discriminant ratio coefficient (DRC = SCC x r) (AMARANTE; CHAVES; ERNANI, 2006).

RESULTS AND DISCUSSION

The starch index and malic acid content at harvest were similar among all genotypes evaluated (Table 1). The lowest flesh firmness was observed in GS fruit (Table 1). The highest SSC was observed in the 103Y ethylene suppressed line (Table 1).

There was no BP at the time of harvest. After three months of storage at 0ºC, BP incidence and index were lower in ethylene-suppressed fruit than wild type fruit (GS) (Figure 1). Accordingly, other studies have shown that BP can be induced by treating apple fruit with ethylene (LÖTZE; THERON; JOUBERT, 2010). In addition, apple fruit treated with an inhibitor of ethylene responses, 1-methylcyclopropene, are less susceptible to BP development in cold storage than untreated fruit (PESIS et al., 2010). Although the mechanisms involved are not well understood, ethylene may trigger BP by accelerating fruit ripening and senescence and possibly the processes leading to BP symptoms development. Ethylene may increase plasma membrane leakiness, enhancing the effect of low tissue Ca^{2+} concentration on fruit susceptibility to BP. Increasing plasma membrane leakiness has been suggested to be involved in BP symptoms development (HO; WHITE, 2005; SAURE, 2005).

Although suppression of ethylene biosynthesis reduced fruit susceptibility to BP, wild type and ethylene-suppressed lines had statistically similar Ca^{2+}, Mg^{2+}, K^{+} and N concentrations, as well as Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+} and N/Ca^{2+} ratios in fruit cortical tissue (Tables 2 and 3). Accordingly, studies have shown that treating apple trees before harvest with ethylene or the ethylene biosynthesis inhibitors had no effect on fruit nutrient uptake (DRAKE et al., 2005). In all genotypes, fruit with and without BP had similar K^{+} and N concentrations in cortical tissue (Table 2). Pitted fruit showed lower Ca^{2+} and higher Mg^{2+} concentrations (Tables 2), as well as bigger Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+} and N/Ca^{2+} ratios in cortical tissue (Tables 3), compared to sound fruit. The lower Ca^{2+} concentration in pitted fruit can be attributed to different factors such as fruit position in the canopy, number of functional xylems in the fruit, fruit transpiration rates, as well as different concentrations of growth regulators in fruit tissue (HO; WHITE, 2005; SAURE, 2005).

According to the CDA of mineral attributes related to Ca^{2+} concentration and Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+}, and N/Ca^{2+} ratios in the fruit cortical tissue only one canonical discriminate function (CDF_1) can explains 100% of the total data variation. ANOVA of canonical scores showed a highly significant difference ($p < 0.01$) between fruit with and without BP on CDF_1. The Mg^{2+}/Ca^{2+} ratio had the highest values of SCC, *r* and DRC for CDF_1 and, therefore, better define differences between fruit with and without BP than Ca^{2+} concentration alone or the N/Ca^{2+} and K^{+}/Ca^{2+} ratios (Table 4). The Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+}, and N/Ca^{2+} ratios in fruit tissue may play an important role in determining fruit susceptibility to BP (BRAMLAGE; DRAKE; LORD, 1980; LANAUSKAS; KVIKLIENE, 2006; AMARANTE; CHAVES; ERNANI, 2006). However, the mechanisms through which these nutrient ratios affect fruit susceptibility to BP are still poorly understood. According to our results, the Mg^{2+}/Ca^{2+} ratio in fruit cortical tissue better explains fruit susceptibility to BP than Ca^{2+} alone or K^{+}/Ca^{2+} and N/Ca^{2+} ratios. Our results show that the average Mg^{2+} concentration in cortical tissue of fruit with BP (222.6 μmol 100 g fw^{-1}) was higher than in fruit without BP (182.9 μmol 100 g fw^{-1}). Since cortical Ca^{2+} was higher in fruit without BP than in fruit with BP, our results suggest that high Mg^{2+} uptake may enhance the effect of low Ca^{2+} uptake on increasing fruit susceptibility to BP. Higher content of Mg^{2+} could compete with Ca^{2+} for binding sites at the plasma membrane surface. Greater Mg^{2+} 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 and BP development. Although other studies suggested that K^+/Ca^{2+}, and N/Ca^{2+} ratios are related to fruit susceptibility to BP (BRAMLAGE; DRAKE; LORD, 1980; LANAUSKAS; KVIKLIENE, 2006), our data showed no clear relationship between these nutrient ratios and BP incidence.

Suppression of ethylene biosynthesis decreases fruit susceptibility to BP. The Mg^{2+}/Ca^{2+} ratio in fruit tissue is a better attribute to estimate or predict fruit susceptibility to BP than Ca^{2+} concentration alone or as part of K^+/Ca^{2+} or Mg^{2+}/Ca^{2+} ratios.

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Table 1. Sarch index, flesh firmness, soluble solids content (SSC) and malic acid equivalents of wild type (GS), ACO-silenced (68G), and ACS-silenced (103Y) 'Greensleeves' apples at harvest.

Genotype	Starch (1-6)	Firmness (N)	SSC (%)	Malic acid (%)
GS	3.12 a*	62.2 b	11.5 ab	0.607 a
68G	3.07 a	66.6 a	10.4 b	0.589 a
103Y	3.19 a	68.7 a	12.0 a	0.630 a
CV (%)	9.8	4.9	2.8	6.2

* Mean values with different letters are significantly different according to Tukey's test (5%).

Table 2. Concentration of Ca^{2+}, Mg^{2+}, K^{+} and N in cortical tissue of wild type (GS), ACO-silenced (68G), and ACS-silenced (103Y) 'Greensleeves' apples stored for three months at 0 ºC.

Genotype	Ca^{2+} (µmol 100^{-1} CFW)		Mg^{2+} (µmol 100^{-1} CFW)		K^{+} (mmol 100^{-1} CFW)		N (mmol 100^{-1} CFW)	
	No BP	BP	No BP	BP	No BP	BP	No BP	BP
GS	84.5 Aa*	61.0 Ba	191.6 Ba	213.8 Aa	2.55 Aa	2.77 Aa	4.40 Aa	4.22 Aa
68G	79.7 Aa	58.1 Ba	188.5 Ba	227.2 Aa	2.53 Aa	2.64 Aa	3.79 Aa	4.53 Aa
103Y	77.5 Aa	59.9 Ba	168.8 Ba	226.9 Aa	2.38 Aa	2.45 Aa	3.88 Aa	3.97 Aa
CV (%)	2.70	3.41	7.03	7.87	8.44	8.47	7.58	14.39

* Different uppercase or lowercase letters show statistical difference between fruit without and with BP for the same plant line (GS, 68G, or 103Y) or statistical differences between plant lines (GS, 68G, and 103Y) according to Tukey's test (5%), respectively.

Table 3. Ratio of Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+}, N/Ca^{2+} in cortical tissue of of wild type (GS), ACO-silenced (68G), and ACS-silenced (103Y) 'Greensleeves' (GS), ACO-silenced (68G), and ACS-silenced (103Y) 'Greensleeves' apples stored for three months at 0 ºC.

Genotype	Mg^{2+}/Ca^{2+}		K^{+}/Ca^{2+}		N/Ca^{2+}	
	No BP	BP	No BP	BP	No BP	BP
GS	2.27 Ba*	3.51 Aa	30.1 Ba	45.4 Aa	52.1 Ba	69.1 Aa
68G	2.36 Ba	3.91 Aa	31.8 Ba	45.5 Aa	47.6 Ba	77.9 Aa
103Y	2.18 Ba	3.79 Aa	30.8 Ba	40.8 Aa	50.1 Ba	66.4 Aa
CV (%)	7.12	10.20	12.50	9.92	8.03	14.96

* Different uppercase or lowercase letters show statistical difference between fruit without and with BP for the same plant line (GS, 68G, or 103Y) or statistical differences between plant lines (GS, 68G, and 103Y) according to Tukey's test (5%), respectively.

Table 4. Canonical discriminant analysis of mineral variables (Ca^{2+} and Mg^{2+}/Ca^{2+}, K^{+}/Ca^{2+}, and N/Ca^{2+} ratios) assessed in cortical tissue of fruit with and without visible symptoms of BP. Fruit were harvested from shaded trees, cold stored at 0ºC for three months and then segregated for the presence of BP symptoms. The values of standardized canonical coefficients (SCC), canonical correlation (*r*) between

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canonical discriminant function 1 (CDF_1) and the original variables, and parallel discriminant ratio coefficients (DRC) for CDF_1 were calculated for each mineral variable.

Attribute	SCC	*r*	DRC
Ca^{2+}	1.270	-0.289	-0.367
Mg^{2+}/Ca^{2+}	**2.828**	**0.665**	**1.881**
K^{+}/Ca^{2+}	-1.360	0.466	-0.633
N/Ca^{2+}	0.285	0.418	0.119

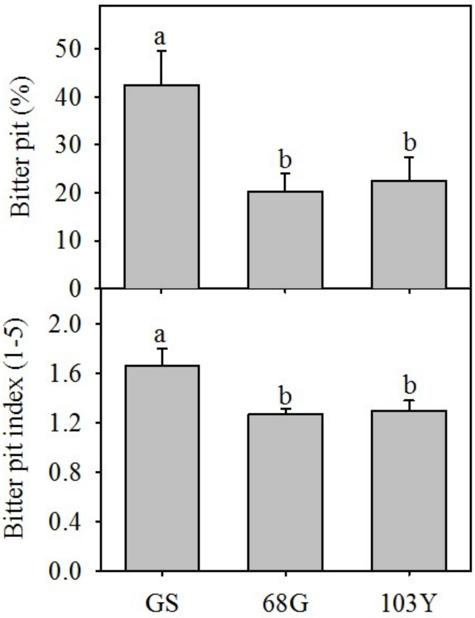

Figure 1. BP incidence (A) and severity (B) of wild type (GS), ACO-silenced (68G), and ACS-silenced (103Y) 'Greensleeves' apple fruit stored for three months at 0ºC. Mean values are compared by Tukey's test (p = 0.05). Different letters show statistical differences between plant lines (GS, 68G, and 103Y). Values represent the mean of four replicates ± SE.

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