Malic Acid Promotes Cracking of Sweet Cherry Fruit

Andreas Winkler, Max Ossenbrink, and Moritz Knoche¹

Institute for Horticultural Production Systems, Leibniz-University Hannover, Herrenhäuser Straße 2, 30419 Hannover, Germany

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ABSTRACT. When mature sweet cherries (*Prunus avium* L.) came into contact with sweet cherry juice, cracking dramatically increased. The objectives of our study were: 1) to quantify the cracking of fruit in cherry juice, 2) to determine which constituent(s) of the juice especially promote cracking and, 3) to establish its/their mode of action in promoting cracking. Artificial juice was made up as an aqueous solution of the same five pure chemicals and at the same relative concentrations as the five major osmolytes of real sweet cherry juice. Artificial and real juice was used at half-isotonic concentrations as the real juice from that batch of fruit. Cracking of sweet cherries placed in either artificial or real juice was more rapid and occurred for lower net water uptakes than of fruit placed in half-isotonic polyethylene glycol 6000. The crack-promoting component in sweet cherry juice was malic acid. Further tests with malic acid, and other organic acids, and with different concentrations of malic acid, with and without pH control, and with the enantiomers of malic acid, showed the effects were primarily related to the pH of the incubation solution. Leakage of anthocyanin from discs of flesh was increased in the presence of malic acid and greater in hypotonic than hypertonic solutions, suggesting that malic acid increases the permeability of the plasma membrane and tonoplast and weakens the cell walls. Malic acid may be an important link (amplifier) in a reaction chain that begins with the bursting of individual epidermal cells and ends with the formation of macroscopic skin cracks.

Rain cracking is a problem for sweet cherry production in all countries where this very high-value crop is grown (Christensen, 1996). Despite considerable research effort the mechanistic basis of the phenomenon is still poorly understood. The fruit's water relations are thought to play a critical role in cracking (Considine and Kriedemann, 1972; Measham et al., 2009; Sekse, 1995, 2008; Sekse et al., 2005). Recent investigations have established that stage III sweet cherry fruit have a surprisingly low turgor (Knoche et al., 2014; Schumann et al., 2014). The mechanism by which low turgor is maintained despite a massive accumulation of carbohydrates and, hence, a very negative osmotic potential is at present unknown. Barring the pit, a sweet cherry has a similar mechanical constitution as a grape (*Vitis vinifera* L., *Vitis labrusca* L.), which is also very vulnerable to rain cracking (Considine and Brown, 1981). As in sweet cherries, grape turgor is very low in postveraison fruit (Lang and Düring, 1990; Wada et al., 2008, 2009). The low turgor in grapes is accounted for by the accumulation in the apoplast of solutes at osmotic concentrations closely matching those in the symplast. The rough balance in the osmotic potentials in symplast and apoplast results in similar pressures in these two compartments and thus flaccidity (Wada et al., 2008). This conclusion is based on the following experimental evidence. First, at harvest maturity there is no significant difference in osmolarity between apoplastic fluid extracted from grapes via the stalk using a pressure bomb and that of the expressed juice of crushed fruit (almost totally symplastic in origin—very large, thin-walled flesh cells), whereas in immature grapes they are substantially different (Lang and Düring, 1991). This finding is interpreted as being the result of a loss of

compartmentation during maturation (Lang and Düring, 1991). Second, Tilbrook and Tyerman (2008) observed significant cell death within an intact, mature grape berry using microscopy and vitality staining. The membrane's loss of osmotic competence allows diffusion of symplastic solutes into the apoplast. Third, using tissue centrifugation (Wada et al., 2008) and a pressure plate apparatus (Wada et al., 2009) the grape apoplast was selectively sampled. The subsequent compositional analyses also revealed that symplastic solutes accumulate in the apoplast of mature grapes (Wada et al., 2008, 2009).

For sweet cherries, comparable, direct evidence for the presence of apoplastic solutes that would account for the lack of turgor is not yet available. However, grape berry and sweet cherry are morphologically and physiologically similar with respect to cracking and therefore the above explanation may also apply to sweet cherry. First, the bursting of cells as a consequence of excessive water uptake possibly through microcracks (Glenn and Poovaiah, 1989; Peschel and Knoche, 2005) and of significant cell-wall degradation during maturation (Kondo and Danjo, 2001) would result in solute leakage into the apoplast. Second, we observed macerated tissue surrounding the pit of many sweet cherry cultivars at maturity (M. Knoche, unpublished data). We interpret this observation as the loss of compartmentation in this region of the fruit.

The question arises what would be the consequences of such leakage? In the work we report here, we observe a surprising and dramatic increase in cracking when sweet cherry fruit are brought into direct contact with the expressed juice of sweet cherries.

The objectives of this study were to investigate what consequences the leakage of cell contents into the fruit's apoplast might have. We were particularly interested in 1) quantifying cracking of sweet cherry fruit when incubated in different osmotica, including in artificial sweet cherry juice containing the same five dominant chemical moieties found in real cherry juice; 2) identifying any “active,” crack-promoting constituent in real cherry juice; and 3) establishing its mode of action in increasing cracking.

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¹Corresponding author. E-mail: moritz.knoche@obst.uni-hannover.de.

Material and Methods

PLANT MATERIAL. ‘Adriana’ and ‘Sam’ sweet cherry fruit were harvested at commercial maturity based on color, size, and taste from greenhouse-grown or field-grown trees grafted on ‘Gisela 5’ rootstocks (*Prunus cerasus* L. × *Prunus canescens* Bois) at the Horticultural Research Station of the Leibniz University in Ruthe (lat. 52°14’N, long. 9°49’E). Fruit were cultivated according to current European regulations for integrated fruit production. In a limited number of experiments, off-season ‘Bing’ fruit was obtained from a market. Here rootstock and harvest date are unknown. Fruit of uniform size and color were randomly selected. All fruit were free from visible defects. To restrict water uptake to the fruit surface, the pedicels were cut about 5 mm above the pedicel/fruit juncture and the juncture and the pedicel end sealed using silicone rubber (3140 RTV Coating; Dow Corning, Midland, MI). Thereafter fruit was placed in cold storage overnight at 2 °C. Next morning the silicone had cured and fruit were equilibrated to room temperature for one hour or so before initiating experiments.

GENERAL EXPERIMENTAL PROCEDURE. Cracking susceptibility was determined using a modified protocol for determining the cracking index first described by Verner and Blodgett (1931) and adapted by Christensen (1996). Briefly, two groups of 25 fruit each were incubated in 500 mL of treatment solutions at 22 °C. Fruit were removed from treatment solutions at regular time intervals, inspected for macroscopically visible cracks (by the naked eye) and the noncracked fruit returned to the incubation solution. Visual inspection detects cracks that extend into the flesh, but not microcracks limited to the cuticle. The experiment was ended after the last fruit cracked or fruit began to rot. There was no detectable change in osmolarity of the incubation solution caused by fruit cracking in the course of an experiment. Also, pH remained essentially constant (within 0.5 pH units) in presence of organic acids without pH adjustment. However, pH of nonbuffered solutions [i.e., those containing water only, carbohydrates, polyethylene glycol (PEG) 6000, or those of organic acids when titrated to pH values > pH 6] decreased by up to 3 pH units during incubation due to cracking.

Water uptake was quantified gravimetrically. Fruit was incubated in treatment solutions at 22 °C, removed from solution usually at 30 and 60 min after incubation, carefully blotted with tissue paper, weighed, and then reincubated. The rate of water uptake was calculated on an individual-fruit basis as the slope of a linear regression through a paired dataset of cumulative water uptake vs. time. As individual fruits cracked during the course of the experiment, these were excluded from further incubation and measurement. The only exception was for fruit incubated in 300 mM malic acid without pH control, where all fruit had cracked within the first 30 min of incubation. The minimum number of individual fruit replicates was 11.

EXPERIMENTS. Water uptake and fruit cracking were compared in ‘Sam’ sweet cherries following incubation in PEG 6000, real sweet cherry juice extracted from the same batch of fruit using a spaghetti press, or in artificial juice comprising the five most abundant osmolytes of sweet cherries (Herrmann, 2001). Together, these five osmolytes account for 98% of the osmolarity of sweet cherry juice. Relative contributions to total osmolarity and absolute final molar concentrations were 41.2% and 432 mM for glucose, 37.5% and 394 mM for fructose, 7.3% and 77 mM for sorbitol, 6.7% and 70 mM for malic acid, and 5.4% and 56 mM potassium as potassium malate (Herrmann,

Fig. 1. Time course of water uptake (A) and cracking of ‘Sam’ sweet cherry fruit (B). Percentage of cracked fruit as a function of water uptake (C). Dashed horizontal line indicates 50% cracking (B and C).

2001). The PEG 6000 was used for comparison. All solutions were prepared and diluted as necessary such that the final incubation solutions were half-isotonic to the real juice as determined by water vapor pressure osmometry (VAPRO® 5520 and 5600; Wescor, Logan, UT). Fruit incubated in deionized water served as controls. Cracking was quantified at selected time intervals as described above. In a subsequent experiment, the relative contributions of the individual osmolytes of cherry juice to cracking was identified by incubating ‘Sam’ sweet cherry in solutions containing only one of the osmolytes at half of its respective concentration in the juice. Concentrations and osmolarities of the various constituents were: 15.9 mM and 0.1 MPa malic acid, 98.1 mM and 0.5 MPa glucose, 89.4 mM and 0.4 MPa fructose, 17.5 mM and 0.1 MPa sorbitol, and 18.3 mM and 0.1 MPa potassium malate. Fruit incubated in half-isotonic artificial juice (1.2 MPa) and in

Table 1. Effect of sweet cherry juice, artificial juice, polyethylene glycol 6000 (PEG 6000), and deionized water on water uptake and cracking of ‘Sam’ sweet cherry fruit. Artificial juice comprised the five most abundant osmolytes reported in real sweet cherry juice [glucose, fructose, sorbitol, potassium malate, and malic acid (Herrmann, 2001)]. All solutions were prepared half-isotonic (1.2 MPa) relative to the osmolarity of the expressed juice of this batch of sweet cherries (2.4 MPa). Cracking was indexed by the amount of water taken up for 50% of fruit to crack (WU₅₀) or by the time to 50% fruit cracking (T₅₀).

Treatment	Rate of water uptake [mean ± SE (mg·h ⁻¹)]	Cracking	
		WU ₅₀ [mean ± SE (mg)]	T ₅₀ [mean ± SE (h)]
Water	4.9 ± 0.4 ^z	26.1 ± 5.6	5.4 ± 1.1
PEG 6000	0.9 ± 0.1*	28.9 ± 6.3	31.2 ± 6.8
Sweet cherry juice	2.0 ± 0.3*	4.9 ± 0.4	2.5 ± 0.2
Artificial juice	1.9 ± 0.2*	4.7 ± 1.2	2.5 ± 0.6

^zSignificance of difference of rate of water uptake to water control indicated by *, Dunnett test at $P \leq 0.05$.

Table 2. Effect of the most abundant osmolytes in sweet cherry fruit on water uptake and cracking. The concentrations and osmolarities correspond to those in a half-isotonic ‘Sam’ sweet cherry juice. Cracking was indexed by the amount of water taken up for 50% of fruit to crack (WU₅₀) or by the time to 50% fruit cracking (T₅₀).

Treatment	Osmolarity (MPa)	Rate of water uptake [mean ± SE (mg·h ⁻¹)]	Cracking	
			WU ₅₀ [mean ± SE (mg)]	T ₅₀ [mean ± SE (h)]
Water	0.0	3.5 ± 0.4 ^z	23.9 ± 1.1	6.8 ± 0.3
Artificial juice	1.2	2.1 ± 0.2*	7.5 ± 1.5	3.6 ± 0.7
Malic acid	0.1	4.8 ± 0.5	9.2 ± 2.6	1.9 ± 0.5
Glucose	0.5	3.6 ± 0.3	41.0 ± 4.7	11.4 ± 1.3
Fructose	0.4	3.3 ± 0.2	24.2 ± 7.4	7.3 ± 2.2
Sorbitol	0.1	3.8 ± 0.4	25.7 ± 1.6	6.8 ± 0.4
Potassium malate	0.1	3.9 ± 0.5	26.2 ± 9.1	6.7 ± 2.3

^zSignificance of difference of rate of water uptake to water control indicated by *, Dunnett test at $P \leq 0.05$.

Fig. 2. Effect of L(-) and D(+) enantiomers or a racemic mixture (DL) of both enantiomers of malic acid on cracking of ‘Bing’ sweet cherry fruit. Dashed horizontal line indicates 50% cracking.

deionized water served as controls. Cracking was assessed regularly as described previously.

Possible differences among the effects of L(-)- and D(+)-enantiomers or a racemic mixture (DL) of malic acid on cracking were identified by incubating ‘Bing’ sweet cherries in 70 mM L(-), D(+) and DL malic acid and monitoring fruit cracking. This concentration corresponds to the malic acid concentration present in real and in our artificial juice (Herrmann, 2001). Fruit were inspected at regular time intervals. Because there were no significant differences in cracking between the enantiomers (see Results section), the racemic mixture of DL malic acid was used in all subsequent experiments.

To control whether the effects on water uptake and cracking were specific for malic acid, a range of mono, di- and tri-carboxylic acids (all at 70 mM, except for fumaric acid at 40 mM

due to its lower solubility limit) were screened for their effects on water uptake and cracking using ‘Bing’ sweet cherry fruit. The acids investigated comprised malic, tartaric, succinic, fumaric, maleic, formic, acetic, propionic, and citric acids. Deionized water served as control. Cracking was monitored by regular inspection.

The effect of malic acid concentration (1, 3, 10, 30, 100, and 300 mM) with and without pH control on water uptake and cracking were established in ‘Sam’ sweet cherries. In the first experiment, the concentration effect was investigated without pH control (range pH 1.9–3.3). In the second (subsequent) experiment, pH was maintained constant between concentrations by adjusting all solutions to pH 3.3 using KOH. Fruit incubated in pure water served as control. Water uptake and fruit cracking were monitored as described above.

The effect of pH on water uptake and cracking was studied in ‘Adriana’ sweet cherries incubated in malic or citric acid or in phosphate buffer. For malic and citric acid (70 mM), pHs were adjusted to pH 3, 4, 5, 6, and 7 using KOH. The pH values < pH 3 were achieved by using malic and citric acid at their respective pH (pH 2.3 and pH 2.1 at 70 mM for malic and citric acid, respectively). The phosphate buffer (70 mM K₂HPO₄) was prepared by titration using H₂SO₄. Fruit incubated in deionized water served as control. Fruits were inspected for cracks up to 97 h.

To identify the mechanistic basis of the effect of malic acid on fruit cracking, its effect on membrane leakage in the presence and absence of osmotic stress was studied in ‘Bing’ sweet cherry. The leakage of anthocyanins from discs of sweet cherry flesh served as an indicator of membrane damage. In the first experiment, the time course of anthocyanin efflux was established. Discs (8 mm diameter, 2 mm thick, three discs per replicate, $n = 10$) were excised from the cheek of a fruit using a biopsy punch and parallel razorblades. Discs were blotted,

Table 3. Effect of selected mono, di- and tricarboxylic acids (concentrations all 70 mM, except fumaric acid 40 mM) on water uptake and cracking of 'Bing' sweet cherry fruit. Cracking was indexed by the amount of water taken up for 50% of fruit to crack (WU₅₀) or by the time to 50% fruit cracking (T₅₀).

Treatment	Water uptake rate [mean ± SE (mg·h ⁻¹)]	Cracking	
		WU ₅₀ [mean ± SE (mg)]	T ₅₀ [mean ± SE (h)]
Water	47.2 ± 3.3 ^a	233.7 ± 14.3	4.9 ± 0.3
Formic acid	57.1 ± 11.3	141.4 ± 7.2	2.5 ± 0.1
Acetic acid	52.1 ± 10.9	129.6 ± 17.6	2.5 ± 0.3
Propionic acid	41.4 ± 3.9	124.9 ± 3.4	3.0 ± 0.1
Succinic acid	40.8 ± 7.7	99.6 ± 4.2	2.4 ± 0.1
Malic acid	59.1 ± 7.2	126.6 ± 4.5	2.1 ± 0.1
Tartaric acid	50.0 ± 6.8	97.5 ± 8.6	2.0 ± 0.2
Maleic acid	52.5 ± 8.3	94.5 ± 0.9	1.8 ± 0.0
Fumaric acid	61.2 ± 5.9	63.9 ± 8.5	1.0 ± 0.1
Citric acid	53.8 ± 7.2	99.7 ± 7.4	1.9 ± 0.1

^aAll rates of water uptake not significantly different from water control, Dunnett test at $P \leq 0.05$.

rinsed and incubated in sucrose solution (osmolarity 3.1 MPa) with and without 70 mM malic acid (osmolarity 3.0 MPa). These solutions were about isotonic to juice extracted from the same batch of 'Bing' fruit (3.0 ± 0.2 MPa). After 1, 2, 4, 8, and 24 h, discs were removed from the incubation solution and the incubation medium sampled for anthocyanin content. To correct for differences in pH between the different incubation solutions, malic acid at a final concentration of 70 mM was also added to the controls (after removal of the discs and before photometry) such that control and treatment solutions contained the same amount of malic acid and hence, had the same pH of pH 2.3. This was necessary, because the absorption spectrum of anthocyanin depends on pH. The absorption at 520 nm was determined using a photometer (Specord 210; Analytik Jena, Jena, Germany). In the second experiment, osmotic stress was imposed by incubating discs in sucrose solutions having osmolarities of 0.9, 1.7, 2.6, 3.4, 4.3, and 5.2 MPa with and without 70 mM malic acid. At low sucrose concentrations, we expect cell walls to be strained due to increased turgor following water uptake from the hypotonic donor solutions. If malic acid weakened cell walls so they were more likely to rupture, anthocyanin efflux would be higher into hypotonic than into hypertonic solutions. Discs were removed from the incubation solutions after 8 h and the anthocyanin leakage quantified as described above. The number of replicates was 10.

DATA ANALYSIS. Data are presented as means ± SE. When error bars are not visible in a graph, they are smaller than the plotting symbols. Data on rates of water uptake were log-transformed, subjected to analysis of variance and mean comparisons performed using the Dunnett test (packet mult-comp 1.3–1, procedure glht, R 3.0.2; R Foundation for Statistical Computing, Wien, Austria). Linear and nonlinear regression analyses were performed using SigmaPlot (version 12.5; Systat Software, San Jose, CA). Fruit cracking typically increased with time in a sigmoidal manner. This relationship is best described by a four-parameter sigmoidal regression model:

$$\text{Cracking (\%)} = y_0 + \frac{a}{1 + e^{\frac{-(\text{time}(h) - x_0)}{b}}}$$

Equating the percentage of cracking to 50% and solving for time allowed the half time (T₅₀ in hours) of fruit cracking to be estimated from:

$$T_{50}(h) = -\ln\left(\frac{a}{50 - y_0} - 1\right) * b + x_0$$

A high T₅₀ represents fruit that cracks slowly. The percentage of fruit cracking was also expressed as a function of water uptake by simply replacing time (in hours) by the amount of water taken up (in milligrams) in the preceding equations (Weichert et al., 2004). The amount of water uptake at any one time is calculated as the product of the rate of uptake multiplied by the duration of incubation in water assuming that cumulative water uptake increases linearly with time. This is a fair assumption for the time periods of incubation in our experiments (Beyer et al., 2005). In analogy with a semilethal dose of a toxic compound resulting in 50% mortality (LD₅₀), the water uptake required for 50% of the fruit to crack (WU₅₀ in milligrams) is obtained from the T₅₀. This WU₅₀ characterizes the mechanical stability of a fruit in a given solution. Because the WU₅₀ is corrected for the amount of water taken up, it is a useful parameter in comparing cracking susceptibility of genotypes, treatments, and conditions. A high WU₅₀ is interpreted as fruit having a high cracking resistance or a low cracking susceptibility irrespective of its water uptake rates. Thus, the WU₅₀ is a measure of the "intrinsic" cracking resistance of a fruit in a given system.

Results

Cumulative water uptake increased linearly with time (Fig. 1A). Incubating fruit in real or artificial juice or in a PEG 6000 solution decreased rates of uptake as compared with the deionized water control. There were no differences in rates of water uptake between real and artificial juice, but both rates were higher than that with PEG 6000 (Fig. 1A; Table 1). The percentage of cracked fruit increased rapidly with time and was always higher for fruit incubated in solutions containing real or artificial juice followed by that of fruit incubated in water or PEG 6000 (Fig. 1B; Table 1). Calculating water uptake at 50% cracking revealed there was little difference in WU₅₀ between fruit incubated in PEG 6000 or in deionized water (Fig. 1C; Table 1). However, WU₅₀ was dramatically lower for fruit incubated in real or artificial juice. Again, there was no difference in WU₅₀ between real and artificial juice (Fig. 1C; Table 1).

Compared with deionized water, water uptake was essentially independent of the presence of any of the major osmolytes

Fig. 3. Effect of concentration of malic acid on water uptake and cracking of 'Sam' sweet cherry fruit. (A) Time course of water uptake. (B) Rate of water uptake as a function of concentration. Solutions were used with (pH 3.3) or without pH control. The 300 mM malic acid treatment without pH control was excluded from regression analysis, because all fruit had cracked and the high rate of uptake may have resulted from cracking. (C) Time course of cracking. (D) Time to 50% cracking (T_{50}) as a function of concentration. (E) Fruit cracking as a function of the amount of water taken up. (F) Water uptake to 50% cracking (WU_{50}) as a function of concentration. (G) pH values of malic acid at different concentrations with (pH 3.3) or without pH control. (H) WU_{50} as a function of pH of the malic acid solutions. Dashed horizontal lines in C and E indicate 50% cracking.

in sweet cherry juice (Table 2). Also, most osmolytes examined (glucose, fructose, sorbitol, and potassium malate) had little effect on the time to 50% cracking or on the intrinsic cracking

susceptibility (Table 2). However, fruit was markedly more susceptible to cracking as indexed by decreases in WU_{50} and T_{50} , when incubated in malic acid. Indeed, there was little difference in WU_{50} and T_{50} between malic acid and artificial juice containing malic acid at the same molar concentration (Table 2). Interestingly, compared with the water control, incubation in potassium malate had no effect on WU_{50} or T_{50} .

The effect of malic acid on cracking was independent of the enantiomer (Fig. 2). Compared with the water control, the L(-)- and D(+)-enantiomers and the racemic mixture (DL) of malic acid increased cracking to the same extents.

There was little effect of the organic acids on the rate of water uptake but, compared with the water controls, all mono, di- and tricarboxylic acids increased cracking as indexed by decreases in WU_{50} and T_{50} (Table 3). Fumaric acid gave the lowest WU_{50} and T_{50} , formic acid the highest and malic acid was intermediate (Table 3).

Varying the concentration of malic acid had little effect on the time course of water uptake (Fig. 3A). Only the highest malic acid concentration markedly increased the rate of uptake without pH control (pH 1.9). There was little difference in T_{50} between the lower malic concentrations (Fig. 3A and B). When pH was held constant, rates of water uptake decreased only slightly as the malic acid concentration increased. Fruit cracking, as indexed by decreasing T_{50} was positively related to malic acid concentration without pH control, but not when pH was maintained constant. Only the highest malic acid concentration increased T_{50} (Fig. 3C and D). The WU_{50} , decreased with increasing malic acid concentration without pH control, whereas there was no effect of concentration when pH was held constant (Fig. 3E and F). Plotting the effects of malic acid concentration on the WU_{50} as a function of pH revealed that the effects were essentially a linear function of pH for all concentrations with and without pH adjustment (Fig. 3G and H).

The pHs of malic acid and citric acid solutions had little effect on rates of uptake, but not when incubated in phosphate buffer. Here, uptake decreased as pH of the phosphate buffer increased (Fig. 4A and B). Fruit cracking

Fig. 4. Effect of pH of malic acid, citric acid, and phosphate buffer on cracking of 'Adriana' sweet cherry fruit. (A) Time course of water uptake from malic acid. (B) Rates of water uptake as affected by pH. (C) Time course of fruit cracking. (D) Time to 50% cracking (T_{50}) as affected by pH of the incubation solutions. (E) Fruit cracking as affected by the amount of water taken up. (F) Effect of solution pH on the amount of water uptake needed for cracking of 50% of fruit (WU_{50}). Dashed horizontal lines in C and E indicate 50% cracking. Dashed lines in D and F indicate extrapolated regression lines above pH 5.

as indexed by T_{50} was most severe at low pH, and decreased as pH increased (Fig. 4C and D). Only in citric acid did the T_{50} remain low with rising pH. Qualitatively similar relationships were obtained between WU_{50} and pH. The effects of malic acid and phosphate buffer were essentially identical (Fig. 4E and F). The lower T_{50} for citric acid at higher pHs, indicates that fruit are more susceptible to cracking, compared with malic acid or phosphate buffer of corresponding pHs (Fig. 4F).

When discs of cherry flesh were incubated in isotonic sucrose solutions, anthocyanin efflux into the solution increased with time in the presence of malic acid, but markedly less in its absence (Fig. 5A). The increase in anthocyanin leakage in the presence of malic acid was greater in hypotonic solutions (lower osmolarities) and tended to decrease as osmolarity increased (Fig. 5B). It is worth noting that in hypertonic sucrose solutions (where the bathing solution had

higher osmolarity than juice from the flesh), anthocyanin leakage was still increased by malic acid, whereas only little anthocyanin efflux was observed in the absence of malic acid (Fig. 5B).

Discussion

Our results demonstrate that sweet cherry juice causes rapid fruit cracking for low amounts of water uptake. The effect of the juice on cracking can be reproduced using an artificial juice composed of the five most abundant osmolytes in real juice which include malic acid. It can also be reproduced using malic acid on its own (Fig. 1; Table 1). Thus, malic acid is clearly identified as the major crack-promoting component in sweet cherry juice.

A comparison of the enantiomers of malic acid (Fig. 2), of various related mono, di-, and tricarboxylic acids (Table 3), the effects of concentration of malic acid with and without pH control (Fig. 3) and that of pH (Fig. 4) demonstrates that cracking is largely independent of the type of acid and is instead primarily related to solution pH. The only deviations occurred with malic acid and citric acid at pH > 4. These pHs are outside of the typical buffer range of these acids and hence, pH control at the site of action in the fruit's apoplast (pH 3.6) may have been insufficient.

Increased cracking at low pH has also been reported for cherry tomatoes (Lichter et al., 2002). As in sweet cherry, cracking in tomato decreases as pH rises. The mechanistic basis for increased cracking at low pH is not known. Several factors

may be involved including effects on the cell walls and/or plasma membranes. Malic acid also increased anthocyanin leakage into a hypotonic sucrose solution, indicating increased membrane permeability compared with the control. Furthermore, the increase in leakage was greater as osmolarity decreased (water potential became less negative and turgor increased), suggesting a weakening of the cell walls and the bursting of cells. Potential mechanisms of cell-wall weakening include 1) the cleavage of neutral sugar side-chains of cell-wall pectin that would weaken the cross-linking between the cellulosic cell wall and the pectins (Brummel, 2006), 2) a pH-dependent activation of polygalacturonases (Chun and Huber, 1998), and/or 3) a desorption, and complexation of cell-wall bound Ca^{2+} (Glenn and Poovaiah, 1989). These reactions weaken the cell-to-cell adhesion and decrease cell wall cross-linking (Demarty et al., 1984; Hepler, 2005).

Fig. 5. (A) Time course of anthocyanin leakage from discs excised from the flesh of mature 'Bing' sweet cherry fruit in the absence and presence of malic acid. (B) Effect of osmolarity of the incubation solution on anthocyanin leakage in the presence and absence of malic acid. The vertical dashed line indicates the osmolarity of the fruit.

We do not have an explanation for the effect of glucose on cracking (Table 2). Osmotic effects on water uptake can be excluded because the WU_{50} , which is corrected for water uptake, increased. Also, the osmolarity of the glucose solution was too low to alter the driving force for water uptake and consequently, there was no significant effect on rates of water uptake. Compared with the control treatment, fruit incubated in glucose solution required more water to crack which would be interpreted as mechanically more robust, less susceptible fruit. Direct effects of glucose on the mechanical properties of the fruit skin that forms the structural backbone of the sweet cherry (Brüggenwirth et al., 2014), are unlikely, particularly within the short time period of the experiment. Any indirect effects for example on strain of the fruit skin would be essentially osmotic and, therefore, equally expected for fructose. Fructose was used in the same experiment at similar osmolarity, but had no effect on the WU_{50} or T_{50} (Table 2). Clearly, further experiments are necessary to reproduce and clarify this effect in greater detail.

PRACTICAL CONSEQUENCES. The scenario described here may occur also in sweet cherry fruit under orchard conditions. First, there is evidence that bursting of individual cells precedes macroscopic cracking (Glenn and Poovaiah, 1989) and also that loss of membrane integrity occurs, at least in the flesh surrounding the pit. Second, microcracks in the cuticle impair its barrier function, resulting in increased water uptake (Knoche and Peschel, 2006) and exposure of cells to water results in cell

bursting and release of anthocyanins (Simon, 1977). The bursting of individual cells would also release malic acid into the apoplast. The appearance of malic acid in the apoplast, even at low concentration, will increase membrane permeability and weaken cell walls. This response may initiate a chain reaction that could contribute to macroscopic fruit cracking.

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