Research Article

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Effects of exogenous compound sprays on cherry cracking: skin properties and gene expression

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Abstract

BACKGROUND: Cherry fruit cracking is a costly problem for cherry growers. The effect of repeated sprayings (gibberellic acid – GA_3; abscisic acid – ABA; salicylic acid – SA; glycine betaine – GB, and *Ascophyllum nodosum* – AN) combined with $CaCl_2$, on 'Sweetheart' cherry fruit-cracking characteristics was investigated. Cracking was quantified in terms of cracking incidence, crack morphology, confocal scanning laser microscopy, cuticular wax content, cell-wall modification, and cuticular wax gene expression.

RESULTS: All spray treatments reduced cracking compared with an untreated control (H_2O), with fewer cheek cracks. The least cracking incidence was observed for ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits, indicating an added benefit compared to spraying with $CaCl_2$ alone. In addition, GB + $CaCl_2$-treated fruits showed higher fruit diameter. ABA + $CaCl_2$ and GB + $CaCl_2$ sprays showed higher wax content and higher cuticle and epidermal thickness compared with the control, including increased expression of wax synthase (ABA + $CaCl_2$) and expansin 1 (GB + $CaCl_2$).

CONCLUSION: In general, factors that improve the cuticle thickness appear to be important at the fruit-coloring stage. At the fruit-ripening stage, larger cell sizes of the epidermis, hypodermis, and parenchyma cells lower cracking incidence, indicating the importance of flexibility and elasticity of the epidermis.

Supporting information may be found in the online version of this article.

Keywords: *Prunus avium* L.; sweet cherry; cracking index; crack type; cuticular waxes; gene expression

INTRODUCTION

Fruit cracking is a physiological disorder that depends on biochemical, anatomical, genetic, environmental, and cultural factors.[1–6] Climate-change predictions suggest that there could be an increased frequency of excessive rainfall that could cause a higher incidence of cherry cracking.[7] Although the mechanism that leads to cherry cracking remains unclear,[8] cracking is currently assumed to be the result of a net influx of water into the fruit, causing an increase in fruit volume and surface area, resulting in a strained fruit skin, which eventually ruptures (reviewed by Knoche and Winkler[9] Three types of cherry fruit macro-cracks may occur: in the stem cavity region (SCR), in the stylar scar region (SSR), and in the cheek region (CR).[10] Fruit with small SCR and SSR cracks are tolerated by consumers as long as there is no fungal infection, but fruit with CR cracks are rejected.[9]

Growth regulators have been applied to limit cracking.[8] Calcium plays an important role in cell-wall physiology,[11] reducing cherry cracking.[12–15] In sweet cherry fruits, foliar application of gibberellic acid (GA_3) either increased,[16] had no effect[17,18] or decreased cherry cracking.[19,20] Salicylic acid (SA) application increased cherry firmness.[21,22] Exogenous ABA foliar application reduced cracking in 'Bing' cherry fruits.[23] Abscisic acid (ABA) foliar application increased cuticle lipid biosynthesis in *Arabidopsis thaliana*,

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which reduced cuticle permeability.[24] Abscisic acid is reported to play an active role in wax metabolism through the activation of several genes related to biosynthesis and transport of cutin and waxes.[24–26] Glycine betaine (GB) and *Ascophyllum nodosum* (AN), a seaweed-based biostimulant, are promising new spray compounds that might reduce cherry cracking. Glycine betaine is a quaternary ammonium compound that helps to maintain membrane integrity in the presence of several stresses, including excessive salt, cold, heat, and freezing.[27,28] *Ascophyllum nodosum* is reported to increase mineral uptake, biomass, yield, fruit quality,[29–32] and reduce cherry fruit cracking.[33]

How growth regulators affect cherry fruit cracking is mostly unclear. One hypothesis is that growth regulators increase resistance to cracking because of higher cuticle thickness. The cherry cuticle is covered by epicuticular waxes acting as a barrier against water uptake and pathogens.[34,35] Moing *et al.*[2] suggested that cuticle properties may play an important role in cracking susceptibility. During the last stage of fruit growth, cutin and wax levels remained constant, despite the increasing volume of the fruit.[36–38] The reduction of cuticle thickness per area may result in the formation of microcracks, increasing the sensitivity to fruit cracking.[37,39] *PaLTPG1*, *PaATT1*, *PaWINB*, *PaWS*, and *PaKCS6* have been identified as being involved in sweet cherry-cuticle formation and wax biosynthesis.[40,41] The involvement of these genes in the development of cherry cracking has been demonstrated, apart from *PaLTPG*. *PaLTPG* expression decreased during fruit development, which suggests no relation with cracking tolerance.[6] Next to the cuticle thickness, other histological parameters might also be affected by growth regulators. A larger mesocarp size was observed in cracking-tolerant cherry cultivars.[42] Smaller hypodermis cell size[43] with generally larger cells, was reported in susceptible cultivars.[1] A recent study reported that macrocracking is caused by increased mesocarp cell volume accompanied by cell bursting.[44]

Growth regulators might also increase resistance against cracking because of higher epidermal flexibility and elasticity. In tomato, cell-wall modification during fruit development involves polygalacturonase, pectin methylesterase, expansin, and β-galactosidase (β-Gal) activity.[45] Antisense suppression of β-Gal in tomato resulted in increased cracking.[46] Expansins contribute to the extension of cell walls by breaking down the links between cellulose microfibrils and xyloglucans molecules.[47] Expression of expansin genes has been characterized during fruit growth in pericarp and mesocarp tissue[45,48,49] and was associated with reduced fruit cracking in litchi[48] and apple.[49] In sweet cherry fruits, Balbontín *et al.*[6] mentioned that expansin (*PaEXP1*) expression was higher in cracking-resistant cultivars than in sensitive sweet-cherry cultivars.

The aim of this study was to investigate how foliar application of growth regulators affects fruit-cracking incidence and cracking characteristics. 'Sweetheart' cherries were sprayed repeatedly with growth regulators during fruit development from yellow / orange to light red (FC) and from light red to dark red (FR). Skin characteristics were assessed and linked to cracking incidence at harvest to gain insight into the physiological background of cracking resistance. For this purpose, histological parameters (thickness and area of cuticle, epidermis, and hypodermis), confocal scanning laser microscopy[34] to visualize the cuticle architecture, soluble cuticular wax, crack-type morphology, and expression of genes involved in cuticular wax biosynthesis (*PaWS*) and wall modification (*PaEXP1* and *Paβ-Gal*) were evaluated at two stages of fruit development (FC and FR). We show that growth regulators affect cracking incidence by affecting wax content, cuticle and epidermal thickness and cell size, and discuss this. The effects of growth regulators on the expression of cuticular wax synthesis and expansin genes during both coloring stages are also discussed, providing indications regarding ways in which foliar applications might affect cracking incidence.

MATERIAL AND METHODS

Plant material

Experiments were carried out in a commercial orchard located at Carrazedo de Montenegro (41° 33′ N, 7° 17′ W, altitude 682 m), Portugal in 2016. The soil characteristics are: 13 g kg^{-1} of organic matter content, with high K_2O (125 mg kg^{-1}) and medium P_2O_5 (75 mg kg^{-1}) content, medium texture, and pH 5.5. Late-maturing, 6-year-old 'Sweetheart' sweet cherry trees (*Prunus avium* L.), grafted on 'Gisela 6' rootstocks, were used. Trees were spaced 4.5 m between rows and 2.0 m from each other in the row (about 864 trees ha^{-1}), trained under a vertical axis system.[50] Trees were drip-irrigated daily between May and September, 4 h per day (drippers 1 m apart in line with a 4 L h^{-1} flow rate) and pruned in summer. Trees were also fertilized periodically according to recommendations provided by a certified soil analysis laboratory of University of Trás-os-Montes e Alto Douro (UTAD).

Experimental design and treatments

For each treatment, 15 trees were selected, but only nine trees were used to compare treatment effects as the other trees were assigned as border row trees to prevent carry-over effects. The treatments applied were: untreated control (distilled water), biostimulant with seaweed *Ascoplyllum nodosum* (AN) at 0.5 mL L^{-1}, gibberellic acid (GA_3) at 10 mg L^{-1}, 10 µmol L^{-1} abscisic acid (ABA), salicylic acid (SA) at 1 mmol L^{-1}, glycine betaine (GB) at 1 mL L^{-1} and calcium chloride ($CaCl_2$) at 5 g kg^{-1} ($CaCl_2$ control). All treatments included 5 g kg^{-1} $CaCl_2$, except for the untreated control, and were mixed with a wetting agent (1 mL L^{-1}, Sticman, Lusosem). All cherry trees were sprayed with a mean volume of 2.5 L of spraying solution per tree. Treatments, except $CaCl_2$, were applied 30, 49, and 56 days after full bloom (DAFB), corresponding to the beginning of fruit development (shuck split), the transition from green to yellow, and the transition from yellow to orange color. At 56, 62, and 69 DAFB, distilled water (only for untreated control trees) and $CaCl_2$ was applied. Calcium chloride alone was applied on different DAFB from other treatments, due to their specific effect on increasing fruit firmness, while the other compounds have an effect on fruit development at the level of cell division. All treatments were applied three times to the same tree, nine trees per treatment for each of the seven treatments for a total of 63 trees. The following treatments were applied: untreated control, $CaCl_2$ control, AN+$CaCl_2$, GA_3+$CaCl_2$, ABA+$CaCl_2$, SA+$CaCl_2$ and GB+$CaCl_2$. For each treatment, 50 fruits were harvested at either the transition from yellow / orange to light red at 60 DAFB (FC) or from light red to dark red at 77 DAFB (FR) per tree. This amounted to 450 FR and 450 FC fruits in total. A schematic representation of the experimental design is presented in Fig. 1.

All fruits were randomly divided into two sub-batches per development stage. Fruits of the first sub-batch were used to determine fruit diameter (width), soluble solid content, cracking index, and soluble cuticular wax content, and for histological analysis. Fruits of the second sub-batch were immediately frozen in

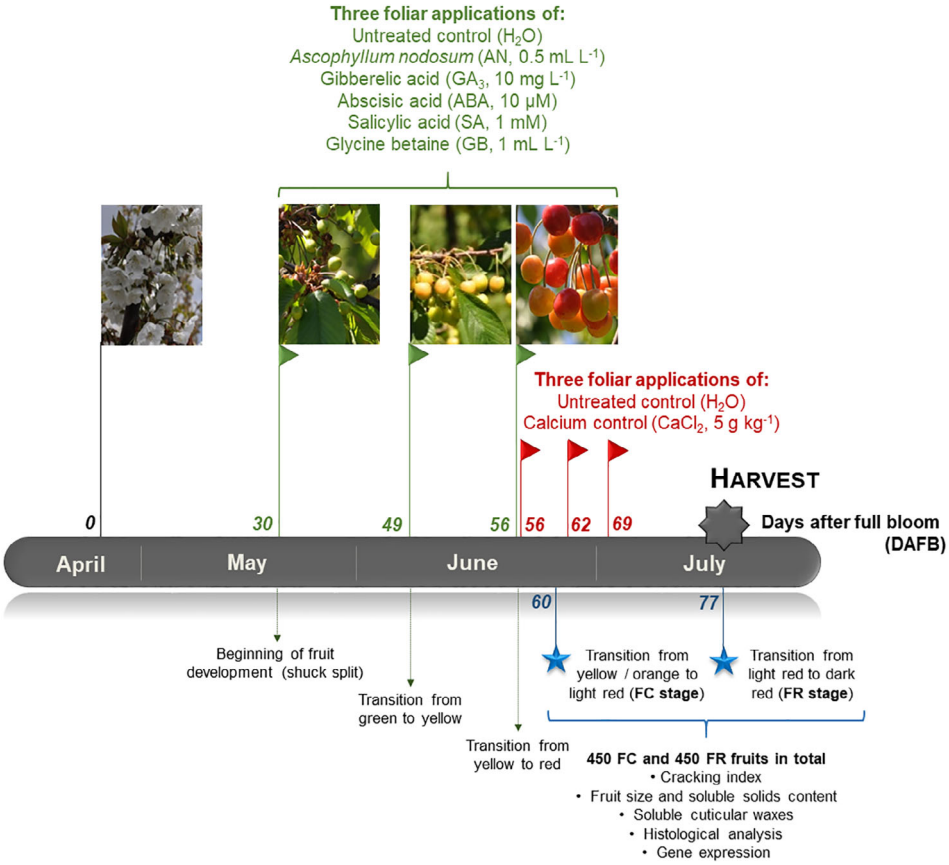

Figure 1. Experimental design overview and timeline.

liquid nitrogen and stored at −80 °C for subsequent assessment of gene expression.

Fruit cracking index

The cracking index (CI in %) was determined as described by Christensen.[51] Fifty fruits without defects at FR stage were immersed in 2 L containers filled with distilled water (20 ± 1 °C) for 6 h. Cracked fruits were removed and counted and fruits without cracks were re-incubated. After 2, 4, and 6 h, the fruits were observed for macroscopic cracks, with the CI calculated according to:

$$CI = \frac{(5a+3b+c)*100}{250}$$

where *a*, *b*, and *c* represent the number of cracked fruits after 2, 4, and 6 h, respectively. The measurements are presented as average values ($n = 3$) with standard errors (SE). Crack type (CT as a percentage) was expressed as the number of cracks of a particular type (SCR, SSR, or CR) compared with the total number of cracks.

Fruit size and soluble solids content

Fruit size was determined by measuring the diameter (width) in mm of 30 fruits using a digital calliper (500–196-30, Mitutoyo, Hampshire, UK), and was presented as the average of 30 fruits with the SE shown. The same 30 fruits were divided afterwards into three groups of 10 fruits each. The juice from 10 fruits was extracted with an electrical extractor (ZN350C70, Tefal Elea, Hong Kong, China) for 1 min. Soluble solid content (SSC, in °Brix) was determined using a digital refractometer (PR-101, Atago, Tokyo, Japan). Soluble solid content was expressed as the average of three replicates, with SE shown.

Fixation and sectioning for histological analysis

Cross sections were prepared by fixation in formalin-acetic acid-alcohol (FAA, 5:5:90 v/v), for 48 h. Afterwards, the cross sections were dehydrated in increasing ethanol concentrations (700 mL L^{-1}, 800 mL L^{-1}, 900 mL L^{-1}, 950 mL L^{-1} and 1 L L^{-1}) doe 1 h. Fruit samples were placed in xylene for 1 h, after which the samples were embedded overnight in liquid paraffin, using a paraffin embedding station (ModelEG1160, Leica-Microsystems, Wetzlar, Germany). Sections of 8 and 15 µm (for bright field and confocal microscopy, respectively) were cut using a rotary microtome (RM2255, Leica-Microsystems).

Bright-field microscopy

Two fruits were embedded in one paraffin block with three paraffin blocks used per treatment. From each paraffin block one tissue section was selected with the best embedding. Tissue sections of 8 µm thickness stained with Sudan III/IV (1 mL L^{-1} solution in 700 mL L^{-1} ethanol) and 0.1 mL L^{-1} methylene blue were imaged using an Eclipse 50i upright microscope equipped with a Fi3 camera. The samples were analyzed using Plan Ph1 DL 10×/0.25 and Plan Fluor Ph2 DLL 40×/0.75 dry objectives and digital zooms. The images obtained with lower magnification (10×) were used to identify crack morphology in mature cherries, and those obtained with higher magnification (40×) were used to measure histological parameters of fruits without cracking symptoms in

both development stages. Histological parameters were quantified, applying Nikon NIS-Elements D imaging software. The thickness of the cuticle and epidermal cell walls, as well as the thickness of epidermis, hypodermis and parenchyma cells, was measured along the axis perpendicular to the fruit surface. The size of epidermal, hypodermal and parenchyma (adjacent to hypodermis) cells was determined (Fig. 1(s)). Histological measurements were taken from six individual cells per image. Two images were taken per tissue section for each of the three paraffin blocks. Data are presented as average values ($n = 36$) with SE shown.

Confocal microscopy

Three-dimensional confocal scanning laser microscopy was used to observe the cuticle architecture of mature-stage cherry fruit exocarp. Tissue sections that were 15 μm thick were stained with Calcofluor white[34] and Auramine O[52] (0.01% w/v in 0.05 M Tris/HCl, pH 7.2). The samples were analyzed using a TCS SP8 confocal laser scanning microscope (DMI 6000, Leica Microsystems, Germany) with HC PL APO CS2 63×/1.20 water objectives operated by LAS 2.0.2.15022 software (Leica Microsystems). Calcofluor white was excited by a 405 UV diode and emission was collected between 415–448 nm. Auramine O was excited using a 458 nm argon laser and emission was collected between 491–563 nm. Z-stack images with a step size of 0.4 μm were obtained using a bidirectional scan rate of 400 Hz in sequential mode to avoid cross talk between the two fluorophores.

Soluble cuticular waxes content determination

Soluble epi- and intra-cuticular waxes were quantified as described by Hamilton.[53] Briefly, two fruits without peduncle were dewaxed with 50 mL of 750 mL L^{-1} chloroform and 250 mL L^{-1} methanol for 2 min at 25 °C. This procedure was repeated nine times for each spray treatment. Results are shown in μg g^{-1} of fresh weight (FW) and expressed as the average of three replicates with SE shown.

RNA isolation and cDNA synthesis

Total RNA was isolated from fruit exocarp pooling three times a set of three fruits per treatment using the RNAqueous™ phenol-free total RNA isolation kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). The RNA integrity was evaluated by electrophoresis in a 10 g L^{-1} agarose gel (80 V for 90 min). All samples were diluted to 100 ng μL^{-1} before reverse transcribing the total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Samples were stored at −20 °C before further analysis.

Quantitative real-time polymerase chain reaction (PCR) analysis

Expression of *PaEXP1*, *Paβ-Gal* and *PaWS* was measured by quantitative real-time PCR (qRT-PCR) during fruit development. Actin (*PaAct)* was used as a housekeeping gene.[6,54,55] Primer sequences, annealing temperatures (Ta) and amplicon size for each gene are shown in Table S1. Quantitative real-time PCR was performed in the StepOnePlus Real-Time PCR system (Applied Biosystems). Each PCR reaction had a final volume of 10 μL constituted by 1 μL of the cDNA sample, 0.25 μL of each primer (10 μmol L^{-1}) and 5 μL of Sybr® Select Master Mix (Applied Biosystems) using the following program: initial denaturation for 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at Ta and 15 s at 72 °C. This was followed by a melting-curve analysis to exclude interference of primer dimers, DNA contaminants, and other nonspecific products. Quantitative PCR amplifications were performed in duplicate and analyzed using StepOnePlus Real Time PCR software (Applied Biosystems). Only threshold quantification cycle (Ct) values, leading to a Ct mean with a standard deviation below 0.5, were considered. A negative control was included on each plate to assess the presence of primer-dimers and to check for potential contamination. Expression was normalized by the average of housekeeping gene, according to Pfaffl.[56] The $2^{-\Delta\Delta Ct}$ method,[57] was used to calculate relative mRNA levels. Results are expressed as the average of three replicates with SE shown.

Statistical analysis

Statistical analysis was performed using SPSS V.25 (SPSS-IBM, Armonk, New York, NY, USA). Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by *post hoc* Duncan's multiple range tests ($P < 0.05$), establishing treatment effects. The ANOVA requirements, namely the normal distribution of the residuals, were evaluated by means of the Shapiro–Wilk's test, and the homogeneity of variance, using the Bartlett's tests. Dependent variables were analyzed using ANOVA with or without the Welch correction, depending on whether homogeneity of variance was observed or not. Spearman's rank correlation was also performed between cracking incidence and skin property parameters.

RESULTS

Fruit diameter, cracking incidence, and crack-type morphology

Fruit diameter was affected by spray treatments ($P < 0.001$) (Fig. 2(a)), while soluble solids content (SSC) was not affected (data not shown). GB + $CaCl_2$ sprays showed the highest fruit diameter, up to 9% and 8% compared with $CaCl_2$ control and untreated control, respectively.

Cracking index values were affected by spray treatments ($P < 0.001$) (Fig. 2(b)). Application of $CaCl_2$, AN + $CaCl_2$, GA_3 + $CaCl_2$ and SA + $CaCl_2$ reduced CI values by around 50% compared with the untreated control. ABA + $CaCl_2$ and GB + $CaCl_2$ were the most effective treatments for reducing cracking. The $CaCl_2$ control, GA_3 + $CaCl_2$, ABA + $CaCl_2$ and the GB + $CaCl_2$ sprays diminished the percentage of CR type cracks compared with the untreated control (Fig. 2(c)). Compared to the $CaCl_2$ control, the ABA + $CaCl_2$ was the most effective treatment in reducing CR type cracks.

$CaCl_2$ control fruits and GA_3 + $CaCl_2$- and ABA + $CaCl_2$-treated cherries exhibited numerous but small exocarp disruptions (Fig. 3). AN + $CaCl_2$ and SA + $CaCl_2$-treated cherries showed a higher percentage CR cracks and a lower percentage of SSR cracks compared with the untreated control (Fig. 2(c)). AN + $CaCl_2$-treated cherry showed mainly deep and wide fractures while SA + $CaCl_2$-treated cherries had broad and shallow basins with a layer of suberized cells. The cracks of GB + $CaCl_2$-treated cherries usually formed craters with suberized external cell layers (Fig. 3). Lower cuticle thickness in untreated control (Fig. 4(a)) and $CaCl_2$ control fruits (Fig. 4(b)) was observed applying 3D confocal microscopy. A thicker cuticle layer was visible for ABA + $CaCl_2$- (Fig. 4(c)) and GB + $CaCl_2$-treated fruits (Fig. 4(d)). ABA + $CaCl_2$-treated fruits showed (yellow colored) lipidic material in intercellular spaces below the epidermis and between hypodermal cells (Fig. 4(c)).

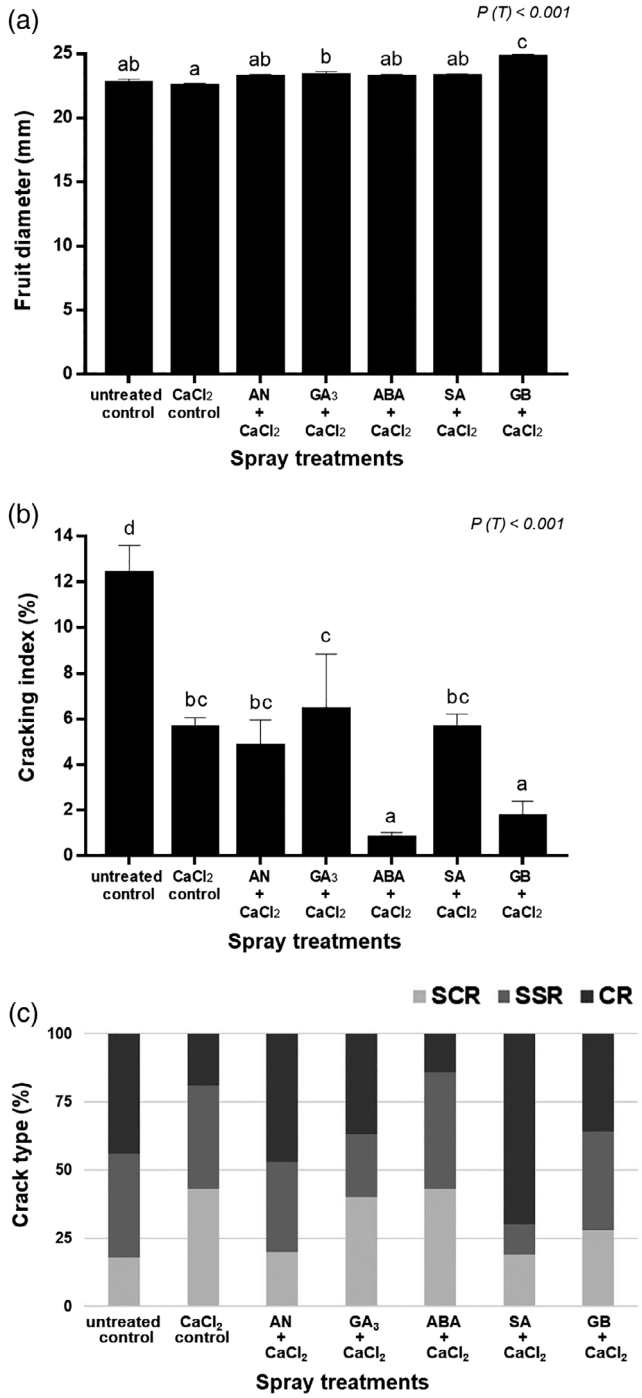

Figure 2. (a) Fruit diameter (mm), (b) cracking index (%) and (c) crack-type incidence (%) of 'Sweetheart' fruits as a function of spray treatments (T) at the fruit ripening (FR) stage. Data are expressed as the mean ± SE ((a) $n = 30$ and (B, C) $n = 3$, each with 50 fruits). Different letters indicate significant differences ($P < 0.05$) among treatments, using Duncan's test.

Fruit histology

At both development stages, all treatments showed higher ($P < 0.001$) fruit-cuticle thickness compared with the untreated control. Comparing with $CaCl_2$ control, ABA + $CaCl_2$-, SA + $CaCl_2$- and GB + $CaCl_2$-treated cherries displayed higher ($P < 0.001$) fruit-cuticle thickness. Moreover, AN + $CaCl_2$, ABA + $CaCl_2$, SA + $CaCl_2$ and GB + $CaCl_2$ increased ($P < 0.001$) epidermal cell-wall thickness

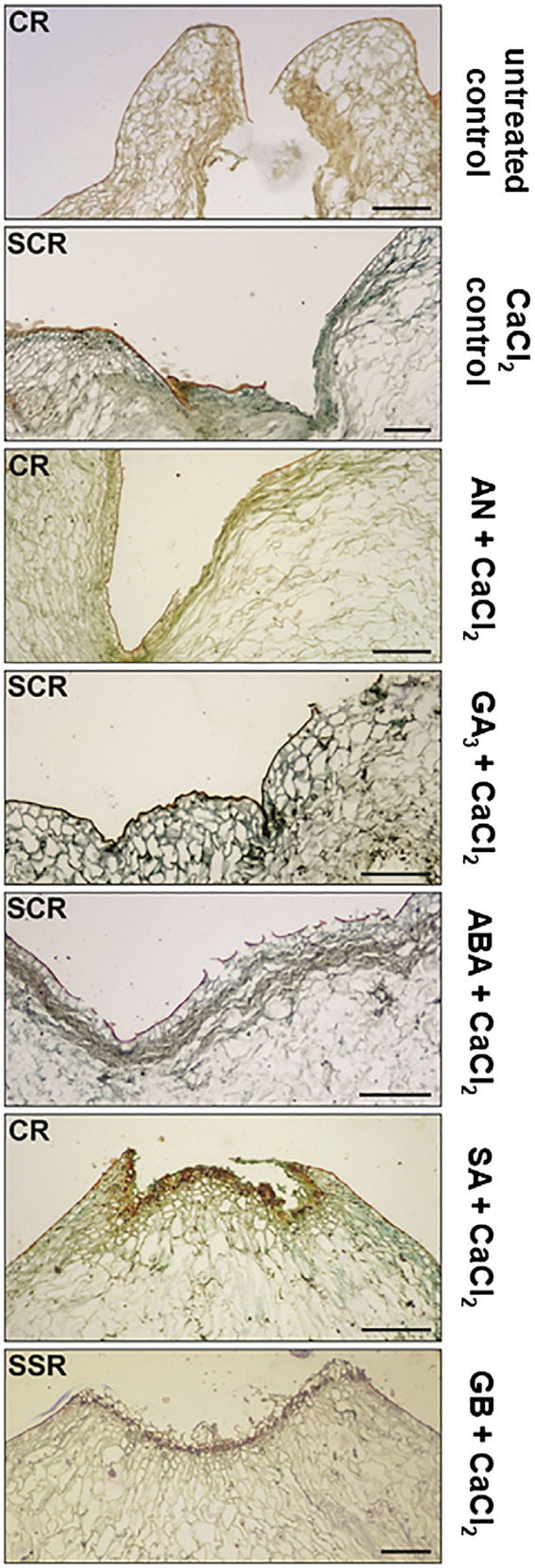

Figure 3. The light microscope images of dominating types of cracks (SCR: stem cavity region; SSR: stylar scar region; CR: cheek region) in 'Sweetheart' cherries exposed to different treatments. Light micrographs of sections stained with Sudan III/IV and methylene blue. Scale bars = 200 μm.

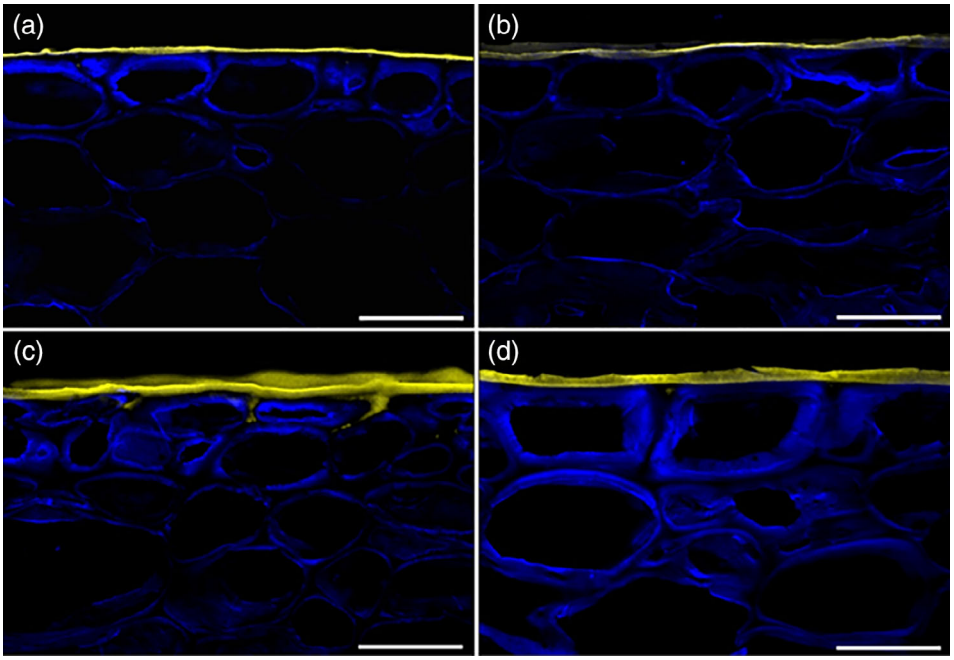

Figure 4. Confocal images of the fruit-ripening (FR) stage fruit sections stained with Auramine O (yellow: cutin / suberin / lignin), and with Calcofluor white (blue. celullose). (a) untreated control fruits; (b) $CaCl_2$ control fruits; (c) ABA + $CaCl_2$-treated fruits; (d) GB + $CaCl_2$-treated fruits. Scale bars = 40 μm.

compared with the untreated and $CaCl_2$ controls (Table 1). Epidermal thickness and cells area were higher ($P < 0.01$) in SA + $CaCl_2$-treated cherries for cherries at the FC stage compared to the untreated and $CaCl_2$ controls. Fruits treated with ABA + $CaCl_2$ showed lower ($P < 0.05$) hypodermis cell thickness and hypodermal cell area at the FC but not the FR stage compared with the untreated and $CaCl_2$ controls. At the FC stage, AN + $CaCl_2$, and ABA + $CaCl_2$-treated fruits had lower hypodermis cell area, while GB + $CaCl_2$-treated cherries showed higher ($P < 0.05$) parenchyma cell area, compared with the untreated and $CaCl_2$ controls. At the FR stage, AN + $CaCl_2$, and GB + $CaCl_2$-treated cherries showed higher epidermal cell thickness and larger cells area values ($P < 0.001$) compared with the untreated and $CaCl_2$ controls. The AN + $CaCl_2$, ABA + $CaCl_2$, and GB + $CaCl_2$-treated cherries showed higher ($P < 0.001$) hypodermal thickness and cells area at the FR stage compared to the untreated control. Finally, parenchyma cell area was larger ($P < 0.01$) for cherries treated with AN + $CaCl_2$, ABA + $CaCl_2$, SA + $CaCl_2$ and GB + $CaCl_2$.

Soluble cuticular wax content

The average soluble cuticular wax content was higher at the FC stage compared with the FR stage, indicating a decrease in soluble cuticular wax content during fruit development ($P < 0.001$, Fig. 5). Soluble cuticular wax content was affected by spray treatments ($P < 0.001$, Fig. 5). At the FC stage, ABA + $CaCl_2$-treated cherries showed higher and GA_3-treated cherries lower soluble cuticular wax content compared with the untreated and $CaCl_2$ controls. At the FR stage, SA + $CaCl_2$ and GB + $CaCl_2$-treated fruits showed higher soluble cuticular wax content compared with the untreated control, but not with the $CaCl_2$ control.

Expression of cell-wall modification and cuticular wax biosynthesis genes

Gene expression was affected by spray treatments (Fig. 6, $P < 0.001$). *PaEXP1*, *Paβ-Gal* and *PaWS* gene expression was higher at the FC stage compared with the FR stage (Fig. 6, $P < 0.001$). *PaEXP1* gene expression did not vary between treatments except at the FC stage, when it was higher for GB + $CaCl_2$-treated fruits (Fig. 6(a)). Expression of *Paβ-Gal* increased for AN + $CaCl_2$- and GB + $CaCl_2$-treated fruits at the FC stage, but it was higher for $CaCl_2$ control-treated cherries at the FR stage (Fig. 6(b)). *PaWS* showed the largest variation among treatments. *PaWS* gene expression increased in the untreated control and SA + $CaCl_2$ treatments while it decreased in the ABA + $CaCl_2$ and GB + $CaCl_2$ treatments when comparing the FC with the FR stage. At the FC stage, *PaWS* gene expression was highest for the ABA + $CaCl_2$ and GB + $CaCl_2$ treatments, while at the FR stage it was lowest for the GB + $CaCl_2$, ABA + $CaCl_2$, and AN + $CaCl_2$ treatments (Fig. 6(c)).

DISCUSSION

Reduced cracking incidence by combining $CaCl_2$ with ABA or GB

Foliar application of $CaCl_2$ is common practice in cherry orchards and was found to be effective in reducing the incidence of cracking.[13,15] Reduced cracking incidence (Fig. 2(b)) was also observed in our experiment, with ABA + $CaCl_2$-treated fruits showing the least incidence of cracking (Fig. 2(b)). This indicates that spraying of ABA in combination with $CaCl_2$ has an added benefit to reduce cracking compared to spraying with $CaCl_2$ alone. Reduced cracking incidence for ABA was found by Balbontín *et al.*[23] for 'Bing' cherry fruits. In the past, agricultural use of ABA was limited by high production costs.[58] Pyrabactin, a low-cost synthetic analogue of ABA, has been shown to activate the ABA pathway.[59] This indicates that application of ABA analogues, preferably in combination with $CaCl_2$, might be a promising strategy to limit cherry fruit cracking. Another promising strategy to limit cherry cracking is to combine $CaCl_2$ with GB. This combination reduced cherry cracking almost to the same degree as $CaCl_2$ in combination with ABA (Fig. 2(b)). Interestingly, the largest diameter was observed

Table 1. Histological parameters of 'Sweetheart' fruits at both development stages (DS – FC: fruit color change and FR: fruit ripening) as a function of spray treatments

Development stage (DS)	Treatment (T)	Cuticle thickness (μm)	Epidermal cell wall thickness (μm)	Epidermal cell thickness (μm)	Epidermal cell area (μm^2)	Hypodermal Cell thickness (μm)	Hypodermal cell area (μm^2)	Parenchymal cell area (μm^2)
FC	untreated control	2.5 ± 0.1 a	3.2 ± 0.1 a	29.6 ± 1.2 bcd	750 ± 45 ab	27.5 ± 1.6 b	1104 ± 97 ab	2685 ± 163 a
	$CaCl_2$ control	3.8 ± 0.1 c	4.9 ± 0.1 b	27.0 ± 0.9 abc	867 ± 42 bc	27.7 ± 1.2 b	1128 ± 79 ab	2790 ± 164 a
	AN + $CaCl_2$	4.0 ± 0.1 cd	4.7 ± 0.2 b	29.9 ± 0.9 cd	846 ± 34 bc	24.9 ± 1.0 ab	867 ± 42 a	2721 ± 171 a
	GA_3 + $CaCl_2$	3.3 ± 0.1 b	3.5 ± 0.2 a	26.2 ± 0.8 a	742 ± 37 ab	28.1 ± 1.6 b	1034 ± 86 ab	2722 ± 191 a
	ABA + $CaCl_2$	5.6 ± 0.2 f	5.0 ± 0.1 b	26.7 ± 0.6 ab	713 ± 38 ab	23.2 ± 1.2 a	871 ± 81 a	2654 ± 211 a
	SA + $CaCl_2$	4.3 ± 0.1 d	6.1 ± 0.2 d	31.9 ± 1.4 d	910 ± 52 c	24.4 ± 1.2 ab	944 ± 68 a	2461 ± 177 a
	GB + $CaCl_2$	4.8 ± 0.1 e	5.8 ± 0.2 c	26.2 ± 0.6 a	659 ± 37 a	28.6 ± 1.3 b	1244 ± 108 b	3486 ± 243 b
	P (T)	***	***	***	**	*	*	*
FR	untreated control	3.0 ± 0.1 a	3.2 ± 0.1 a	23.4 ± 0.4 a	610 ± 18 a	23.2 ± 1.5 a	1035 ± 92 a	2520 ± 151 a
	$CaCl_2$ control	3.7 ± 0.1 b	3.4 ± 0.2 a	26.0 ± 0.6 ab	855 ± 40 b	27.6 ± 1.6 abc	1352 ± 122 abc	2928 ± 189 ab
	AN + $CaCl_2$	3.5 ± 0.1 b	5.6 ± 0.2 c	32.1 ± 0.9 c	1105 ± 67 d	32.8 ± 1.4 d	1411 ± 86 bcd	3564 ± 232 bc
	GA_3 + $CaCl_2$	3.4 ± 0.1 b	4.2 ± 0.2 b	28.0 ± 0.6 b	954 ± 55 bc	24.4 ± 1.0 ab	1206 ± 78 abc	2993 ± 180 ab
	ABA + $CaCl_2$	5.2 ± 0.1 d	5.3 ± 0.2 c	25.6 ± 0.7 ab	934 ± 44 bc	29.1 ± 2.8 bcd	1499 ± 171 cd	3709 ± 265 c
	SA + $CaCl_2$	4.3 ± 0.1 c	5.7 ± 0.2 c	25.2 ± 1.2 ab	837 ± 52 b	25.7 ± 1.0 ab	1120 ± 75 ab	3439 ± 324 bc
	GB + $CaCl_2$	4.9 ± 0.1 d	5.6 ± 0.1 c	32.2 ± 0.7 c	1059 ± 45 cd	31.3 ± 1.6 cd	1797 ± 166 d	3446 ± 266 bc
	P (T)	***	***	***	***	***	***	**
	P (DS)	*ns*	*ns*	*ns*	***	*ns*	***	***
	P (T)	***	***	***	***	**	***	*
	*P (DS*T)*	***	***	***	***	***	**	**

*$P < 0.05$ by Duncan's test.
**$P < 0.01$ by Duncan's test.
***$P < 0.001$ by Duncan's test.
Values are means ± standard error ($n = 36$). Means flanked by the same letter are not significantly different at $P < 0.05$ (Duncan's test).
Abbreviation: *ns*, not significant.

for GB + $CaCl_2$-treated fruits (Fig. 2(a)), which also had less CI (Fig. 2(b)). In fact, a negative correlation ($R = -0.65$, $P < 0.01$) was found between fruit size and CI. In general, a positive correlation was found between size and CI for several cultivars.[60] The application of Ca has been linked with reduced cracking but also lower fruit size.[12] The higher fruit diameter for GB + $CaCl_2$-treated fruits therefore seems to be related to the effect of GB. Although confirmation of the added benefit of adding GB to $CaCl_2$ sprays needs be to gathered for other cherry cultivars, foliar application of GB + $CaCl_2$ might be an attractive (and affordable) option to limit the incidence of cherry cracking.

Incidence of cheek cracks decreased for $CaCl_2$ and ABA + $CaCl_2$-treated fruits

Cheek region (CR) cracks, the type of cracks that negatively affect consumer acceptance were mostly[9] correlated ($R = 0.50$, $P < 0.001$) with CI. The CR cracks decreased most in fruits treated with $CaCl_2$ and ABA + $CaCl_2$ and to a lesser extent in GA_3 + $CaCl_2$- and GB + $CaCl_2$-treated fruits (Fig. 2(b)). Although fruits treated with $CaCl_2$, GA_3 + $CaCl_2$, ABA + $CaCl_2$ and GB + $CaCl_2$ showed numerous SSR and SCR cracks, no disruptions of the exocarp were observed (Fig. 3). In ABA + $CaCl_2$-treated fruits, cuticular material filled intercellular spaces of the epidermis and hypodermis (Fig. 4 (c)), which may contribute to the cuticle thickness (Table 1) and in that way may help reduce CR cracks in these fruits (Fig. 2(c)).

Cracking incidence is affected by wax content, and cuticle and epidermal thickness

Calcium chloride control fruits are characterized by increased cuticle thickness at both development stages compared with the untreated control (Table 1). ABA + $CaCl_2$ and GB + $CaCl_2$-treated fruits showed lower CI than $CaCl_2$ control fruits (Fig. 2(b)). It is likely that the higher cuticle thickness of ABA + $CaCl_2$ and GB + $CaCl_2$ compared with the $CaCl_2$ control fruits (Table 1) limited CI. In fact, a correlation coefficient (R) of −0.85 ($P < 0.001$) and − 0.72 ($P < 0.001$) was found between CI and cuticle thickness at the FC and FR stage, respectively. Our findings fit with those of Demirsoy and Demirsoy,[61] who found a negative correlation between cuticle thickness and fruit cracking in eight sweet cherry cultivars.

Thickness of the cuticle was correlated with soluble cuticular wax content at the FC ($R = 0.48$, $P < 0.05$) and at the FR stage ($R = 0.51$, $P < 0.05$) (Table 1 and Fig. 5). However, no correlation was found between cracking incidence and soluble cuticular wax content. For instance, $CaCl_2$ control fruits, characterized by a low cracking incidence, did not show higher soluble wax content compared with the untreated control (Fig. 5). However, the

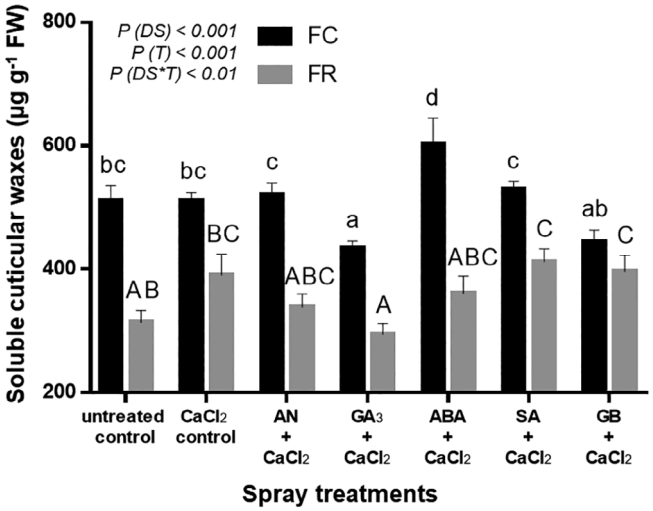

Figure 5. Soluble cuticular waxes content of 'Sweetheart' fruits at the fruit color change (FC) and the fruit ripening stager (FR) as function of spray treatments (T) with DS indicating development stage. Data are expressed as the mean ± SE ($n = 9$). Different lowercase letters indicate significant differences ($P < 0.05$) among treatments at FC and different uppercase letters indicate significant differences ($P < 0.05$) among treatments at FR stage using Duncan's test.

lower cracking incidence of ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits might be due to increased soluble wax content, either at the FC stage (ABA) or the FR stage (GB) (Fig. 5).

Epidermal cell-wall thickness might be related to cracking incidence as $CaCl_2$ (FC stage), ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits had higher epidermal cell-wall thickness compared with the untreated control (Table 1). However, SA + $CaCl_2$-treated fruits showed the highest epidermal cell-wall thickness but did not show lower CI (Fig. 2(b)). Brüggenwirth *et al.*[62] mentioned that the cuticle is the penetration barrier, but the epidermis and hypodermis layers represent the structural backbone in a cherry skin, which might contribute to cracking resistance. In fact, most histological parameters showed negative correlations with CI. At the FC stage, a negative correlation was found between CI and thickness of epidermal cell wall ($R = -0.55$, $P < 0.05$) (Fig. 2(b), Table 1). The thickness of the epidermal cell wall ($R = -0.65$, $P < 0.01$), epidermis cell area ($R = -0.59$, $P < 0.01$), hypodermis cell area ($R = -0.52$, $P < 0.05$) and parenchyma cell area ($R = -0.58$, $P < 0.01$) showed negative correlations with CI at the FR stage (Table 1). These findings suggest that, at an early stage of fruit development (FC), it is mostly the thickness of cuticle that plays an important role limiting CI, as only one correlation was found with histological parameters. At the FR stage, larger epidermis, hypodermis, and parenchyma cells seem to lower CI. This agrees with Kertesz and Nebel,[43] who mentioned that cultivars more tolerant to cracking have larger hypodermis cell size. Our findings contrast with those of Yamaguchi *et al.*,[1] who claimed that larger hypodermis cells were found in CI susceptible cultivars. Our data support the idea that cuticle- and epidermal thickness, soluble wax content, and cell size are related to CI, but that complex interactions exist that need to be elucidated in further research.

Cracking resistance is related to increased cuticular wax synthesis and expansin activity during the fruit coloring stage

Higher *PaEXP1* expression was observed in GB + $CaCl_2$-treated fruits at the FC stage (Fig. 6(a)). This might indicate that

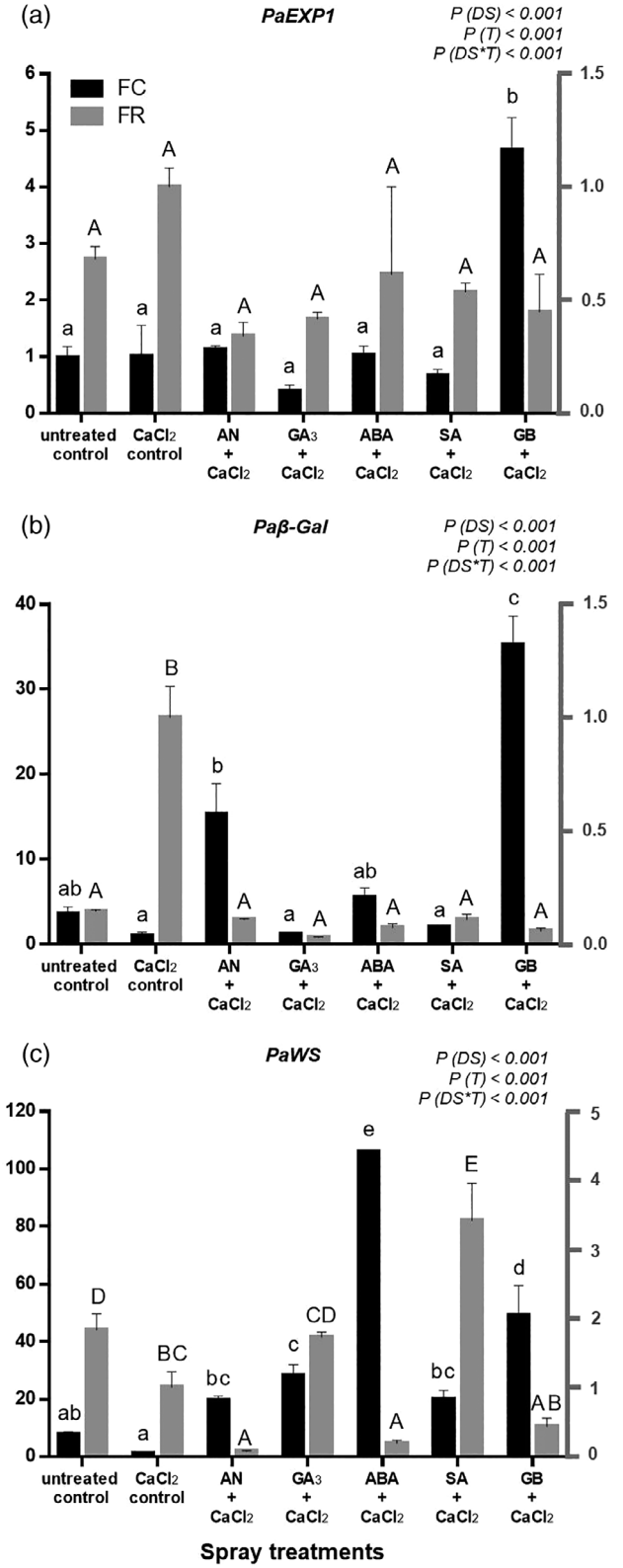

Figure 6. (a) Normalized relative expression of *PaEXP1* for 'Sweetheart' fruits at the fruit color-change (FC) and fruit ripening stage (FR) as function of spray treatments (T) with DS indicating development stage. (b) Normalized relative expression of *Paβ-Gal*. (c) Normalized relative expression of *PaWS*. Data are expressed as the mean ± SE of three replicates expressed in arbitrary units. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments at FC and different uppercase letters indicate significant differences ($P < 0.05$) among treatments at FR stage using Duncan's test.

GB + $CaCl_2$-treated fruits at the FC stage have greater cell-wall elasticity, which might explain the lower CI (Fig. 2(b)). Balbontín *et al.*[6] showed higher *PaEXP1* expression for a cracking-resistant cultivar compared to a susceptible cultivar but this was encountered at the FR stage, not the FC stage. Brüggenwirth and Knoche[63] showed less cracking susceptibility for 'Regina' cherries due to a higher elasticity value, implying a stiffer skin and higher pressure when fractured, than 'Burlat' cherries.

The CI was correlated with *PaWS* expression at the FC stage ($R = -0.64$, $P < 0.01$). Increased expression of *PaWS* in combination with increased soluble wax content for ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits at the FC stage (Fig. 5, Fig. 6(c)) and lower CI (Fig. 2(b)) all point towards the importance of the wax metabolism with regard to conferring cracking resistance. It appears that wax biosynthesis is especially important during the FC stage as the higher *PaWS* expression for SA + $CaCl_2$-treated fruits at the FR stage (Fig. 6(c)) did not result in lower CI (Fig. 2(b)). Balbontín *et al.*[6] showed a higher *PaWS* expression in the FC stage for the cracking resistant cultivar compared to a susceptible cultivar, which supports our results.

Higher levels of *Paβ-Gal* transcripts were found in fruits treated with AN + $CaCl_2$ and GB + $CaCl_2$ at the FC stage and for $CaCl_2$ treated fruits at the FR stage (Fig. 6(b)). However, AN + $CaCl_2$-treated fruit were not characterized by higher CI (Fig. 2(b)) and higher expression of *Paβ-Gal* was also not found for ABA + $CaCl_2$-treated fruits. which showed the lowest CI (Fig. 2(b)). This casts doubt about the role of *Paβ-Gal* conferring cracking tolerance.

The role of ABA-inducing genes related to biosynthesis and transport of wax was described earlier.[24–26] The contribution of GB inducing *PaEXP1* and *PaWS* expression at the FC stage, which might result in higher elasticity and higher wax content, is, however, new. We examined the expression of just a few CI-related genes. No doubt genes involved in cuticle deposition, cell-wall metabolism during fruit growth, maturation, softening, and sugar transport might also play an important role in conferring cracking resistance in the developing sweet cherry exocarp.[41]

CONCLUSIONS

All spray treatments reduced the incidence of cracking in comparison with the untreated controls, with the least cracking observed for ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits, including fewer cheek cracks. Foliar application of ABA and GB in association with $CaCl_2$ is therefore likely to be a promising cherry-cracking mitigation strategy. Higher wax content, cuticle and epidermal thickness at both development stages was related to higher cracking tolerance in ABA + $CaCl_2$- and GB + $CaCl_2$-treated fruits. Higher cracking tolerance due to ABA and GB spray treatments is likely due to increased cuticular wax synthesis (ABA + $CaCl_2$) and increased flexibility and elasticity of the epidermis (GB + $CaCl_2$). For GB + $CaCl_2$-treated fruits, this likely resulted in fruits with a larger diameter. At the fruit-ripening stage, the larger cell size of the epidermis, hypodermis and parenchyma cells lowers the incidence of cracking, indicating the importance of the flexibility and elasticity of the epidermis.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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