Effect of Receiver pH on Infinite Dose Diffusion of $^{55}\text{FeCl}_3$ across the Sweet Cherry Fruit Exocarp

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ABSTRACT. Recent studies established that some ferric salts, including FeCl_3 , decrease water permeability of the sweet cherry (*Prunus avium* L.) fruit exocarp and fruit cracking, presumably by a pH-dependent precipitation reaction that blocks high-flux pathways across the fruit surface. The objectives of our study were the following: to establish the effect of receiver pH on penetration of $^{55}\text{FeCl}_3$ through excised exocarp segments (ES) and isolated cuticular membranes (CM) and to localize any Fe precipitates in the epidermal system of mature sweet cherry fruit. Penetration was studied using an infinite dose diffusion system where ^{55}Fe penetrated from donor solutions of ferric salts (10 mM, pH 2.2–2.6) or EDTA- Na-Fe(III) (10 mM, pH 5.0) across an interfacing ES or CM into aqueous receiver solutions of pH values ranging from 2.0 to 6.0. For receiver pH 2.0, ^{55}Fe penetration of the ES from a 10 mM FeCl_3 donor (pH 2.6) was linear with time, but for receiver pH ≥ 3.0 , penetration was low and insignificant. Increasing the pH of the water receiver from 2.0 to 6.0 in the course of an experiment resulted in an immediate halt of penetration regardless of whether ^{55}Fe penetration occurred from FeCl_3 (pH 2.6), $\text{Fe(NO}_3)_3$ (pH 2.6), or $\text{Fe}_2(\text{SO}_4)_3$ (pH 2.4) as donor solutions (all at 10 mM). Only from EDTA- Na-Fe(III) (pH 5.0) ^{55}Fe penetration continued to occur albeit at a decreased rate ($\sim 30\%$). At receiver pH 2.0, the $^{55}\text{FeCl}_3$ flux through stomatous ‘Sam’ ES averaged 10.4 ± 2.3 $\text{pmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and was positively correlated to stomatal density. Conventional and analytical electron microscopy (energy dispersive X-ray analysis, electron spectroscopic imaging, and electron energy loss spectroscopy) identified ferric precipitates in periclinal and anticlinal cell walls of epidermal cells underlying the cuticle, but not within the cuticle. These data indicate that the lack of ^{55}Fe penetration from donor solutions of ferric salts through the ES into a receiver solution at pH ≥ 3 and the previously reported decrease in water uptake and cracking as a response to immersing fruit in solutions of ferric salts are the result of a precipitation reaction at the cuticle/cell wall interface in the sweet cherry exocarp. Although spray application of ferric salts is prohibitive for ecotoxicological reasons, understanding their mechanism in decreasing water uptake and fruit cracking may be helpful in the search for alternate compounds that are effective and ecotoxicologically acceptable.

Rain cracking of sweet cherry fruit is an important limitation in crop production worldwide and is thought to be related to water uptake through the fruit surface. This uptake occurs by diffusion through the cuticle and by viscous flow along an aqueous continuum across the sweet cherry fruit exocarp that is referred to as the polar pathway (Weichert and Knoche, 2006a). Polar pathways are formed by hydration and orientation of polar functional groups in the cuticular membrane [CM (Beyer et al., 2005; Schönherr and Bukovac, 1970; Schönherr and Schmidt, 1979; Schönherr, 2006)] and allow for rapid water uptake into the fruit (Weichert and Knoche, 2006a).

Incubating sweet cherry fruit in aqueous solutions of AgNO_3 , CuCl_2 , HgCl_2 , AlCl_3 , EuCl_3 , and FeCl_3 was recently shown to markedly decrease water uptake and subsequent cracking (Beyer et al., 2002; Weichert et al., 2004). Unfortu-

nately, none of these salts can be used in horticultural practice to control cracking. Nevertheless, understanding their mechanism in decreasing water uptake and fruit cracking may be helpful in identifying compounds that are equally effective as the above salts, but also acceptable from an ecotoxicological perspective.

Of the salts cited above, the most information is available on FeCl_3 . The data demonstrated that decreased water uptake is related to decreased permeability of the polar pathways (Weichert and Knoche, 2006b), which most likely results from a pH-dependent precipitation in the exocarp. Aqueous solutions of ferric salts [e.g. FeCl_3 , $\text{Fe(NO}_3)_3$, and $\text{Fe}_2(\text{SO}_4)_3$] are strongly acidic. During penetration into the exocarp, ferric ions encounter a microenvironment of increasing pH until the pH of the fruit’s apoplast is reached (Weichert and Knoche, 2006b). The increase in pH causes formation of colloidal gels and precipitates, comprising complex hydrated amorphous ferric-oxides and -hydroxides, most likely FeO(OH) (Greenwood and Earnshaw, 1990) that are expected to form a significant barrier to water transport across the sweet cherry exocarp (Weichert and Knoche, 2006b).

Indirect evidence for this pH-dependent precipitation reaction comes from experiments by Weichert and Knoche (2006b) where diffusion of $^3\text{H}_2\text{O}$ was studied using an infinite dose diffusion system (Bukovac and Petracek, 1993). In this

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system, $^3\text{H}_2\text{O}$ diffusion was followed from a donor solution containing FeCl_3 (pH 2.6) through an interfacing ES into a water receiver of pH values adjusted to pH 2.0 to 6.0. In presence of FeCl_3 in the donor, $^3\text{H}_2\text{O}$ diffusion through the ES into the receiver markedly decreased at receiver pH ≥ 3.0 , but there was no effect of FeCl_3 on $^3\text{H}_2\text{O}$ diffusion at the receiver pH 2.0 (Weichert and Knoche, 2006b). According to the above hypothesis, we would expect formation of precipitates at receiver pH ≥ 3.0 that must have decreased water uptake and fruit cracking.

To obtain direct evidence for this mechanism, we now studied the effect of receiver pH on penetration of ^{55}Fe from ferric salts through excised ES or isolated CM into a receiver solution using the infinite dose diffusion system, and the occurrence of ferric or ferrous precipitates in the sweet cherry exocarp.

Materials and Methods

PLANT MATERIAL. Mature sweet cherry (*Prunus avium*) cultivars Adriana, Sam, and Hedelfinger grafted on 'Alkavo' (*P. avium*) rootstocks were sampled from commercial orchards near Halle and experimental orchards of the Institute for Plant Breeding at Dresden-Pillnitz, and of the Lehr- und Versuchsanstalt Gartenbau at Erfurt, Germany. Fruit were selected for freedom from defects and uniformity of size and maturity based on color. The ES consisting of cuticle, epidermis, hypodermis, and some layers of mesocarp tissue were excised from the cheek region of 'Adriana' and 'Sam' sweet cherries using a razor blade. For the experiment on the effect of stomatal density (d_{sto}), ES from 'Sam' fruit were also excised from stem cavity and the apical area of the fruit excluding the stylar scar to maximize the range in stomatal density (Peschel et al., 2003). ES were stored in 1 or 2 g·L⁻¹ NaN_3 at 5 °C until use. CM were isolated from the cheek of 'Adriana' sweet cherry fruit by incubating ES in pectinase and cellulase as described previously (Weichert and Knoche, 2006b).

DIFFUSION STUDIES. Diffusion of $^{55}\text{FeCl}_3$ (specific activity 2.6 Gbq/mg, 99% radiochemical purity; PerkinElmer Life and Analytical Sciences, Boston) across sweet cherry ES and CM was determined using the infinite dose technique where diffusion is monitored under quasi steady-state conditions from a dilute donor through the interfacing ES or CM into a receiver (Bukovac and Petracek, 1993). ES or CM were mounted in Plexiglas holders (2 or 5 mm inner diameter) using silicone rubber (3140 RTV coating; Dow Corning, Midland, MI) and subsequently inspected for microscopic cracks in the cuticle at 100× magnification. ES or CM having microscopic cracks was discarded. Holders with crack-free ES or CM were mounted between two glass half-cells of the diffusion apparatus using silicone grease (Baysilone-Paste hochviskos; GE Bayer Silicons, Leverkusen, Germany). Generally, the morphological outer side of the ES faced the donor. Diffusion cells were placed in a thermostated water bath held at 25 °C and positioned on a multistirring plate. Unless specified otherwise, donor solutions were prepared 24 h before the initiation of an experiment using 10 mM FeCl_3 (pH 2.6, $\text{FeCl}_3 \times 6\text{H}_2\text{O}$; Carl Roth, Karlsruhe, Germany). Donor solutions were spiked with $^{55}\text{FeCl}_3$ to yield concentrations of radioactivity ranging from 0.8 to 2.6 $\times 10^6$ cpm/mL, equivalent to 0.07 to 0.24 μM $^{55}\text{FeCl}_3$. Deionized water with pH adjusted using HCl or NaOH served as receiver solution because earlier studies established that some buffers interact with the Fe-dependent decrease in water

transport (Weichert and Knoche, 2006b). The pH of receiver and donor solution was checked at the beginning and end of each experiment and remained within 0.1 pH units in all experiments. Diffusion experiments were initiated by adding 5 mL of donor and receiver solution to donor and receiver cells of diffusion units, respectively. The time course of penetration of ^{55}Fe was followed by repeated sampling of the receiver solution. Aliquots (1 mL) were removed from receiver solutions, radioassayed by liquid scintillation spectrometry [scintillation cocktail (Ultima Gold™ XR; PerkinElmer Life and Analytical Sciences); counter (LS 6500; Beckman Instruments, Fullerton, CA)]; and replaced by fresh solution. Steady-state flow rates [F (mol·h⁻¹)] were determined from the slope of a linear regression line fitted through plots of cumulative ^{55}Fe penetration versus time. From F the flux [J (mol·m⁻²·s⁻¹)] was calculated by dividing by the cross-sectional area of the ES or CM exposed in the diffusion cell [A (m²)].

The effect of receiver pH on the time course of ^{55}Fe -transport from a 10 mM FeCl_3 donor solution (pH 2.6) was studied using stomatous ES excised from 'Sam' sweet cherry fruit. Deionized water with pH adjusted to 2.0, 3.0, 4.5, and 6.0 using HCl or NaOH served as receiver. Because significant penetration was only detected at a receiver pH 2.0, the diffusion experiment was continued for the pH 2.0 treatment by increasing receiver pH to 6.0 at 47.5 h after initiation of the experiment. The minimum number of replication was four.

The effect of stomatal density on ^{55}Fe -transport from a 10 mM FeCl_3 donor solution (pH 2.6) was established in a two-phase experiment using astomatous 'Adriana' and stomatous 'Sam' ES. Stomata number was established by microscopy on an individual ES basis. 'Adriana' sweet cherry has a very low stomatal density (Peschel et al., 2003). When using holders having an inner diameter of the orifice of only 2 mm and ES or CM from 'Adriana' sweet cherry, it was possible to select astomatous specimen by microscopy. During phase I of the experiment [J^I ('Sam', 0–48 h; 'Adriana', 0–96 h)], the pH of the water receiver was adjusted to 2.0. For phase II [J^{II} ('Sam', 49–168 h)] the water receiver of pH 2.0 was replaced by a water receiver of pH 6.0 in 'Sam' ES. The astomatous 'Adriana' ES were discontinued because no detectable penetration occurred despite a 3-fold increase in concentration of radioactivity and extended sampling intervals. At the end of the experiment, the ES exposed in the holder was excised and transferred to a scintillation vial. After adding cocktail, radioactivity was quantified by liquid scintillation counting. Fluxes (J) were calculated from equilibrium flow rates as described above. The effect of pH on ^{55}Fe -transport was indexed by the change in J [ΔJ ($\Delta J = J^I - J^{II}$)].

To investigate whether the decrease in ^{55}Fe penetration from a 10 mM FeCl_3 (pH 2.6) through stomatous Sam ES into deionized water (pH 5.5) depended on the orientation of the ES, penetration was studied through ES in normal (i.e., morphological outside to the donor) versus reversed orientation (i.e., morphological outside to the receiver). The J was established as described above.

The effect of the counter ion on the time course of ^{55}Fe -transport was studied using donor solutions containing FeCl_3 ($\text{FeCl}_3 \times 6\text{H}_2\text{O}$; Carl Roth), $\text{Fe}(\text{NO}_3)_3$ [$\text{Fe}(\text{NO}_3)_3 \times 9\text{H}_2\text{O}$; Sigma-Aldrich, St. Louis], $\text{Fe}_2(\text{SO}_4)_3$ [$\text{Fe}_2(\text{SO}_4)_3 \times \text{H}_2\text{O}$; Sigma-Aldrich], or EDTA-Na-Fe(III) (Sigma-Aldrich; all at 10 mM). Donor solutions were spiked with $^{55}\text{FeCl}_3$ 24 h before initiation of the experiment to yield a concentration of

radioactivity of about 0.8×10^6 cpm/mL. Steady-state fluxes were established at receiver pH 2.0 (J^I , 0–47 h) and subsequently at receiver pH 6.0 (J^{II} , 47.5–95.5 h). The effect of pH on ^{55}Fe transport was indexed by the ratio J^{II}/J^I .

Electron microscopy and elemental analysis

SAMPLE PREPARATION. Unless otherwise specified, ‘Hedelfinger’ fruit were selected for localizing Fe precipitates because this cultivar has a high frequency of stomata (Peschel et al., 2003). Fruit were incubated for 10 h in 10 mM FeCl_3 , removed from solution, thoroughly rinsed with deionized water, and carefully blotted using tissue paper. Small segments (4 mm² area, thickness 1–2 mm) of exocarp and adhering mesocarp tissue were excised using a razor blade. Segments used for Transmission electron microscopy (TEM) were fixed with 3% glutaraldehyde in 0.6 M phosphate buffer (pH 7.4) and 1% OsO_4 in 0.1 M Palade buffer [pH 7.0 (Palade, 1952)], and those used for analytical electron microscopy with 3% glutaraldehyde in 0.6 M phosphate buffer (pH 7.4) only. Subsequently, samples were dehydrated in an ascending series of acetone and embedded in low-viscosity epoxy resin [ERL (Plano, Wetzlar, Germany)].

TEM. Ultrathin sections (50 nm) were investigated with a transmission electron microscope (model EM912 OMEGA; Leo, Oberkochen, Germany) equipped with an incolumn energy filter and an energy dispersive X-ray (EDX) analysis System (Link exIII; Oxford Instruments, High Wycombe, UK). The images were recorded with a 2K slow scan charged-coupled device (CCD)-camera (Proscan elektronische Systeme, Lagerlechfeld, Germany). EDX analysis was performed on exocarp cross sections of 150 to 200 nm thickness. Segments were collected on Cu-grids and analyzed in the spot mode (spot size 100 nm at 80 keV acceleration voltage, emission current 20 μA).

ELECTRON SPECTROSCOPIC IMAGING (ESI) AND ELECTRON ENERGY LOSS SPECTROSCOPY (EELS). The spatial distribution

of Fe and Cl across the exocarp and the valency of the Fe in the precipitates were established using ESI and EELS, respectively. Briefly, ultrathin sections (50 nm) on Cu-grids were investigated in an EM910 OMEGA electron microscope (Leo). Images and spectra were recorded and subsequently processed by digital image analysis (analySIS 3.1; Soft Imaging System, Münster, Germany). For localization of precipitates, Fe or Cl maps were superimposed with the corresponding zero loss images. Superimposing the Fe and Cl signal simultaneously with the zero loss image allowed the colocalization of Fe and Cl in the exocarp.

Electron energy loss spectra were recorded from the sweet cherry fruit exocarp and were compared with spectra of Fe^{2+} and Fe^{3+} standards.

STATISTICS. Data were subjected to analysis of variance (ANOVA). ANOVA (Proc Anova, Proc Glm), multiple comparisons of means, and linear regression analysis (Proc Reg) were carried out using SAS (version 9.1; SAS Institute, Cary,

Fig. 1. Effect of receiver pH on the time course of ^{55}Fe -transport across exocarp segments (ES) excised from mature ‘Sam’ sweet cherry fruit. (A) Penetration at receiver pH 2.0, 3.0, 4.5, and 6.0. (B) Change in ^{55}Fe -transport across ES upon increasing the receiver pH from 2.0 during phase I to 6.0 during phase II.

Fig. 2. Effect of stomatal density (d_{sto}) on the flux (J) of ^{55}Fe through stomatous ‘Adriana’ and stomatous ‘Sam’ sweet cherry fruit exocarp segments (ES). The J for ^{55}Fe -transport was determined (A) at receiver pH 2.0 during phase I (J^I) and (B) at receiver pH 6.0 during phase II (J^{II}). (C) Decrease in J for ^{55}Fe -transport (ΔJ) upon increasing receiver pH from 2.0 to 6.0. Inset: ΔJ as affected by the amount of ^{55}Fe in the ES. The ΔJ was calculated by subtracting the J at a receiver pH 6.0 (J^{II}) from that at pH 2.0 (J^I). Regression equations for the relationship between J and d_{sto} were: receiver pH 2.0: J^I (pmol·m⁻²·s⁻¹) = $0.55 (\pm 2.29) + 7.04 (\pm 1.30) \times d_{\text{sto}}$ (mm⁻²), $r^2 = 0.68^{***}$, $P \leq 0.0001$; receiver pH 6.0: J^{II} (pmol·m⁻²·s⁻¹) = $-0.17 (\pm 0.27) + 0.55 (\pm 0.16) \times d_{\text{sto}}$ (mm⁻²), $r^2 = 0.47^{**}$, $P \leq 0.003$. The corresponding equation for the decrease in ΔJ upon increasing receiver pH from 2.0 to 6.0 was: ΔJ (pmol·m⁻²·s⁻¹) = $0.72 (\pm 2.06) + 6.49 (\pm 1.17) \times d_{\text{sto}}$ (mm⁻²), $r^2 = 0.69^{***}$, $P \leq 0.0001$; that for the relationship between the decrease in ΔJ and the amount of ^{55}Fe in the ES at the end of the experiment: ΔJ (pmol·m⁻²·s⁻¹) = $-7.83 (\pm 2.97) + 1.35 (\pm 0.21) \times \text{Amt } ^{55}\text{Fe}$ (pmol), $r^2 = 0.75^{***}$, $P \leq 0.0001$. Data for ‘Sam’ represent individual ES, those for ‘Adriana’ mean $\pm$ SE of eight stomatous ES.

NC). Unless specified otherwise, regression analysis was performed on data for individual ES. Data in figures are presented as means $\pm$ SE of means except for Fig. 2, A–C, where data points represent individual ES.

Results

Penetration of ^{55}Fe from a FeCl_3 donor across the sweet cherry exocarp increased linearly with time at the receiver pH 2.0 (Fig. 1A). There was essentially no penetration at receiver pH ≥ 3.0 . Replacing the receiver of pH 2.0 by a receiver of pH 6.0 resulted in an immediate halt of penetration (Fig. 1B). The flux (J) calculated from mean flow rates of ^{55}Fe across stomatous ‘Sam’ ES at a receiver pH 2.0 averaged $10.44 \pm 2.34 \text{ pmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at a donor concentration of 10 mM FeCl_3 ($n = 16$ ES).

^{55}Fe penetration from a FeCl_3 donor into a receiver of pH 2.0 was a linear function of stomatal density (Fig. 2A). The regression line for the relationship between J and d_{sto} passed through the origin and predicted the absence of ^{55}Fe penetration in an astomatous system ($d_{\text{sto}} = 0 \text{ mm}^{-2}$). Indeed, for astomatous ‘Adriana’ CM and ES, the J were very low (2.01 ± 0.66 and $2.73 \pm 1.12 \text{ pmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for ‘Adriana’ CM and ES, respectively; both at receiver pH 2.0). Increasing receiver pH from 2.0 to 6.0 for stomatous ‘Sam’ ES resulted in a drastic decrease in J (Fig. 2B). This decrease was again a linear function of stomatal density (Fig. 2C). The y-axis intercept of the regression line represented the decrease in J for a hypothetical astomatous ES ($d_{\text{sto}} = 0 \text{ mm}^{-2}$) and did not differ from zero. The decrease in J upon increasing the receiver pH was closely related to the amount of ^{55}Fe associated with the ES at the end of the experiment (Fig. 2C, inset).

The effect of receiver pH was independent of the orientation of the ES relative to the $^{55}\text{FeCl}_3$ donor. When water at pH 5.5 served as the receiver, the J for ^{55}Fe transport from FeCl_3 averaged 0.11 ± 0.03 and $0.09 \pm 0.03 \text{ pmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for normal versus reverse orientation of the ES, respectively.

There was no significant difference in J for ^{55}Fe penetration from FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, and $\text{Fe}_2(\text{SO}_4)_3$, or EDTA-Na-Fe(III) at the receiver pH 2.0 (Fig. 3, Table 1). However, increasing receiver pH from 2.0 to 6.0 nearly stopped ^{55}Fe penetration from the salts FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, and $\text{Fe}_2(\text{SO}_4)_3$. In contrast, ^{55}Fe penetration from EDTA-Na-Fe(III) continued to increase, albeit at a decreased rate [$\sim 30\%$ (Table 1)].

TEM revealed a thin cuticle of about 1 μm thickness on cell walls that averaged about 3 μm in thickness in the periclinal region of epidermal cells (Fig. 4A). Micrographs from sections of fruit incubated in 10 mM FeCl_3 showed electron dense particles in anticlinal and periclinal cell wall regions, particularly at the cuticle/cell wall interface (Fig. 4B). The frequency of these electron-dense particles decreased as distance from the cuticle increased (Fig. 4B). Using ESI and EDX, these particles were identified as Fe precipitates (Figs. 4, C–G and 5). There was no detectable Fe signal (red) in the cuticle (Fig. 4C). Cl maps (green) generated by ESI revealed the presence of Cl throughout the cuticle, the cuticle/

cell wall interface, and the cell wall region (Fig. 4D). The overlay map of Cl (green) and Fe (red) indicated by the yellow color that some Fe may be associated with Cl (Fig. 4E). Comparing energy loss spectra of the Fe precipitates in the sweet cherry fruit exocarp with those of Fe^{2+} and Fe^{3+} standards demonstrated that precipitates contained ferric but not ferrous Fe (Fig. 6).

Discussion

Our data on ^{55}Fe penetration and the localization of ferric iron in the epidermal cell walls are consistent with the hypothesis of a pH-dependent precipitation reaction in the sweet cherry exocarp. There was little penetration of ^{55}Fe from donor solutions of the salts FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, and $\text{Fe}_2(\text{SO}_4)_3$ into receiver solutions at pH values ranging from 3.0 to 6.0 because of formation of ferric precipitates in the exocarp. However, penetration was up to 36-fold higher at the receiver pH 2.0 where precipitates would not form. The immediate cessation of ^{55}Fe penetration from FeCl_3 into the receiver upon increasing the receiver pH from 2.0 to 6.0 demonstrates that the ES in the infinite dose system must have equilibrated rapidly with the pH of the receiver solution and the ferric precipitates formed instantaneously.

Support for a causal relationship between the formation of ferric precipitates as indexed by the absence of significant Fe penetration into the receiver and the effect of ferric salts on water uptake comes from a detailed comparison of data on ^{55}Fe penetration (this study) and previously published data on $^3\text{H}_2\text{O}$ diffusion through ES, osmotic water uptake, and cracking in intact fruit (Beyer et al., 2002; Weichert et al., 2004; Weichert

Fig. 3. Effect of counterion, chelator, and receiver pH on the time course of ^{55}Fe -transport across ‘Sam’ exocarp segments excised from mature sweet cherry fruit. During phase I, ^{55}Fe -transport was determined at receiver pH 2.0, during phase II, at receiver pH 6.0. For flux estimates, see Table 1.

Table 1. Effect of counter ion, chelator, and receiver pH on penetration of ^{55}Fe through excised exocarp segments of ‘Sam’ sweet cherry fruit. During phase I of the experiment, permeability was established at a receiver pH 2.0 (J^I), in phase II, at a receiver pH 6.0 (J^{II}).

Donor	Donor pH	MW (g·mol ⁻¹)	J (pmol·m ⁻² ·s ⁻¹)		J ^{II} /J ^I (ratio)
			J ^I	J ^{II}	
			Receiver pH 2.0	Receiver pH 6.0	
FeCl ₃	2.6	270	8.64 ± 2.36 a ^z	0.24 ± 0.09 b	0.03 ± 0.01 b
Fe(NO ₃) ₃	2.6	404	10.38 ± 3.19 a	0.33 ± 0.14 b	0.02 ± 0.01 b
Fe ₂ (SO ₄) ₃	2.4	400	12.04 ± 0.86 a	0.41 ± 0.19 b	0.03 ± 0.02 b
EDTA-Na-Fe(III)	5.0	367	13.17 ± 2.72 a	9.06 ± 1.43 a	0.71 ± 0.07 a

^zMean separation in columns by Tukey’s Studentized range test at $P \leq 0.05$.

Fig. 4. Cross sections and elemental distribution maps of Fe and Cl in the sweet cherry fruit exocarp. (A) Anticlinal and periclinal cell wall region of the epidermis of 'Sam' sweet cherry. Zero loss images (B and E) and elemental distribution maps of Fe [C (red)], Cl [D (green)] and the overlay image of Fe and Cl [E (yellow)] in periclinal and anticlinal regions (F and G) of the sweet cherry exocarp following incubation of 'Hedelfinger' fruit in 10 mM FeCl_3 . Elemental distribution maps were obtained by electron spectroscopic imaging (ESI). For details see materials and methods; CM = cuticular membrane, CW = cell wall.

and Knoche, 2006b). First, receiver pH greater than or equal to 3.0 had a marked negative effect on penetration of ^{55}Fe from FeCl_3 (Fig. 1) and on water transport in presence of FeCl_3 (Beyer et al., 2002; Weichert and Knoche, 2006b). Second, astomatous ES (and CM) were essentially impermeable to ^{55}Fe from FeCl_3 (Fig. 2) and we did not detect any effect of FeCl_3 on water transport in astomatous systems (Weichert and Knoche, 2006b). Third, ^{55}Fe penetration across the exocarp into the

receiver at pH 2.0 (Fig. 2) and $^3\text{H}_2\text{O}$ penetration were positively related to stomatal density (Weichert and Knoche, 2006b). When increasing the pH of the receiver from 2.0 to 6.0, ^{55}Fe penetration from the donor into the receiver markedly decreased. Similarly, $^3\text{H}_2\text{O}$ penetration into the receiver markedly decreased when adding FeCl_3 to the donor (Weichert and Knoche, 2006b). In both experiments, the ferric ion in the donor must have penetrated into and precipitated within the exocarp, resulting in a marked decrease of ^{55}Fe and $^3\text{H}_2\text{O}$ penetration through the exocarp into the receiver solution. Fourth, the marked decrease of ^{55}Fe penetration upon increasing receiver pH from 2.0 to 6.0 (Fig. 1B) and the decrease in $^3\text{H}_2\text{O}$ penetration caused by FeCl_3 (Weichert and Knoche, 2006b) were independent of the orientation of the ES. Fifth, FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, and $\text{Fe}_2(\text{SO}_4)_3$ decreased water permeability of stomatous ES and intact fruit (Beyer et al., 2002). Accordingly, there was little ^{55}Fe penetration from these salts at a receiver pH 6.0, which mimics the pH of the cell wall space underlying the CM (Fig. 3; Marschner, 1995). In contrast, ^{55}Fe penetration occurred from EDTA-Na-Fe(III) in the donor at receiver pH 6.0 and the chelate had no effect on water uptake into sweet cherry fruit (H. Weichert, unpublished data). Thus, experimental conditions that induced formation of ferric precipitates in the exocarp markedly decreased ^{55}Fe and $^3\text{H}_2\text{O}$ penetration through the exocarp into the receiver and water uptake and cracking of detached sweet cherry fruit (Weichert et al., 2004). These three conditions were the following: 1) a stomatous exocarp, 2) free ferric ions in the donor, and 3) a receiving compartment (i.e., a receiver solution of the infinite dose system or the apoplast of an intact fruit) having a pH ≥ 3.0 .

It may be argued that the ligand exchange between the radiolabeled $^{55}\text{FeCl}_3$ used for spiking and the non-radiolabeled Fe of the EDTA-Na-Fe(III) complex may have been incomplete and that this would have resulted in penetration of $^{55}\text{FeCl}_3$ rather than of EDTA-Na- ^{55}Fe . However, the following arguments indicate that this was unlikely the case in our study. First, for EDTA-Na-Fe(III) (stability constant K , $\log K = 25.7$; Furia, 1972), half times of ligand exchange of ^{55}Fe and

Fig. 5. Energy dispersive X-ray analysis (EDX) of cross sections of the sweet cherry fruit exocarp after incubation in 10 mM FeCl_3 . EDX spectra were obtained in the cuticle (A) and the epidermal cell wall region with Fe deposits (B). The Cu peaks were caused by the supporting grid.

Fig. 6. Electron energy loss spectra (EELS) of Fe precipitates in the periclinal cell wall region of the epidermis of the sweet cherry fruit exocarp (solid line). The Fe^{2+} (dashed line) and Fe^{3+} signal (dotted line) originated from $\text{Fe}(\text{OH})_2$ and $\text{Fe}(\text{OH})_3$ that served as standards of known valency for comparison.

EDTA–Na–Fe(III) depended on pH and decreased from 1480 to 740 min as pH increased from 1.6 to 2.5 (Jones and Long, 1952). Thus, at pH 2.5 two half-times would have past when donor solutions were prepared 24 h before the experiment, as was done in our study. In our study, pH of the EDTA–Na–Fe(III) donor solution was markedly higher and, therefore, half-times most likely were even shorter. Second, if ligand exchange was incomplete, the $^{55}\text{FeCl}_3$ in the donor solution would have precipitated immediately in the donor as complex hydrated amorphous ferric-oxides and -hydroxides. Under these circumstances, ^{55}Fe would not be detectable in the donor. Third, the data obtained for $^{55}\text{FeCl}_3$ penetration demonstrate conclusively that even if $^{55}\text{FeCl}_3$ had remained in the donor in solution, the high pH of the receiver would have caused precipitation in the ES and the absence of detectable penetration into the receiver solution (Fig. 1). However, this was not the case. Thus, incomplete ligand exchange was unlikely to be a factor in our study.

The close relationship between ^{55}Fe penetration from FeCl_3 and stomatal density or between the FeCl_3 induced decrease in

water permeability and stomatal density (Weichert and Knoche, 2006b) deserves some further comments. In sweet cherry fruit, as in many other species (Franke, 1964; Schlegel et al., 2005; Schönherr and Bukovac, 1970; Schönherr, 2006), polar pathways are primarily associated with stomata (Weichert and Knoche, 2006a). Because water transport across the exocarp occurs predominantly via these polar pathways (Weichert and Knoche, 2006a) and FeCl_3 decreased water uptake (Weichert and Knoche, 2006b), penetration of ^{55}Fe ions from ferric salt solutions must also occur along these pathways. This conclusion is supported by two further arguments. First, the ferric ion carries three charges and thus, is excluded from penetration along the lipophilic pathway (Schönherr et al., 2005). Second, comparison of the size exclusion limits of polar pathways [between 0.47 and 1.15 nm; estimated from reflection coefficients (σ) for sucrose ($\sigma = 0.74$) and polyethylene glycol 1500 ($\sigma = 1.0$; Weichert and Knoche, 2006a)] and the hydrodynamic radius of the ferric ion (0.41 nm; Beyer et al., 2002) revealed that ferric ions are accommodated by the polar pathway. Unfortunately, we were not successful in obtaining cross sections through a stoma to provide direct evidence for preferential precipitation at stomata. Although ‘Hedelfinger’ sweet cherry has a high number of stomata relative to fruit from other cultivars, stomatal density on sweet cherry fruit is still low compared with leaves (for ‘Hedelfinger’ fruit, up to 1.71 mm^{-2} ; Peschel et al., 2003). Attempts using SEM and EDX that allow stomata to be localized more easily were not entirely conclusive in identifying preferential precipitation of Fe in the region of the stomatal pore (M.J. Bukovac, W. Schröder, and M. Knoche, unpublished data), possibly because of signal attenuation/absorption by the cuticle. Nevertheless, we would expect ferric precipitates to form at the stomatal apparatus in an identical manner as demonstrated here for the region between stomata, thereby accounting for decreased water uptake and fruit cracking (Weichert et al., 2004; Weichert and Knoche, 2006b).

It should be noted that the presence of these precipitates in the region between stomata and the absence of effects of FeCl_3 on water penetration through the CM between the stomata reported earlier (Weichert and Knoche, 2006b) are not contradictory. The data from this and our earlier study (Weichert and Knoche, 2006b) indicate that the ferric precipitates form an additional resistor arranged in series to the polar pathway or the CM. According to Ohm’s law, total resistance equals the sum of individual resistances when arranged in series. From these considerations, a larger effect of ferric precipitates is expected for penetration along high-flux pathways such as the polar pathway, but a small and possibly non-detectable effect on high-resistance pathways such as the cuticle (Weichert and Knoche, 2006b). This observation would also explain why FeCl_3 did not decrease transpiration of sweet cherry fruit (Beyer et al., 2002).

In summary, the data of this and our earlier studies established that under laboratory conditions, water uptake and cracking of sweet cherry fruit can be effectively controlled by a pH-dependent precipitation reaction in the fruit exocarp where the precipitates block high-flux pathways for water uptake. In contrast to non-specific coating strategies, this reaction has no side effect on transport of water vapor and most likely other gases. Unfortunately, spray application of the salts investigated so far is prohibitive for ecotoxicological reasons. However, because the mechanistic basis underlying this effect is now identified, the search for the effective and acceptable ‘silver bullet’ can now begin.

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