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Cell wall swelling, fracture mode, and the mechanical properties of cherry fruit skins are closely related

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Abstract

Main conclusion **Cell wall swelling, fracture mode (along the middle lamellae vs. across cell walls), stiffness, and pressure at fracture of the sweet cherry fruit skin are closely related.**

Skin cracking is a common phenomenon in many crops bearing fleshy fruit. The objectives were to investigate relationships between the mode of fracture, the extent of cell wall swelling, and the mechanical properties of the fruit skin using sweet cherry (*Prunus avium*) as a model. Cracking was induced by incubating whole fruit in deionised water or by fracturing exocarp segments (ESs) in biaxial tensile tests. The fracture mode of epidermal cells was investigated by light microscopy. In biaxial tensile tests, the anticlinal cell walls of the ES fractured predominantly *across* the cell walls (rather than *along*) and showed no cell wall swelling. In contrast, fruit incubated in water fractured predominantly *along* the anticlinal epidermal cell walls and the cell walls were swollen. Swelling of cell walls also occurred when ESs were incubated in malic acid, in hypertonic solutions of sucrose, or in water. Compared to the untreated controls, these treatments resulted in more frequent fractures *along* the cell walls, lower pressures at fracture (p_{fracture}), and lower moduli of elasticity (E, i.e., less stiff). Conversely, compared to the untreated controls, incubating the ES in $CaCl_2$ and in high concentrations of ethanol resulted in thinner cell walls, in less frequent fractures *along* the cell walls, higher E and p_{fracture}. Our study demonstrates that fracture mode, stiffness, and pressure at fracture are closely related to cell wall swelling. A number of other factors, including cultivar, ripening stage, turgor, $CaCl_2$, and malic acid, exert their effects only indirectly, i.e., by affecting cell wall swelling.

Keywords Biaxial tensile test · Cracking · Epidermis · Modulus of elasticity · *Prunus avium*

Abbreviation

ES	Exocarp segments
E	Modulus of elasticity
p_{fracture}	Pressure at fracture
$\varepsilon_{\text{fracture}}$	Strain at fracture

Introduction

Rain cracking is a common phenomenon in a large number of fruit crops—especially of species that bear soft, fleshy fruit. Sweet cherry (*Prunus avium*) is a prominent example of this, where cracking limits the production in all areas which experience rainfall during the fruit-ripening season (Christensen 1996). The close relationship between rainfall and fruit cracking has long been recognised (von Wetzhausen 1819). This correlation led to the plausible hypothesis that cracking is caused by excessive water uptake by the fruit. The most popular explanation of rain cracking is based on the simple idea that rainwater is taken up into the fruit by osmosis. This causes the fruit's volume to increase and thus its skin to be stretched (Considine and Kriedemann 1972; Considine and Brown 1981). Then, so the explanation goes, when the skin is stretched beyond some upper limit of extensibility, it tears open.

Today, some 30 or 40 years later, surprisingly little further information is available on the mechanism of fruit

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skin failure. This is despite the enormous economic importance of rain cracking to the cherry industry and also to a large number of other fruit-growing industries producing rain cracking susceptible fruits, including grapes (*Vitis vinifera*) and tomatoes (*Solanum lycopersicum*).

In sweet cherry, the epidermis and underlying hypodermal cell layers represent the load-bearing structure of the skin (Brüggenwirth et al. 2014; Brüggenwirth and Knoche 2016a, b, c). In principle, fracture of these layers may occur in one or the other of two distinct modes: (1) separation of adjacent cells *along* the middle lamellae (fracture is *along* cell walls, fracture mode *along*) or (2) by rupture of cells walls (fracture is *across* the cell walls, fracture mode *across*; Niklas 1992). The first of these fractures *along* cell walls is likely indicative of pectin degradation in the middle lamellae, possibly as a result of softening during ripening, while the cellulose and matrix glucans of the cell walls are not necessarily affected (Brummell 2006). In the second mode, if the strength of cell to cell adhesion exceeds the strength of the cell walls themselves, then fracture will likely occur *across* the cell walls.

Softening is a characteristic of the fruit-ripening process, and increased activity of pectinases has been reported in sweet cherry fruit as early as 30 days after full bloom (Barrett and Gonzalez 1994; Kondo and Danjo 2001). Also, fruit cell walls often swell during ripening, and swelling is accompanied by fruit softening (Redgwell et al. 1997a). Swelling of cell walls also occurs in sweet cherry fruit when epidermal cells plasmolyse and turgor decreases (Grimm and Knoche 2015). Furthermore, swelling of cell walls (isolated) and cracking susceptibility have both been reported to increase during the course of fruit development (Christensen 1996; Redgwell et al. 1997a). However, to our knowledge, relationships between cell wall swelling, fracture mode and the mechanical properties of the fruit skin have not been investigated. That fracture mode and fruit firmness of parenchyma tissue may be related has been demonstrated in pear (De Belie et al. 2000). Thus, when firmness is high, cells rupture *across* cell walls, but when firmness is low they rupture *along* the middle lamellae (De Belie et al. 2000). Also, strong cell to cell adhesion results in fracture *across* cell walls in apple flesh, whereas the cells separate *along* the middle lamellae when cell to cell adhesion is weak (Ng et al. 2013).

Using sweet cherry as a model, the objectives of this study were to investigate whether there are relations between: (1) the mode of fracture of the fruit skin, (2) the swelling of the cell walls, and (3) the mechanical properties of the fruit skin. We employ a biaxial tensile test first described by Bargel et al. (2004) to quantify the mechanical properties of the fruit skin.

Materials and methods

Plant material

Fruit of seven cultivars of sweet cherry 'Adriana', 'Burlat', 'Early Korvic', 'Hedelfinger', 'Merchant', 'Regina', 'Sam', and 'Samba' and one cultivar of sour cherry (*Prunus cerasus*) 'Morellenfeuer' were obtained from field-grown or glasshouse-grown trees at the Horticultural Research Station of the Leibniz University in Ruthe (lat. 52°14′N, long. 9°49′E). Sweet cherry cultivars were grafted on Gisela 5 rootstocks (*Prunus cerasus* × *P. canescens*), the sour cherry on 'Maxma Delbard' rootstock (*P. avium* × *P. mahaleb*). Fruit used in the experiments were free from visual defects, uniform in size, and processed on the day of sampling. Unless otherwise stated, fruit were used at commercial maturity based on size and colour.

Immersion assay

Fruit was induced to crack by incubation in deionised water (Christensen 1996). Two samples of 25 fruit each were prepared as follows. The receptacle, stem/fruit junction and the cut pedicel end were sealed with silicone rubber (3140 RTV Coating; Dow Corning, Midland, MI, USA). After curing for at least 2 h, fruit were incubated in deionised water. At regular intervals, fruits were removed from the water and individually inspected for macroscopic cracks.

Elastometer

Biaxial tensile tests were carried out on exocarp segments (ESs) using an elastometer and the procedure described by Brüggenwirth et al. (2014). Briefly, a brass washer (12 mm i.d.) was mounted on one of the two shoulders of the fruit using a cyanoacrylate adhesive (Loctite 406; Henkel/Loctite Deutschland, Munich, Germany). After curing, an ES was excised by cutting underneath the washer using a razor blade. This technique produced an ES with a maximum thickness of 2.4 ± 0.02 mm where the in vivo strain of the skin was maintained (Knoche and Peschel 2006; Brüggenwirth et al. 2014). The ES comprised cuticle, epidermis, hypodermis, and some adhering mesocarp (flesh) tissue. The ES was then mounted on the elastometer with the cuticle facing outwards. The inner (cut) surface was in contact with silicone oil (Wacker AK10, Wacker Chemie, Munich, Germany). Pressure was applied to the inner side of the ES by displacing the silicone oil using a motorised piston. This caused the ES to bulge outwards. The oil pressure and the extent of bulging were measured using a pressure transducer (Type 40PC100G, Honeywell International, Morristown, NY, USA) and a displacement

transducer (KAP-S/5N; AST Angewandte System Technik, Wolnzach, Germany). Pressure was increased up to the point of $p_{fracture}$ (kPa). The strain (ε) was calculated from the change in surface area (ΔA in mm^2) relative to the initial surface area of the bulging ES (A_0 in mm^2) and is given in Eq. 1.

$$\varepsilon = \frac{\Delta A}{A_0} \quad (1)$$

The strain at $p_{fracture}$ is referred to as the fracture strain ($\varepsilon_{fracture}$; $mm^2\ mm^{-2}$). The E (MPa) was estimated from the height of the bulging ES (h; mm), the radius of the orifice (r; mm), the pressure (p; MPa), and the thickness of the load-bearing layer ($t = 0.1$ mm) in Eq. 2 (Brüggenwirth et al. 2014).

$$E = \frac{p \times r^2 \times (r^2 + h^2)}{h^3 \times t \times 2} \quad (2)$$

Experiments were conducted at 22 °C.

Determining fracture mode and cell wall thickness

Fracture modes were quantified in cracked fruit following immersion assays and also in fractured skins following biaxial tensile tests. Skin samples were prepared for microscopy by removing tissue from one side of a crack using a sharp razor blade. Because of the uniform curvature, skin was excised from one of the two shoulders of the fruit. The skin segments were mounted on a microscope slide using silicone oil (Wacker AK10; Wacker Chemie). Immersion in oil prevented water uptake and bursting of cells and also evaporative water loss and shrinkage. Samples were viewed at 200× or 250× using a fluorescence microscope (BX-60; Olympus, Hamburg, Germany; Axioplan; Carl Zeiss Microscopy, Jena, Germany). Calibrated digital images were taken (camera: DP73; Olympus). The fracture mode was quantified by counting the number of epidermal cells that fractured *along* their anticlinal cell walls and expressing this as a percentage of the total number of epidermal cells adjacent to the crack (i.e. those fracturing *along* the cell walls + those fracturing *across* the cell walls). For each treatment, one crack per fruit on a total of ten fruit was investigated. This procedure meant that a total of 250–350 epidermal cells were inspected for fracture mode per treatment.

Cell wall thickness was quantified on a separate set of images taken from the same samples at 400× (BX-60; Olympus) or 500× (Axioplan; Carl Zeiss Microscopy). Cell wall thickness was measured at the thinnest point of the cell wall between two adjacent cells using image analysis software (Cell-P; Olympus Soft Imaging Solution, Münster, Germany). Thus, our thickness data represent the thickness of the two primary cell walls including the middle lamellae between two neighbouring cells.

Experiments

Fracture mode, cell wall thickness and mechanical properties were investigated for all sweet cherry cultivars except 'Early Korvic' and for one sour cherry cultivar (see above).

Relationships between the final stage of development, fracture mode, cell wall thickness, and mechanical properties of the skin were studied in 'Regina'. The stage of development was indexed by the change in colour from an immature, inedible pale red (27.7 ± 0.6° Hue, mass 10.2 ± 0.4 g) to an edible, dark-red fruit (11.9 ± 0.9° Hue, mass 10.5 ± 0.3 g). Colour is a good indicator of ripening sweet cherry (Hansen 2011; Brüggenwirth and Knoche 2016a). It is closely related to sugar content, whereas changes in mass or firmness or relationships with time after full bloom are often variable. For the fruit used in our experiments, the relationship between osmolarity and colour was: osmolarity (mmol kg^{-1}) = −18.37 (± 2.0) × hue angle (°) + 1478 (±39.5); $r^2 = 0.80$***. Colour was quantified using the CIE 1976 L^*, a^*, b^* scale (CR-200 chromameter; Minolta, Osaka, Japan), and the hue angle was calculated (McGuire 1992). The fruit skin was induced to crack either by incubating whole fruit in water or by straining the ES samples in the elastometer. Fracture mode and cell wall thickness were quantified as described above.

The effect of turgor on the mechanical properties in biaxial tensile tests, the fracture mode, and the cell wall thickness of ES was studied in 'Regina' and 'Early Korvic'. Turgor was varied by incubating the ES for 14 h in a series of sucrose solutions of increasing osmolarity (0, 1, 2.5, 4, and 5 MPa equivalent to 0, 0.4, 0.8, 1.1, and 1.3 M) and in aqueous ethanol at concentrations of 0, 25, 50, 75, and 100% (v/v). After incubation mechanical properties, fracture mode and cell wall thickness were determined.

The effects of malic acid and $CaCl_2$ on the mechanical properties, fracture mode, and cell wall thickness were studied in 'Regina'. The ESs were incubated for 14 h in 70 mM malic acid or 100 mM $CaCl_2$, removed from the solution, blotted, and then tested in the elastometer. The malic acid concentration is equivalent to that found in sweet cherry (Herrmann 2001). Untreated ES served as controls. Fracture mode and cell wall thickness were determined.

Cell wall thickness was also investigated along cracks induced by incubation of whole fruit in deionised water. Digital images were prepared and cell wall thickness quantified using image analysis in the three zones: ahead of

the crack (zone I), in the zone of the crack tip (zone II), and in the older zone of a crack (zone III).

Data analyses

Data analyses were restricted to those ES that fractured at the centre of the orifice of the elastometer. Any ESs that fractured at the edge were excluded because of possible artefacts caused by the mounting procedure (Brüggenwirth et al. 2014). The data were subjected to analysis of variance (AOV). Percentage data were arcsine transformed before AOV. Mean comparisons were made using Tukey's Studentized range test ($P < 0.05$, packet multcomp 1.2–12, procedure glht, R 2.13.1; R Foundation for Statistical Computing, Vienna, Austria). Regression analyses were carried out with Sigma Plot (version 12.5; Systat Software, San Jose, CA, USA). Significance of the coefficients of determination (r^2) at the 0.05, 0.01, and 0.001 probability levels is indicated by *, **, and ***, respectively. The data are presented as means ± standard errors of means (SE). The number of replicates was ten.

Results

Fruit subjected to the biaxial tensile tests cracked mostly *across* epidermal cell walls. Cell walls were thinner, and only the fractured cells were colourless (Fig. 1a). In contrast, fruits immersed in water cracked predominantly by fracturing *along* their anticlinal cell walls causing two adjacent cells to separate along the intervening middle lamellae. Cell walls were thick, and all cells were colourless, indicating plasma membranes had ruptured and anthocyanin leakage had occurred (Fig. 1b). Averaged across cultivars, the frequency of fracture *along* epidermal cell walls was 2.1-fold higher in the immersion assays (mean 75.0%, range 47.7–89.6%) than in the biaxial tensile tests (mean 35.4%, range 15.5–59.7%; Table 1).

Plotting frequencies of fracture *along* epidermal cell walls of the same species and cultivar but from two consecutive seasons, one against the other, yielded a highly significant 1:1, linear relationship (slope of 1.00 ± 0.01; $r^2 = 0.99$***). This indicates differences in fracture mode among cultivars, and species were highly reproducible between seasons (Fig. 2a). This consistency also held for differences between incubation assays and biaxial tensile tests ($r^2 = 0.77$**; Fig. 2b). Interestingly, the effect of cultivar and that of technique were accounted for by their effects on the thickness of the anticlinal epidermal cell walls. The thicker these cell walls, the more frequently did fracture occur *along* cell walls. This relationship could be described by the linear regression equation: $\text{Fracture}_{\text{along}}$ (%) = 24.9 (±2.1) × Thickness (µm) −51.2 (±9.8), $r^2 = 0.95$*** (Fig. 2c). The sour cherry cultivar 'Morellenfeuer' followed the same relationships as the sweet cherry cultivars (Fig. 2b, c).

Thickness of epidermal cell walls increased during the early stages of ripening (as indexed by the lower hue angle, i.e., darker colour) but then remained constant (Fig. 3a). The fracture mode of the epidermis was again a linear function of cell wall thickness ($r^2 = 0.97$**). Furthermore, during ripening, the stiffness of the fruit skin as indexed by the elastic modulus E ($r^2 = 0.80$*) and the strength as indexed by the value of p_{fracture} ($r^2 = 0.99$***) both decreased, as cell wall thickness increased (Fig. 3b–d). There was no relationship between the value of $\varepsilon_{\text{fracture}}$ and the thickness of the cell wall (Fig. 3d, inset).

Immediately after excision, the cell walls of epidermal cells were thin (Fig. 1c). The cell walls thickened when the cells plasmolysed upon incubation in hypertonic sucrose solution (Fig. 1d). The presence of red anthocyanins in the vacuoles indicated that the plasma membranes remained intact. In contrast, ES incubated in aqueous ethanol had thinner cell walls than those in water (Fig. 1e, f). In both treatments, the ESs were discoloured indicating that plasma membranes were damaged and anthocyanins had leaked out (Fig. 1e, f).

In the biaxial tensile tests with ES from the sucrose treatments, increasing osmolarity of the sucrose solutions increased the percentage of cells that fractured *along* cell walls to 90% in the fully plasmolysed stage (Fig. 4a). The only exception to this was in the water control (0 MPa sucrose), where the cells died and the majority of cells separated *along* their cell walls. In the in vivo condition, in about 57% of cases, the epidermal cells fractured *along* the cell walls. This is similar to the effects of nearly isotonic sucrose (2.8 ± 0.1 MPa; Fig. 4a). Increasing the concentration of ethanol in the incubation solution decreased the percentage of epidermal cells fracturing *along* their cell walls. The relationship was linear and described by the equation: $\text{Fracture}_{\text{along}}$ (%) = −0.82 (± 0.06) × ethanol (%) + 93.5 (±3.2), $r = 0.95$*** (Fig. 4b). Plotting fracture modes determined after incubation in sucrose and ethanol vs cell wall thickness revealed a common, linear and highly significant relationship despite the quite unrelated treatments (Fig. 4c). There was a weak negative relationship between the modulus E and cell wall thickness which was mostly due to the high and variable E at low cell wall thickness (Fig. 4d). The value of p_{fracture} decreased consistently as cell wall thickness increased (Fig. 4e). However, the value of $\varepsilon_{\text{fracture}}$ did not depend on cell wall thickness (Fig. 4f).

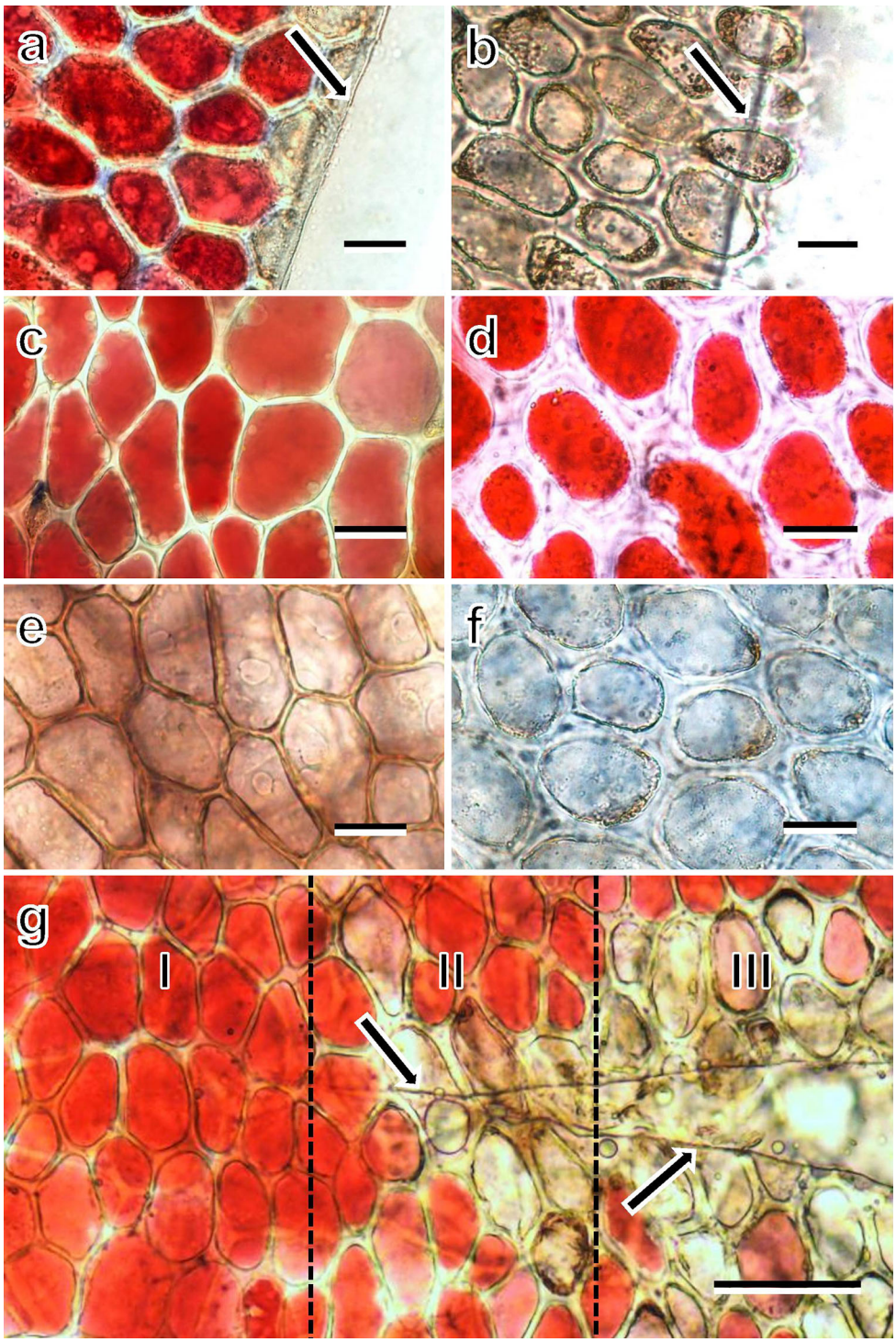


Fig. 1 **a**, **b** Light microscope view of crack in the fruit skin subjected to a biaxial tensile test in the elastometer (**a**) or induced by immersing fruit in deionized water (**b**, **g**). **c**–**f** Changes in thickness of anticlinal epidermal cell walls of sweet cherry immediately after excision (**c**, control), after incubating in hypertonic sucrose solution (**d**), in 75% aqueous ethanol (**e**), and in deionized water (**f**). Cell walls are non-swollen in **a**, **c**, **e**, but swollen in **b**, **d**, **f**. Fruit was incubated for 14 h. **g** Detailed view of crack in fruit skin. Zone I represents an intact area of skin ahead of a crack. Zone II shows the crack tip as indexed by the ruptured cuticle. Zone III demonstrates older region along a crack. Along the crack, cell wall swelling increased and the fraction of dead cells increased. *Arrows* in **a**, **b**, and **g** indicate edge of crack in cuticle. *Bars* 25 µm (**a**–**f**) and 100 µm (**g**)

Incubation in malic acid caused cell walls to swell and resulted in more fractures *along* the cell wall, higher values of $\varepsilon_{\text{fracture}}$, and lower values of E and of p_{fracture} compared to the control. In contrast, incubating ES in $CaCl_2$ resulted in thinner cell walls, less frequent fracture *along* cell walls, and lower value for $\varepsilon_{\text{fracture}}$. Also, the values of both E and p_{fracture} were higher than in the untreated control (Table 2).

Pooling data from all experiments confirmed the linear and highly significant relationships for the epidermal cells between: fracture mode, cell wall thickness, and the mechanical properties E and p_{fracture} (Fig. 5a–c). From the regression equations describing these relationships, the following predictions may be made. For an extrapolated cell wall thickness of about 8 µm, fracture is expected to

Table 1 Fracture mode of anticlinal epidermal cell walls of sweet cherry fruit in immersion and tensile tests as affected by cultivar

Cultivar	$Fracture_{along}$ (%)			
	$Immersion_{test}$	Biaxial $tensile_{test}$	$Mean_{test}$	Ratio
Adriana	47.7 ± 4.4	15.5 ± 2.9	31.6 ± 3.65 c[a]	3.1
Burlat	88.5 ± 3.8	38.3 ± 4.6	63.4 ± 4.2 ab	2.3
Hedelfinger	75.1 ± 5.7	32.0 ± 7.3	53.6 ± 6.5 abc	2.3
Merchant	79.2 ± 4.2	51.2 ± 4.8	65.2 ± 4.5 ab	1.5
Regina	89.6 ± 5.7	59.7 ± 4.2	74.7 ± 5.0 a	1.5
Sam	64.3 ± 4.8	22.7 ± 4.3	43.5 ± 4.6 bc	2.8
Samba	80.4 ± 2.9	28.6 ± 3.2	54.5 ± 3.1 abc	2.8
$Mean_{cultivar}$	75.0 ± 4.5 a	35.4 ± 4.5 b		2.1

Fracture was induced by immersing whole fruit in water ('immersion') or by a biaxial tensile test of the fruit skin using an elastometer. The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*; $n = 10$)

[a] Significant main effects for test and cultivars by two factorial analysis of variance, mean separation by Tukey's Studentized range test at $P < 0.05$

occur exclusively *along* the cell walls. At this point, the values of E and $p_{fracture}$ would be close to zero (Table 3). There was only a weak positive relationship between the value of $\varepsilon_{fracture}$ and cell wall thickness (Fig. 5d).

When fruit cracked during water immersion, the cell walls in the zone ahead of the crack (zone I) were thin, like those in the surrounding epidermis away from the crack. Epidermal cells in zone I were intact as indexed by the presence of large vacuoles containing anthocyanin (Table 4; Fig. 1g). In the zone at the tip of a crack (zone II), the cuticle had ruptured. Immediately below the crack tip, the cell walls began to swell and to separate one from another. Individual epidermal cells also showed discolouration indicating death. In the older zone of a crack (zone III), the crack had split the skin. Cell walls of epidermal cells on either side of crack were very swollen, and nearly all the cells along the crack were dead (Table 4; Fig. 1g).

Discussion

Our study establishes several new findings:

1. The mode of fracture of sweet cherry fruit skin is related to the thickness of the epidermal cell walls which reflects swelling of cell wall and middle lamella in our system;
2. The mechanical properties of the fruit skin depend on cell wall thickness. The closest relationships are obtained for $p_{fracture}$, followed by E which is markedly more variable. The value of $\varepsilon_{fracture}$ is little affected by cell wall thickness. The effects of cultivar, ripening stage, sucrose, ethanol, $CaCl_2$, and malic acid are largely accounted for by their effects on cell wall thickness.
3. Fruit that crack in water had thicker cell walls and more often showed fracture *along* the cell walls, compared to ES fractured in the biaxial tensile test.

Fracture mode, cell wall swelling, and mechanical properties

The mode of fracture clearly depended on the thickness of the epidermal cell walls. Thicker cell walls fractured more frequently *along* the cell wall, and thinner walls more frequently fractured *across* the cell wall. This observation was consistent across all cultivars, ripening stages, and treatments (sucrose, ethanol, $CaCl_2$, or malic acid). Variation in cell wall thickness was caused by different degrees of cell wall swelling (Redgwell et al. 1997a; Grimm and Knoche 2015). Cell wall swelling is a characteristic of the ripening process in soft, fleshy fruits (Hallett et al. 1992; Redgwell et al. 1997a; Brummell 2006) including sweet cherry (Kondo and Danjo 2001; Grimm and Knoche 2015). It is preceded by biochemical modifications of the cell wall during maturation and ripening. Swelling occurs within hours after removal of turgor when the slight pressure of the symplast on the cell wall is released, for example, by plasmolysis or by destroying plasma membrane integrity using ethanol (Figs. 4, 5). This behaviour is indicative of a low swelling pressure relative to cell turgor (Saladié et al. 2007; Schumann et al. 2014; Grimm and Knoche 2015).

The $p_{fracture}$ and—to a lesser extent—the E of the ES are closely related to the thickness of the anticlinal epidermal cell walls (Figs. 4, 5). This is in agreement with our earlier

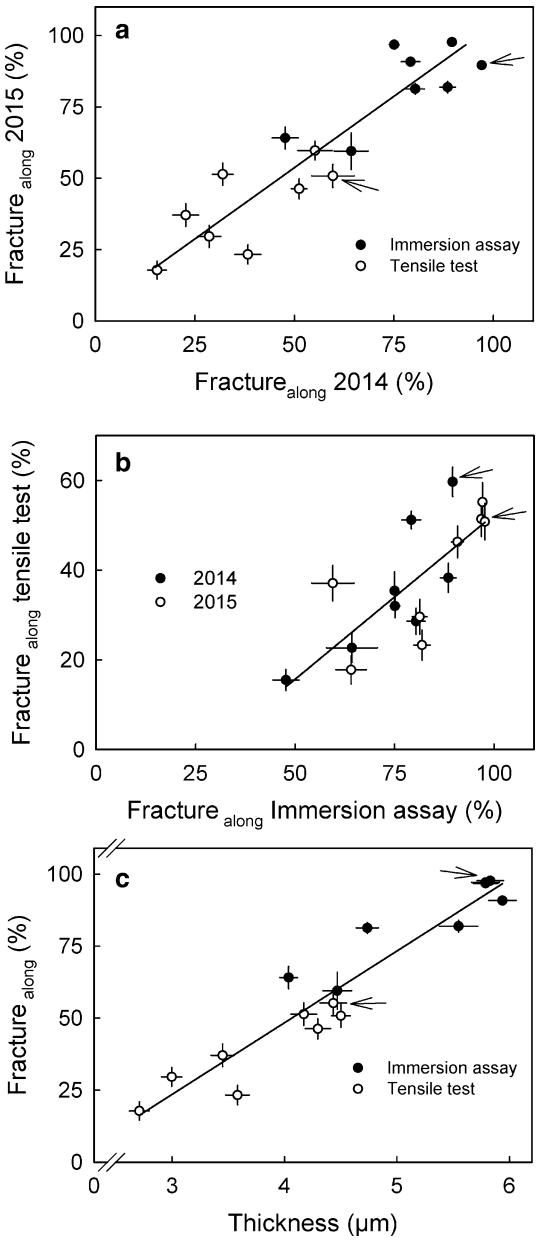


Fig. 2 Effect of cultivar and season on the mode of fracture and thickness of anticlinal epidermal cell walls of sweet and sour cherry fruit. Cracks were induced in immersion assays or in biaxial tensile tests using the elastometer. **a** Reproducibility of fracture mode across cultivars and assays (immersion of whole fruit or biaxial tensile skin test) in two successive seasons. **b** Relationship between fracture mode in biaxial tensile tests and immersion assays. **c** Relationship between the fracture mode and cell wall thickness (data from 2015 only). Data symbols represent mean ± SE for seven sweet cherry and one sour cherry cultivars (indicated by *arrow*). The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*). Values are mean ± SE ($n = 10$)

finding that the epidermis and hypodermis together represent the structural backbone of the sweet cherry skin (Brüggenwirth et al. 2014; Brüggenwirth and Knoche 2016a, b).

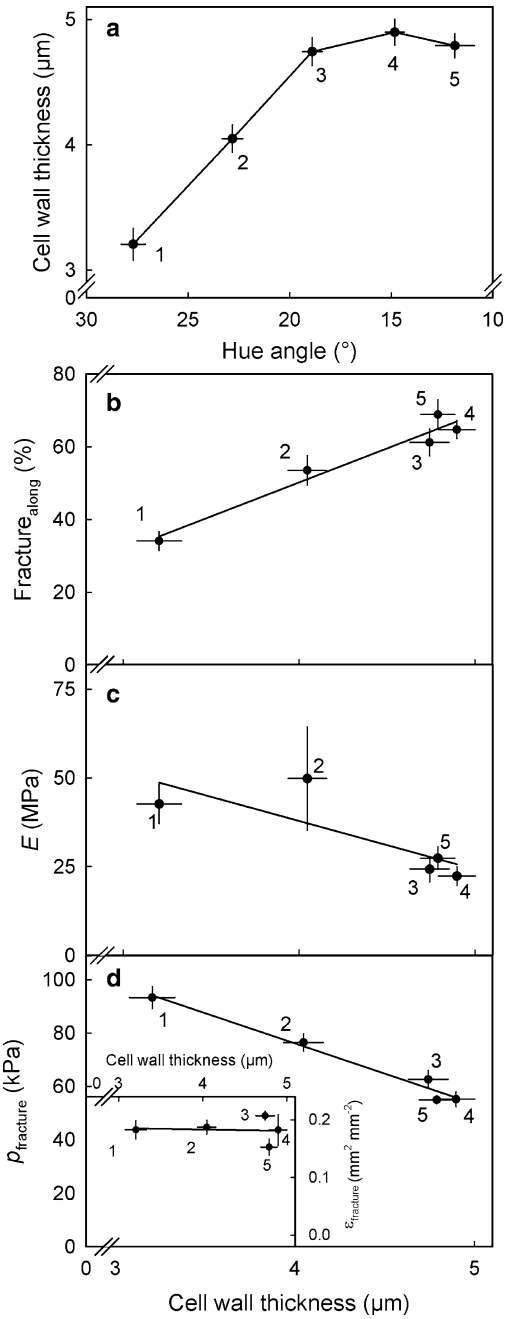


Fig. 3 Relationship between the thickness of anticlinal epidermal cell walls of 'Regina' sweet cherry and the mechanical properties of the skin tested in biaxial tensile tests. Fruits were sampled during the final stage of maturation and ripening. **a** Relationship between cell wall thickness and maturity as indexed by the change in colour. The colour is expressed as the hue angle. High hue angles imply less mature, pale red fruit; low hue angles imply more mature, darker fruit. **b–d** Relationship between the mechanical properties of the fruit skin and cell wall thickness. **b** Fracture mode, **c** modulus of elasticity (E), **d** pressure at fracture (p_{fracture}) and *Inset* strain at fracture ($\varepsilon_{\text{fracture}}$). Numbers next to data symbols refer to the stages of maturation ranging from less mature ('1') to fully mature fruit ('5'). The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*). Values are mean ± SE ($n = 10$)

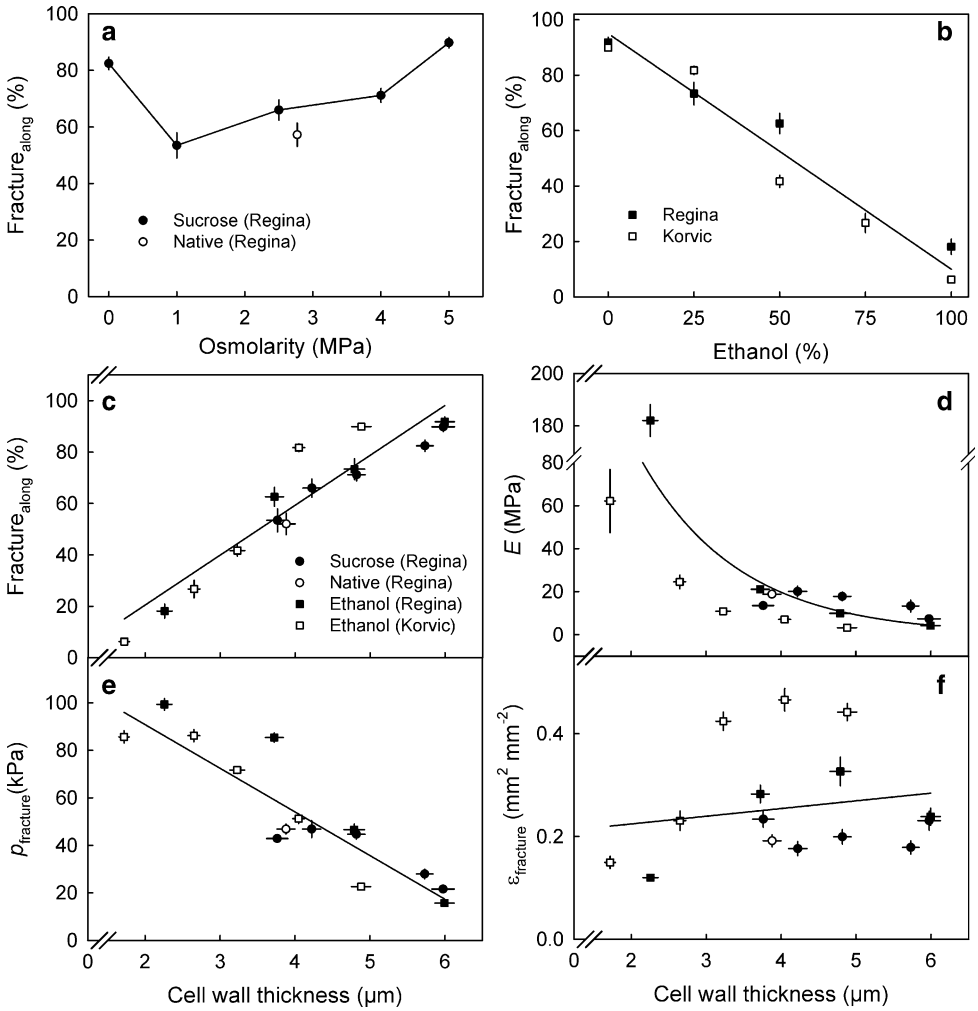


Fig. 4 **a**, **b** Effects of the concentrations of ethanol or sucrose solutions on the mode of fracture of the skin of sweet cherry fruit. **c** Same data as in **a**, **b**, but fracture mode redrawn as a function of the thickness of anticlinal epidermal cell walls. The fracture mode was determined on fruit skin segments subjected to a biaxial tensile test. The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*). Values are mean ± SE ($n = 10$)

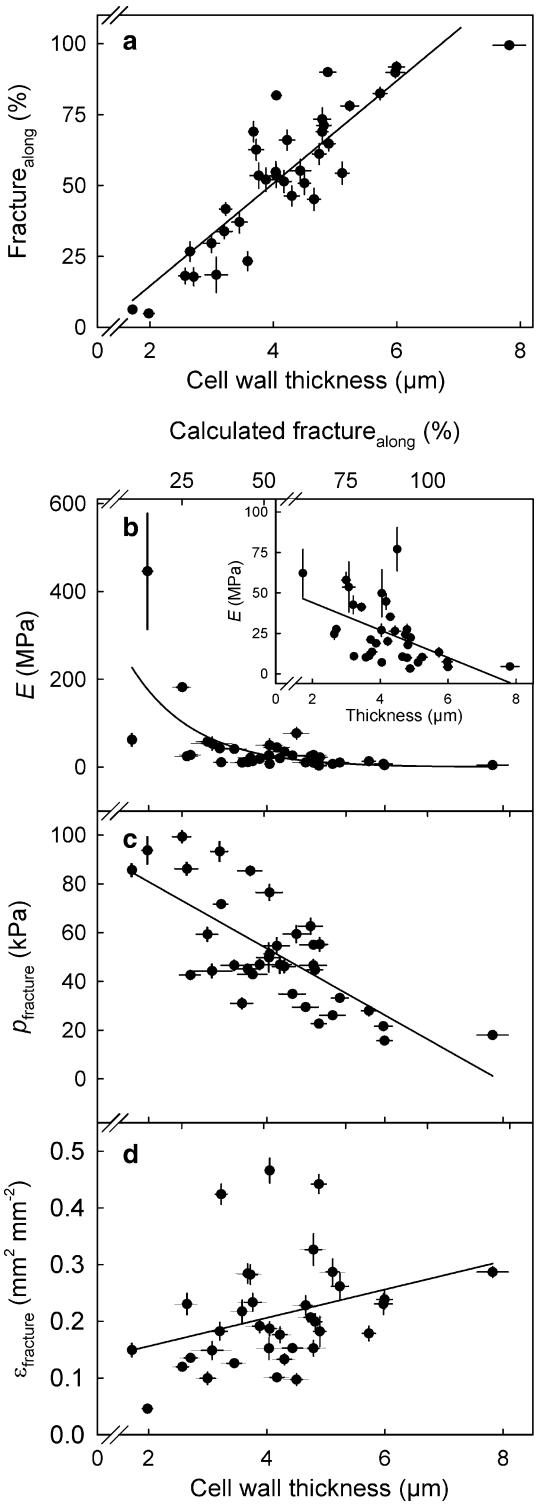
Table 2 Effect of malic acid (70 mM) and $CaCl_2$ (100 mM) on the thickness of anticlinal epidermal cell walls, the fracture mode, the modulus of elasticity (E), the pressure at fracture ($p_{fracture}$), and the strain at fracture ($\varepsilon_{fracture}$) of the sweet cherry fruit skin

Treatment	Thickness (µm)	Fracture$_{along}$ (%)	E (MPa)	$p_{fracture}$ (kPa)	$\varepsilon_{fracture}$ (mm^2 mm^{-2})
Control	5.2 ± 0.1 b[a]	78.0 ± 1.8 b	10.3 ± 1.1 b	33.2 ± 1.8 b	0.26 ± 0.02 a
Malic acid	7.8 ± 0.3 a	99.4 ± 0.4 a	4.5 ± 0.5 c	18.0 ± 1.2 c	0.29 ± 0.01 a
$CaCl_2$	4.0 ± 0.1 c	54.7 ± 3.7 c	27.0 ± 3.9 a	49.8 ± 6.0 a	0.15 ± 0.02 b

The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*; $n = 10$). The control represents the cell wall in the in vivo state

[a] Mean separation within columns by Tukey's Studentized range test, $P < 0.05$

It may be argued that the decrease in $p_{fracture}$ of the fruit skin upon plasmolysis and cell death was due to the removal of turgor rather than to the swelling of cell walls. These effects are difficult to separate in our system because removal of turgor is accompanied by swelling of the cell wall in this and earlier studies (McClendon 1981; Grimm and Knoche 2015). Also, effects of turgor on mechanical properties have often been reported in the literature (Niklas 1992; De Belie et al. 2000; Oey et al. 2007; Knoche et al. 2014; Brüggenwirth and Knoche 2016a). However, when skin sections were incubated in ethanol, the turgor was destroyed, but cell walls remained thin due to a slight dehydration by the ethanol (Fig. 4). The mechanical properties of these skins did not differ from turgescent skins of comparable cell wall thickness indicating that cell wall swelling and not the lack of turgor was the dominating factor. Also, cell turgor in the outer mesocarp of mature sweet cherry is very low (17–64 kPa; Knoche et al. 2014)



◀**Fig. 5** **a** Relationship between the fracture mode and the thickness of anticlinal epidermal cell walls of the skin of sweet cherry. Data points represent pooled data from all experiments conducted. **b–d** Mechanical properties of the skin as affected by the thickness of epidermal cell walls. **b** Modulus of elasticity (*E*). *Inset E* redrawn on a different scale (outlier for 100% ethanol treatment omitted). **c** Pressure at fracture ($p_{fracture}$) and **d** strain at fracture ($\varepsilon_{fracture}$). Because fracture mode and cell wall thickness were linearly related, a fracture mode calculated using the regression equation and line depicted in **a** was included as a second *x*-axis in **b–d**. The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*). Values are mean ± SE ($n = 10$)

relative to the osmotic potential of the fruit (−2500 to −4000 kPa), and the turgor of the epidermis is most likely even lower (Grimm and Knoche 2015). This too makes an effect of turgor unlikely.

The stiffness of the fruit skin as indexed by *E* was also related to cell wall thickness. Compared to $p_{fracture}$, however, variability in *E* was considerably higher, particularly for thinner cell walls (Fig. 5; Table 3). When cell walls are thin (at low water content), the intrinsic properties of the non-swollen polymer dominate and cause significant variability. Variability decreases as swelling progresses, and the effect of cell wall swelling becomes more and more dominant over that of any compositional and/or structural differences in the cell wall under the various treatments.

The above discussion demonstrates that the effects observed in our experiments (i.e. those of cultivar, ripening stage, sucrose, ethanol, $CaCl_2$ and malic acid on the $p_{fracture}$ and on *E*) were largely accounted for by their effects on cell wall swelling. Clearly, some specific effects of individual treatments must have remained as indicated by the larger scatter and decreased r^2 when comparing the relationships depicted in Figs. 4 and 5. However, these treatment-specific effects were minor.

From the regression equations fitted through plots of $p_{fracture}$ vs. mode of fracture as indexed by the frequency of fracture *along cell* walls, some further interesting conclusions may be drawn (Fig. 5; Table 3). The pressure required for 100% failure *along* cell walls is about 20 kPa, whereas that required for 100% fracture *across* cell walls is about four times higher, about 81 kPa. Furthermore, only non-swollen cell walls will fracture *across* cell walls. In swollen walls, it would appear that the pressure required to overcome cell to cell adhesion (i.e. the middle lamella) must fall below the pressure required for fracture *across* cell walls.

Our findings on the striking effect of cell wall swelling on the mechanical properties are consistent with those observed in hydrated onion epidermal cell walls (Ha et al. 1997; Zamil et al. 2015). In onion (*Allium cepa*), as in sweet cherry, increased hydration decreased the modulus of elasticity (Zamil et al. 2015). This effect was related to the hydration and swelling of pectins (Ha et al. 1997; Zamil et al. 2015). The same argument applies with sweet cherry, where fracture mode and cell wall thickness are closely related.

Table 3 Parameter estimates and standard errors (SE) of linear regression equations describing the relationships between the modulus of elasticity (E), pressure at fracture ($p_{fracture}$) and strain at fracture ($\varepsilon_{fracture}$) and the mode of fracture of the sweet cherry fruit skin (Fig. 5b–d)

Dependent variable	Independent variable	Parameter estimates		Coefficient of determination
		Slope	Intercept	
E (MPa)	Cell wall thickness (μm)	−8.5 ± 2.5	61.1 ± 10.9	0.52**[a]
	$Fracture_{along}$ (%)	−1.6 ± 0.4	127.5 ± 26.4	0.52***
$p_{fracture}$ (kPa)	Cell wall thickness (μm)	13.7 ± 2.2	108.2 ± 9.5	0.73***
	$Fracture_{along}$ (%)	−0.6 ± 0.1	81.9 ± 7.2	0.62***
$\varepsilon_{fracture}$ (mm^2 mm^{-2})	Cell wall thickness (μm)	0.025 ± 0.013	0.11 ± 0.06	0.32
	$Fracture_{along}$ (%)	0.002 ± 0.001	0.10 ± 0.03	0.54**

The fracture mode was quantified by expressing the number of epidermal cells that fractured *along* their anticlinal cell walls as a percentage of the total number of epidermal cells fractured (i.e. *along* + *across*; $n = 28$)

[a] Significance of coefficient of determination at $P < 0.05$, 0.01, and 0.001 indicated by *, **, and ***, respectively

Table 4 Cell wall thickness of different zones of a crack (see Fig. 1g) in the fruit skin induced by immersing fruit in deionized water ($n = 10$)

Position	Zone	Cell wall thickness (μm)
Ahead of crack (intact)	I	3.6 ± 0.1 c[a]
Crack tip with cuticle rupture	II	5.3 ± 0.2 b
Cell wall rupture with separation of cells	III	6.1 ± 0.1 a

[a] Mean separation by Tukey's Studentized range test, $P < 0.05$

Hypothetical mechanism of failure

The physicochemical process of the absorption of water in the cell wall and/or middle lamellae that led to swelling must have been preceded by biochemical changes in the wall and/or the middle lamellae. The consequence of these changes is a higher frequency of cells fracturing along cell walls at reduced pressure. Separation along cell walls is indicative for reduced cell to cell adhesion. Mechanistically, cell to cell adhesion is realised by (1) calcium ion crosslinking of low-esterified homogalacturonan (HG), (2) alkali-labile covalent ester bonds, and (3) alkali-resistant covalent or intermolecular linkage (Jarvis et al. 2003; Zamil et al. 2014).

It is well established that marked changes occur in the pectin fraction of ripening sweet cherry fruit (Batisse et al. 1996; Basanta et al. 2013; Salato et al. 2013). Cell adhesion decreases during ripening due to enzymatic and non-enzymatic processes, resulting in the disassembly of the middle lamella and the solubilisation of pectins (Hallett et al. 1992; Redgwell et al. 1997a; Brummell 2006). Enzymatic processes contributing to the solubilisation of pectins include the cleavage of homogalacturonans by polygalacturonases (Jarvis et al. 2003), the demethylesterification of homogalacturonans by polygalacturonases and pectin methylesterases (Roy et al. 1992; Tieman and Handa 1994), and the removal of neutral side chains that anchor pectins to matrix glycans and cellulose in the primary cell wall (Paniagua et al. 2014; Redgwell et al. 1997b). The effect of malic acid on decreasing cell adhesion may be accounted for by increased activity of polygalacturonases caused by a decrease in the extracellular pH (Chun and Huber 1998). Non-enzymatic mechanisms of pectin degradation stimulated by acids include breakdown due to reactive oxygen species (ROS), produced by acids such as ascorbate, causing the cleavage of neutral galactose residues (Dumville and Fry 2003) and removal and complexation of cell wall bound Ca^{2+}, resulting in the loosening of the crosslinks (Glenn and Poovaiah 1989; Tibbits et al. 1998; Brummell 2006). Accordingly, the effect of Ca^{2+} would be attributable to increased crosslinking, resulting in stronger cell walls (Jarvis 1984; Roy et al. 1994).

Swelling may occur due to (1) a deesterification of pectins in the primary cell wall and the middle lamella which increases the charge density, causing electric repulsion and hydration (Jarvis 1991) and/or (2) the filling of intermicrofibrillar spaces in the primary cell wall that were previously filled with pectins (Redgwell et al. 1997a). In addition, osmotic processes due to increased osmolarity in the apoplast (Wada et al. 2008, 2009) may be visualised that cause water to partition into the cell wall. Pectin gels are capable of large changes in hydration and stiffness. This alters the mechanical behaviour of tissues which would also be consistent with our observations on the sweet cherry skin (Levesque-Tremblay et al. 2015). Clearly, the above arguments are speculative and alternate

mechanisms cannot be excluded. However, at present the interrelationship of cell wall swelling, fracture pressure, and mode of fracture makes failure of a middle lamella, which is weakened by ripening processes and cell wall swelling, the most likely explanation. Further investigations are needed on the interaction of cell wall constituents, i.e., the pectins in the middle lamellae, and water.

Implications for fruit cracking

Fracture surfaces of fruit that cracked in an immersion assay due to water uptake differed from those that cracked from strain in the biaxial tensile test. First, cell walls were thinner in the biaxial tensile tests, compared to in the immersion assays (Fig. 1). Second, in the tensile test, fracture occurred more frequently *across*, rather than *along* the cell walls. Third, cell walls below the surface of an intact fruit skin were thinner immediately after excision than cell walls located along cracks induced by incubating fruit in water (Fig. 1g). In addition, cracking occurs at lower strain values in immersion assays where fruit is incubated in water compared to in biaxial tensile tests (Brüggenwirth and Knoche 2016b). These observations suggest that (1) the two fracture processes differ between fruit cracking in an immersion assay and skin rupture in tensile tests and (2) cell wall swelling in fruit incubated in water is an early, critical, and essential step in a chain reaction of events that ultimately leads to cracking.

Based on these observations and literature data, the following scenario may be hypothesized. First, microcracks in the cuticle result from a lack of synchronisation of fruit growth and cuticle deposition (Knoche et al. 2004; Peschel and Knoche 2005), coupled with exposure of the strained cuticle to surface wetness (Knoche and Peschel 2006). Microcracks impair the cuticle's barrier function (Kerstiens 1996) and so focus water uptake in the region immediately underlying the microcrack. This causes individual cells just below a microcrack to burst, thereby releasing cell contents into the apoplast (Simon 1977; Glenn and Poovaiah 1989). Besides glucose, fructose, and anthocyanins, a number of acids including malic, citric, and ascorbic acid are also released (Herrmann 2001; Demir 2013). Plasma membranes of adjacent cells are damaged by these acids (Winkler et al. 2015), leading to a chain reaction of further bursting of cells in the vicinity. Exposure of the cell walls to acids leads to pectin solubilisation and removal of Ca^{2+} crosslinks (Dumville and Fry 2003; Brummell 2006). The lack of turgor, the pectin solubilisation, and the extraction of Ca^{2+} increases swelling (Grimm and Knoche 2015), resulting in decreased cell wall stiffness, decreased pressure at fracture, and decreased cell to cell adhesion (Figs. 4, 5). The weaker cell walls cannot any longer withstand the strain of the fruit skin (Grimm et al. 2012). The low turgor pressure at the whole fruit level is sufficient to result in the formation of macrocracks by rupture *along* cell walls (Knoche et al. 2014). The continuation of this process at the crack tip and the phenomenon of stress concentration at a crack tip will result in crack elongation in much the same way as a 'zipper' or a 'ladder' is propagated in a piece of knitted fabric (Fig. 1g). The swelling of cell walls and, in consequence, the decrease in stiffness and lower pressure at fracture allow a microcrack in a strained cuticle to extend into a macrocrack through the load-bearing fruit skin that subsequently causes the entire fruit to crack deep into the flesh.

The data presented here demonstrate that swelling of cell walls precedes failure of the fruit skin in water-induced cracking, but not necessarily when inducing fracture in a biaxial tensile test. Cell wall swelling favours fracture of epidermal cell walls *along* cell walls, decreases stiffness of the cell wall, and decreases the pressure at fracture. Factors affecting fracture modes and mechanical properties—such as cultivar, ripening stage, and turgor as manipulated by incubation in sucrose or aqueous ethanol, $CaCl_2$ or malic acid—do so primarily by altering cell wall swelling. Future studies are required to identify the cell wall constituent(s) responsible for swelling and the mechanism of swelling in sweet cherry. Based on the arguments presented above, the pectins are the most likely candidate. Of particular interest is the mechanism responsible for the weakening of cell to cell adhesion and the subsequent separation of adjacent cells. A better understanding of these processes is a prerequisite to the development of suitable countermeasures and possibly and ultimately to mitigate the rain cracking of fruit. Cultural means include the application of foliar sprays of Ca-salts that, as we have shown, prevent the swelling of cell walls in the load-bearing skin. Also, approaches that modify the processes of pectin solubilisation are well documented for apple (*Malus* × *domestica*; Atkinson et al. 2012), tomato (Brummell and Harpster 2001; Wen et al. 2013), and strawberry (*Fragaria* × *ananassa*; Quesada et al. 2009; for recent review see Sénéchal et al. 2014). Such strategies could be useful in modifying cracking susceptibility of the sweet cherry in the long term in breeding programs.

Author contribution statement MK obtained the funds to support the study. MK and MB planned the experiments. MB conducted the experiments. MB and MK analysed the data and wrote, revised, and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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