Rain damage in strawberries: identifying the drivers and understanding the mechanisms

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Gartenbauwissenschaften (Dr. rer. hort.)

genehmigte Dissertation

von

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2024

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Tag der Promotion: 05.04.2024

Abstract

Strawberries are extensively cultivated and enjoyed for their appealing sensory attributes and nutritional benefits. Rain can significantly reduce its quality. Rain damage is characterized by water soaking and cracking. Both disorders increase the risk of decay, causing significant economic losses. Despite their importance, disorders have yet to receive comprehensive investigation of the underlying physiological mechanisms involved. The objectives of this study were (1) to identify the mechanisms and factors of water movement through the fruit's surface, (2) to characterize and to identify the factors affecting microcracking of the cuticle, (3) to identify the mechanism and the factors affecting cracking in strawberry, (4) to identify the triggers, factors, and mechanism of water soaking in strawberry and (5) to establish the effects of calcium (Ca) and other monovalent, divalent, and trivalent cations on water soaking.

Permeances for osmotic water uptake and transpiration were higher than in other soft fruit. High osmotic uptake permeance may be attributed to a thin cuticle and viscous water flow through microcracks and polar pathways. The abscission zones of the petals and stamina, and microcracks in the calyx and receptacle served as preferential pathways for osmotic uptake. Microcracking increased with fruit development, and it was significantly promoted by surface wetness. Cuticle thickness decreased while the cuticle mass per fruit remained constant during fruit expansion. Necked strawberries were more susceptible to cracking than normal-shaped fruit. Frequency of cracking was size-dependent. Cracks (macro and micro) were mainly oriented latitudinal in the proximal region of the neck and longitudinal in the mid and distal regions of the neck. Growth strain was the main driver of cracking, and this was further exacerbated by water uptake. Water-soaked fruit showed show pale, deliquescent patches of skin. Water uptake markedly increased water soaking. Incubation in dilute citric and malic acids increased plasma membrane permeability and increased water soaking. Cuticular microcracks were observed in water-soaked areas. Ca mitigates water soaking by decreasing cuticular microcracking, leakage from plasma membranes, and possibly increased cross-linking of cell wall constituents. Our study has identified the mechanism of rain damage, which is based on the "Zipper" model that accounts for rain-cracking in sweet cherries. Rain damage in strawberries involves cuticular microcracks, localized uptake by viscous flow, cell bursting, and release of organic acids in the apoplast. These events trigger a chain reaction that extends a microcrack into a macrocrack and, as skin cell destruction progresses, causes water soaking.

Keywords: water movement, microcracking, cuticle, water soaking, Zipper model.

Zusammenfassung

Erdbeeren sind beliebt wegen ihres Geschmacks und ihrer Nährstoffe, jedoch kann Regen ihre Qualität mindern. Regenschäden durch Water soaking und Aufreißen verursachen Fäulen und wirtschaftliche Verluste. Die zugrunde liegenden Mechanismen sind bislang nicht bekannt. Ziele des Vorhabens waren (1) die Mechanismen und Faktoren der Wasserbewegung durch die Fruchtoberfläche zu identifizieren, (2) die Mikrorissbildung in der Kutikula zu charakterisieren und Einflussfaktoren zu identifizieren, (3) den Mechanismus und die Einflussfaktoren der Rissbildung zu identifizieren, (4) die Auslöser, Einflussfaktoren und Ursachen von Water soaking aufzuklären und (5) den Einfluss von Calcium (Ca) und anderen Kationen auf Water soaking zu quantifizieren.

Die Permeabilität der Fruchthaut für osmotische Wasseraufnahme und Transpiration ist höher als bei Früchten anderer Arten. Die hohe Permeabilität bei der Wasseraufnahme ist auf eine dünne Kutikula und viskosen Fluss durch Mikrorisse und polare Penetrationswege zurückzuführen. Die Abszissionszonen der Blütenblätter und Staubblätter sowie Mikrorisse im Kelch und Fruchtknoten dienen als bevorzugte Pfade für die osmotische Aufnahme. Mikrorisse nehmen während des Fruchtwachstums zu und werden durch Feuchtigkeit verstärkt. Die Kutikuladicke verringert sich während der Entwicklung, die Kutikulamasse je Frucht bleibt konstant. Erdbeeren mit ausgeprägtem Hals sind anfälliger für Risse, die vor allem bei größeren Früchten auftreten. Die Risse waren im proximalen Hals der Breite nach und im mittleren und distalen Hals der Länge nach ausgerichtet. Das Wachstum der Früchte ist der Hauptauslöser für Rissbildung, die durch Wasseraufnahme durch die Fruchthaut verstärkt wird. Früchte mit Symptomen von Water soaking zeigen helle, wässrige Hautflecken. Wasseraufnahme verstärkt die Symptome. Inkubation in Zitronen- und Apfelsäure erhöht die Membranpermeabilität und Water soaking. Mikrorisse in der Kutikula treten in Bereichen mit Water soaking auf. Calcium reduziert Water soaking durch Verringerung von Mikrorissbildung und Membranleakage und möglicherweise durch Stärkung der Zellwandvernetzung. Mit unserer Studie wurde die Ursache von Regenschäden durch Water Soaking und Aufreißen identifiziert. Wie bei dem Reißverschlussmodell (sog. Zipper-Model) der Süßkirschen entstehen Regenschäden bei Erdbeeren durch die Bildung von Mikrorissen in der Kutikula, lokale Wasseraufnahme, ein Platzen von Zellen und die Freisetzung organischer Säuren in den Apoplasten. Diese Ereignisse lösen eine Kettenreaktion aus, die einen Mikroriss zu einem Makroriss erweitert und/oder bei fortschreitender Zerstörung der Fruchthautzellen zu Water soaking führt.

Schlagwörter: Wasserbewegung, Mikrorisse, Kutikula, “Water soaking’’ , Zipper Modell.

Table of Contents

Abbreviations

A_{fruit}	Surface area of the fruit
$AlCl_3$	Aluminum chloride
ANOVA	Analysis of variance
$BaCl_2$	Barium chloride
Ca	Calcium
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
$CaSO_4$	Calcium sulfate
$CHCl_3$	Chloroform
CM	Cuticle membrane
CPP	Cell pressure probe
$CuCl_2$	Cupric chloride
DAFB	Days after full bloom
DCM	Dewaxed cuticular membrane
EGTA	Ethyleneglycol-bis(β-aminoethyl),N,N′,N′-tetraacetic acid
ES	Epidermal skin segments
$FeCl_3$	Ferric chloride
F_f	Rate of water uptake
F_t	Transpiration rate
HCl	Hydrogen chloride
J_f	Flux density of osmotic uptake
J_t	Flux density of transpiration
KCl	Potassium chloride
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
$LaCl_3$	Lanthanum chloride
LiCl	Lithium chloride
MeOH	Methanol
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganous chloride
NaCl	Sodium chloride
NaN_3	Sodium azide

NH_4Cl	Ammonium chloride
ns	Non-significant effect
P	Pression
PE	Polyethylene
PEG	Polyethylene glycol
P_f	Permeance for osmotic water uptake
P_t	Permeance to water vapor
R	Universal gas constant
RH	Relative humidity
RMSE	Root mean squared error
$SrCl_2$	Strontium chloride
T	Absolute temperature
t	Wall thickness
$\bar{V}_w$	Molar volume of water
x_{max}	Maximum width
y_{max}	Maximum length
ΔC	Difference in water vapor concentration
Δa_w	Water activity
ρ_w	Density of water
σ	Tangential stress in a wall
$\Delta\Psi$	Gradient in water potential
Ψ_{fruit}	Fruit water potential
Ψ_{water}	Water potential of incubation media
Ψ_Π	Osmotic solute potential
Ψ_p	Turgor or hydrostatic pressure

1. General introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is the most commercialized berry worldwide (Hancock, 2020), an average of 9 million tons were produced in the last years (FAO, 2021). Strawberries are produced and consumed widely due to their organoleptic and nutritional traits in the fresh market and the industry (Husaini and Neri, 2016). Hence, high fruit quality is demanded. However, strawberries are very perishable, they have an average shelf life of 7 days under cold room storage (Ayala-Zavala et al., 2004). Fruit production is susceptible to abiotic and biotic factors. One important factor that diminishes its quality is rain, particularly in regions with a high incidence of rainfall during harvest time.

Open-field production is the main type of cultivation system (Neri et al., 2012; Hancock, 2020). Rain damage arises frequently in this kind of production system provoking several economic losses. As a solution to this problem, in the last years open field cultivation has been shifting to protected cultivation in tunnels or greenhouses to increase yields, fruit quality and face the challenges of climate change (Menzel et al., 2014; Claire et al., 2018; Neri et al., 2012). However, protected cultivation requires high investments into structures, and has a high environmental impact, particularly in terms of energy and fertilizers consumption (Khoshnevisan et al., 2013; Claire et al., 2018). Furthermore, some cultivars do not adapt to protected cultivation encountering problems related to a high incidence of powdery mildew and red spider mite, and calcium deficiency (Grijalba et al., 2015; Menzel et al., 2015; Demchak, 2009; Xiao et al., 2001).

Rain damage in strawberries is described mainly as water soaking and cracking (Herrington et al., 2011). Both disorders increase the incidence of rot, mainly grey mold, and stem-end rot (Menzel et al., 2017). Consequently, harvesting, grading, post-harvest, and fruit quality are compromised (Herrington et al., 2009). Despite their importance, rain damage disorders of strawberries have not been studied in detail. The physiological mechanism involved are largely unknown.

The objective of this study is to identify the mechanism, factors, and drivers of water soaking and cracking of strawberry. The development of mitigation strategies through culture or breeding must be based on the understanding of these disorders.

The following is an overview of the literature available, in order to provide the background information needed to detect the gaps of knowledge and elucidate the specific objectives of this dissertation.

1.1. Rain damage in strawberries

Cracking and water soaking are typical disorders of rain damage (Fig. 1A, B) (Herrington et al., 2011), but pollination is also affected, resulting in misshaping fruits, after periods of heavy rain or over-head irrigation (Menzel, 2021). Rainfall can cause severe losses of more than 50% of the strawberry crop (Menzel et al., 2017; Herrington et al., 2011). However there was no direct relationship between the incidence of rain damage and the amount of precipitations, this further implies that only small amounts of rainfall may be required to cause damage (Herrington et al., 2009).

Water soaking and cracking increase the incidence of decay particularly of *Botrytis cinerea* (Fig. 1C). Cultivars vary their susceptibility to rain damage however, nearly all cultivars are affected when the fruit is mature (Menzel, 2021; Herrington et al., 2009). The two major disorders may occur in different regions of the fruit surface, the stem, neck, shoulder, and tip (Herrington et al., 2009).

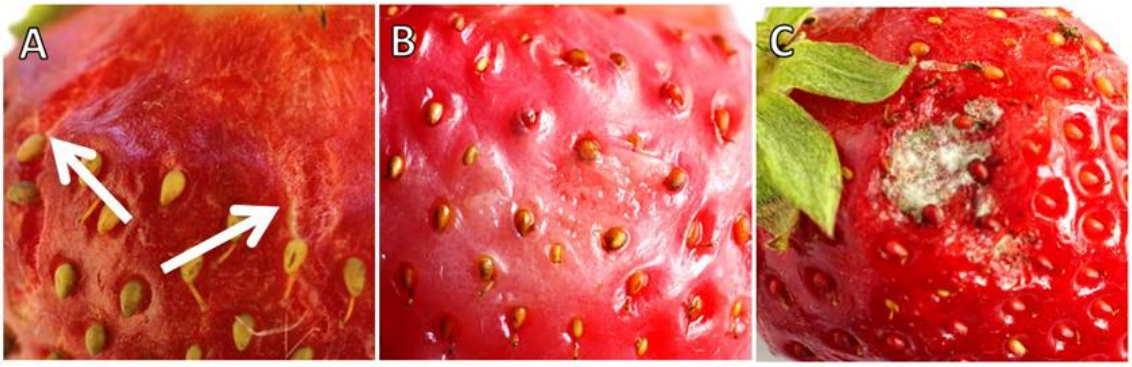

Fig. 1. Principal rain damages on Strawberry fruit (a) Cracking, (b) Water soaking, and (c) Grey mold caused by *Botrytis cinerea* that often is a consequence of cracking and water soaking (Photos taken by Martin Brüggenwirth, 2017)

1.1.1. Water Soaking

Water soaking occurs on the strawberry fruit surface. Water-soaked tissue can take on many appearances, often soggy and lighter colored or translucent (Fig. 1B). Often desiccated water-soaked tissue that shrinks is described as surface "etching", this is mainly observed as the achenes rise to the same level as the surrounding damaged tissue (Herrington et al., 2013; Herrington et al., 2011). Water soaking is the most frequent rain damage disorder (Herrington et al., 2013). There is no comparable phenomenon in other crops. Water soaking is hypothesized to be related to water uptake due to a wet surface and possibly guided and speeded up by the rupture of cells (Herrington et al., 2013), however definitive evidence is lacking.

1.1.2. Cracking

Cracking is a physiological disorder of fleshy fruits that can be observed easily (macrocracks) by the naked eye. Macrocracks are usually preceded by minute fractures (not visible to the naked eye) in the cuticle that do not extend into the flesh called microcracks (Fig. 2) (Knoche and Winkler, 2017). Macro and microcracks compromise fruit quality causing a higher incidence of fruit rots, high rates of water transport in uptake (during rain) and in transpiration (pre- and postharvest), loss of firmness, and impaired appearance (shrivel) (Opara et al., 1997; Knoche and Peschel, 2006; Knoche and Peschel, 2002; Beyer et al., 2005).

Herrington et al. (2009) observed macrocracks in strawberries that extend longitudinally from the shoulder of the fruit, concentric around the neck, and the combination of concentric and longitudinal called "star-cracking". "Desiccated seed or seed etching" were rain damages also associated with cracking and they were restricted close to the point of achene attachment. The mechanism of the formation of cracking is unknown. Rain is a factor but not the only one involved in the phenomenon (Herrington et al., 2009; Knoche and Winkler, 2017). Due to the lack of detailed studies on strawberries, the following section is based on research made on sweet cherry and other cracking-susceptible crops.

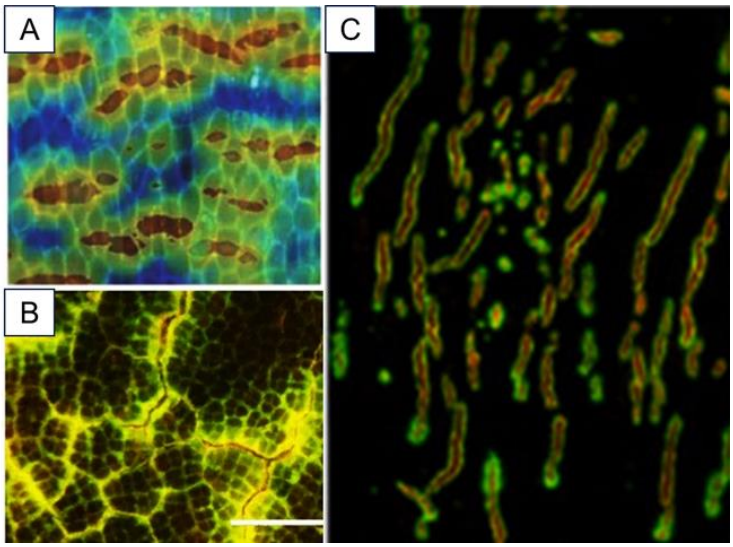

Fig. 2. Fluorescence micrographs of microcracks observed after incubation in 0.1% acridine orange (fluorescent tracer) in the surface of (A) sweet cherry, (B) apple, and (C) plum. Modify from Peschel and Knoche (2005), Knoche et al. (2018), Knoche et al. (2019).

1.1.3. Zipper model.

The mechanism of rain cracking has been studied extensively in sweet cherry in the latest decades. The zipper model proposed by Winkler et al. (2016) is the current hypothesis that provides a plausible explanation for cracking that is consistent with all the experimental evidence. The model is based on a series of events that lead to a macrocrack formation (Fig. 3).

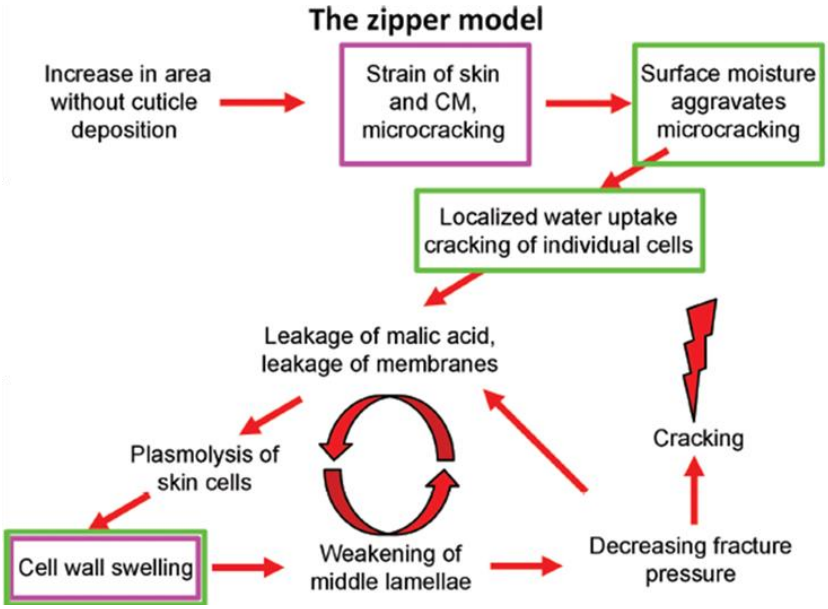

Fig. 3. Sketch of zipper model that explains processes involved in rain cracking of sweet cherry fruit (Modify from Knoche and Winkler (2019)).

I. Microcracks on the skin develop as a consequence of the stress during fruit growth (Peschel and Knoche, 2005). Furthermore, surface wetness after rain or the exposure of the skin to high humidity promotes the formation of microcracks in the cuticle membrane (CM) of sweet cherries since the fracture force of the hydrated CM decreases (Knoche and Peschel, 2006).

II. Water uptake occurs through microcracks in the cuticle and focuses water uptake on a specific region of the epidermis. The symplast of the skin cell takes up water osmotically. Flesh cells are parenchyma cells that are structurally weak and crack easily (Grimm and Knoche, 2015).

III. The bursting of individual cells releases cell constituents among those, malic acid into the apoplast. Malic acid extract cell wall-bound Ca weakens cell walls and increases the permeability of the plasma membrane of neighboring cells (Winkler et al., 2015). Leakage of cells triggers a chain reaction causing other cells to lose solutes into the apoplast (Winkler et al., 2016)

IV. Cell turgor is lost when epidermal cells are plasmolyzed by juice from the flesh that generates a swelling of cell walls (Grimm and Knoche, 2015). The swelling of cell walls decreases stiffness, fracture tension, and cell adhesion thereby allowing neighboring cells to separate along their cell walls (Brüggenwirth and Knoche, 2017).

V. The process of cell bursting continues and promotes the crack to extend. The microcrack extends into a macrocrack. The skin becomes ‘‘unzipped", this could be compared as a ‘‘ladder" will spread in a piece of fine, knitted fabric (Winkler et al., 2016).

1.2. Fruit morphology and growth

1.2.1. Fruit morphology

Botanically, a strawberry is a pseudocarp or a false fruit comprised out of a succulent receptacle carrying achenes, which are the real fruit (Fig. 4). A single flower has many carpels; from each carpel, an achene derives making the strawberry an aggregate fruit (Darrow, 1966; Hancock, 2020). The shape of the fruit varies from globose, to oblate, to conical, with or without a neck. Achenes are distributed in spirally arranged rows on the receptacle; their position on the surface varies across cultivars, ranging from very depressed to shallow (Darrow, 1966). Mature and pollinated achenes are ovate and about 1 mm in length; they are composed of a hard and thick

pericarp, a thin testa, an endosperm consisting of one cell layer, and a small embryo (Perkins-Veazie, 1995). The strawberry receptacle comprises the epidermis, cortex, bundle zone, and pith (Darrow, 1966) (Fig. 4).

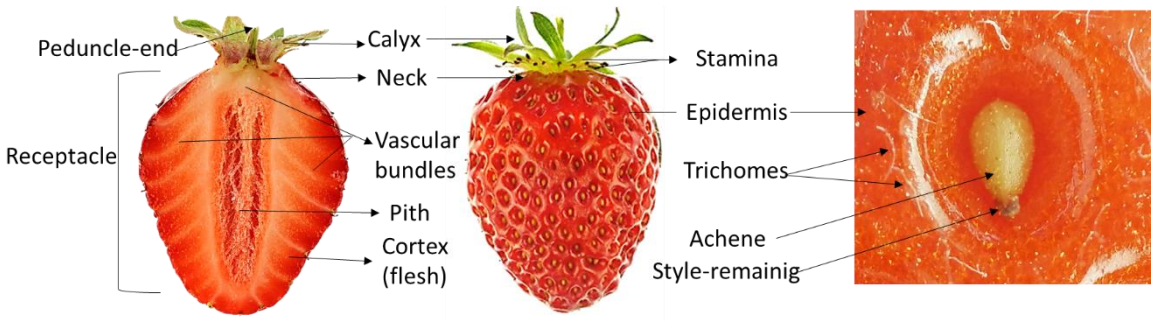

Fig. 4. Components of strawberry fruit.

The epidermis is pubescent covered by a slightly waxy cuticle; it comprises a single layer of tangentially elongated polygonal cells and trichomes (hairs) (Mauseth, 2016) characterized to be long, pointed, and with thick-walled cells (Polito et al., 2002; Szczesniak and Smith, 1969). Stomata were rarely found in the epidermis of some cultivars (Blanke, 2000; Perkins-Veazie, 1995). Beneath the epidermis, there is not a distinct hypodermis, unlike most fleshy fruits. Instead, a sub-epidermis of cortical cells was found (Polito et al., 2002). The cortex is a fleshy part; it is formed by rounded cells with long intercellular spaces. As the cells enlarge, the tissue becomes parenchymatous, characterized by thin-walled, isodiametric, vacuolate cells (Szczesniak and Smith, 1969; Polito et al., 2002).

The bundle zone consists of a central ring of vascular bundles with branch vessels connected to the achenes. These vessels are the xylem and phloem (Darrow, 1966). The xylem consists of dead cells with secondary thickening in the form of rings, spirals, and nets. Bundles supply nutrients to achenes (Antoszewski, 1973) and offer mechanical support to the receptacle (Sharma et al., 2019). The pith is localized at the center and composed of thin-walled cells that frequently pull apart during the growth of the fruit generating large cavities since the cortex often grows much quicker than the pith (Szczesniak and Smith, 1969).

1.2.2. Fruit development and growth

Strawberry fruit is the most competitive sink in the plant, accumulating 20‑40% of the total plant dry weight (Hancock, 2020); it develops rapidly compared to other soft fruits. It takes from 20 to 40 days after anthesis to get a ripe fruit (Darrow, 1966). The growth pattern is sigmoidal or double sigmoidal depending upon conditions, cultivars, and sample frequency

(Perkins-Veazie and Huber, 1988; Nitsch, 1950). The double sigmoidal growth curve shows two periods of rapid growth. The first period is related to the achene development (endosperm and embryo), and the initial growth of the receptacle. The second period is associated with cell enlargement of the receptacle. Achene's embryo maturation occurs before the final growth of the receptacle (Perkins-Veazie and Huber, 1988).

Cells in the cortex and pith accounted for most of the berry size (Hancock, 2020). The size increase is due to a combination of cell division and expansion. The length of the cell division period is uncertain in strawberries and varies among cultivars. Cell division has been reported to stop 7 days after a petal fall and 15 days after anthesis in the cortex (Cheng and Breen, 1992; Perkins-Veazie, 1995). While Havis (1943) found that after anthesis only 15 to 20 % of the pith growth and about 10% of the cortex growth was due to cell division.

Fruit development is affected by factors such as the number of achenes on the receptacle and the level of fertilization of the carpels (Nitsch, 1950; Abbott et al., 1970). At least 30% of the carpels need to be successfully pollinated to develop a well-shaped ripe fruit (Hancock, 2020). Receptacle growth is mainly regulated by the auxin synthesis in the achenes (Nitsch, 1950). Auxin is translocated basipetal through the phloem of vascular bundles from the achenes to the peduncle (Antoszewski, 1973).

The final size of the fruit depends on endogenous factors such as the position of flower buds, the duration of cell division in each layer of the fruit, the degree of cell enlargement, the number of cells, and the size of intercellular air spaces and inherent differences in achene activity and receptacle sensitivity. Exogenous factors that affect fruit size are the temperature at the time of planting or during fruit set, and plant nutrition (Darrow, 1966; Cheng and Breen, 1992; Perkins-Veazie, 1995).

Strawberries are non-climacteric; ripening occurs within the fruit development. Fruit ripening is triggered by a gradual decline in free auxin in the receptacle as a result of achene maturation (Archbold and Dennis, 1984). Ripening includes the degradation of chlorophyll, the accumulation of anthocyanins, softening that is partially mediated by cell wall-hydrolyzing enzymes, the metabolism of sugars and organic acids, and the production of flavoring compounds. The process is rapid, generally occurring within 5 to 10 days after the receptacle turns white, depending on temperature (Sharma et al., 2019; Perkins-Veazie, 1995).

As strawberries ripen different changes occur principally in the cortical parenchyma cells (Neal, 1965). At petal fall, cells have thick cell walls, plastids with starch grains, and small vacuoles; tubular proliferations of the tonoplast (vacuole membrane) start to appear and become extreme at ripeness (Knee et al., 1977), these tubular proliferations may be sites involved in anthocyanin synthesis (Grisebach, 1982). After 14 d of petal fall, cell walls commence to swell and consist of outer diffuse, and inner more densely fibrillar layers (Knee et al., 1977). At the white stage (21 d after petal fall), cells are expanded and vacuolated, plastids have degenerated, and most of the starch has disappeared (Szczesniak and Smith, 1969; Perkins-Veazie, 1995). During ripening, cells are enlarged, and vacuolated; cell walls are extremely swollen and separated at the middle lamella; cellular junctions turned into small projections of the cells which were connected at the tips (Neal, 1965; Knee et al., 1977; Polito et al., 2002).

Softening on strawberries is principally caused by the cell wall disassembly and the reduction of cell adhesion, resulting from the dissolution of the middle lamella of the cortical parenchyma cells (Brummell and Harpster, 2001; Santiago-Doménech et al., 2008). Cell wall disassembly in strawberries is consistently attributed to solubilization of pectins, slight depolymerization of covalently bound pectins, a loss of galactose and arabinose, and a reduction in the hemicellulosic content (Knee et al., 1977; Santiago-Doménech et al., 2008; Posé et al., 2019; Posé et al., 2011); therefore many studies focused in the role of pectinolytic enzymes, e.g., pectate lyases, polygalacturonase, and pectin methyl esterase (Santiago-Doménech et al., 2008; Liu et al., 2023; Paniagua et al., 2016).

1.3. Water movement through fruit surface

The movement of water to and from the fruit occurs through the surface as transpiration and osmotic uptake or also through the vascular system. Water movement is an important factor in many surface disorders in soft fruits, e.g. cracking and shriveling, in cherries (Christensen, 1996; Schlegel et al., 2018), grapes, (Considine and Kriedemann, 1972), gooseberries, jostaberry, and black currants (Khanal et al., 2011), and tomatoes (Frazier, 1947). Water movement may play a role in shriveling, cracking, and water-soaking of strawberries; however, little is known about water movement through the strawberry fruit skin.

1.3.1. Transpiration

Transpiration or water loss is a physical process that involves the diffusion of water molecules out of the plant tissue or organ into the surrounding air. Transpiration is proportional to the water vapor pressure gradient between the surface of the commodity and the surrounding air, and inversely proportional to the resistance to transpiration e.g. the cuticle, stomata, and suberized surfaces (Díaz-Pérez, 2019).

Transpiration rate (F_t, kg s^{-1}) is usually determined gravimetrically, assuming that the weight loss due to respiration is negligible, therefore the weight change is due to water loss (Díaz-Pérez, 2019; Knoche, 2015). F_t on fruits may be influenced by morphological characteristics, development (surface area), maturity stage, surface injuries, presence of stems/pedicels or calyx, cultivars, and environmental factors such as storage temperature, relative humidity, air movement, and atmospheric pressure (Díaz-Pérez, 2019; Athoo et al., 2015; Becker and Knoche, 2011; Burghardt and Riederer, 2006).

To compare transpiration properties from different species, cultivars, systems, and experiments, the permeance to water vapor of the fruit surface (P_t; m s^{-1}) can be useful. P_t can be calculated, using F_t described by Fick's law of diffusion (Eq. 1) (Becker and Knoche, 2011); where Δa_w represents the gradient in water activity across the fruit skin (dimensionless), that is used as the driving force (Lange et al., 1982); because the humidity above dry silica is practically zero, Δa_w equals the water activity of the strawberry juice, which is approximately one. ρ_w is the density of water (kg m^{-3}), A_{fruit} is the surface area of the fruit (m^2). F_t is only diffusional since a pressure difference across the cuticle is absent, (Knoche, 2015).

$$P_t = \frac{F_t}{A_{fruit} \cdot \rho_w \cdot \Delta a_w} \qquad \textbf{[Eq.1]}$$

1.3.2. Osmotic water uptake

Osmotic water uptake occurs through the wetted fruit surface after rain or dew. The mechanism is mainly mass flow and to a smaller extent diffusional. The driving force for osmotic uptake is the gradient in water potential ($\Delta\Psi$) between the water potential of the fruit (Ψ_{fruit} in MPa) and the water potential of the incubation solution (Ψ_{water} = 0 MPa) (Beyer et al., 2005). Ψ_{fruit}

comprises the osmotic solute potential (Ψ_Π) and turgor or also known as hydrostatic pressure (Ψ_p). Gravitational and matric potentials are negligible.

Osmotic solute potential reaches negative values due to the accumulation of solutes in the fruit; usually is measured from the juice obtained using a garlic press and reflects the osmotic properties of the flesh's symplast, since the volume is greater compared to the volume of the skin and apoplast area (Beyer et al., 2005). Hydrostatic pressure or turgor has positive values and tends to limit the ingress of water. A gradient of Ψ_p represents the driving force for water flow in the xylem and influences the mechanical integrity of the fruit (Matthews and Shackel, 2005). Knoche et al. (2014) established that the turgor in mature sweet cherries is very low, between three orders of magnitude lower. This may be also the case for strawberries, Pomper and Breen (1995) estimated, based on water potential and osmotic potential measurements, low values of turgor (~0.05 MPa) for ripe strawberries probably due to the presence of apoplastic solutes. The negligible values of turgor in ripe fruits allow the assumption that $\Psi_{fruit} \approx \Psi_\Pi$.

In analogy to transpiration, the permeance for osmotic water uptake (P_f; m s^{-1}) can be determined using the filtration permeability (Eq. 2) (House, 1974); where F_f represents the rate of water uptake (kg s^{-1}), A_{fruit} the surface area (m^2), $\Delta\Psi$ the difference in water potential (MPa), ρ the density of water (kg m^{-3}), R the universal gas constant (m^3 MPa K^{-1} mol^{-1}), T the absolute temperature (K), and $\bar{V}_w$ the molar volume of water (m^3 mol^{-1}) .

$$P_f = \frac{F_f}{A_{fruit} \cdot \Delta\Psi} \cdot \frac{RT}{\rho \bar{V}_w} \qquad \textbf{[Eq.2]}$$

P_t and P_f may be determined on a whole-fruit basis and represent the weighted mean of all parallel water pathways contributing to water movement through the surface of the fruit. As a consequence of the different movement mechanisms for transpiration and uptake: P_f is more variable than P_t, also P_t may be less susceptible to surface defect, but more susceptible to changes in temperature than P_f, (Beyer and Knoche, 2002; Becker and Knoche, 2011; Knoche, 2015), whether it is the case in strawberries is still unknown.

1.3.3. Vascular flow.

Vascular flow through the pedicle/stem has been studied in various fruits such as sweet cherries (Brüggenwirth et al., 2016), apples (Lang, 1990), grapes (Düring et al., 1987), plums (Khanal et al., 2021) and recently also in strawberries (Winkler et al., 2021).

It has been established that the net balance of the xylem, phloem, and transpiration flows of strawberries change during development. Xylem inflow was 70–80% of total water inflow at early stages, towards maturity this decreased to ~36%. In contrast, phloem inflow increased from 20 - 30% of the total at early stages, rising to ~64% towards maturity. The outflow of water increases towards maturity due to transpiration, the calyx accounted for ~50% of total fruit transpiration. The permeance of the calyx seems to be high and is probably accounted for the high density of trichomes and stomata on the abaxial surface (Winkler et al., 2021).

During the development of strawberries, the functionality of the xylem decreases. From this perspective, the pseudocarp strawberry does not differ from the true fruit of many other fruit crop species. The decrease in functionality may be attributed to stretching or breakage of the xylem caused by growth strain (Düring et al., 1987; Lang and Ryan, 1994; Grimm et al., 2017). As a consequence of this phenomenon, the import of calcium (Ca) into the fruit through the xylem is also expected to decrease (Winkler et al., 2021).

1.3.4. Preferential pathways

Water movement through the surface may occur through several parallel pathways: the cuticle, stomata, lenticels, microcracks, abscission zones, stylar scar, the pedicel-fruit juncture, and the pedicel/stem-end (Knoche and Measham, 2013). The contribution of each pathway to transpiration and uptake may differ depending on the mechanism of water movement (Knoche, 2015). For strawberries, pathways may include the cuticle, calyx, microcracks, abscission zones of petals and stamina, stem/receptacle juncture, and the stem end. Quantitative information, however, is lacking.

The cuticle is the primary barrier to water transport. Abrading the cuticle increased the rates of transpiration and uptake for grapes (7 and 73 fold) and sweet cherries (5 and 33 fold) (Knoche and Winkler, 2017; Becker and Knoche, 2011). Water uptake through the cuticle takes place along a polar pathway in the cutin matrix. These polar pathways offer an aqueous continuum of pores across the cuticle that allows transport by viscous flow (Franke, 1964; Weichert and Knoche, 2006).

Microcracks are important pathways for water movement. In transpiration, they can increase rates but they have a higher effect on rates of water uptake because they allow water to bypass the cuticle and enter the fruit (Grimm et al., 2012a; Grimm et al., 2012b; Peschel and Knoche, 2005; Knoche, 2015). Surface wetness or high humidity after an episode of rainfall enhanced

microcracking in sweet cherry and grape (Knoche and Peschel, 2006; Becker and Knoche, 2012b)

Abscission zones form when flowering parts separate and/or fall off during the early stages of fruit development. Occasionally, small areas of periderm form at the abscission points. In crops like currants, jostaberry, and gooseberry, the dried remnants of the flowering organs persist and remain attached to the apex of the fruit. These structures can act as preferential routes for water uptake and have been found to contribute to approximately 30% of the water uptake (Khanal et al., 2011). In grape berries, the periderms of the abscission zones and lenticels on the pedicel represented regions of preferential water uptake that contributed 21,3% (abscission zones) and 18,2% (lenticels) of the total uptake, respectively (Becker et al., 2012). The specific contribution of these structures in transpiration has not been thoroughly assessed yet.

The juncture between pedicel/stem and fruit may be a region of preferential water movement, particularly for uptake. In this region, the cuticle is discontinuous (Knoche and Winkler, 2017). A high density of microcracks may be expected due to higher stress concentration (Considine and Kriedemann, 1972; Peschel and Knoche, 2005) and, possibly, extended periods of surface wetness after rainfall (Knoche and Winkler, 2017). The relative contribution to water uptake increases as maturity proceeds. Weichert et al. (2004) established that by sealing the pedicel/fruit juncture in sweet cherry, water uptake decreased by 46% on average for different cultivars, while in grape berries, Becker et al. (2012) determined that the stem-fruit junction contributed to 24,9% of the total water uptake.

1.4. Role of cuticle in fruit skin disorders

1.4.1. The Cuticle and its functions

The cuticle membrane (CM) is a non-cellular, polymeric membrane that covers the outer primary surfaces of organs of terrestrial plants. The lipophilic CM is comprised of polymeric cutin, waxes (epi or intra-cuticular), and polysaccharides encrusting the outer cell wall. In some species, cutan and flavonoids occur in significant amounts (Martin, 1964; Jeffree, 1996; Domínguez et al., 2011). Järvinen et al. (2010) established that the cutin of strawberry is highly resistant to depolymerization probably due to the presence of a high amount of cutan-type compounds. The predominant component of the cutin was 9(10),16- dihydroxy hexadecanoic acid. Wax components of the strawberry have not been reported yet.

The CM is deposited on the outer cell wall of the epidermal cells and separated from the cell wall by a pectin layer. The CM acts as a barrier and gateway between the fruit and its environment. Therefore, it limits water movement and restricts gas exchange (Schönherr, 1976; Domínguez et al., 2011; Jeffree, 1996) and offers protection against pathogens, pests, and mechanical injury from the environment (Martin, 1964; Arya et al., 2021). Some CMs may also reflect the UV-B light and allow self-cleaning (the lotus effect) with their very high water repellency (Barthlott and Neinhuis, 1997). To carry out these functions effectively, the cuticle must maintain its integrity throughout the fruit development.

1.4.2. Cuticle deposition, stress and strain during fruit growth.

Stress refers to the forces acting on a material per unit of area, while strain is the resulting fractional deformation (compression or stretching) of the material due to those forces. During fruit growth, the fruit skin is subject to significant stress and strain, this strain also involves the cuticle (Lai et al., 2016; Khanal and Knoche, 2017). The dermal tissues can accommodate the strain generated during the fruit expansion by cell division and enlargement. However, this becomes a challenge for the polymeric cuticle that leans on the deposition of new cuticular material.

The strain that accumulates in the cuticle can be reversible (elastic and viscoelastic) or irreversible (plastic) strain. The type of strain accumulated depends on the relationship between the rate of cuticle deposition and the rate of increase in fruit surface area. In tomatoes (Knoche and Peschel, 2007b) and apples (Knoche et al., 2011; Lai et al., 2016), cuticle deposition continues throughout the entire growth. The deposition of new cuticular material through the addition of cutin and the incorporation of intracuticular wax fixes the elastic strain and transforms it into a plastic strain (Khanal et al., 2013; Khanal et al., 2014).

On the other hand, in sweet cherries (Knoche et al., 2004), grape berries (Becker and Knoche, 2012a), ribes berries (Khanal et al., 2011), mango (Athoo et al., 2021) and plums (Knoche and Peschel, 2007a) cuticle deposition is limited to early stages of development and decreases or even ceases in later stages. Consequently, as the fruit matures, there is a decline in cuticle thickness, an increase in elastic strain, and an increasing risk of microcracking. Contigiani et al. (2018) reported a cuticular thickness of 0.6 µm for mature “Albion” strawberries using transmission electron microscopy. Such a thin cuticle may be the result of an imbalance between cuticle deposition and fruit surface area expansion; however, definitive evidence is missing.

1.4.3. Fruit skin disorders related to microcracking in the cuticle.

The impairment of the cuticle through microcracks may become a problem during the harvesting and post-harvesting phases (Lara et al., 2014) Microcracks allows the development of many skin-related disorders such as shriveling and skin browning in plum and litchi (Stösser and Neubeller, 1985; Knoche et al., 2019; Underhill and Simons, 1993), maturity bronzing in banana (Williams et al., 1990), cracking of fleshy fruit (Opara et al., 1997; Herrington et al., 2011; Considine and Kriedemann, 1972; Skene, 1980), russeting in apple, pear and mango (Athoo et al., 2021; Khanal et al., 2013; Scharwies et al., 2014), and skin spot in apple (Winkler et al., 2014).

Microcracking of the cuticle occurs in a specific pattern depending on the species. In sweet cherries and plums, failure of the cuticle is most common above the periclinal cell walls of epidermal cells (Peschel and Knoche, 2005; Knoche and Peschel, 2007a). On the other hand, in apples, bananas, and litchi microcracks follow the pattern of anticlinal cell walls of groups of epidermal cells (Curry, 2009; Grimm et al., 2012b; Knoche et al., 2018). There is no information on microcracking for strawberries.

It is important to point out that the cuticle is responsible for the barrier properties of the fruit skin. The mechanical backbone of the skin, however, is formed by the underlying dermal cell layers (Brüggenwirth et al., 2014; Khanal and Knoche, 2017; Bargel and Neinhuis, 2004).

2. Gap of knowledge

Despite the economic importance of rain damage in strawberries, little is known about the underlying physiological mechanisms of the two major rain-related disorders, water soaking and cracking. Essential aspects of these disorders are poorly understood and require detailed investigation. These include the following:

(1) Water movement through the fruit surface, driven by transpiration (in dry air), and water uptake through osmosis (when the fruit surface is wet), plays a crucial role in various fruit skin disorders (Opara et al., 1997; Knoche, 2015). However, the understanding of the mechanism of water movement through the strawberry fruit skin, along with any preferential pathways and related factors, remains limited.

(2) Strawberry fruit develops over a short period (20-40 days), suggesting that the fruit skin is subject to very high rates of strain (Darrow, 1966; Considine, 1982; Khanal et al., 2011; Grimm et al., 2012b). Consequently, extensive microcracking may be expected which compromises fruit quality and induces fruit skin disorders (Knoche and Lang, 2017). Although the importance of cuticle integrity, to our knowledge, there is no information on cuticle deposition and microcrack formation in strawberries.

(3) The mechanism of cracking in strawberries is unknown. Due to the shape and uneven surface topography of strawberries, the mechanism of failure may be more complex and not completely explained by the theoretical model of a ‘thin-walled pressure vessel’ applicable to smooth-skinned fruits like cherries and grapes (Considine and Brown, 1981; Considine, 1982; Considine and Kriedemann, 1972).

(4) Water soaking on the strawberry fruit surface is the most frequently occurring rain damage. The mechanism of water soaking is not known. A model that may elucidate the water-soaking mechanism is the zipper model developed for sweet cherries (Winkler et al., 2016). However, definitive evidence on the mechanism is lacking.

(5) Calcium applications improved firmness and mechanical properties of strawberries (Dunn and Able, 2006; Langer et al., 2019; Lara et al., 2004; Cieniawska et al., 2023). However, there is no published information on the possible benefits of calcium in water soaking. Sprays of Ca salts are reported to decrease susceptibility to cracking in sweet cherries, due to the hypothetical similarities between the mechanism of cracking in sweet cherries (Winkler and Knoche, 2019) and water soaking in strawberries, such beneficial effects may not be unlikely.

3. Objectives

The objectives of this dissertation were:

(1) To identify the mechanisms of water movement through the surface of detached strawberry fruit (Chapter 4.1).
(2) To identify the dominant pathways of water movement through the strawberry fruit surface, and the external factors affecting the rates of movement (Chapter 4.2).
(3) To characterize microcracking of the cuticle of strawberry skin and to identify the key factors affecting cuticular microcracking (Chapter 4.3).
(4) To identify the mechanism of cracking and the factors affecting cracking in strawberry (Chapter 4.4).
(5) To identify the triggers, factors, and mechanisms underlying the water soaking disorder in strawberry fruit (Chapter 4.5).
(6) To establish the effects of Ca and other monovalent, divalent, and trivalent cations on water soaking in strawberry (Chapter 4.6).

4. Results

4.1. Strawberry fruit skins are far more permeable to osmotic water uptake than to transpirational water loss

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Figures 6(A-C), and 9(A-E) were part of Master thesis. Eckhard Grimm: Investigation, Methodology Martin Bruggenwirth: Conceptualization, Funding acquisition. Moritz Knoche: Conceptualization, Funding acquisition, Formal analysis, Project administration, Writing original draft, Writing review and editing.
Journal	PlosOne
Impact factor	3.752(2022)
Date of publication	13.05.2021
DOI	10.1371/journal.pone.0251351

PLOS ONE

RESEARCH ARTICLE

Strawberry fruit skins are far more permeable to osmotic water uptake than to transpirational water loss

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OPEN ACCESS

Citation: Hurtado G, Grimm E, Brüggenwirth M, Knoche M (2021) Strawberry fruit skins are far more permeable to osmotic water uptake than to transpirational water loss. PLoS ONE 16(5): e0251351. https://doi.org/10.1371/journal.pone.0251351

Editor: Hernâni Gerós, Universidade do Minho, PORTUGAL

Received: January 5, 2021

Accepted: April 24, 2021

Published: May 13, 2021

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: All funding for this study was supported by grant KN 402/19-1 from the Deutsche Forschungsgemeinschaft. The publication of this article was funded by the Open Access fund of Leibniz Universität Hannover. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the

Abstract

Water movements through the fruit skin play critical roles in many disorders of strawberry (*Fragaria* × *ananassa* Duch.) such as water soaking, cracking and shriveling. The objective was to identify the mechanisms of fruit water loss (dry skin, transpiration) and water uptake (wet skin, osmosis). Fruits were held above dried silica gel or incubated in deionized water. Water movements were quantified gravimetrically. Transpiration and osmotic uptake increased linearly with time. Abrading the thin cuticle (0.62 g m^{-2}) increased rates of transpiration 2.6–fold, the rates of osmotic uptake 7.9-fold. The osmotic potential of the expressed juice was nearly the same for green and for white fruit but decreased in red fruit stages. Fruit turgor was low throughout development, except for green fruit. There was no relationship between the rates of water movement and fruit osmotic potential. The skin permeance for transpiration and for osmotic uptake were both high (relative to other fruit species) but were two orders of magnitude greater for osmotic uptake than for transpiration. Incubating fruit in isotonic solutions of osmolytes of different sizes resulted in increases in fruit mass that depended on the osmolyte. The rate of osmotic uptake decreased asymptotically as molecular size of the osmolyte increased. When transpiration and osmotic uptake experiments were conducted sequentially on the same fruit, the rates of transpiration were higher for fruit previously incubated in water. Fluorescence microscopy revealed considerable microcracking in a fruit previously incubated in water. Our findings indicate that the high permeance for osmotic uptake is accounted for by an extremely thin cuticle and by viscous water flow through microcracks and along polar pathways.

Introduction

Strawberry is a highly perishable commodity. The quality of strawberries at retail is often compromised by pre- and postharvest factors. Preharvest factors include the exposure of fruit to rain in the course of growth and development. Classical disorders related to rain exposure are fruit cracking and water soaking [1]. Both disorders are often followed by fruit rot, such as

manuscript. There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

grey mold. During the harvesting and subsequent postharvest storage, transport and handling, fruit water loss due to transpiration is critical. Ultimately it results in shrivel and in compromised appearance. In addition, the mass loss that occurs along the marketing chain requires fruit containers to be 'over-packed' so as to ensure a pre-specified weight for the consumer. Compromised fruit quality and overpacking of fruit containers both cause significant financial loss.

Water movement through the fruit surface is likely to be a critical factor in all the above disorders. Although the mechanistic bases of strawberry fruit cracking and water soaking have not been investigated in great detail, research on similar disorders in other fruitcrop species indicates the involvement of water uptake directly through the fruit surface and, possibly also of water uptake through the fruit vascular system [2]. Such research includes the rain cracking of soft, fleshy fruits such as apples (*Malus* × *domestica* Borkh.) [2,3], blueberries (*Vaccinium corymbosum* L.) [4], plums (*Prunus domestica* L.) [5], tomatoes (*Solanum lycopersicum* L.) [6], citrus lemon (*Citrus* ×*limon* (L.) Burm. f.) [7], grape berries (*Vitis vinifera* L.) [8], sweet cherries (P*runus avium* L.) [9], jostaberry (*Ribes nidigrolaria* B.), gooseberry (*Ribes uva-crispa* L.), and black currant (*Ribes nigrum* L.) [10]. For strawberry fruit, water loss through the fruit skin is likely to be a critical factor in shriveling and compromised appearance. For sweet cherry, shrivel-type phenomena such as 'orange peel' disorder are caused by skin dehydration, with the latter exacerbated if fruit are allowed to transpire excessively [11–13].

The above arguments indicate that water loss through the strawberry fruit surface by transpiration (in dry air) and water uptake by osmosis (when the fruit surface is wetted) are likely to be the major determinants in the shriveling, cracking and water soaking of strawberries. Little is known about water movement through the strawberry fruit skin. It is worth noting that a strawberry 'fruit' has an unusual morphology. It is primarily comprised of receptacle tissue—a strawberry is a pseudocarp or false fruit, not a true fruit comprised of mostly pericarp tissue. The actual fruits of a strawberry are the tiny achenes embedded in the surface of an expanded receptacle. The strawberry is also unusual in that it develops over quite a short period of time, suggesting that the fruit skin is subjected to particularly high rates of strain. A better understanding of these underlying factors should be helpful in developing improved strategies for strawberry breeding, cultivation and handling so as to reduce or eliminate fruit quality impairments.

The objective of this study is to identify the mechanism(s) of water movement in transpiration (loss in dry air) and osmosis (uptake when wetted) through the surface of detached strawberry fruit.

Materials and methods

Plant material

Strawberry fruit were harvested from commercial plantings at Gleidingen, Bad Nenndorf, research plots of the Horticultural Research Center in Cologne-Auweiler and from the green house and growth chamber facility at the Campus Herrenhausen of Leibniz University, Hannover, Germany. Temperature and relative humidity (RH) of the growth chamber were set at 20/16˚C and 60/80% RH during a 16 h day/night photoperiod.

Unless specified otherwise, fruit were harvested randomly and at commercial ripeness (>80% of the fruit surface red). Fruit of the same size, shape and color and free of visible defects were selected. Fruit was processed fresh on the day of sampling or held at 2˚C and 80% RH for no longer than 2 d. Previous studies showed that holding fruit for up to 2 d under these conditions had no effect on rates of water uptake or transpiration. Unless otherwise specified, the calyx was removed from the fruit by carefully pulling and the resulting hole sealed using a

fast-curing silicone rubber (Silicone rubber, SE 9186 Clear; Dow Corning Corp., Midland, USA). The number of individual fruit replicates was 15 unless otherwise specified.

General procedure

For transpiration experiments, fruits were incubated for 1.5 h in a polyethylene (PE) box, usually above dry silica gel (RH~0%; [14]) and weighed individually at 30-min intervals. The rate of transpiration (F_t; mg h^{-1}) was calculated on an individual fruit basis as the slope of a linear regression line fitted through a plot of fruit mass versus time.

For osmotic uptake experiments, fruits were incubated individually in deionized water for 1.5 h. Osmotic uptake was determined gravimetrically. Fruits were carefully blotted using soft tissue paper and then weighed at 30-min intervals. The rate of osmotic uptake was calculated (F_f; mg h^{-1}) on an individual fruit basis from the slope of a linear regression line fitted through a plot of fruit mass versus time.

All experiments were carried out in a temperature controlled laboratory at 22˚C.

Experiments

The time courses of osmotic uptake and transpiration were established in 'Clery' fruits. To ensure the repeated handling and blotting of fruit in the osmotic uptake experiment did not damage the fruit surface, rates of osmotic uptake of fruit blotted and weighed multiple times (at 0, 0.5, 1, 1.5 and 3 h) and of fruit blotted and weighed just once (at 3 h) were compared.

The effect of the cuticle on water movement was studied by abrading the cuticle from the fruit surface of 'Florentina' using sand paper (grain 400). Non-treated fruit served as control. Weighing intervals were modified in water uptake assays to avoid bursting of cells and leakage of osmolytes (osmotic water uptake) and excessive dessication (transpiration). Osmotic uptake was determined at 1 min intervals for up to 3 min for fruit with an abraded cuticle. Control fruit was measured at intervals of 5 min for up to 15 min.

The effects of fruit size were investigated by selecting 'Clery' fruit of differing mass. Fruit surface area was calculated from fruit dimensions determined from calibrated photographs (Lumix DMC-G80; Panasonic Corporation, Osaka, Japan) by image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions, Münster, Germany). Briefly, the fruit was assumed to represent a truncated cone capped by two halves of rotational prolate ellipsoids. Using this approximation fruit surface area (A) was calculated from the upper and lower diameters of the cone, cone height and the heights of the two rotational prolate ellipsoids, one on either end. The flow rates of transpiration (F_t) and osmotic uptake (F_f) were quantified as described above. The flux densities (kg m^{-2} s^{-1}) of water in transpiration (J_t) and in osmotic uptake (J_f) were calculated by dividing the transpiration flux and osmotic uptake flux by the corresponding fruit surface area.

The effects of juice osmotic potential on osmotic water uptake and transpiration were studied using 'Florentina' fruit of similar mass (mean 11.7 ± 0.1 g, range: 10.3–13.5 g) harvested at commercial ripeness, as indexed by color. Rates of osmotic uptake and transpiration were measured as described above. The osmotic potential of the expressed juice was determined by water vapor pressure osmometry (VAPRO 5600; Wescor, Utah, USA) on an individual fruit basis. The flow per unit osmotic potential was calculated by dividing F_t and F_f by the osmotic potential. This procedure normalizes for differences in driving force.

The effect of fruit development stage on transpiration and on osmotic uptake was studied in 'Clery' strawberry. Fruit growth was followed gravimetrically. Digital photographs were taken and the fruit surface area quantified from fruit dimensions using the model described above. The changes in color (CM-2600 d, orifice 3 mm diameter; Konica Minolta, Tokyo,

Japan) and in osmotic potential of the expressed juice (VAPRO 5600; Wescor, Utah, USA) were monitored at 5-d intervals until fully ripe, beginning at 8 d after full bloom (DAFB). Rates of transpiration and osmotic uptake were determined. The skin permeances in transpiration and in osmotic uptake were calculated as described below.

The fruit water potential (Ψ_{fruit}) was also determined using water-uptake experiments. 'Florentina' fruit were incubated in mixed solutions of increasing concentrations of fructose and glucose in equal molar ratios. These two monosaccharides represent the most abundant osmolytes in strawberry juice and together account for 63.5% of the osmolytes present [15]. Osmolarities of the incubation solutions were 0, 250, 500, 750 and 1000 mmol kg^{-1}. The time courses for the osmotic uptake experiment were established at 0, 0.5, 1, 1.5 and 30 h for all hypertonic solutions ($\geq$500 mmol kg^{-1}). For the hypotonic solutions, i.e. the water control and the solution of 250 mmol kg^{-1}, incubation was terminated after 1.5 h–longer incubations led to extensive fruit cracking. Rates of osmotic uptake were calculated for each incubation interval (0 to 0.5 h, 0.5 to 1 h, 1 to 1.5 h and 1.5 h to 30 h). A linear regression was fitted through a plot of the rate of uptake during each interval versus the osmotic potential of the incubation solution. From the regression parameters obtained, the hypothetical osmotic potential of a solution that would result in zero change in fruit mass was calculated. At this null point the Ψ_{Π} of the incubation solution would exactly equal the fruit water potential (Ψ_{fruit}) and a driving force for net water uptake is absent [16].

The effect of the molecular size of the osmolytes on net osmotic uptake into 'Florentina' strawberry was established by preparing incubation solutions at osmolarities that were isotonic to the Ψ_{Π} of juice expressed from the same batch of fruit. The osmolytes and their molecular masses were glycerol (92 g mol^{-1}), glucose (198.2 g mol^{-1}), sucrose (342.3 g mol^{-1}), polyethylene glycol (PEG) 1500 (1500 g mol^{-1}) and PEG 6000 (6000 g mol^{-1}).

The relationship between osmotic uptake and transpiration was studied by sequentially incubating 'Laetitia' fruit, first in water and then above silica gel and vice-versa. Thereafter, fruit were incubated in acridine orange (0.1%) (Carl Roth, Karlsruhe, Germany) for 5 min, then rinsed with deionized water and carefully blotted. The fruit surface was then inspected under incident white light and incident fluorescent light using a binocular microscope (Leica MZ10F with filter GFP plus 480–440 nm excitation, $\geq$510 nm emission; Leica Microsystems GmbH, Wetzlar, Germany).

Water potential, osmotic potential and turgor

To quantify fruit water potential (Ψ), the osmotic potential (Ψ_{Π}) and turgor (Ψ_p) were established in developing 'Clery' fruit grown in a growth chamber. The developmental stage of a fruit was indexed by the change in fruit color (CM-2600 d; Konica Minolta, Tokyo, Japan). Fruit was held at 4˚C for a maximum of 1 h before turgor measurement. The Ψ_{Π} was determined by water vapor pressure osmometry (VAPRO 5600; Wescor, Utah, USA) from the juice expressed using a garlic press. The Ψ_p of the cells of the outer flesh were determined using a cell pressure probe (CPP; [17,18]). The capillary was carefully inserted into the cells (< 0.5 mm below the fruit surface) under a horizontal microscope. Following volume correction, the peak pressure of the system was recorded. This pressure was taken as an estimate of Ψ_p. For a detailed description of the protocol the reader is referred to [18]. The water potential of a cell was calculated as the algebraic sum of the osmotic potential (negative) and the turgor (positive).

Calculating the permeances for osmotic water uptake and transpiration

The skin permeance for transpiration (P_t; m s^{-1}) was calculated as described earlier using flow rates determined in 'Clery', 'Florentina' and 'Laetitia' strawberry [19]. The P_t was calculated

from the rate of transpiration (F_t; kg s^{-1}) divided by the product of the fruit surface area (A; m^2), the density of water (ρ_w; kg m^{-3}) and the driving force for transpiration. In analogy to [20], the gradient in water activity ($\Delta\alpha_w$;dimensionless) across the fruit skin was used as the driving force (Eq 1). Because the humidity above dry silica is practically zero, $\Delta\alpha_w$ equals the water activity of the strawberry juice, which is approximately one.

$$P_t = \frac{F_t}{A_{fruit} \cdot \rho_w \cdot \Delta a_w} \quad (1)$$

The permeance for the opposite process of osmotic water uptake (P_f, m s^{-1}) was determined on the same cultivars using Eq 2. An alternate expression for the permeance in osmotic water uptake is the filtration permeability [21]. In Eq 2, F_f represents the rate of osmotic uptake, A the fruit surface area, and $\Delta\Psi$ (MPa) the difference in water potential between the water potential of the fruit (Ψ_{fruit}) and that of the incubation solution(Ψ_Π). For fruit incubated in water ($\Psi_\Pi = 0$) the driving force for osmotic uptake is essentially equal to Ψ_{fruit}. For mature fruit, Ψ_{fruit} was approximately equal to the osmotic potential of the expressed juice of the fruit (Ψ_Π). The value of Ψ_Π was determined by water vapor pressure osmometry (VAPRO 5600; Wescor, Utah, USA) following expression of the juice using a garlic press. The parameters R, T, V_w and ρ_w are all constants where R (m^3 MPa mol^{-1} K^{-1}) represented the universal gas constant, T (K) the absolute temperature, V_w (m^3 mol^{-1}) the molar volume of water and ρ_w (kg m^{-3}) the density of water. The permeance estimates P_t and P_f so obtained are directly comparable [19,22].

$$P_f = \frac{F_f}{A_{fruit} \cdot \Delta\Psi} \cdot \frac{RT}{\rho \cdot \bar{V}_w} \quad (2)$$

Mass of cuticle, cutin and wax

Mass of cuticle, cutin and wax was determined in 'Florentina' strawberries. Epidermal segments comprising cuticle, epidermis, and some adhering flesh were excised using a biopsy punch (6 mm diameter; Kai Europe, Solingen, Germany). The cuticular membrane (CM) was enzymatically insolated [23] by incubating in 50 mM citric acid buffer containing pectinase (90 ml l^{-1}; Panzym Super E flüssig, Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark) and cellulase (5 ml l^{-1}; Cellubrix L; Novozymes A/S) at room temperature. To prevent microbial growth, NaN_3 was added at a final concentration of 30 mM. The isolated CMs were carefully cleaned from adhering cellular debris using a soft, camel-hair brush and desorbed in deionized water. Achenes were manually removed. Samples of CMs (n = 10) were dried above silica gel for 48 h and weighed. Subsequently, CMs were extracted by incubation in $CHCl_3$/MeOH (1:1, v/v) for 24 h at room temperature. The dewaxed CMs (DCMs) were dried above silica gel for 48 h and their mass determined. CM mass per unit fruit surface area were calculated. The wax mass per unit area was calculated by subtracting the cutin mass per unit area from the cuticle mass per unit area. The experiment was carried out using 12 replicates.

Data analyses

All experiments were conducted and analyzed using completely randomized designs. Data were analyzed by analysis of variance and linear regression. Means were compared using Tukey's studentized range tests ($p < 0.05$) using R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Unless individual observations are shown, i.e. Figs 2–4, 5C and 9A, data are presented as means ± standard errors. All data shown in figures and tables are available in the S1 Dataset.

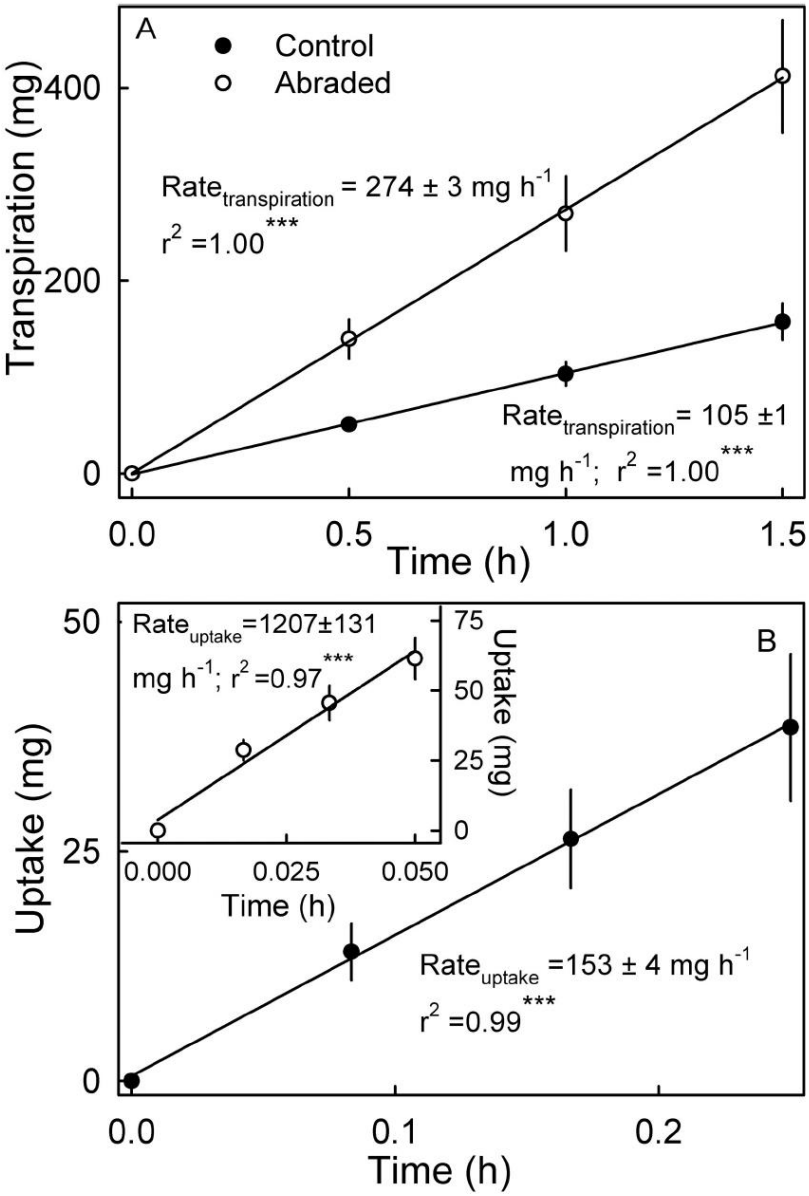

Fig 1. Effect of abrading the cuticle on water movement. (A) Transpiration and (B) osmotic water uptake. Significance of coefficients of determination (r^2) at $P < 0.001$ indicated by ***.

https://doi.org/10.1371/journal.pone.0251351.g001

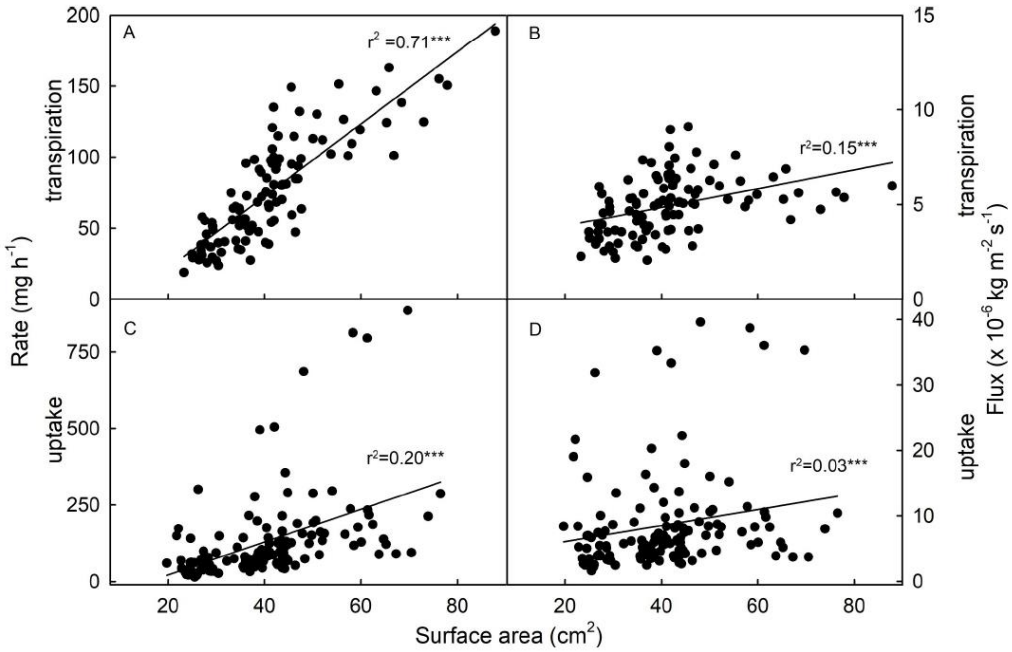

Fig 2. Relationship between fruit surface area and water movement. (A) Flow rates and (B) flux densities of transpiration from strawberry fruit. (C) Flow rates and (D) flux densities of osmotic uptake into strawberry fruit. Significance of coefficients of determination (r^2) at $P < 0.001$ and indicated by ***.

https://doi.org/10.1371/journal.pone.0251351.g002

Results

Osmotic water uptake rate and transpiration rate through the surface of mature strawberries increased linearly with time (Fig 1). There were no significant differences between the rates of osmotic uptake of fruit that were repeatedly weighed and blotted (91.8 ± 13.6 mg h^{-1}) and fruit that were weighed and blotted only once (124.8 ± 13.9 mg h^{-1}; $P < 0.115$). Abrading the cuticle increased the amount of water transpired or taken up osmotically compared to the control (Fig 1). The strawberry cuticle was extremely thin as indicated by a low mass per unit area of cutin and wax (Table 1). The wax content averaged 19.2%.

Not surprisingly, the flow rates (mass of water, per hour, per fruit) for transpiration and for osmotic uptake were positively and significantly related to fruit surface area. But this surface-area relationship was closer for transpiration than for osmotic uptake, as indexed by a higher coefficient of determination for transpiration (Fig 2A and 2C). Also, not surprisingly, dividing these water flow rates by the corresponding fruit surface areas revealed that the water flux densities (mass of water, per hour, per unit area of fruit surface) for transpiration and for osmotic uptake were markedly less dependent on fruit surface area (Fig 2B and 2D). For a representative dataset, there was no significant correlation between fruit size and fruit osmotic potential ($r = 0.01^{ns}$). This indicates the absence of a confounding interaction between size and osmotic potential for fruit of the same color maturity.

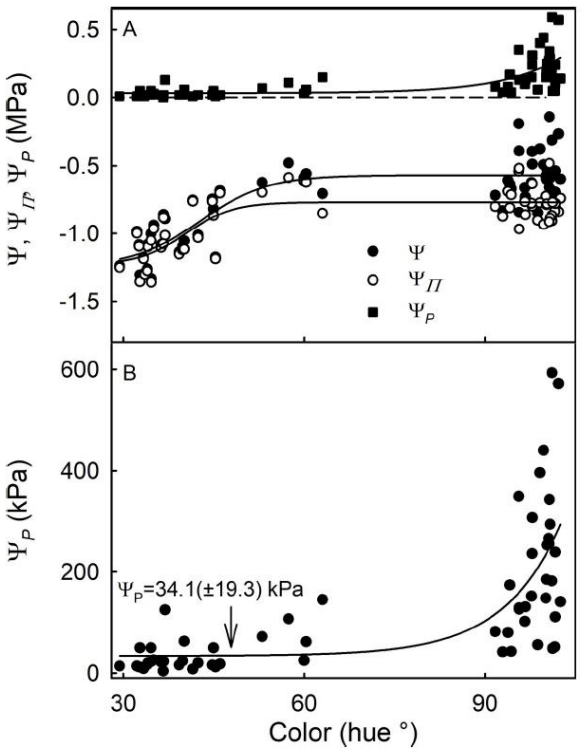

Fig 3. Water potentials in developing strawberries. (A) Calculated fruit water potential (Ψ), osmotic potential (Ψ_Π) and cell turgor (Ψ_P) of developing strawberry fruit; (B) Ψ_P in A but redrawn on a different scale. The value of Ψ was calculated as $\Psi = \Psi_\Pi + \Psi_P$. (Bars represent SE). The arrow indicates the Ψ_P at maturity.

https://doi.org/10.1371/journal.pone.0251351.g003

The osmotic potential of the expressed fruit juice was nearly constant for unripe fruit ranging from green (hue angle >90˚) to white (hue angle ≈ 60˚), but decreased and became more negative as the fruit turned red (hue angle <60˚) (Fig 3A). Fruit turgor (Ψ_P) was very low throughout most of the fruit-development period, compared to the negative values of fruit osmotic potential. Only during the early stages of development, when the fruit were still green, was cell turgor pressure significantly higher, when average values were about 200 kPa, with occasional peak values of up to 600 kPa being recorded (Fig 3B).

There was no relationship between the rate of transpiration or the rate of uptake and the osmotic potential of expressed juice for fruit on commercial ripeness (Fig 4A and 4B).

Skin permeances for transpiration (P_t) and for osmotic uptake (P_f) follow a log normal distribution as indexed by approximately symmetrical frequency distributions when plotted on a log scale and by linear normal probability plots (Fig 5A–5C). The permeance for osmotic uptake was 226-times larger than that for transpiration (Table 2). This huge dissimilarity is not unique to 'Clery' strawberry, but was of similar magnitude in 'Florentina' and 'Laetitia' (Table 1).

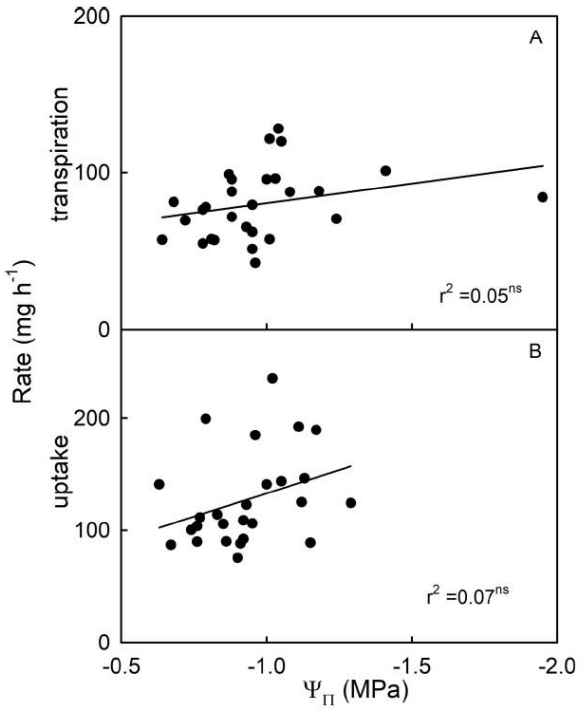

Fig 4. Relationship between osmotic potential (Ψ_Π) of a strawberry fruit's expressed juice and water movement. (A) Rates of transpiration and (B) Rate of osmotic water uptake. Coefficients of determination (r^2) not significant at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0251351.g004

The increase in strawberry fruit mass and, hence, in surface area during development followed a sigmoidal pattern with time (Fig 6A- main graph). Color change as indexed by the decrease in hue angle from green (>90˚) to white (≈60˚) and finally to fully red (<44˚) occurred at about 27 days after full bloom (DAFB) (Fig 6A—Inset). This corresponds to the phase of maximum mass growth rate and of maximum decrease (more negative) in osmotic potential (Fig 6B). Rates of transpiration and of osmotic uptake both increased markedly during development (Fig 6C- main graph). Meanwhile, rates of transpiration (per fruit) decreased during development, while rates of osmotic uptake (per fruit) increased markedly after the fruit turned red (Fig 6C- inset). The permeances (related to flux densities) for transpiration and osmotic uptake decreased as development progressed (Fig 6D). The permeance for osmotic uptake was markedly and consistently higher than that for transpiration—by about two orders of magnitude.

Rates of osmotic uptake depended on the osmotic potential of the incubation solution (Fig 7). Decreasing the incubation osmotic potential decreased the rate of osmotic uptake (Fig 7B). Interestingly, positive rates of osmotic water uptake were recorded from isotonic, and even from hypertonic solutions (Fig 7A). The rates of osmotic uptake were consistently higher during the first experimental interval (0.25 h), before decreasing towards an asymptote and then

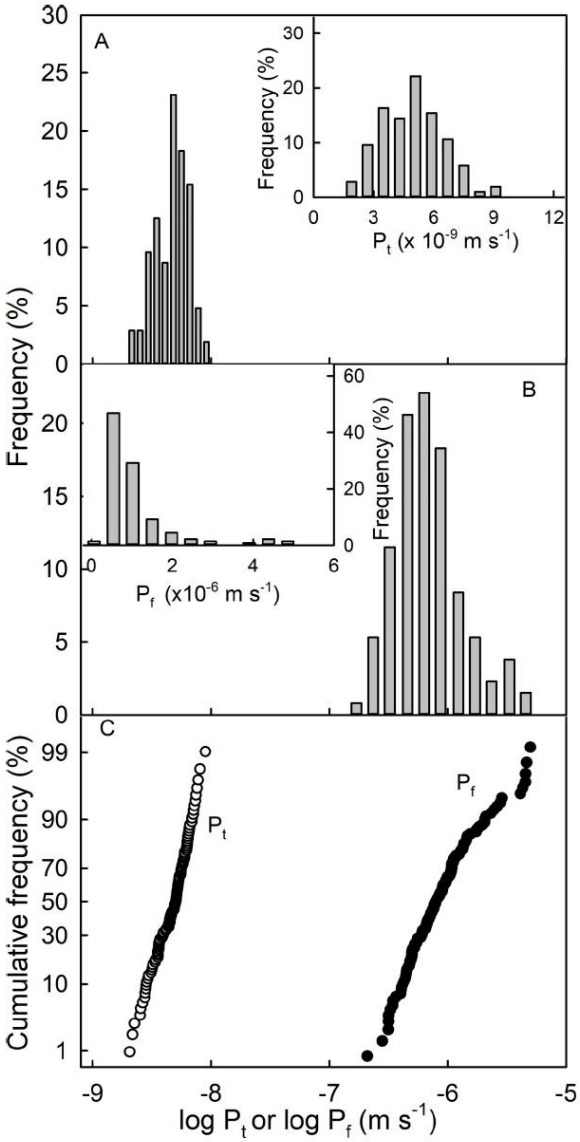

Fig 5. Frequency distributions of skin permeances of strawberries. (A) Log-transformed permeance for transpiration (P_t) (main graph) and un-transformed P_t (inset). (B) Log-transformed permeance for osmotic uptake (P_f) (main graph) and un-transformed P_f (inset). (C) Normal probability plot of the log-transformed permeance of P_t and P_f.

https://doi.org/10.1371/journal.pone.0251351.g005

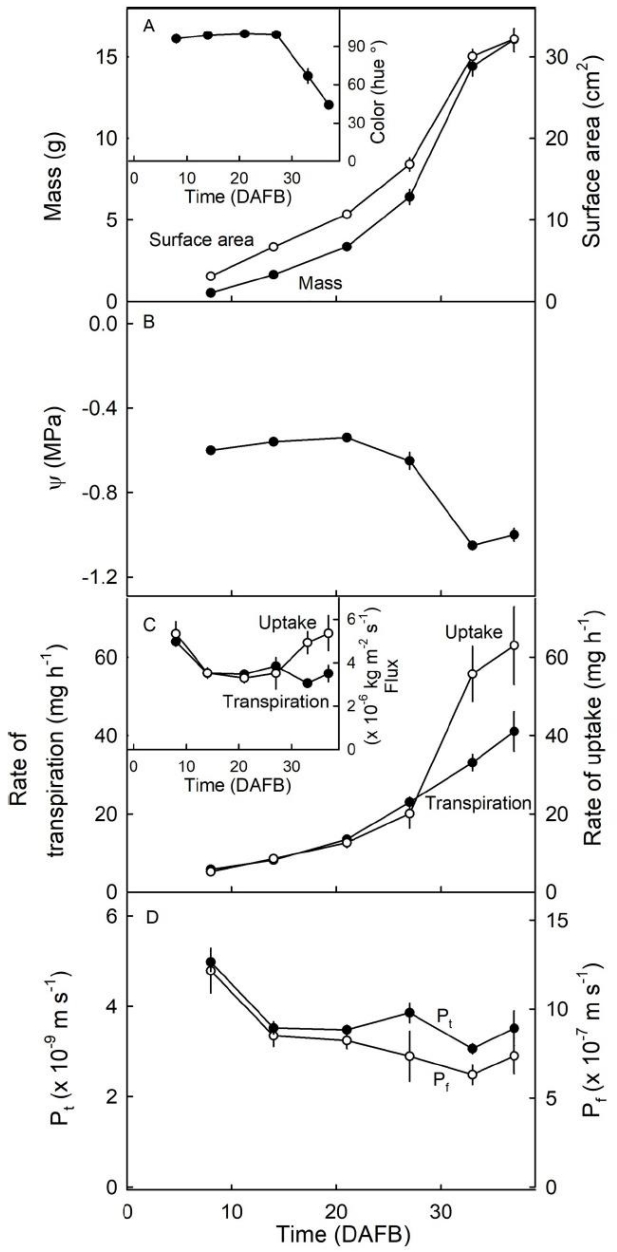
A
B
C
D
Mass (g)
Surface area (cm^2)
Color (hue °)
Time (DAFB)
Surface area
Mass
ψ (MPa)
Rate of transpiration (mg h^{-1})
Rate of uptake (mg h^{-1})
Uptake
Transpiration
Flux (x 10^{-6} kg m^{-2} s^{-1})
P$_t$ (x 10^{-9} m s^{-1})
P$_f$ (x 10^{-7} m s^{-1})
P$_t$
P$_f$
Time (DAFB)

Fig 6. Time course of strawberry development. Change in fruit mass (A), surface area (main graph) and color as indexed by the Hue angle (inset), (B) water potential (Ψ), and (C) flow rates of osmotic uptake and transpiration (main graph) and flux densities of transpiration and osmotic uptake (inset) and permeances of the skin for osmotic uptake (P_i) and for transpiration (P_t) (D) (Bars represent SE).

https://doi.org/10.1371/journal.pone.0251351.g006

remaining about constant for up to 30 h (Fig 7B). Fitting a linear regression through a plot of osmotic uptake rate vs. the osmotic potential of the incubation solution, allowed fruit water potential to be estimated from the x-axis intercept. At this point, the driving force for osmotic water uptake is zero because fruit water potential (unknown) equals the osmotic potential of the incubation solution (known). This calculation revealed very negative values for fruit water potential during the first incubation interval. Fruit water potential then gradually increased (became less negative) as it approached the osmotic potential of the expressed fruit juice. The values found for Ψ_{fruit} were consistently more negative than those measured for the osmotic potential of the expressed fruit juice (Ψ_{Π}) (Fig 7D). This difference amounted to about -0.6 MPa.

Incubating fruit in isotonic solutions composed of osmolytes of different molar mass, resulted in osmotic water uptake at rates that depended on the molar masses of the osmolytes (Fig 8A). Osmolytes having molar masses of 1500 g mol^{-1} or lower resulted in rates of osmotic uptake of > 0 mg h^{-1}. The rate of osmotic uptake decreased asymptotically as molecular size increased (Fig 8B).

When conducting transpiration and osmotic-uptake experiments sequentially on the same fruit, the rates of water movement recorded depended on the order in which the experiments were conducted (Fig 9A). That is, the rate of transpiration was consistently higher when recorded after first recording the osmotic uptake rate, and lower if recorded before. Incubating fruit in the fluorescence tracer acridine orange revealed considerable microcracking after fruit were incubated in water (i.e. as per an osmotic-uptake experiment). The microcracks were in the areas of epidermis lying between the achenes and were ring-shaped and centered on the achenes (Fig 9D and 9E) and in the depressions of the achenes (Fig 9F and 9G). In contrast, fruit that had not been incubated in deionized water (i.e. as per a transpiration experiment) had markedly fewer or no microcracks (Fig 9B and 9C).

Discussion

Mature strawberries have very low turgor

Low fruit turgor pressure is not unique for mature strawberries but has been reported also in the mature fleshy fruit of many species including of grape berries [26–30], sweet cherry, plum, currents and tomato [18,31]. For strawberries, low turgor pressures are also in agreement with calculations made by [32], who estimated turgor pressures of 0.2 (unripe fruit) to 0.05 MPa (ripening fruit) by subtracting measured fruit osmotic potential from measured fruit water potential.

Low turgor pressures occur in many species of ripe fruit, despite the usually very negative osmotic potential of their expressed juices. As a consequence, in mature fruit, the water potential is essentially equal to the osmotic potential. However, in the unripe fruit of many fruit species, the turgor is usually markedly higher. Our findings for strawberries (pseudocarps) follow this general pattern found for true fruit. Thus, for grape berries, a transient peak in turgor coincides with veraison [33]. In sweet cherry, peak turgors occur at the onset of color change, when rates of fruit growth and rates of accumulation of carbohydrates are at maximum [31].

Accumulation of apoplastic solutes in the cell wall space of grape berries is responsible for the lack of turgor at maturity [33,34]. This is also the case in strawberries. The apoplast of ripe

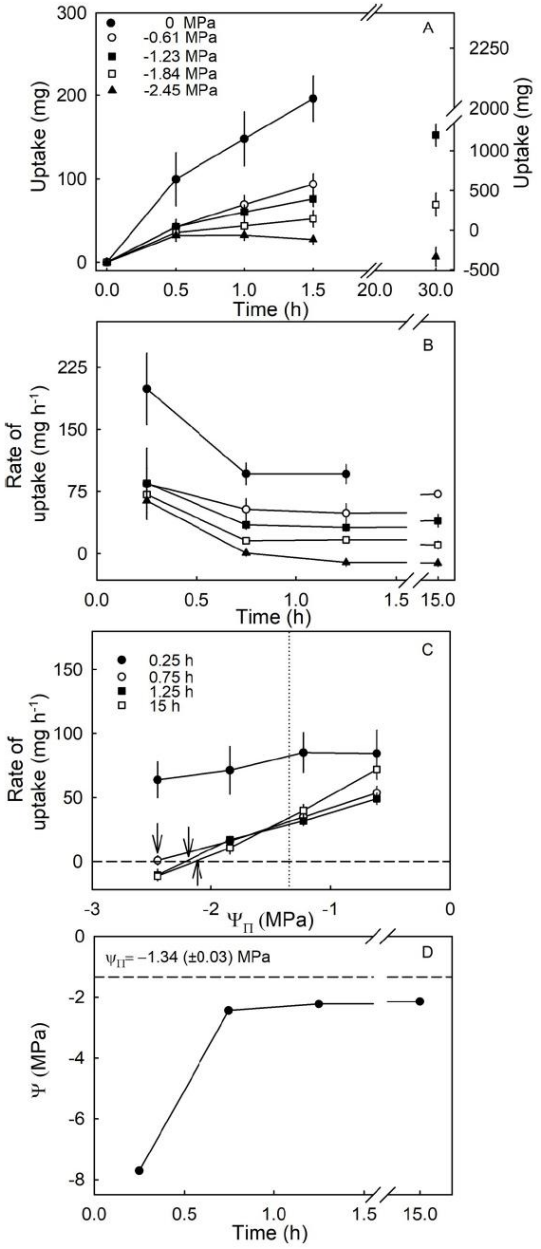
A
0 MPa
-0.61 MPa
-1.23 MPa
-1.84 MPa
-2.45 MPa
Uptake (mg)
Time (h)
B
Rate of uptake (mg h-1)
Time (h)
C
0.25 h
0.75 h
1.25 h
15 h
Rate of uptake (mg h-1)
ΨΠ (MPa)
D
ψΠ= −1.34 (±0.03) MPa
Ψ (MPa)
Time (h)

Fig 7. Effect of osmotic potentials (Ψ_{Π}) of the incubation solution on osmotic uptake. (A) Time course of cumulative uptake (B) and of change in rate of uptake. (C) Relation between rate of uptake and the osmotic potential of the incubation solution. The arrows indicate the osmotic potential at zero water uptake. At this point the osmotic potential of the incubation solution equals the calculated fruit water potential. The vertical dotted line indicates the osmotic potential of the expressed juice. (D) Time course of change in the calculated fruit water potential. Water potential was estimated from the osmotic potential of the incubation solutions for zero water uptake. (Bars represent SE). Glucose and fructose at a molar ratio of 1:1 were used as osmolytes in the incubation solution because these two carbohydrates represent the most abundant osmolytes in strawberries.

https://doi.org/10.1371/journal.pone.0251351.g007

strawberries contains high levels of solutes, but not that of unripe ones [32]. The consequences of this are two-fold: 1) High concentrations of apoplastic solutes generate a negative water potential in the apoplast and the decreased turgor, in turn, facilitates phloem transport and thus phloem water inflow into the fruit. 2) The resulting lack of turgor implies that fruit water potential and osmotic potential are essentially equal and very low (negative) in mature fruit. In

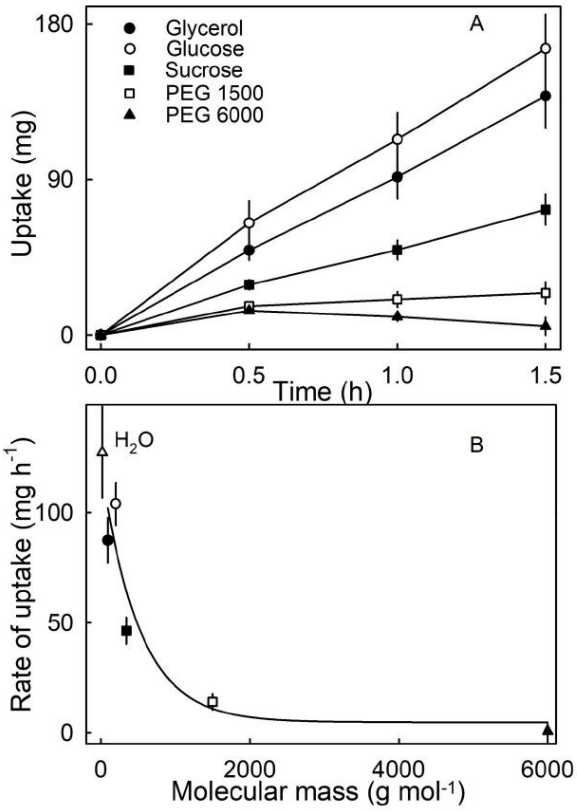

Fig 8. Effect of different molecular mass of isotonic solutions on osmotic uptake. (A) Cumulative uptake. (B) Rates of uptake. (Bars represent SE).

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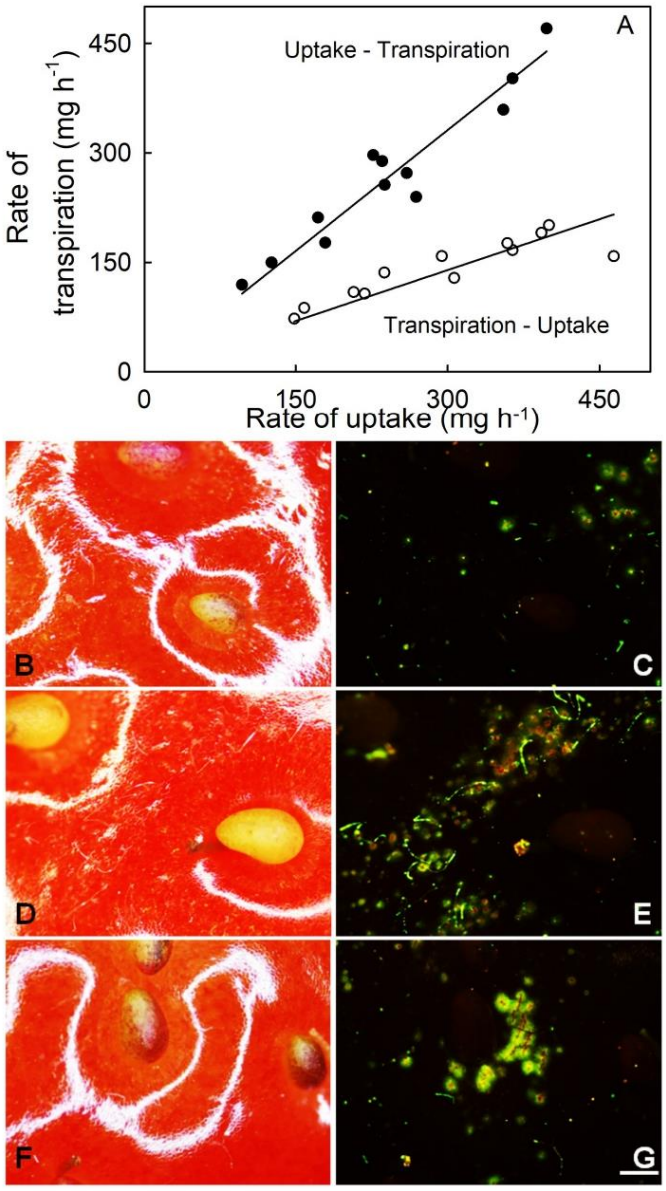

Fig 9. Effect of sequence when determining both water uptake and transpiration on water movement and on formation of microcracks. (A) Relationship between rate of osmotic uptake (F_o) and rate of transpiration (F_t) for fruit subjected to the osmotic uptake determination first, followed by transpiration ('uptake→transpiration') and vice-versa

('transpiration→uptake'). The regression equations were: $F_t = 1.10$ (±0.03) x F_p, $r^2 = 0.92^{***}$ (uptake before transpiration); $F_t = 0.47$ (±0.02) x F_p, $r^2 = 0.71^{***}$ (transpiration before uptake). The intercepts were not significant, so regression lines were forced through the origin. Significance of slope parameter at $P < 0.001$ indicated by ***. Microscopic view of a strawberry fruit surface under incident bright (B, D, F) and fluorescent light (C, E, G) following incubation in acridine orange solution (0.1%) for 5 min. Fruit was subjected to 1.5 h of transpiration (B and C) or to 1.5 h of osmotic uptake (D to G). Following water uptake, ring microcracking was plainly evident in the epidermis around the achenes (E) and in the achene depressions themselves (G). Scale bar 500 μm.

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sweet cherry, the negative fruit water potential results in water being pulled into the fruit osmotically even in the absence of transpiration [35]. Whether this is also the case in strawberry is currently unknown.

Strawberry skins are highly permeable to water and their permeance for osmotic uptake very greatly exceeds that for transpiration

When compared with other fruitcrop species, the permeances of strawberry fruit skin recorded here for transpiration and also for osmotic uptake are the highest ever recorded (see compilation in Table 1). Thus, the strawberry skin is not a very effective barrier to the movement of water.

To the best of our knowledge, the surface of a mature strawberry fruit is covered by the thinnest fruit cuticle ever reported (Table 1). There was no significant relationship between cuticle thickness and cuticle permeance. The extremely thin cuticle implies an extreme likelihood that surface defects will result if the fruit skin is subjected to high rates of strain during growth. The intracuticular waxes rather than the epicuticular waxes are considered to form the primary barrier to water movement across plant surfaces [36].

So, an extremely thin cuticle indicates an extreme dearth of intracuticular waxes and thus an extremely water-permeable skin. Moreover, our results reveal numerous cracks in the cuticle, particularly after the strawberry fruit skin has been in contact with surface water. Thus, an extremely thin cuticle along with a predisposition to microcracks indicates the barrier properties of a strawberry fruit's skin will be very limited in the dry and still further impaired by any exposure to surface wetness.

Table 1. Mass of the cuticular membrane (CM), dewaxed CM (DCM) and permeances for transpiration (P_t) and for osmotic uptake (P_f) of a range of ripe fruitcrop species.

Species	Cultivar	Mass per unit area (g m^{-2})			P_t (x 10^{-9} m s^{-1})	P_f (x 10^{-9} m s^{-1})	Ratio P_f/P_t
		CM	DCM	Wax			
Strawberry	Clery	-	-	-	4.9±0.1	1108.0±86.1	226
	Florentina	0.62±0.02	0.50±0.01	0.12±0.01	4.4±0.2	863.4±37.7	196
	Laetitia	-	-	-	6.4±0.3	860.1±68.1	134
Grape [19,24]	Chardonnay	3.90±0.07	-	-	2.2±0.1	7.7±0.9	4
	Müller-Thurgau	3.30±0.03	-	-	2.5±0.1	8.9±1.5	4
	Riesling	4.56±0.04	3.21±0.09	1.35±0.08	1.6±0.1	4.1±1.2	3
Cherry [22,25]	Sam	1.31±0.04	0.94±0.04	0.37±0.01	1.8±0.2	44.5±19.1	25
	Hedelfinger	1.20±0.04	0.92±0.0	0.28±0.02	3.1±1.1	135.3±3.0	44
	Adriana	1.09±0.02	0.73±0.02	0.36±0.02	2.2±0.6	30.7±2.8	14
Tomato [22]	Sun Rise	8.08±0.30	-	-	1.0±0.2	15.2±3.2	15
Black currant [10]	Zema	5.04±0.04	4.09±0.03	0.95±0.01	-	77.0±4.0	-
Gooseberry [10]	Rote Triumph	5.62±0.08	5.03±0.08	0.59±0.00	-	52.0±1.0	-
Jostaberry [10]	Jostine	4.85±0.05	3.81±0.04	1.04±0.01	-	33.0±3.0	-

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Table 2. Permeance for transpiration (P_t) and permeance for osmotic uptake (P_f) of the skins of ripe strawberry fruit cv. Clery.

Permeance x 10^{-9} (m s^{-1})	Mean	Median	SE	Range		CV (%)	Number of observations (n)
				Min	Max		
Transpiration (P_t)	4.9	5.1	0.1	2.1	9.1	30.9	104
Osmotic uptake (P_f)	1108.0	793.3	86.1	209.1	5104.1	89.0	131

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It is interesting to note that the permeance to osmotic uptake exceeded that to transpiration by more than two orders of magnitude. The average ratio P_f/P_t is about 190 for strawberry. This ratio is much larger than for other fruitcrop species (Table 1) where P_f generally exceeds P_t but where the ratio is much less extreme (i.e. P_f/P_t is about 4 for skins of grape berries and about 27 for skins of sweet cherries) [19,22]. This observation can be adduced as evidence for the involvement of a viscous flow, syn. mass flow, component in osmotic uptake–i.e. along a pathway with a liquid water continuum [21]. Viscous water flow along a liquid continuum across the lipophilic cuticle is very rapid compared with diffusion of individual water molecules across the cuticle. The latter occurs by sorption to the cuticle, diffusion across the cuticle and desorption of individual water molecules at the innerside of the cuticle. For polar penetrants such as the water dipol, the affinity for the lipophilic cuticle is low [37].

The involvement of viscous flow in osmotic uptake is consistent with the following observations:

1. Microcracks occur on the strawberry surface as indexed by penetration of the fluorescence tracer acridine orange (Fig 9). Simulating microcracking by abrading the cuticle increased the rate of transpiration 2.6-fold and the rate of osmotic uptake 7.9 fold. That microcracks are important in osmotic uptake is also inferred from the 'sequence effect' when osmotic uptake and transpiration were determined sequentially—i.e. osmotic uptake before transpiration vs. transpiration before osmotic uptake. Transpiration was markedly increased when osmotic uptake was measured before transpiration. Incubation in water induces cuticular microcracking and so impairs the cuticle's barrier function and increases transpiration. In grape berries, simulated microcracking increased the rates of water uptake 47-fold compared with non-treated control fruit [38]. Similar data were reported by [39] for sweet cherry where water uptake rates were almost double than those of control fruit.
2. Polar pathways occur in cuticles of some fruit crops [16]. These provide an aqueous continuum across the lipophilic cuticle that allows penetration of polar substances by viscous flow [40,41]. These pathways are not physical holes in the cuticle but polar domains that accommodate polar penetrants [42]. They result from the orientation of polar functional groups in the cuticle [43]. Evidence for the presence of polar pathways in strawberry skin comes from the effect of osmolyte molecular size on the rate of osmotic uptake from isotonic solution. The experiment provides evidence for an increase in osmotic uptake that depends on the molecular size of the bathing osmolyte. Because the osmolytes we chose are polar (and polar osmolytes are excluded from lipophilic pathways) their penetration must have occurred via a polar pathway. And this penetration was size-selective. The smallest non-penetrating osmolyte was PEG 1500 (1500 g mol^{-1}) and the largest penetrating osmolyte was sucrose (342 g mol^{-1}). The size selectivity of the polar pathways of a strawberry fruit skin is in close agreement with that reported previously [16, 17].

From the above observations we infer that viscous flow through microcracks and polar pathways across the extremely thin cuticle accounts for the high permeability in osmotic uptake as compared to transpiration. This interpretation is consistent with a lower coefficient

of determination for the relationship between flow rate and fruit surface area in osmotic uptake as compared to transpiration. Apparently, the occurrence of microcracks and the frequency of polar pathways–both involved in osmotic water uptake–is independent of area. In contrast, transpiration occurs primarily by diffusion across the cuticle and hence, bears a closer relationship with surface area.

Conclusions

The high permeability of strawberry fruit skins to water is of significant commercial importance. The very high permeability for transpiration (water loss) accounts for the special susceptibility of strawberries to postharvest water loss during handling, transport and shelf life. The initial water loss results in loss of shine which makes the fruit less appealing to the consumer. Further water loss causes visible shriveling. The high permeability for osmotic uptake contributes to the very limited 'rainfastness' of strawberries in the field, where unprotected strawberry fruit are highly susceptible to skin cracking and water soaking [1]. It is likely that rapid water uptake and the bursting of cells also contribute to these problems. Further research is needed to identify causal relationships and mechanisms. The findings reported in this paper are an important prerequisite.

Supporting information

S1 Dataset. Excel file containing all data produced in figures and tables throughout the manuscript.
(XLSX)

Acknowledgments

We thank Dr. Ludger Linnemannstöns, Heike and Karl Walter Brüggenwirth and Bendix Meyer for the gift of fruit, Hana Weiß, Marcel Pasta and Peter Grimm-Wetzel for technical support and Drs. Sandy Lang and Andreas Winkler for helpful comments on an earlier version of this manuscript.

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Visualization: Grecia Hurtado.

Writing – original draft: Grecia Hurtado, Moritz Knoche.

Writing – review & editing: Grecia Hurtado, Moritz Knoche.

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Supplementary material

Electronic File E1. Dataset. Excel file containing all data produced in figures and tables throughout the manuscript. (XLSX) (access through: https://doi.org/10.25835/5p308h3f)

4.2. Detached, wetted strawberries take up substantial water in the calyx region

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Figures 1,2 (a,b),4,7,8 and Table.1 were part of Master thesis. Moritz Knoche: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing original draft, Writing review and editing.
Journal	Scientific Reports
Impact factor	4.996(2022)
Date of publication	08.03.2023
DOI	10.1038/s41598-023-31020-0

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Detached, wetted strawberries take up substantial water in the calyx region

Grecia Hurtado & Moritz Knoche✉

In strawberry, surface disorders like 'water soaking', 'cracking' and 'shrivel' impair fruit quality of this high value crop. Water movement through the fruit surface is implicated a role in these disorders. The objective was to identify the pathways of water uptake and water loss (transpiration) and to identify factors affecting these flows. Water movement was quantified gravimetrically in detached fruit. Cumulative transpiration and uptake increased linearly with time. During ripening, fruit osmotic potential and water potential became slightly more negative. Rates of transpiration and water uptake and their corresponding permeances were constant during early ripening but increased as the fruit turned red. The permeance for osmotic water uptake was more than 10-times that for transpiration. Sealing selected regions of the fruit surface with silicone rubber allowed identification of the petal and staminal abscission zones in the calyx region and cuticular microcracks of the calyx region and receptacle as high flux pathways particularly for water uptake (osmotic). These results were confirmed by acridine orange infiltration and fluorescence microscopy. Increasing the relative humidity (RH) decreased the rate of transpiration, while increasing temperature increased both transpiration and water uptake. There was no effect of storing fruit (2 °C, ~ 80% RH) for up to 10 days. Our results identify petal and staminal abscission zones and cuticular microcracks as high flux pathways for water uptake.

Strawberry is a highly perishable, high-value fruit crop that is grown worldwide. The fruit is subject to rapid deterioration preharvest in the field and postharvest during transit, storage, and sale. Surface disorders such as water soaking, cracking, loss of shine and shrivel impair the fruit quality causing significant economical losses[1–4]. Water movement through the fruit surface, i.e. uptake into the fruit and water loss by transpiration from the fruit, is implicated a role in these disorders. Water soaking results from localized water uptake through microscopic cracks ('microcracks') on the fruit surface causing a bursting of cells. Microcracks are minute cracks in the cuticle invisible to the naked eye that do not extend into the underlying epidermis[5,6]. As a consequence, the fruit skin develops irregular, pale and deliquescent patches[7]. The macroscopic cracking of strawberries has not been studied in detail, but field observations suggest that water uptake after rainfalls may be involved[8]. Loss of shine, reduced calyx apperance[3,9] and shrivel[10] are typical symptoms of reduced turgidity as a result of water loss by transpiration.

Despite of its importance, little is known about the movement of water through the surface of strawberries. The mechanisms of transpirational water loss and osmotic water uptake have only recently been identified[6]. However, the predominant pathways of transpirational water loss and osmotic water uptake are largely unknown, nor have the external factors been identified that affect the rates of these water exchanges through the fruit surface.

The objective of our study was to identify the dominant pathways of water movement through the strawberry fruit surface, and also the external factors affecting the rates of those water movements.

Results

Transpiration increased linearly with time indicating constant rates in both the short and the long-term for fruits of all size classes (Fig. 1a). For osmotic water uptake, uptake increased linearly with time only until about 8 h when uptake rates decreased slightly for the medium- and large-size fruit (Fig. 1b-inset). Uptake rates were generally more variable than transpiration rates. Both transpiration and uptake rates were positively related to fruit size (Fig. 1a,b).

Transpiration and water uptake changed with ripening (Fig. 2). As ripening progressed, as indexed by a decrease in hue angle from white (82.7° hue) to dark red (34.3° hue), the osmotic potential of the expressed fruit

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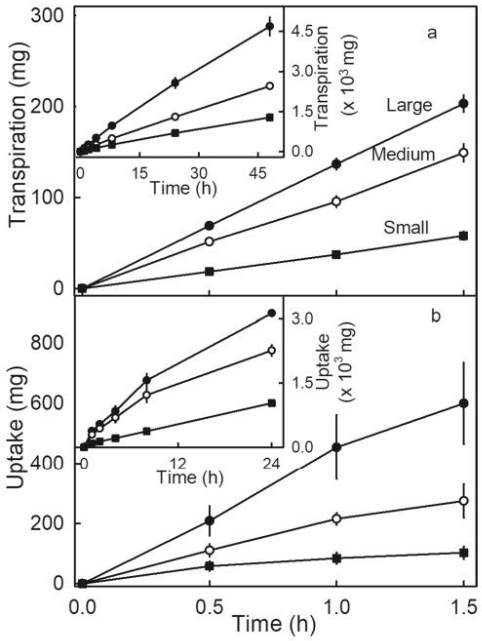

Figure 1. Short-term (main graph) and long-term (insets) time course of (**a**) transpiration and (**b**) water uptake of large, medium, and small strawberry fruit (cv. Clery).

juice and the water potential of the whole fruit became slightly more negative (Fig. 2a). The rates of transpiration and water uptake were constant up to a hue angle of about 45° then increased as the fruit turned red (Fig. 2b). Estimating the fruit surface area from fruit shape and assuming conical shape allowed calculation of the permeance of the fruit surface. The permeances for both transpiration and water uptake increased as the fruit turned red (Fig. 2c). The permeance values for transpiration were consistently lower than those for osmotic water uptake, by more than an order of magnitude, irrespective of the stage of ripening.

Preferential pathways for water movement are pathways, where rapid movement occurs through small cross-sectional areas. Consequently, these areas represent high flux pathways in regions of preferential water movement. To identify preferential pathways of transpiration and water uptake, several experiments were conducted comprising a complexity of treatments. The first experiment was a two-phase one, where the initial rates of water movement were established during phase I. Then, in phase II, parts of the fruit surface were selectively sealed or excised before incubation was continued and rates of water movement were re-evaluated. A second group of fruit remained untreated during phase II and served as the controls.

The first experiment focused on the calyx region. During phase I, both transpiration and water uptake increased linearly with time. During phase II, in one group of fruit, the entire calyx was excised including the abscission zones of petals and stamina, and the resulting wound was sealed using silicone rubber (Fig. 3). This treatment reduced the rate of transpiration and, even more, the rate of water uptake. Transpiration and water uptake in the untreated controls continued to increase (Fig. 4, Table 1). In the second comparison, the same treatments were applied for phase II but the cut surfaces (wounds) remained unsealed. Following excision without sealing, the rates of transpiration increased slightly, whereas the rates of water uptake more than doubled (Table 1). In the third comparison, we addressed the role of the cut peduncle end. Here, the cut end was sealed for phase II. It was found that sealing the cut peduncle end had no effect on either the rate of transpiration or the rate of water uptake (Table 1).

In the second experiment on preferential water pathways, the portion of the fruit surface responsible for the increased water movement in the calyx region was identified by sealing a progressively increasing portion of the fruit surface—beginning at the proximal end of the fruit (Fig. 3). Selective sealing of the sepals and peduncle surface had almost no effect on the rate of transpiration but reduced the rates of osmotic water uptake by 13% (Table 2). When (in addition to the sepals) the abscission zones of the petals and stamina, and the junction between the calyx and receptacle were sealed, the rate of transpiration decreased slightly, however, the rate of water uptake decreased much more, by about 23% (Table 2). When the entire calyx was removed, including the

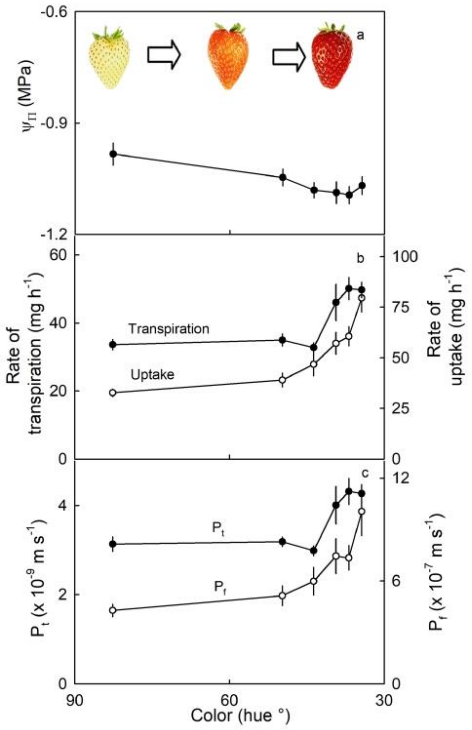

Figure 2. Changes during ripening of strawberry fruit indexed by (**a**) osmotic potential (Ψ_Π), (**b**) the rates of transpiration and uptake, and (**c**) the permeances for transpiration (P_t) and water uptake (P_f) of 'Florentina' strawberry. The time of ripening was indexed by the change in color.

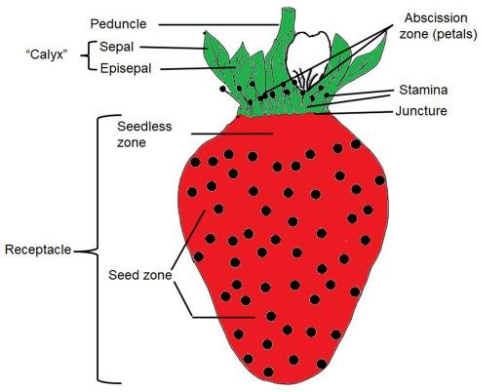

Figure 3. Sketch illustrating the nomenclature used to describe the different regions and organs of a strawberry fruit addressed by the sealing treatments. We refer to the portion of the receptacle that lacks seeds (strictly, the zone lacking the single-seeded achenes) as the 'seedless' zone.

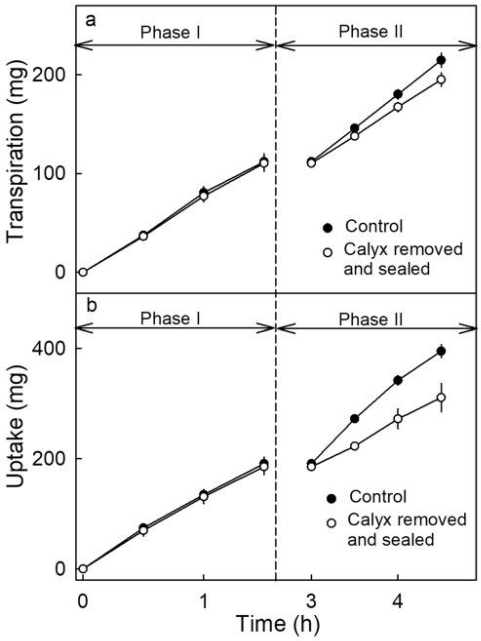

Figure 4. Effect of sealing selected regions of the surface of ripe strawberry cv. Sonsation on water movement in (**a**) transpiration or (**b**) water uptake. The experiment was conducted in two phases using a repeated measures design. During phase I (0 to 1.5 h), water movement was quantified in non-sealed fruit with calyx and 5 mm of the peduncle. For phase II (3–4.5 h), sepals and stamina were removed and the cut surface of the sepals, the abscission zones of the petals, the stamina, the junction calyx-receptacle, the peduncle surface including the peduncle end were all sealed ('Calyx removed and sealed'). Fruit that remained without treatment during phase II served as control. For flow rates see Table. 1.

Treatment	Rate of transpiration (mg h^{-1})			Rate of water uptake (mg h^{-1})		
Phase I/Phase II	Phase I	Phase II	Δ Rates	Phase I	Phase II	Δ Rates
Control/Control	75.8 ± 5.5	68.5 ± 4.8	−7.3 a[a]	126.7 ± 7.7	136.4 ± 7.9	9.8 a[a]
Control/sepals, abscission and stamina zone cut, and cut surface, junction calyx-receptacle, and peduncle end sealed	74.4 ± 5.5	56.7 ± 4.7	−17.7 b	123.5 ± 9.5	85.2 ± 17.8	−38.3 b
Control/Control	84.4 ± 6.5	82.2 ± 6.1	−2.2 a	129.7 ± 11.2	171.6 ± 17.1	41.9 a
Control/sepals, abscission and stamina zone cut no sealing	80.8 ± 6.5	92.7 ± 6.2	11.9 b	141.1 ± 12.3	376.3 ± 39.7	235.2 b
Control/Control	86.3 ± 5.9	85.3 ± 5.9	−1.0 a	149.4 ± 8.3	180.6 ± 7.5	31.2 a
Control/peduncle-end sealed	79.2 ± 3.8	76.7 ± 3.7	−2.4 a	160.6 ± 12.6	169.6 ± 21.6	9.0 a

Table 1. Rates of water uptake and transpiration along different pathways of strawberry cv. Sonsation. The experiment was conducted in two phases using a repeated-measures design. During phase I (incubation for 0–1.5 h) uptake and transpiration were determined without any treatment ('control'). During phase II (incubation in water for 3–4.5 h), the remains of floral parts were excised, or parts of the fruit surface sealed using silicone rubber. Phase II treatments comprised the excision of sepals and stamina and subsequent sealing of the cut surfaces, the calyx-receptacle junction and peduncle surface including the peduncle end ('Control/sepals and stamina cut, and junction calyx-receptacle and peduncle end sealed'), the excision of sepals and stamen without subsequent sealing of the cut surfaces ('Control/sepals and stamina cut, no seal'), and sealing of the cut peduncle end ('Control/peduncle-end sealed'). Fruit without excision and without sealing served as controls ('Control/control'). [a]Mean separation within main effect by Dunnett's test at $p = 0.05$. Each treatment was compared with its control.

Treatment	Rate of transpiration		Rate of water uptake	
	(mg h^{-1})	(%)	(mg h^{-1})	(%)
a. Control	97.7 ± 6.0	100	258.7 ± 27.6	100
b. Sepals sealed	95.9 ± 3.6	98.2	225.5 ± 19.4	87.2
c. Sepals, abscission zone of petals, stamina, and junction sealed	91.2 ± 5.8	93.3	199.7 ± 18.0	77.2
d. Seedless zone sealed	72.9 ± 3.8	74.6	177.1 ± 15.2	68.5

Table 2. Rates of water movement for transpiration and osmotic uptake along different pathways through the strawberry fruit surface (cv. Sonsation). Fruits were progressively sealed, starting from the proximal end. Treatment **a** ('Control'): Fruit without sealing served as control. The sealing treatments were: Treatment **b** ('Sepals sealed'): sepals and the peduncle surface sealed, open remained the abscission zones of petals, stamina, the junction between calyx and receptacle, and the seedless zone of the receptacle. Treatment **c** ('Sepals, abscission zones, stamina, and junction sealed'): sepals, abscission zones of petals and stamina, peduncle and junction calyx-receptacle cut end all sealed. Treatment **d** ('Seedless zone sealed'): seedless zone of the receptacle, sepals, abscission zone of petals and stamina, calyx-receptacle junction, peduncle removed and sealed.

sepals, the abscission zones, and the junction between calyx and receptacle, and the seedless zone was sealed, the rate of transpiration was reduced by about 25%, and the rate of water uptake by about 31% (Table 2).

From this experiment, the relative contributions of the various pathways of transpiration and water uptake could be calculated (Table 3). Most of the transpiration (93%) occurred through the surface of the receptacle, including that of the seedless zone. However, the receptacle and seedless zone together accounted for only 77% of water uptake. The remaining 23% of water uptake occurred in the region of the sepals, the petal and staminal abscission zones, and the calyx-receptacle junction (Table 3).

The distinct role of the water uptake pathways in the various structures located at the proximal end of the fruit (the petal and staminal abscission zones, and the calyx-receptacle junction) was not unique to 'Sonsation' but was also observed in the other cultivars examined (Table 4). Only in 'Elsanta' was the magnitude of the contribution to total water uptake made by these pathways almost negligible. It is worth noting here that the rates of osmotic water uptake differed significantly among the cultivars examined by a factor of 4. Rates were highest in 'Clery' and lowest in 'Joly' (Table 4).

Acridine orange dye and fluorescent microscopy were used to identify in more detail the sites of water uptake. This revealed staining of the trichomes on the sepals and episepals (Fig. 5a–d). Trichome staining was more pronounced on the abaxial as compared to on the adaxial surfaces (Fig. 5a, b). The base of the calyx on the receptacle was pentangular in shape. Dye infiltrated microcracks were clearly visible; these extended radially from the vertices of the pentagon up into the seedless zone of the receptacle (Fig. 5e–h). Infiltration was also observed in the petal abscission zones and in the bases of some stamina (Fig. 6a, b). Dye infiltration resulted from microcracks in the region of the staminal bases (Fig. 6c, d) and the calyx-receptacle junction (Fig. 6e, f). Numerous microcracks also occurred in the depressions in which lay the seeds on the receptacle (Fig. 6g, h).

Increasing the relative humidity (RH) decreased the rate of transpiration ($r^2 = 0.98^{***}$). Fruit incubated at high humidity had the lowest rates of transpiration (Fig. 7a-main graph). When the recorded rates of transpiration were normalized for the driving force at each of the different humidities, the rates became nearly independent of humidity. This indicates that skin permeance was largely unaffected by the RH (Fig. 7a-inset).

Increasing the temperature increased the rates of transpiration and water uptake (Fig. 7b, c). The increase was exponential. Varying the temperature also varied the driving force for transpiration and water uptake. After normalizing for the different driving forces, the rates of transpiration decreased slightly but the relationship between normalized transpiration rate and temperature was not significant (Fig. 7b-inset). This indicates that skin permeance to water was also largely unaffected by temperature.

Pathway	Rate of transpiration		Rate of uptake	
	(mg h^{-1})	(%)	(mg h^{-1})	(%)
Sepals and peduncle surface	1.8	1.9	33.2	12.8
Abscission zone, stamina, junction calyx-receptacle,	4.7	4.8	25.8	10.0
Seedless zone	18.2	18.7	22.6	8.7
Receptacle (with achenes)	72.9	74.7	177.1	68.5
Whole fruit	97.7	100	258.7	100

Table 3. Relative contribution of different pathways to whole fruit transpiration and water uptake into strawberry fruit cv. Sonsation. The pathways were: sepals, the peduncle surface, abscission zone of petals, stamina, the junction between calyx and receptacle, and the seedless zone of the receptacle. Data is calculated from data in Table 2.

Cultivar	Rates of uptake			
	Whole fruit	Whole fruit with abscission zone and junction sealed	Abscission zone and junction	
	(mg h^{-1})	(mg h^{-1})	(mg h^{-1})	(%)
Sonsation	208.2 ± 17.0	134.7 ± 9.0	73.5	35.3
Clery	349.9 ± 51.7	254.6 ± 25.3	95.4	27.3
Florentina	127.8 ± 5.1	81.7 ± 3.9	46.1	36.1
Dream	86.6 ± 13.3	70.5 ± 9.2	16.1	18.6
Joly	115.1 ± 17.4	83.7 ± 6.7	31.4	27.3
Elsanta	221.9 ± 28.8	212.1 ± 34.3	9.8	4.4
Grand Mean	181.0 ± 14.3a	141.2 ± 9.8 b	45.4	24.8

Table 4. Rates of osmotic water uptake into detached strawberries of selected cultivars. Water uptake was quantified on a whole fruit basis and after sealing abscission zones of petals and stamina, and junction calyx-receptacle ('abscission zone and junction sealed') using silicone rubber. The contribution of the abscission zone and the junction was calculated by subtracting the uptake in the sealing treatment from that of the non-sealed control. Non-sealed fruit served as control. [a]Mean separation within main effects by Tukey's test, P = 0.05. No interaction between main effects was found by ANOVA analysis.

There were no significant effects of holding fruit in storage at 2 °C and ~ 80% RH for up to 10 days on the rates of transpiration or osmotic water uptake (Fig. 8). During storage, the total mass loss increased linearly with time, reached about 5% of the initial fruit fresh mass by 10 d (Fig. 8-inset). Cumulative mass loss had no effect on the rate of mass loss. Similarly, holding fruit for 24 h under different conditions of RH at 22 °C had no significant effect on the rates of water loss or uptake (Fig. 9).

Discussion

Our results indicate two general findings. (1) That the water fluxes into and out of a strawberry fruit occur along a number of distinct parallel pathways. Of these pathways and flows, preferential water movements occur in the calyx region, particularly for osmotic water uptake. And (2) both transpirational loss and osmotic water uptake exhibit behaviors typical of purely physical processes—there being no evidence of strong temperature sensitivity nor of responsive changes in water permeance.

Preferential parallel pathways for water uptake. Our study identified several of parallel pathways through which in particular osmotic water uptake occurred. The preferential high flux pathways were: (1) the junction region between calyx and receptacle, (2) the abscission zones of the petals and stamina, and (3) distinct microcracks in the receptacle. Compared to the entire fruit surface, these pathways have a small cross sectional area for water uptake and therefore represent high flux pathways. The above conclusions are based on the following observations.

First, selective sealing of these areas with silicone rubber markedly reduced osmotic water uptake. Conversely, when the calyx region was excised without sealing, osmotic water uptake increased markedly indicating that a strong driving force for uptake was present and that the vasculature in this region was still functional.

Second, fruit incubated in a solution of the fluorescent tracer acridine orange revealed various pathways for rapid water uptake that 'bypassed' the cuticle on the fruit surface. Acridine orange does not penetrate the cuticle, so tissue penetration must be via openings in the cuticle. The pathways for tissue penetration include microcracks: in the seed depressions on the receptacle; in the seedless zone; at the calyx-receptacle junction; and at the bases of the staminal and petal abscission zones. It is speculated that enhanced penetration in the abscission zones is due to the associated vascular bundles remaining open following the abscission. The role in water uptake of the trichomes on the abaxial surface of the calyx is unclear. The trichomes were stained and occasionally also some epidermal cells at the trichome bases. This indicates that dye penetration occurred. Thus, trichomes are also sites of preferential entry of water. The proportional contribution of water uptake at trichomes to total water uptake by the fruit is not known. Water uptake along these pathways is relatively rapid because the cuticle is bypassed as a penetration barrier.

The causes of microcracking in strawberries are unknown. Potential explanations include: (1) The development of cuticular growth strains during organ development[11,12]. Such strains occur particularly when cuticular deposition rates fail to keep pace with surface area expansion rates during organ development[13]. And that: (2) The microcracking of an already-strained cuticle is exacerbated by surface wetness as has been shown in a number of fruit crops species including sweet cherry, apple, mango, and grape[14–17]. In spite of the botanical distinction (a strawberry is not a true fruit) research shows that in many ways its growth and development are more closely similar to those of a true fruit than they are to a floral accessory organ, a receptacle. We note that the region of the calyx-receptacle junction is likely to suffer from extended periods of surface wetness making cuticular microcracking all the more likely.

Transpiration and water uptake are physical processes. Transpiration and water uptake are physical processes that do not require metabolic energy and so are relatively temperature insensitive. We found that

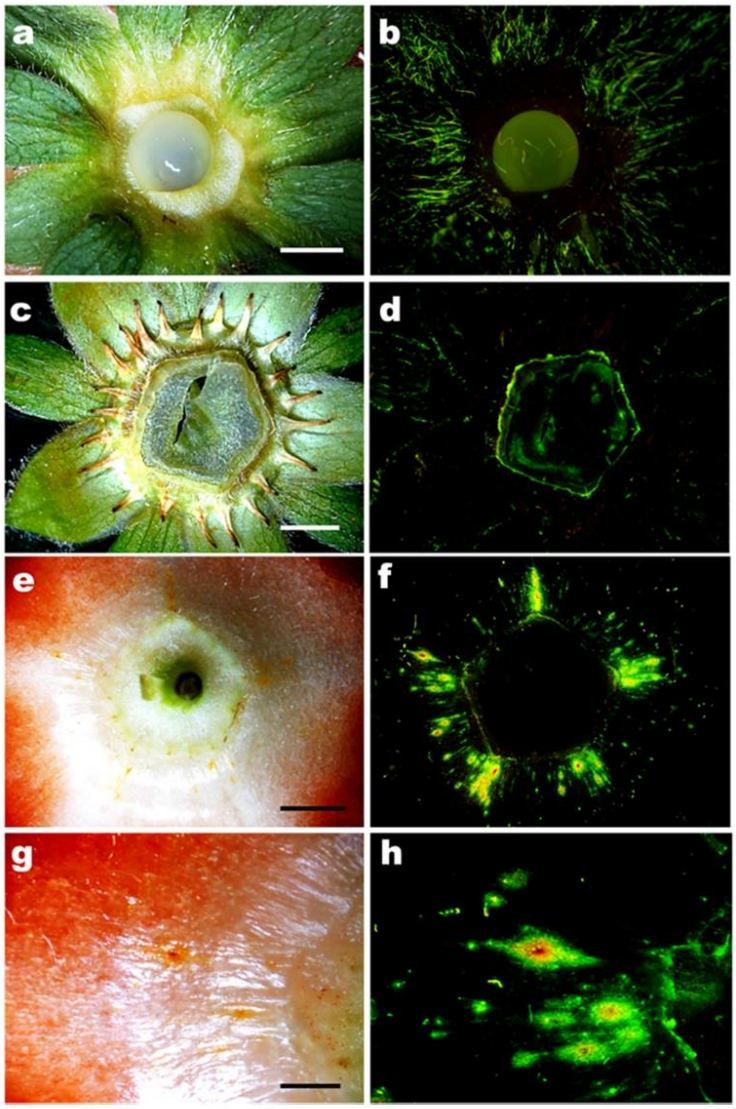

Figure 5. Micrographs of the calyx region of ripe strawberries cv. Sonsation after incubation in the fluorescent tracer acridine orange. Images were taken in incident bright light (**a, c, e, g**) and incident fluorescent light (**b, d, f, h**). **a, b** abaxial surface of sepals and episepals with numerous trichomes stained by acridine orange. The opaque dot in the center is a drop of silicone rubber that was applied for sealing the peduncle end; **c, d** adaxial surface of sepals and episepals; **e, f** top view of the seedless zone of the receptacle below the calyx showing fluorescing microcracks in the vertices of the pentagon-shaped base; **g, h** same as **e, f** but microcracks at higher magnification. Scale bar in a,c,e = 3 mm, and g = 1 mm.

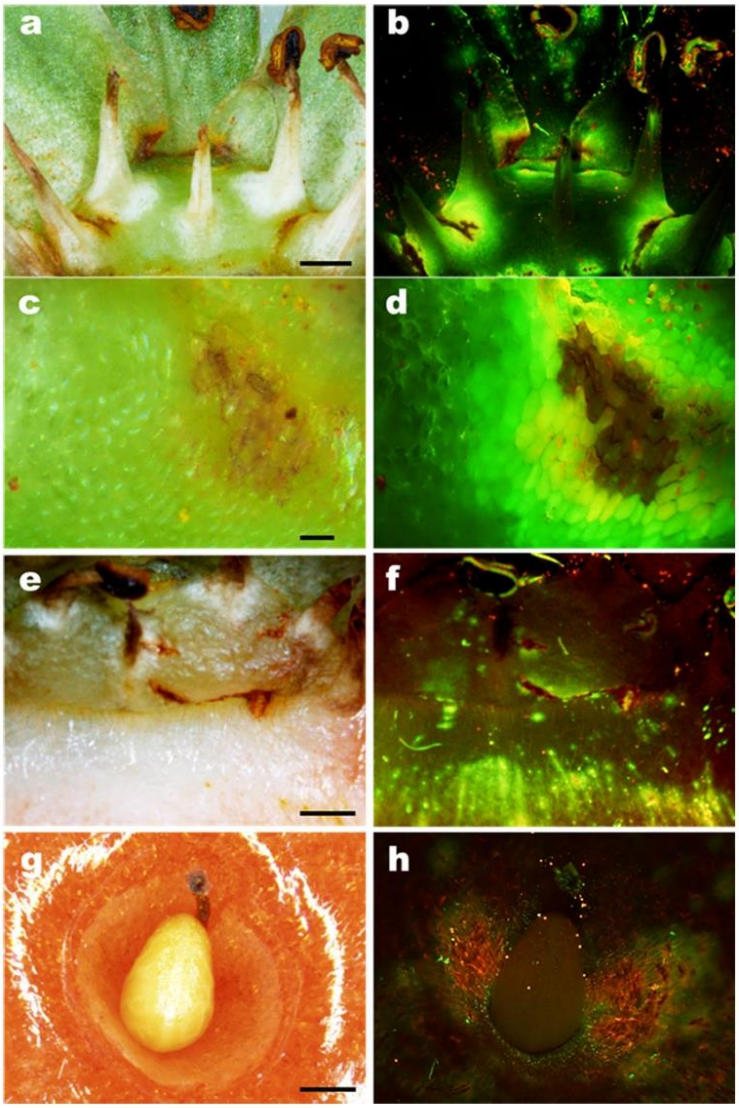

Figure 6. Micrographs of microcracks in the cuticle on the surface of ripe strawberries cv. Clery after incubation in the fluorescent tracer acridine orange. Images were taken in incident bright light (**a, c, e, g**) and incident fluorescent light (**b, d, f, h**). **a, b** microcracks at the base of stamina and the abscission zone of petals; **c, d** detailed view of microcrack at the base of a stamen; **e, f** microcracks at the calyx-receptacle junction and in the seedless zone of the receptacle; **g, h** microcracks in the depression of an achene on the surface of strawberry fruit. Scale in a,e = 1 mm, c = 0.1 mm and g = 0.5 mm.

water movement followed a general transport equation where the rate of movement is a function of the cross-sectional area through which the movement occurs, the driving force that causes the movement, and the ease of

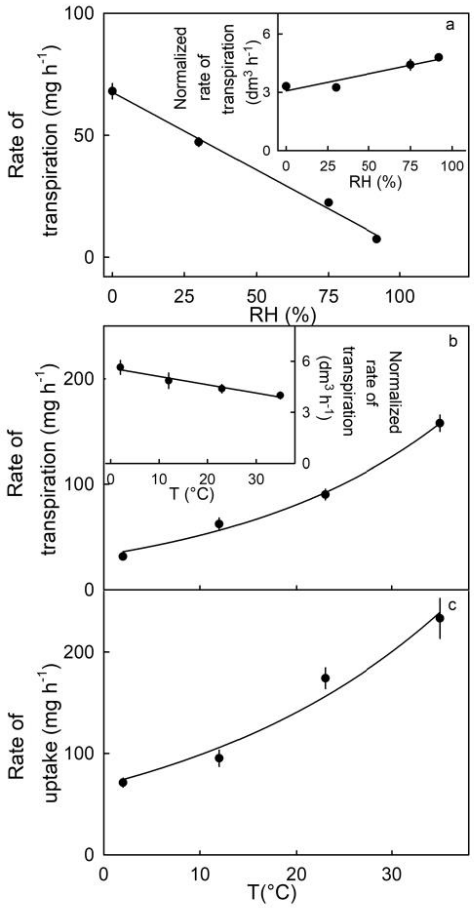

Figure 7. (**a**) Effect of relative humidity (RH) on the rate of transpiration (main graph) and on the normalized rate of transpiration (inset). (**b**) Effect of temperature (T) on the rate of transpiration (main graph) and on the normalized rate of transpiration (inset) and (**c**) on the rate of water uptake of 'Laetitia' strawberry. Normalized transpiration rates were calculated by dividing the rate of transpiration by the difference in water vapor concentration between the inside of the fruit[20] – assumed to be a saturated atmosphere – and the outside atmosphere above dry silica gel – assumed to be 0 g m^{-3}.

movement—a hydraulic conductance term—i.e., the permeance of the fruit skin or a porous pathway within it. This conclusion is inferred from the following findings.

First, rates of transpiration and water uptake were higher for larger fruit compared to for intermediate or small fruit. This is to be expected since the cross-sectional area through which movement occurs is greater in larger fruit. Second, the rate of transpiration was strongly dependent on RH and temperature, while those of water uptake were dependent on temperature only. When accounting for differences in driving force between different temperatures and different RHs, the normalized flow rates were proportional to the skin permeances and nearly independent of temperature and RH. Third, the lack of any effect of pre-storage duration is consistent with the concept of a physical transport process.

Earlier studies have established that the cuticle forms the rate-limiting barrier in water movement[6]. Effects on properties that affect cuticle permeance during a 10-d cold storage period are highly unlikely. For example, it might be argued that the mass loss that occurred during storage should have had an effect on the normalized flow rates. However, the mass loss during storage did not exceed 5% of the fruit's initial fresh mass. In a first approximation, this would yield a decrease in osmotic potential of about 5%. The anticipated increase in osmotic water uptake rate (+5%) would be too small to have been detected in our experiments.

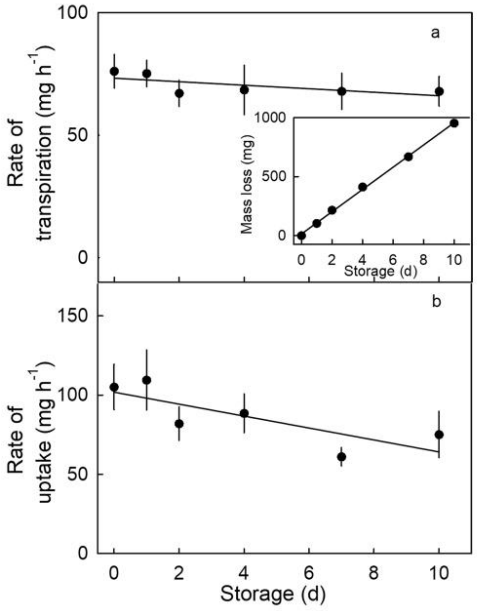

Figure 8. Effect of cold storage duration on (**a**) the rate of transpiration (main graph), mass loss (inset), and (**b**) the rate of water uptake of 'Clery' strawberry following storage.

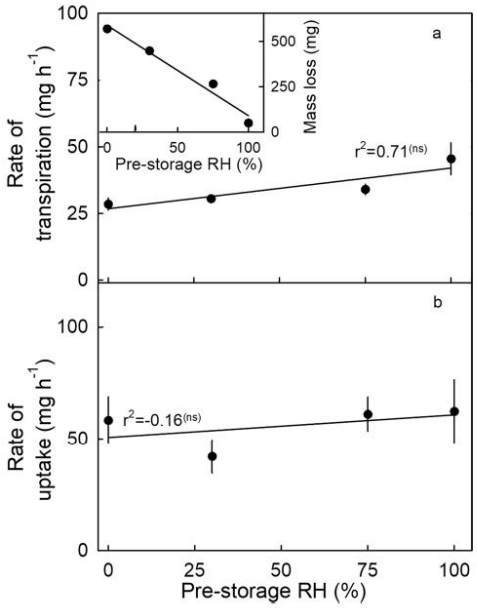

Figure 9. Effect of relative humidity (RH) during a 24 h pre-storage period on (**a**) the rate of transpiration (main graph) and cumulative mass loss (inset) and on (**b**) the rate of uptake of 'Clery' strawberry.

The conclusions drawn here are also consistent with earlier reports. Transpiration occurs primarily by gaseous diffusion. In osmotic water uptake, however, viscous flow along 'porous pathways' is expected to occur in parallel to diffusion through the cuticle[18]. In strawberries, these parallel pathways include microcracks, abscission zones, a leaky junction between receptacle and calyx, and (possibly) ruptured stomata and broken trichomes. Viscous flow through such openings is rapid because it bypasses the cuticle as the primary barrier. The occurrence of microcracks on the fruit surface is highly variable and highly localized within a fruit. Rates are also highly variable between fruit on the same parent plant or between fruits on neighboring plants in the same planting. This may explain the variability in rates of osmotic water uptake by fruit that typically exceeds that reported for transpiration. Viscous flow through these openings also accounts for the higher permeance for osmotic water uptake, as compared to that for transpiration in both this and an earlier study[6].

Conclusion

Our study provides evidence for multiple and high flux pathways for osmotic water uptake into strawberry fruit. Water uptake along these pathways occurs by viscous flow. Uptake by viscous flow is rapid compared to diffusion through an intact cuticle. Potential consequences of this rapid uptake are the development of macroscopically visible cracks resulting in cracked fruit and, most likely, the initiation and subsequent spreading of water soaking on the fruit surface. In addition, macroscopic and microscopic cracks account for the high susceptibility of strawberries to fruit rots. Since microcracks are exacerbated by surface water, the avoidance of surface wetness is key to reducing fruit rots and increasing fruit quality. These benefits can be expected to be enhanced if strawberry production is moved from field to protected cultivation.

Material and methods

Plant material. Strawberry (*Fragaria × ananassa* Duch.) fruits were harvested from commercial plantings at Gleidingen (lat. 52°16′N, long. 9°50′E), Ohndorf (lat. 52°21′N, long. 9°21′E), a growth chamber on the Herrenhausen Campus (lat. 52°23′N, long. 9°42′E), and the Horticultural Research Station of the Leibniz University in Ruthe, Germany (lat. 52°14′N, long. 9°49′E). Temperature and relative humidity (RH) of the growth chamber were set at 20/16 °C and 60/80% RH during a 16/8 h day/night photoperiod. Fruits of the following cultivars were used: Clery, Sonsation, Florentina, Laetitia, Dream, Joly, and Elsanta. The use of different cultivars was necessary since strawberries at the optimum ripening stage are available only for a short period of time. The change of cultivars ensured that the effects observed are valid for strawberry in general and not limited to a specific cultivar.

Fruit was harvested randomly at commercial ripeness (> 80% of the fruit surface red)[19]. Uniform fruit was selected based on size, color, and freedom from visual defects. Unless specified otherwise, fruit was processed fresh on the day of collection or after a maximum of 2 d of storage at 2 °C and 80% RH.

Transpiration and uptake. In most of the experiments, the calyx (sepals, episepals), abscission zones of the petals, and stamina (Fig. 3) were carefully removed from the fruit, and the site of attachment of the calyx to the peduncle was sealed using a fast-curing silicone rubber (Silicone rubber, SE 9186 Clear; Dow Corning Corp., Midland, USA). All experiments were carried out in a temperature-controlled laboratory at 22 °C. The number of individual fruit replicates was 15 unless otherwise specified.

For transpiration experiments, fruits were held in a polyethylene (PE) box above silica gel (RH ~ 0%;[20]) for 1.5 h, unless specified otherwise. Fruit was removed from the box and weighed individually at 30 min intervals. The rate of transpiration (F_t; mg h^{-1}) was calculated as the slope of a linear regression line of the relationship between the change in fruit mass and time.

For osmotic water uptake experiments, fruits were incubated individually in beakers (150 mL) with deionized water. A soft foam plug was placed on the beakers to sink the fruit, so as to wet the whole fruit surface. Fruits were removed from water and carefully blotted using soft tissue paper and then weighed at 30-min intervals for 1.5 h. The osmotic water uptake rate represented the change in fruit mass measured on an individual fruit basis. The rate of osmotic uptake (F_i; mg h^{-1}) was calculated as the slope of a linear regression line fitted through the relationship of change in fruit mass and time.

Time course of water movement. Short- and long-term time courses of osmotic water uptake and transpiration water loss were established for large (> 32 g), medium (15 to 32 g), and small (< 15 g) fruit of cv. Clery (Fig. 1). For the short-term measurements, fruit were weighed at intervals of 30 min for up to 1.5 h and for the long-term time course at 0, 1, 2, 4, 8, 24 h, and for transpiration up to 48 h.

Effect of ripening on water movement. The effects of ripeness on osmotic water uptake and transpirational water loss were identified using six stages of ripeness (Fig. 2). The ripeness stages were: white, 1/2 light red, 3/4 light red, 1/2 red, 3/4 red, dark red[21]. Fruits of 'Florentina' were selected based on color (CM-2600 d, orifice 3 mm diameter; Konica Minolta, Tokyo, Japan). The fruit selected ranged from white to dark red. Color was expressed as the hue angle. Rates of water uptake and transpiration were determined as indicated above. Additionally, juice was extracted from the fruit using a garlic press and its osmotic potential quantified by vapor pressure osmometry (VAPRO 5600; Wescor, Utah, USA). The skin permeances for transpiration (P_t; m s^{-1}) and osmotic water uptake (P_i, m s^{-1}) were determined from rates of water movement[22]. Briefly, P_t was calculated from Eq. (1). The rate of transpiration (F_t; kg s^{-1}) was divided by the product of the fruit surface area (A; m^2), the density of water (ρ_w; kg m^{-3}), and the gradient in water activity (Δa_w; dimensionless) across the fruit skin[23]. Since the humidity above dry silica is practically zero, Δa_w equals the water activity of the strawberry juice, which is approximately one.

The value of P_f (m s^{-1}) was determined using the filtration permeability relation in Eq. (2); where F_f (kg s^{-1}) represents the rate of osmotic uptake, A_{fruit} the fruit surface area (m^2), R (m^3 MPa mol^{-1} K^{-1}) the universal gas constant, T (K) the absolute temperature, V_w (m^3 mol^{-1}) the molar volume of water and ρ_w (kg m^{-3}) the density of water and $\Delta\Psi$ (MPa) the difference in water potential between the water potential of the fruit (Ψ_{fruit}) and that of the incubation solution (Ψ)[24]. For fruit incubated in water ($\Psi = 0$) the driving force for osmotic uptake is essentially equal to the water potential of the fruit (Ψ_{fruit}). The fruit water potential equals the sum of the fruit's turgor and the osmotic potential of the expressed juice (Ψ_Π). Because the fruit turgor is negligibly low in strawberry[6], the value of Ψ_Π essentially equals the Ψ_{fruit}.

$$P_t = \frac{F_t}{A_{fruit} \cdot \rho_w \cdot \Delta a_w} \tag{1}$$

$$P_f = \frac{F_f}{A_{fruit} \cdot \Delta\Psi} \cdot \frac{RT}{\rho \cdot \overline{V_w}} \tag{2}$$

Fruit surface area was calculated from a solid geometrical model comprising a truncated cone capped by two halves of rotational prolate ellipsoids[6]. The respective dimensions were estimated from calibrated photographs by image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions, Münster, Germany). The relationship between mass and the measured surface area was plotted and an empirical regression model was fitted. Data from a compilation between different cultivars and development stages ranging from green fruitlets to fully mature fruit were used (see supplementary information). The total number of individual fruit replications was 200.

Identifying high flux pathways for water movement. Preferential pathways of water movement through the surface of 'Sonsation' fruits were identified by selectively sealing different portions of the fruit surface with silicone rubber. Two experiments were carried out with the rates of osmotic water uptake and transpirational water loss being quantified gravimetrically.

The first experiment was conducted in two phases ('control'/'treatment') using a repeated-measures design, comparing each treatment with its control (Fig. 4, Table 1). In phase I, fruit with calyx and peduncle (cut to 5 mm long) was incubated for 1.5 h. Rates of water movement were determined gravimetrically as described above. There was no sealing in phase I ('control'). In phase II, selective sealing using silicone rubber or cutting was applied as follows ('treatment'): (1) sepals (including episepals) and stamina were excised at their base and the cut surfaces, the calyx-receptacle junction, and the peduncle end were sealed (Fig. 3); (2) sepals and stamina were excised and the cut surfaces, the calyx-receptacle junction, and the peduncle end were left un-sealed, and (3) the peduncle end was sealed (Table 1). Each treatment had its own control. Fruit were held in a controlled temperature room for 1.5 h for the silicone rubber to cure. Fruits were then incubated for an additional 1.5 h.

In the second experiment, the portion of the fruit surface responsible for the increased water movement in the calyx region was identified by sealing a progressively increasing portion of the fruit surface—beginning at the proximal end of the fruit (Fig. 3, Table 2). The treatments were (a) 'Control': no sealing; (b) 'Sepals sealed': the sepals and the peduncle surface were sealed. The petal and stamina abscission zones, the calyx-receptacle junction, and the seedless zone of the receptacle were not sealed; (c) 'Sepals, petal and stamina abscission zones and calyx-receptacle junction sealed': the sepals, abscission zone, stamen, peduncle, and calyx-receptacle junction was cut and all surfaces sealed (the 'seedless' zone, strictly, the zone lacking the 'pips' or single-seeded achenes, was not sealed); and (d) 'Seedless zone sealed': the seedless zone of the receptacle was sealed, also the abscission zone and stamens, the calyx-receptacle junction, the peduncle, and sepals were removed and sealed. The cut end of the peduncle was sealed in all these treatments including the control.

Rates of transpiration and osmotic water uptake were calculated as described above. The total number of replications per treatment was 30. From these treatments, the amounts of water transpired or taken up through the sepals and through the peduncle surface or through the abscission zones of petals, stamina, the junction between calyx and receptacle, and through the seedless zone and through the receptacle bearing seeds were obtained by calculating the difference between the respective rates of water movement in phase I and phase II (Table 3).

To identify the sites of water uptake microscopically, 'Sonsation' fruits from the same batch were incubated in 0.1% (w/w) aqueous acridine orange (Carl Roth, Karlsruhe, Germany) for 1.5 h. The cuticle is impermeable to the fluorescent tracer acridine orange. The dye therefore penetrates only in regions where the cuticle barrier is bypassed. Fruits were rinsed with deionized water, blotted using soft tissue paper, and viewed under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany) (Figs. 5, 6).

To establish whether the same preferential pathways for water movement occurred in other strawberry cultivars, the relative contributions to water movement of the various pathways—through the petal and staminal abscission zones, and around the junction between calyx and receptacle, were determined on a whole fruit basis in 'Clery', 'Sonsation', 'Florentina', 'Dream', 'Joly', and 'Elsanta' (Table 4). To do this the abscission zone and the junction were sealed using silicone rubber. Unsealed fruit served as controls. Pathways of water movement were also viewed by fluorescence microscopy. Fruits from the same batch were incubated in a solution of the fluorescence tracer acridine orange at 0.1% (w/w) (Carl Roth, Karlsruhe, Germany) for 1.5 h. Thereafter, fruits were observed under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany).

External factors affecting water movement. Effect of temperature. The effects of temperature (T) on osmotic water uptake and transpiration were investigated in 'Laetitia' strawberry (Fig. 7b,c). Fruits were incubated at 2, 12, 23, or 35 °C. The rates of water movement were calculated as described above. Thereafter, the rates of transpiration were normalized by dividing by the gradient in water vapor concentration between the inside of the fruit

(assumed to be saturated) and the outside atmosphere at the incubation temperature. Because in the transpiration assays the fruit was incubated above dry silica gel and the water vapor concentration above the dry silica gel is practically zero, the driving force for transpiration equals the water vapor concentration at saturation at the respective temperature. The values so obtained will be proportional to the fruit's permeance.

Effect of relative humidity. The effect of relative humidity (RH) on the rate of transpiration in 'Laetitia' strawberry was determined by incubating the fruit at ~ 0, 30, 75, or 92% RH and 22 °C using silica gel, or saturated solutions of $CaCl_2$, NaCl, or KNO_3, respectively (Fig. 7a)[25]. Rates of transpiration were normalized by dividing by the gradient in water vapor concentration between the atmosphere inside the fruit (assumed to be saturated) and the outside atmosphere.

Effect of storage duration. The effect of storage duration of 'Clery' fruit on water movement was investigated in two experiments. In the first, fruits were held in polyethylene (PE) boxes above saturated NaCl (Carl Roth, Karlsruhe, Germany) (RH ~ 76%[25]) at 2 °C for 0, 1, 2, 4, 7, or 10 d (Fig. 8). Fruits were placed in the boxes on foam trays over a metallic mesh to avoid direct contact with the NaCl. The mass loss during storage was determined by weighing fruit before and after the storage period. Thereafter, fruits were equilibrated at room temperature and ~ 40% RH for 2 h before the rates of transpiration and osmotic water uptake were measured as described above.

Second, the effect of inducing a mass loss by incubating 'Clery' fruit for 24 h at different RHs on rates of osmotic water uptake and transpiration was determined (Fig. 9). To induce the mass loss, fruits were pre-incubated in PE boxes above silica gel (0% RH), or above saturated solutions of $CaCl_2$ (30% RH), NaCl (75% RH), or deionized water (100% RH)[25]. The mass loss was determined by weighing fruit before and after the pre-incubation period. The rates of transpiration and water uptake after mass loss induction were determined gravimetrically.

Data analyses. All experiments comply with relevant institutional, national, and international guidelines and legislation. Data were analyzed by analysis of variance (ANOVA) and linear regression. Means were compared using Dunnett's and Tukey's tests ($p < 0.05$) in the statistical software R (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria). Data are presented as means ± standard errors.

Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Received: 13 October 2022; Accepted: 6 March 2023
Published online: 08 March 2023

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Acknowledgements
We thank Dr. Ludger Linnemannstöns, Heike, Karl Walter, Martin Brüggenwirth, and Bendix Meyer for the gift of fruit, Hana Weiß, Marcel Pasta and Peter Grimm-Wetzel for technical support, and Drs. Sandy Lang and Andreas Winkler for helpful comments on an earlier version of this manuscript.

Author contributions
M.K. obtained the funds to support the study. G.H. and M.K. planned the experiments. G.H. conducted the experiments. G.H. and M.K. analyzed the data and G.H. and M.K. wrote, revised, and edited the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. This research was funded in part by a grant from the Deutsche Forschungsgemeinschaft (KN 402/19-1).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-31020-0.

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Supplementary material

Electronic File E2. Dataset. Excel file containing all data produced in figures and tables throughout the manuscript. (XLSX) (access through: https://doi.org/10.25835/5p308h3f)

4.3. Microcracking of strawberry fruit cuticles: Mechanism and factors

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Moritz Knoche: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing original draft, Writing review and editing.
Journal	Scientific Reports
Impact factor	4.996(2022)
Date of publication	08.11.2023
DOI	10.1038/s41598-023-46366-8

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Microcracking of strawberry fruit cuticles: mechanism and factors

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Microscopic cracks in the cuticle (microcracks) are the first symptom of the strawberry fruit disorder 'water soaking' in which the fruit surface appears watery, translucent, and pale. Water soaking severely impacts fruit quality. The objective was to investigate the factors and mechanisms of cuticular microcracking in strawberry. Fluorescence microscopy revealed numerous microcracks in the achene depressions, on the rims between depressions and at the bases of trichomes. Microcracks in the achene depressions and on the rims were either parallel or transversely oriented relative to a radius drawn from the rim to the point of attachment of the achene. In the achene depression, the frequency of microcracks with parallel orientation decreased from the calyx end of the fruit, towards the fruit tip, while the frequency of those with transverse orientation remained constant. Most microcracks occurred above the periclinal cell walls of the epidermal cells. The long axes of the epidermal cells were primarily parallel-oriented. Microcracking increased during fruit development. Cuticle mass per fruit remained constant as fruit surface area increased but cuticle thickness decreased. When fruit developed under high relative humidity (RH) conditions, the cuticle had more microcracks than under low RH conditions. Exposing the fruit surface to increasing RHs, increased microcracking, especially above 75% RH. Liquid-phase water on the fruit surface was markedly more effective in inducing microcracking than high vapor-phase water (high RH). The results demonstrate that a combination of surface area growth strain and water exposure is causal in inducing microcracking of the strawberry cuticle.

The cuticular membrane (CM) is an extracellular lipophilic membrane deposited on all primary above ground plant surfaces. The receptacle of the strawberry, a false fruit, is no exception. The CM comprises epicuticular and embedded cuticular waxes, polymeric cutin and phenolics. It is encrusted with cell-wall polysaccharides[1,2]. The CM fulfills important barrier functions. It helps restrict uncontrolled water and gas exchanges, inhibits pathogen entry and preserves fruit quality[3,4]. Maintaining all these functions requires an intact CM throughout fruit development.

The integrity of fruit cuticles is sometimes compromised by growth strains, resulting from rapid increases in fruit surface area and/or hostile environmental factors. Cuticular microcracks result from the failure of an overly-strained CM. Microcracks are minute fractures in the cuticle, not visible to the naked eye[5]. Microcracking is economically important in commercial fruit production and marketing. Microcracks impair the cuticle's barrier function, thereby compromising fruit quality and, hence, fruit quantity (by necessary rejection). Moreover, microcracking is the first visible symptom in a number of fruit-surface disorders such as russeting in apple and pear[6] and mango[7], cracking in sweet cherry[8] and grapes[9], neck shrivel in plum[10] and desiccation of lychee[11]. In strawberries, microcracking is the first symptom of impending water soaking[12] and of cracking[13]. Microcracking allows rapid water uptake by viscous flow, that bypasses the cuticle barrier. The incidence of water soaking and cracking is high in open-field cultivation of strawberry and is substantially eliminated in protected cultivation[14]. Microcracking also increases fruit transpiration and, hence, accelerates fruit shrivel[12]. Lastly, microcracks serve as entry points for fruit rot pathogens causing diseases such as grey mold and anthracnose[15]. Despite the importance of cuticle integrity to the highly perishable strawberry fruit, remarkably little is known about cuticle deposition and microcrack formation in strawberries.

The objective of this study was: (1) to characterize microcracking of the cuticle of strawberry skin and (2) to identify the key factors affecting cuticular microcracking.

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Results

Characterizing microcracking

Fluorescence microscopy of the strawberry fruit (receptacle) surface revealed numerous microcracks (Fig. 1a). Microcracking occurred in the achene depressions and also on the rims between neighboring depressions (Fig. 1b,c); they also occurred at the base of trichomes as indicated by the orange, yellow or green fluorescence of acridine orange (Fig. 1d). The orange colors indicate high penetration underneath and in the immediate vicinity of a microcrack in the achene depression (Fig. 1b,c), whereas the green color is indicative for low penetration for example at the base of trichomes (Fig. 1d).

Irrespective of whether an achene was situated in the distal or proximal region of the fruit, microcrack orientation in all achene depressions showed a frequency distribution with three dominant peaks: at + 90°, 0° and −90° (Supplementary Fig. S1). This indicates most microcracks are oriented in one of two directions: either transverse to, or parallel to, a radius drawn between the depression rim and the point of attachment of the achene (Fig. 2a). Moving from the proximal (calyx) towards the distal (tip) end of the fruit, crack orientation changed systematically. The frequency of microcracks orientated parallel to the depression radius decreased, while that orientated transversely to it remained about constant (Fig. 2b–d). The frequency distribution of epidermal cell orientation in the achene depressions was simpler—there were no changes, proximal to distal. The long axes of all epidermal cells were oriented parallel to a radius drawn from the point of achene attachment to the rim (Fig. 2).

On the rims between the depressions, microcrack orientations was similar to that in the achene depressions—microcracks were oriented either parallel to, or transverse to, a radius drawn to the point of attachment of the nearest achene. Location on the fruit surface had no effect. Meanwhile, the epidermal cell long-axis orientation was primarily parallel to a radius drawn to the point of attachment of the nearest achene, without major

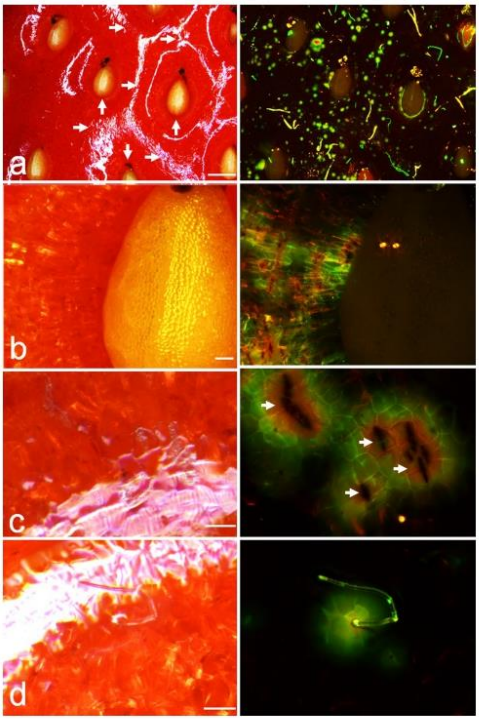

Figure 1. Micrographs of ripe 'Clery' strawberry fruit surface with numerous microcracks in the cuticle. Each row represents a pair of images in incident bright (left column) and fluorescent light (right column). (**a**) Overview of surface; (**b**) detail view of an achene depression; (**c**) detail view of a rim between achene depressions (**d**) detail view of trichomes. Scale bars in a = 1 mm and b,c,d = 0.1 mm. The vertical arrow in (**a**) indicates position of achene depression, the horizontal arrow in (**a**) the position on the rim between two adjacent achene depressions. Arrows in (**c**) point out microcracks. Fluorescence images were obtained following 5 min incubation of fruit in the fluorescent tracer acridine orange. The tracer does not penetrate an intact cuticle, but penetration is restricted to openings in the cuticle such as microcracks. Orange, yellow and green fluorescence indicates decreasing concentrations of fluorescent tracer. For details see materials and methods.

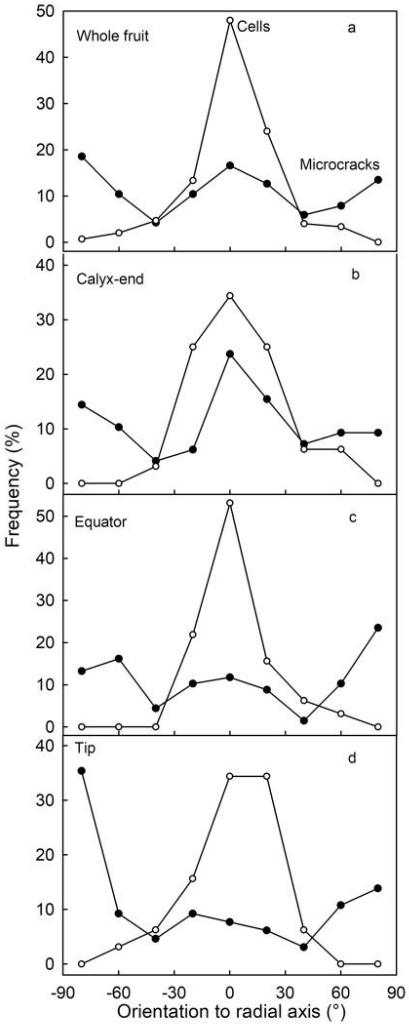

Figure 2. Frequency distribution of orientation of microcracks and epidermal cells in the achene depression of 'Florentina' strawberry. The orientation of microcracks and epidermal cells was measured relative to a radius drawn through the point of attachment of the achene. (**a**). Data were pooled for different regions. (**b**) Calyx region of the fruit (calyx and surroundings) within the seed zone; (**c**) Equator region (maximum fruit diameter and the center of the fruit). (**d**) Tip region of the fruit.

differences between regions of the fruit surface (Fig. 3). There were no systematic differences in the dimensions of the achene depressions between the proximal, equatorial or distal regions of the fruit surface (Supplementary Table S1).

There were no significant relationships between microcrack characteristics and fruit size but microcracking incidence did tend to decrease distally ($P = 0.06$; Supplementary Fig. S2). Microcracking was more frequent above the periclinal cell walls of the underlying epidermal cells, compared with above the anticlinal cell walls (Table 1).

Factors affecting microcracking

During strawberry fruit development, fruit mass and fruit surface area increased sigmoidally with time (Fig. 4a, main graph). At the same time, the receptacle color changed from green (≈100° Hue), to white (≈94° Hue) and

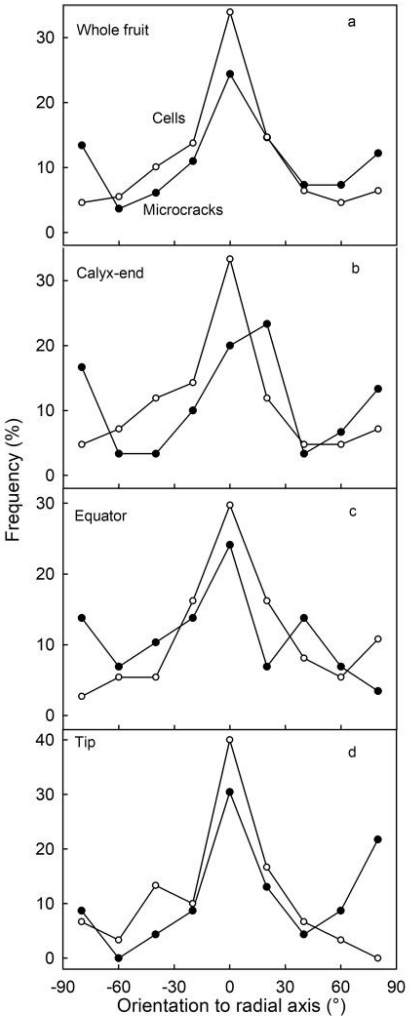

Figure 3. Frequency distributions of orientations of microcracks and epidermal cells on the rim between achene depressions of 'Florentina' strawberry. The orientation of microcracks and epidermal cells was measured relative to a radius drawn through the point of attachment of the achene. (**a**). Data pooled for different regions. (**b**) Calyx region of the fruit (calyx and surroundings) within the seed zone; (**c**) Equator region (maximum fruit diameter and the center of the fruit). (**d**) Tip region of the fruit.

	Frequency distribution (%)	
Zone	Periclinal	Anticlinal
Achene depression	60.2	39.8
Rim between achene depressions	65.7	34.3

Table 1. Frequency distribution of the location of cuticular microcracks above the anticlinal and periclinal cell walls in the achene depression and in the rim between achene depressions of strawberry 'Florentina'.

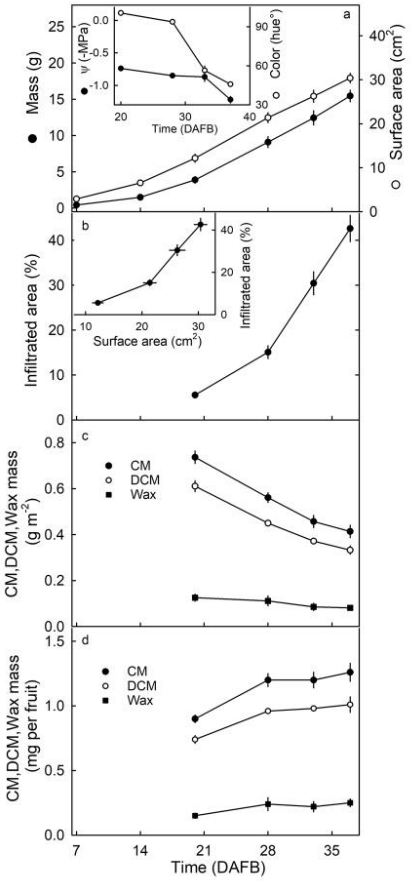

Figure 4. Developmental time course of change in (**a**) fruit mass, (**b**) microcracking of the cuticular membrane, (**c**) mass per unit area of the CM, the dewaxed CM (DCM), and the wax, and (**d**) mass per fruit of the CM, DCM and wax of 'Clery' strawberry. Inset in a: Developmental time course of change in color and osmotic potential (Ψ). Inset in b: Microcracking as a function of fruit surface area. Microcracking was indexed by the area infiltrated with acridine orange. The mass per fruit of the CM, DCM and wax was calculated by multiplying the mass per unit area by the surface area of a whole fruit.

to red (≈45° Hue) as hue angle decreased (Fig. 4a, inset). The change in color from green to white began before fruit ripening, the subsequent change to red occurred during ripening.

Cuticular microcracking as indexed by the percentage of the surface area infiltrated by the fluorescent tracer acridine orange, increased during development (Fig. 4b, main graph). The relationship between the percentage infiltrated area and fruit surface area was biphasic. When small fruit grew, there was little increase in microcracking. However, beyond about 28 DAFB microcracking increased markedly as fruit surface area increased (Fig. 4b, inset).

Change in the mass per unit surface area of the isolated CM served as a proxy for change in cuticle thickness. On this basis, cuticle thickness decreased during development (Fig. 4c). The decrease in unit CM mass was due primarily to a decrease the unit DCM mass. This says that unit cutin matrix mass decreased more than unit wax mass. On a whole-fruit basis, total CM mass increased by 33% from flowering (0 DAFB) to the white stage (28 DAFB) and then remained substantially constant until ripeness (Fig. 4d).

The severity of cuticular microcracking on ripe fruit depended on the RH environment of the fruit during development (Fig. 5a). When fruit were grown at higher RHs, cuticular microcracking increased exponentially (Fig. 5a). This effect was common between the two strawberry cultivars investigated. As RH increased, so too did microcracking, particularly for RH values above 75% (Fig. 5b).

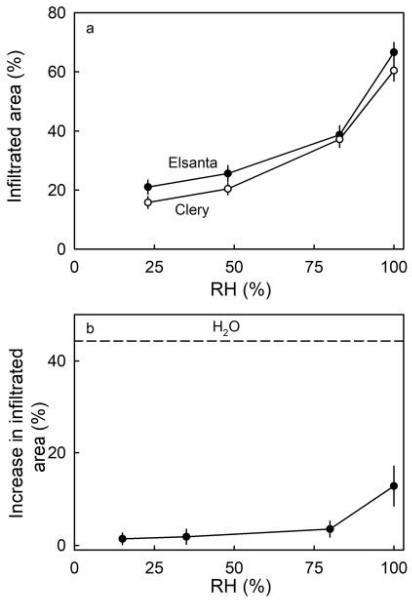

Figure 5. (**a**) Effect of relative humidity during fruit development on microcracking of the cuticle of 'Elsanta' and 'Clery' strawberries. Fruit were incubated in pottles for RH control between 10 days after full bloom and full ripeness. The bottom of the pottles was covered with dry silica gel, $CaCl_2$, NaCl or deionized water. Using this setup, the RH in the pottle equilibrated at about 23% RH when the bottom of the pottle was covered with dry silica gel, at 48% RH when using $CaCl_2$, at 83% RH when using NaCl and at 100% RH when using deionized water. At the ripe stage the percentage of the fruit surface area infiltrated with acridine orange was quantified. For experimental setup see supplementary Fig. S4. (**b**) Effect of relative humidity during a 24 h incubation period on microcracking of the cuticle of ripe 'Clery' strawberry. Microcracking was indexed by the increase in area infiltrated with acridine orange before and 24 h after exposure to water. For experimental setup see supplementary Fig. S4.

Vapor-phase water (RH) was less effective in increasing microcracking than liquid-phase water. Exposure of the fruit surface to liquid water, increased cuticular microcracking rapidly, compared to the opposing fruit surface (control) that remained dry (Fig. 6a). Surface exposure to liquid water also induced microcracking in younger fruit (Fig. 6b). As with the cuticle's response to water vapor exposure (Fig. 5a), susceptibility to liquid water exposure increased towards maturity (Fig. 6b).

Discussion

The main findings of this study were: (1) Growth strain is the primary driver of microcracking of strawberry's very-thin cuticle and (2) exposure of the fruit surface to water—either vapor or liquid—greatly exacerbates cuticular microcracking.

Growth strain is the primary driver for cuticular microcracking

Growth strain causes cuticular microcracking. First, cuticle synthesis and deposition cease at about 28 DAFB, when the fruit has achieved about half its later maximum surface area (at ripeness). The subsequent doubling of fruit surface area to the fully-ripe stage, has the effect of distributing an approximately constant amount of cuticle over about twice the surface area. As a result, the cuticle is significantly strained and this strain is associated with significant thinning and failure. Interestingly, the cessation of cuticle deposition (28 DAFB) coincides with a breakpoint in microcracking incidence and severity. Before this stage, the deposition of cutin and the impregnation of the cutin network with wax partially 'fixed' the accumulating strain[6,16]. The fixation of strain (elastic) essentially prevented/reduced microcracking. Beyond the breakpoint, the microcracking increased markedly, probably as a result of the continuing increases in strain in the face of the absences of any deposition of new cutin and wax and, hence, the absence of any further strain 'fixation'. Unfortunately, the thin, and extremely fragile strawberry cuticle, prevented a strain-relaxation analysis[17,18]. However, it is expected that the increase in strain and the concurrent decrease in structural support by the underlying tissue due to fruit softening, induced

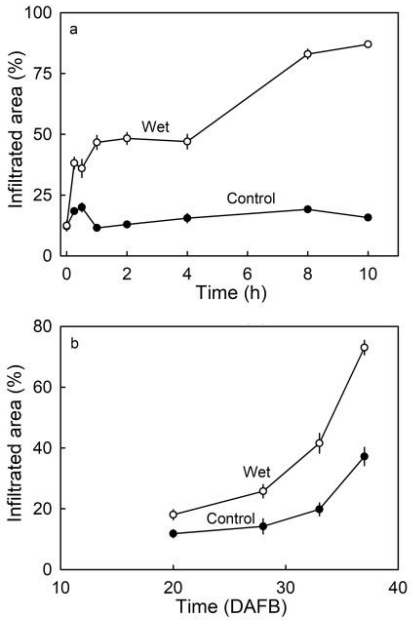

Figure 6. (**a**) Time course of moisture-induced microcracking of the cuticle of 'Elsanta' strawberry. Half of the fruit (along the longitudinal axis) was incubated in deionized water ('Wet'), the other half remained in the air ('Dry') and served as control ('Control'). (**b**) Developmental time course of moisture-induced microcracking of the cuticle of 'Elsanta' strawberry as indexed by the area infiltrated with acridine orange. The fruit was incubated for 4 h in deionized water as described for (**a**).

failure and microcracking of the CM[8]. This cause and effect relationship is not unique to strawberry but is also seen in grapes[19], sweet cherries[20] and plums[21].

The above interpretation is also consistent with the following arguments. First, the strawberry cuticle is the thinnest fruit cuticle ever reported across large number of fruit species[14]. This makes it very susceptible to failure. Second, microcracks tend to be more frequent in the fruit's proximal region (calyx) and equatorial region, than in its distal region (tip) ($P = 0.06$). The proximal and equatorial regions have larger diameters than the narrower tip region, due to the higher rates of radial expansion of the fruit. For similar reasons one would predict larger fruit will show more microcracking than smaller fruit. However, this relationship was not evident in our results—perhaps because of high fruit:fruit variability in microcracking. Third, the unique surface topography of the strawberry causes a characteristic pattern of microcracking. To better understand the pattern of microcracking, it is important to understand the origins of the unusual 'corrugated' topography of the strawberry surface. The corrugation of the surface results from restricted flesh expansion growth in the immediate vicinity of an achene and the lack of such a restriction in the spaces between achenes. The restriction in flesh expansion is probably a result of tensile forces in the radially orientated vascular bundles serving the achenes, with the result that the achenes are 'pulled' into the surface as flesh expansion growth proceeds. This results in the 'funnel type' structure of the achene cavity where the vascular bundles form the center of the funnel. This interpretation is consistent with a pronounced parallel radial orientation of the epidermal cells (in depressions and on the rim) irrespective of the position of the achene on the fruit surface. As a consequence, microcracks form transverse to this extension growth as the cuticle is dragged along by the underlying epidermal cells. This explains the peaks in the orientation frequency at ± 90° relative to any radius drawn from the achene axis.

However, the above explanation does not account for the about 20% incidence of microcracks in the achene depression orientated parallel to the radii (0°) and the about 30% incidence on the rims between the depressions. Interestingly, in the achene depression these parallel-orientated microcracks occurred in both the proximal (calyx) and the equator regions but not in the distal (tip) region. A parallel orientation of the microcracks would result from a 'flattening' of the funnel-shape of the achene depression and thus an increase in the depression diameter. This would be consistent with a tangential strain as fruit volume and, hence, fruit circumference increased[22].

Finally, there was preferential location of microcracks above the periclinal epidermal cell walls (compared with above the anticlinal cell walls) in the achene depressions and on the rims between depressions. This distribution indicates strain is greater above the periclinal walls. This result is similar to that in sweet cherry, where

microcracking is not related to an underlying anticlinal cell wall[8]. This is different from apple, where the pattern of microcracking aligns with the underlying anticlinal epidermal cell walls, indicating that a failure of cell–cell adhesion is a factor in microcracking[23].

Both liquid- and vapor-phase water trigger microcracking

Surface moisture plays a critical role in cuticular microcracking in strawberry fruit. Microcracking was especially increased when the fruit surface was exposed to liquid-phase water and less so, but still significantly, when it was exposed to high concentrations of vapor-phase water. These findings are not unique to strawberry but have also been reported in apple[24], mango[7], grape[9] and sweet cherry[25].

The mechanism of water-induced microcracking is not known. Earlier experiments demonstrated that strawberry fruit incubated in an isotonic polyethylene glycol solution did not take up water but they did develop microcracks[12]. Hence, water uptake by the epidermal cells is judged unlikely to play a direct role. What other factors might be involved in water-induced microcracking?

First, exposure to liquid water, or even to high concentrations of vapor-phase water, hydrates the cuticle[26]. It is already known that hydration of a strained cuticle alters its rheological properties, leading to a general weakening. Both the cuticle's stiffness and its fracture force decrease, while its fracture strain increases. This phenomenon has been demonstrated for the cuticles of sweet cherry[25], tomato[27] and for apple and pear[28].

Second, it is possible that surface wetness (liquid-phase water) may induce swelling of the epidermal cell walls immediately underlying the cuticle. Cell wall swelling would strain the immediately overlying area of cuticle and so could result in microcracking. Epidermal cell wall swelling has been reported in strawberry during ripening[29]. Here, the swelling is accompanied by an increase in intercellular spaces, presumably due to a reduction in cell–cell adhesion and to partial cell separation in the strained fruit skin[29]. In sweet cherry, cell wall swelling results in decreased cell–cell adhesion[30]. Additional evidence for a role for cell wall swelling in microcracking in strawberry comes from an effect of Ca ions on microcracking. Microcracking is reduced if fruit are incubated in a $CaCl_2$ solution[31]. In sweet cherry, Ca ions decrease cell wall swelling by increasing crosslinking of the cell wall constituents. This particularly applies to the homogalacturonans of the middle lamella[32]. As a consequence, cell–cell adhesion is increased and along with the skin's fracture force[30,33]. These interpretations are consistent with the absence of a role for the cuticle in fruit-skin mechanics. It is the epidermal and hypodermal cell layers that represent main structural member in most fruit skins—not the cuticle[34–36].

Lastly, surface wetness (liquid-phase water) decreases the rate of cuticle deposition (both wax and cutin) and the decrease favors cuticle failure during fruit expansion growth. In apple, the genes involved in cuticle synthesis and deposition are downregulated when the fruit surface is exposed to surface moisture[37]. It is not known if such downregulation also occurs in strawberries.

Conclusion

The strawberry cuticle is subject to marked growth strain during fruit development. First, this is because the rate of cuticle deposition in strawberry is low, compared with other fruit crop species, and it is also limited to the early stages of fruit development. Second, strawberries develop to considerable size during quite a short period of time. As a result, the amount of cuticle present at the stage deposition ceases must be stretched over a much larger area during quite a short period of time. In the light of this information, the high incidence of cuticular microcracking in strawberry is hardly surprising. Taking both a whole-fruit perspective, and also a more local achene-depression perspective, the incidences and orientations of microcracking are fully consistent with the idea that growth strain is the dominant driver of cuticular microcracking. Along with this, as in many other fruit crop species, the exposure of the strained cuticle of strawberry to either liquid-phase or vapor-phase water exacerbates microcracking. Projecting these mechanistic understandings to the production issues facing strawberry growers, the cultivation of strawberries in protected environments or in regions with low rainfall and low RH and the maintenance of open canopy structures should all reduce cuticular microcracking and hence also reduce the incidences of water soaking, flesh cracking and fruit rots.

Material and methods

Plant material

Strawberry (*Fragaria* × *ananassa* Duch., 'Clery', 'Elsanta' and 'Florentina') were cultivated in a growth chamber or in a greenhouse on the Herrenhausen Campus of the Leibniz University, Hannover, Germany (lat. 52° 23′ N, long. 9° 42′ E). Plants in the growth chamber were grown in pots (14 cm diam.) filled with a peat/moss-based growing medium (Einheitserde Pikiererde Typ P; Company, Sinntal-Altengronau, Germany). The growing conditions in the growth chamber were set at 20/16 °C day/night temperature, 60/80% day/night relative humidity (RH), and a 16 h photoperiod. In the greenhouse, plants were grown in boxes (size 25 × 120 × 20 cm) with 8–10 plants per box. Fruits were selected for uniformity of size and color and absence of macroscopically visible defects.

Microscopy

Microcracking of the cuticle of strawberry fruit was studied following infiltration with the fluorescent tracer acridine orange using fluorescence microscopy. When incubating fruit in an aqueous solution of acridine orange, penetration of the tracer is restricted to openings in the cuticle including those associated with microcracks, lenticels, and (sometimes) stomata and trichomes[8]. When viewed at the appropriate wavelength, tissues infiltrated with acridine orange exhibit orange, yellow and green fluorescence[8]. Orange, yellow and green fluorescence indicates decreasing concentrations of fluorescent tracer. Fruit was incubated in 0.1% (w/w) aqueous acridine orange (Carl Roth, Karlsruhe, Germany) for 5 min. This incubation time is sufficiently long to allow dye uptake, but not long enough to induce microcracks. After incubation, fruit was rinsed with deionized water, blotted dry

with soft tissue paper, and the fruit surface viewed under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Calibrated images were taken randomly (Camera DP71; GFP-plus filter, 480–440 nm excitation, ≥ 510 nm emission wavelength) in the equatorial region of the fruit, where diameter is at its maximum. The number of individual fruit replicates was 20. Microcracking was indexed by recording the percentage of the surface area in the microscope window infiltrated by acridine orange. The areas infiltrated by acridine orange were quantified using image analysis (cellSens Dimension 1.18; Olympus Soft Imaging Solutions, Münster, Germany). Fluorescing areas associated with achenes and trichomes were subtracted.

Experiments

Characterizing microcracking

Orientations of microcracks and epidermal cells in the achene depressions and on the rims between depressions. The achene depression, and the rim between adjacent depressions, were selected because they are representative locations on the 'corrugated' strawberry fruit surface. The orientations of microcracks and epidermal cells were quantified as the angle made between the microcrack, or of the long axis of an epidermal cell, and a radius drawn from that point to the point of attachment of the achene—like the spokes of a bicycle wheel, with the point of attachment of the achene being the hub (cellSens Dimension 1.18; Olympus Soft Imaging Solutions, Münster, Germany). Depending on the orientation of a microcrack or cell, the angle value might range from − 90° (anticlockwise) to + 90° (clockwise). The exact point of attachment of an achene was determined in a preliminary experiment in which achenes were excised and viewed under a microscope with the lower side up so that the point of attachment was visible (VHX-7000; Keyence, Osaka, Japan). Calibrated images were taken and the maximum length (y_{max}) and maximum width (x_{max}) of the achene determined using image analysis. The point of attachment was then expressed relative to the y_{max} and the x_{max}. This point had the x and y coordinates of $0.5{\cdot}x_{max}$ and $0.7{\cdot}y_{max}$ (Supplementary Fig. S3). This point was then used as the hub for the radius measuring the microcrack or epidermal cell orientation.

The frequency distributions of the angles of orientation of microcracks and epidermal cells in the achene depressions and on the rim between depressions were established in three regions along the fruit's longitudinal axis: in the calyx region (proximal); the equatorial region (maximum fruit diameter and about the center of the fruit), and the tip region (distal).

The dimensions of the achene depressions (length, width, depth) were measured on 3D-images using a digital microscope (VHX-7000; Keyence, Osaka, Japan). The areas of the fruit surface investigated were the proximal, equatorial, and distal regions of the fruit. The fruit used for these measurements were selected to be of almost the same mass (10.9 ± 0.2 g). The number of replicates was 16 per region.

Effect of fruit size on microcracking

This was studied by harvesting 20 fruits of the same ripeness stage (color) but of a broad range of different masses (5.1–26.4 g per fruit). The soluble solids of the expressed juices were quantified (mean 8.4 ± 0.4° Brix; CV = 20.0%) using a refractometer (DR6200-T; A. Kruess Optronic, Hamburg, Germany). To record microcracks, the procedure described above was used.

Microcracking in different regions along the fruit

Microcracking was also quantified in three different regions of the fruit surface along its length: within the region carrying achenes near the calyx (proximal end), near the equator (the point of maximum diameter) and near the tip (distal end). A total of 14 fruits were investigated.

Microcracking above the anticlinal and periclinal epidermal cell walls

To identify the sites of initiation of microcracking, it was assumed the shortest microcracks had only recently been initiated. We selected only those shorter than the dimensions of the underlying epidermal cells. The percentages of short microcracks lying just above the anticlinal cell walls and those just above the periclinal cell walls were recorded in the achene depressions and also on the rim between adjacent depressions.

Factors affecting microcracking

Microcracking and development

The time course of cuticular microcracking was investigated in fruit sampled at 20, 28, 33 and 37 days after full bloom of the respective flower (DAFB). Younger fruit (< 20 DAFB) were too small to measure. Microcracks were quantified following infiltration with acridine orange as described above. Fruit developmental stage was characterized by measuring fruit mass, color and the osmotic potential of the expressed juice. Color was quantified in the equatorial region using a spectrometer (CM-2600 d, orifice 3 mm diameter; Konica Minolta, Tokyo, Japan). The hue angle was calculated[38]. Juice of the fruit was extracted using a 'garlic press' and its osmotic potential quantified by water vapor pressure osmometry (VAPRO5600; Wescor, Logan, Utah, USA).

The change in mass of the CM, the mass of the dewaxed CM (the DCM) and the wax fraction were determined on the same batches of fruit. Epidermal skin segments (ES) comprising cuticle, epidermis, and adhering flesh were excised in the equatorial region of the fruit using a biopsy punch (4 mm diameter; Kai Europe, Solingen, Germany). Care was taken to include not more than one achene per ES. The CMs were enzymatically isolated by incubating the ESs in a solution of pectinase (90 ml l^{-1}; Panzym Super E flüssig, Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark), and cellulase (5 ml l^{-1}; Cellubrix L; Novozymes A/S) buffered in 50 mM citric acid buffer[39]. The pH was adjusted to pH 4.0 using NaOH. Sodium azide (NaN_3) was added at a final concentration of 30 mM to prevent microbial growth. The isolation medium was refreshed once. Six CM discs per fruit were collected from a total of 25 fruits. There was no significant difference in CM area after isolation when only one ES (7.2 ± 0.2

mm^2) was excised per fruit or when several ES (7.4 ± 0.2 mm^2) were excised per fruit. Following isolation, the CMs were rinsed three times with deionized water. Adhering cellular debris was removed by ultrasonication at 35 kHz for 10 min (RK 510; Sonorex Super, Bandelin electronic, Berlin, Germany). Achenes were carefully removed from the CM discs by hand. The CM samples (n = 5 discs per rep) were dried above silica gel for 48 h and weighed on a microbalance (M2P; Sartorius, Göttingen, Germany). Wax was extracted from the CMs by incubating in $CHCl_3$/MeOH (1:1, v/v) for 24 h at room temperature. The DCM were dried above silica gel and re-weighed. The wax mass per unit area was obtained by subtracting the mass of the DCM from that of the CM. The wax content was calculated. The number of replicates was 10.

Relative humidity and development

The effect of the RH during development on cuticular microcracking was established by containing still-attached and growing fruit in plastic pottles until ripeness (120 ml, PET, screw-cap lids). This was done as follows: at 10 DAFB fruitlets were introduced through a 12-mm-diameter hole in the lid of the pottle. The space between the hole and the peduncle was then sealed with soft plastic-foam plug. This prevented damage of the peduncle and also reduced water–vapor transfer into/out of the pottle[40]. Data logger sensors were placed inside the pottle to monitor humidity (MSR147WD, sensor: FH2.3/160; MSR Electronics, Seuzach, Switzerland). Pottle humidities were adjusted using dry silica gel, or saturated solutions of $CaCl_2$, NaCl or deionized water[41]. Using our setup, the RH in the pottle equilibrated at about 23% RH when the bottom of the pottle was covered with dry silica gel, at 48% RH when using $CaCl_2$, at 83% RH when using NaCl and at 100% RH when using deionized water. There was no contact between the salts / solutions and the fruit (Supplementary Fig. S4). The salt solutions and silica gel were refreshed twice a week. Ripe fruit were harvested, and microcracks quantified as described above. The number of replicates was 15, where one replicate represented one plant with one pottle per plant.

Relative humidity and microcracking

The effect of atmospheric RH on microcracking at maturity was determined using ES[25]. Briefly, stainless steel washers (6.4 mm inner diameter) were glued onto the fruit surface in the equatorial region of a mature strawberry using a fast-curing epoxy glue (UHU Plus Schnellfest; UHU). The washer prevented any strain relaxation of the fruit skin before recording microcracking. The extent of microcracking was quantified before and after exposure to the different humidities. Before exposure, acridine orange was applied to the ES surface. After 5 min, the dye solution was removed, the surface rinsed with deionized water and the ES viewed under a fluorescence microscope. The area infiltrated by dye was quantified. Thereafter, the ES were incubated in polypropylene boxes above either dry silica gel, or saturated salt slurries of $CaCl_2$ or NaCl, or deionized water[41] (Supplementary Fig. S4). Fruit that was submerged in deionized water served as control. After 24 h, the ESs glued to the washers were re-inspected for microcracks, as described above. This procedure allowed quantification of the change occurring in the infiltrated area (microcracking area) of each individual ES during incubation at a particular humidity. The number of replications was 10.

Time course of moisture-induced microcracking

A total of 80, visually-similar strawberry fruit were partly submerged in deionized water. They were held in such a way that each fruit's longitudinal axis was exactly horizontal, and its lower half was wet and its identical upper half was dry (control). After 0, 0.25, 0.50, 1, 2, 4, 8, and 10 h, 10 fruit were examined by staining with acridine orange, blotting dry, and inspecting for microcracks using fluorescence microscopy. The fluorescing areas were quantified as described above.

Moisture induced microcracking during fruit development

Moisture effect on microcracking was studied at four stages of fruit development. Fruit was harvested at 20, 28, 33 or 37 DAFB. Individual fruits were incubated for 4 h in deionized water as described above with half being submerged in deionized water and half remaining dry (control). Microcracks were quantified as described above.

Data analyses

All experiments comply with relevant institutional, national, and international guidelines and legislation. All experiments had completely randomized designs. Data were subjected to analysis of variance and regression analysis using R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Means were compared using Tukey's studentized range test at $P < 0.05$. Data in the Tables and Figures are presented as means ± standard errors.

Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Received: 21 June 2023; Accepted: 31 October 2023
Published online: 08 November 2023

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Acknowledgements

We thank Dr. Andreas Winkler and Germaine Malon for technical support and Drs. Sandy Lang and Jannis Straube for helpful comments on an earlier version of this manuscript. We thank Heike, Karl Walter, and Martin Brüggenwirth, and Bendix Meyer for the gift of fruit.

Author contributions

M.K. obtained the funds to support the study. G.H. and M.K. planned the experiments. G.H. conducted the experiments. G.H. and M.K. analyzed the data and G.H. and M.K. wrote, revised, and edited the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. This research was funded in part by a grant from the Deutsche Forschungsgemeinschaft (KN 402/19-1).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-46366-8.

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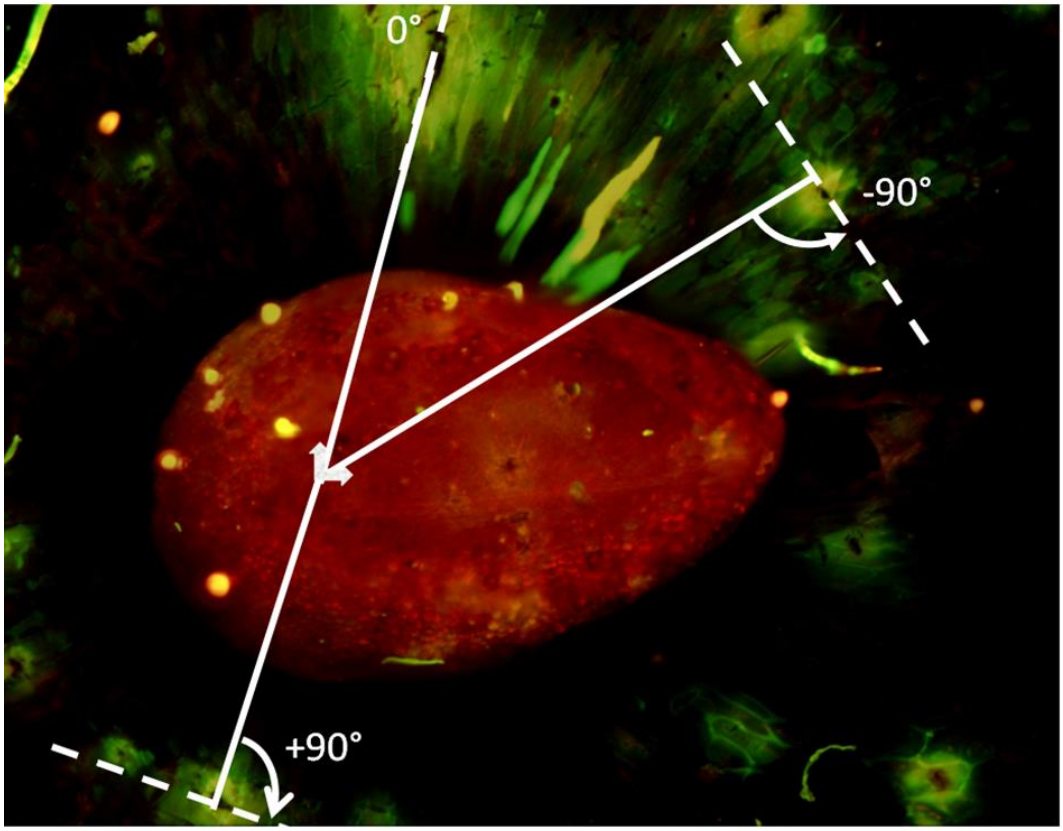
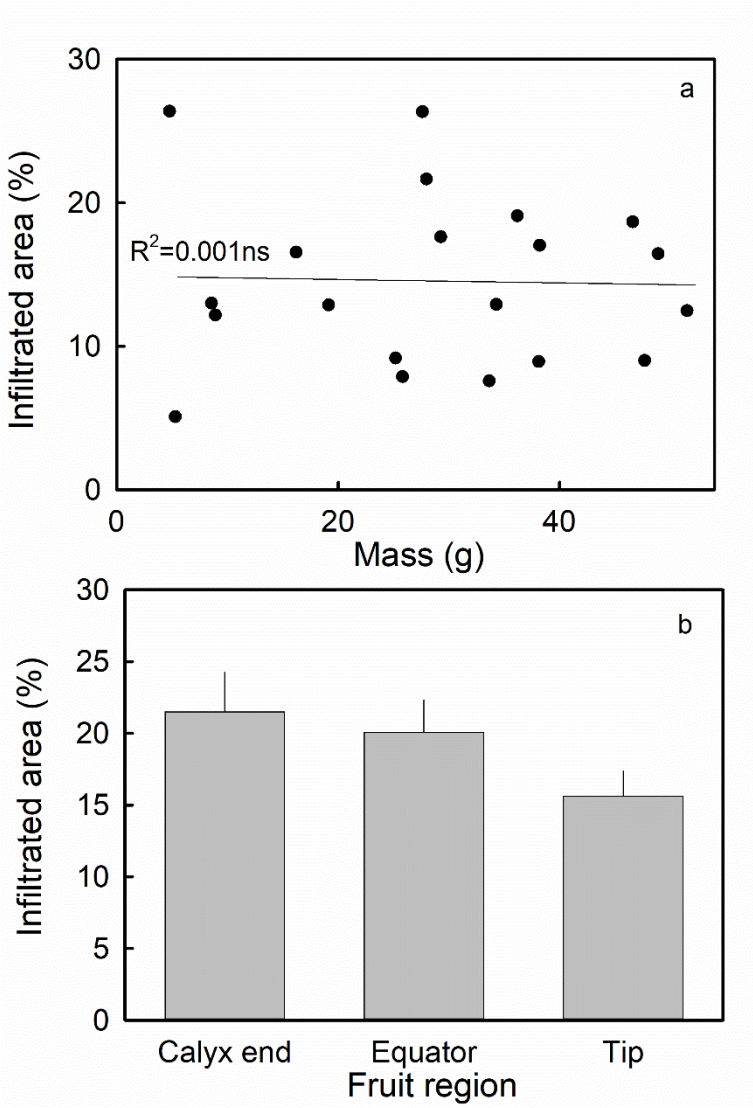
Supplementary material

	Calyx-end	Equator	Tip
Depth (um)	727±39	806±35	798±27
Length (um)	2945±101	3077±110	3091±90
Width (um)	2531±99	2712±109	2625±84
Volume (mm^3)	2.3±0.3	2.8±0.3	2.6±0.2
Ratio (W/L)	0.9±0.0	0.9±0.0	0.9±0.0

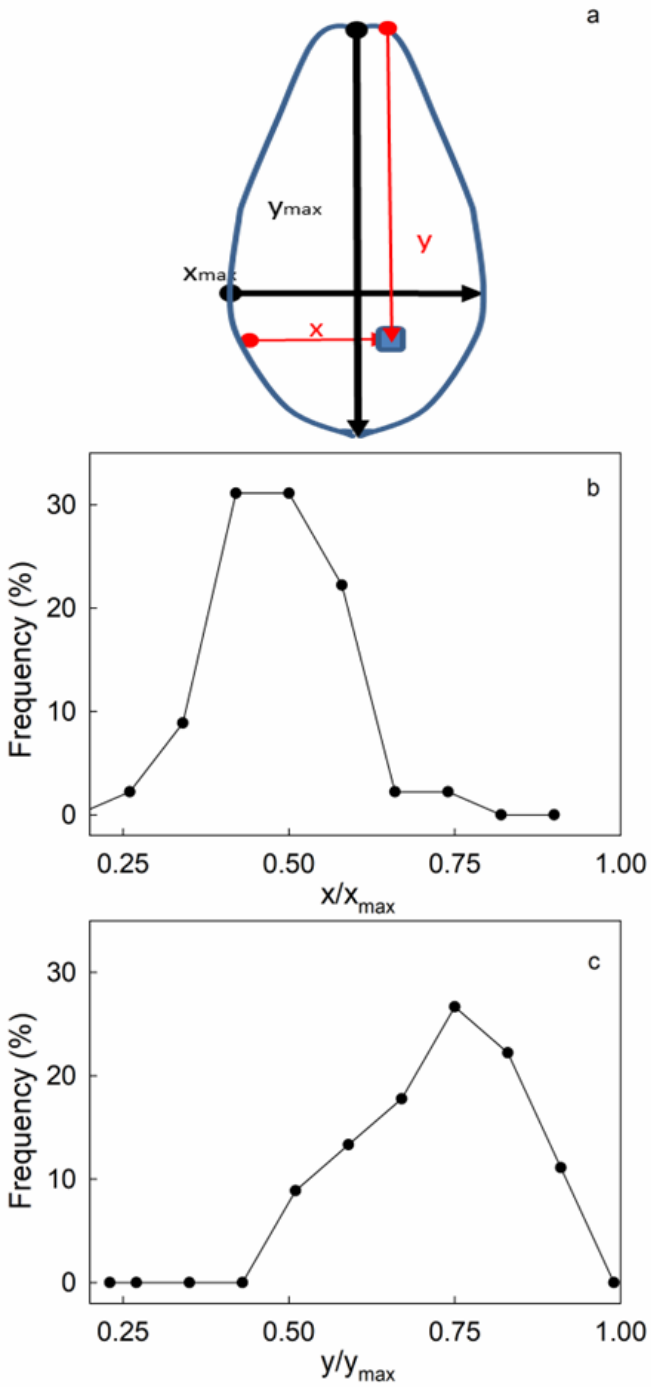
Supplementary Table S1. Dimensions of the cavity depression within the calyx-end (proximal), equator (equatorial), and tip (distal) regions of strawberry fruit ‘Clery’.



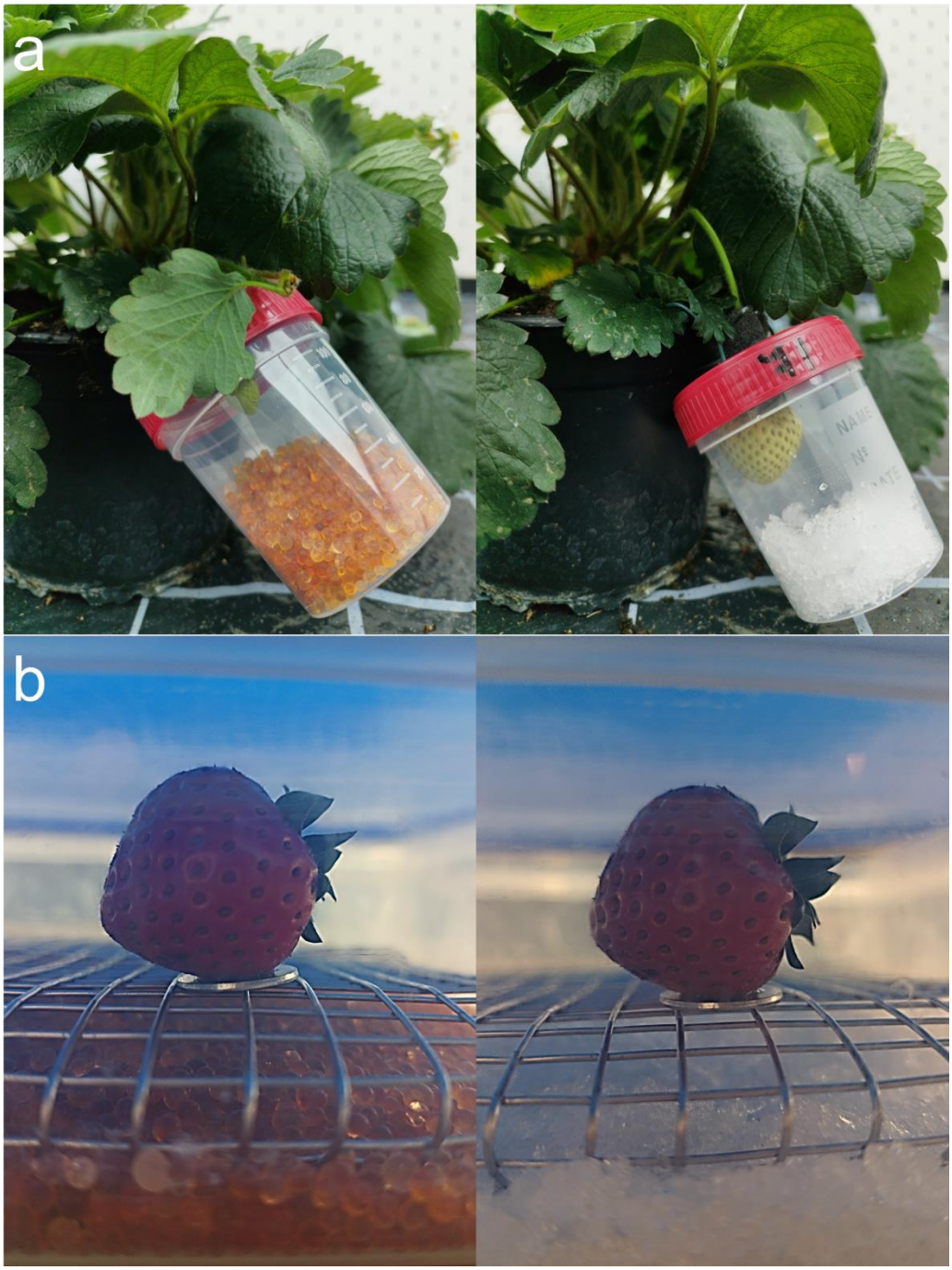
Supplementary Fig. S1. Measurement of the orientations of microcracks as the angle made between the microcracks, and a radius drawn from the point of attachment of the achene.



Supplementary Fig. S2. (a) Effect of fruit size on microcracking of the cuticle. (b) Microcracking of the cuticle in different regions of ripe 'Clery' strawberry. The regions were the calyx-end of the fruit within the seed zone; region of equator (maximum fruit diameter and the center of the fruit), and tip of the fruit. Microcracking was indexed by the area infiltrated with acridine orange.



Supplementary Fig. S3. Frequency distribution of the position of the attachment of the vascular bundle to the achene of strawberry 'Florentina'. (a) Scheme of the measurement of the position relative to the maximum length (Y_{max}) and diameter of the achene (X_{max}). (b) position on the y axis relative to Y_{max}. (c) position on the x axis relative to X_{max}.



Supplementary Fig. S4. (a) Experimental setup of the study on the effect of the relative humidity (RH) during fruit development on cuticular microcracking; (b) Experimental setup of the effect of RH on microcracking at maturity.

Electronic File E3. Dataset. Excel file containing all data produced in figures and tables throughout the manuscript. (XLSX) (access through: https://doi.org/10.25835/5p308h3f)

4.4. Necked strawberries are especially susceptible to cracking

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Moritz Knoche: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing original draft, Writing review and editing.

Journal	PeerJ
Impact factor	3.061(2022)
Date of publication	11.05.2023
DOI	10.7717/peerj.15402

PeerJ

Necked strawberries are especially susceptible to cracking

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ABSTRACT

Fruit cracking is a commercially important disorder that reduces both quantity and quality of strawberries (*Fragaria* × *ananassa* Duch.). The objective was to identify the physiological mechanism of cracking and the factors affecting cracking. Cracking is more common in necked than in normal-shaped fruit. Most macroscopic cracks ('macrocracks') occur in the seedless neck. Large fruit is more cracking susceptible than medium size or small fruit. Macrocrack orientation is predominantly latitudinal in the proximal region of the neck and longitudinal in the mid and distal regions of the neck. The neck region of necked fruit has a thicker cuticle than the body of necked or normal-shaped fruit. The vascular bundles in the neck (seedless) are orientated longitudinally, while those in the body (with seeds) are both longitudinal and radial. Epidermal cells in the neck region are elongated longitudinally, with those in the proximal region of the neck being more elongated than those in the mid or distal regions of the neck. Cuticular microcracking was more severe in necked fruit than in normal-shaped fruit. The orientations of the microcracks matched those of the macrocracks, *i.e.*, latitudinal in the proximal neck and longitudinal in the mid and distal neck regions. Following artificial incisions (blade), gaping was significantly more pronounced in necked than in normal-shaped fruit. Incubation of fruit in deionized water induced macrocracks in about 75% of fruit. Necked fruit cracked more than normal-shaped fruit. Most macrocracks were oriented latitudinally in the proximal neck and longitudinally in the distal neck regions. The results indicate cracking results from excessive growth strains which are further increased by surface water uptake.

Submitted 30 January 2023
Accepted 20 April 2023
Published 11 May 2023

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Academic editor
Sapna Langyan

Additional Information and Declarations can be found on page 18

DOI **10.7717/peerj.15402**

Subjects Agricultural Science, Food Science and Technology, Plant Science
Keywords *Fragaria* × *ananassa*, Splitting, Rupture, Microcrack, Macrocrack, Cuticle

INTRODUCTION

Strawberry fruit is highly perishable. Its quality is often compromised by pre- and postharvest disorders. Impaired fruit quality causes significant economic loss during harvesting, packing and marketing (*Herrington et al., 2009*).

Preharvest disorders arise from environmental factors during growth and development. Among these, rain damage is a critical factor in all regions where rain occurs during the fruiting season (*Blanco, Santos & Romero, 2006*; *Herrington et al., 2009*, *2011*, *2013*; *Menzel, 2021*). The high susceptibility of strawberry to rain damage has caused a shift in

How to cite this article Hurtado G, Knoche M. 2023. Necked strawberries are especially susceptible to cracking. **PeerJ 11:e15402 DOI 10.7717/peerj.15402**

production from the open field to greenhouses or plastic tunnels—but these are more capital intensive (*Menzel, Smith & Moisander, 2014*).

Rain damage in strawberries includes water soaking and cracking (*Herrington et al., 2009*).

Water soaking is a surface disorder that occurs in unprotected field production when strawberries are exposed to rain. Symptomatic fruit show pale, deliquescent patches of skin (*Herrington et al., 2009*, *2013*; *Hurtado & Knoche, 2021*). Recently, the physiological mechanism of water soaking has been elucidated and factors affecting water soaking have been identified (*Hurtado & Knoche, 2021*). To our knowledge there are no such studies on cracking in strawberries, and the physiological mechanism of cracking in strawberries is unknown.

Cracking is a failure of the fruit skin that can be categorized as both microcracking (invisible) and macrocracking (visible). Macrocracks are always preceded by the formation of microcracks. Microcracks are minute fractures whose depth is limited to the thickness of the cuticle (<1 µm). These cannot be observed by the naked eye. Macrocracks are easily observed. They extend through the skin into the flesh (~2–3 mm) (*Knoche & Winkler, 2017*). In strawberries, macrocracks have been observed in the shoulder and in the neck of the fruit (*Herrington et al., 2011*).

Understanding the physiological mechanism of cracking is a necessary prerequisite for developing effective mitigating strategies—either through changes in cultural practice or through breeding or both. For this reason, the objective of this study is to identify the mechanism of cracking and the factors affecting cracking in strawberry fruit.

MATERIALS AND METHODS

Plant material

Strawberry fruits were harvested from a commercial plantation in the open field at Gleidingen (lat. 52°16′N, long. 9°50′E), and from the Horticultural Research Station in Ruthe (lat. 52°14′N, long. 9°49′E), and from a growth chamber at the Herrenhausen Campus (lat. 52°23′N, long. 9°42′E) of the Leibniz University of Hannover, Germany. Temperature and relative humidity (RH) of the growth chamber were set at 20/16 °C and 60/80% RH during a 16/8 h day/night photoperiod. Fruit of the following strawberry cultivars were used: 'Clery', 'Faith', 'Dream', 'Joly' and 'Lambada'.

Unless otherwise specified, uniform and sound fruit were harvested randomly at commercial ripeness (>80% of the fruit surface red) (*Mitcham, Crisosto & Kader, 1996*). Fruit was processed fresh on the day of collection or after a maximum of 2 d of storage at 2 °C and 80% RH.

Macroscopic analysis

The position and orientation of macrocracks on the fruit surface and the shape of the fruit were established on fruit from Gleidingen. Fruit shape was classified into two categories—'normal' and 'necked' fruit—based on the length of the seedless zone *i.e.*, the so-called 'fruit neck' (Fig. 1A). Normal fruit were of conical shape with a very short 'neck' that was frequently covered by the calyx (Fig. 1B). In necked fruit, the conical shape was

elongated, and the neck was more extended (Fig. 1C). Three fruit-size classes were inspected: small <11 g, medium size 11–22 g and large fruit >22 g. The experiment was conducted using 'Faith', 'Dream', 'Joly' and 'Lambada'. The number of replicates was 50 per size class and cultivar.

Fruit were brought to the laboratory and calibrated photographs were taken (Lumix DMC-G80; Panasonic Corporation, Osaka, Japan) and the masses of cracked fruit from the field was determined. The length and width of the neck and of the body of each fruit and the orientations of any macrocracks relative to the fruit's longitudinal axis were measured by image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions, Münster, Germany). This data was also used to establish objective criteria for categorizing fruit as necked *vs* normal shaped fruit.

Cuticular mass

The mass per unit area of the cuticular membrane (CM) of the seedless neck and of the seeded body of both normal-shaped and necked fruit was determined. Epidermal discs comprising cuticle, epidermis, and some adhering flesh were excised using a biopsy punch (4 mm diameter; Kai Europe, Solingen, Germany). The CMs were enzymatically isolated by incubating discs in a solution of 50 mM citric acid buffer containing pectinase (90 ml l^{-1}; Panzym Super E flüssig, Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark), cellulase (5 ml l^{-1}; Cellubrix L; Novozymes A/S) and 30 mM NaN_3 at pH 4.0. To prevent microbial growth, NaN_3 was added (*Orgell, 1955*). Four CM discs per fruit were collected from the neck and from the body in the region of maximum fruit diameter. A total of 30 'Joly' fruit from each shape category was used.

After isolation, CMs were rinsed three times with deionized water. Adhering cellular debris was removed by ultrasonication at 35 kHz for 10 min (RK 510; Bandelin electronic, Berlin, Germany). All achenes were removed manually from the CM discs. The CMs were thoroughly dried above silica gel for 48 h and then weighed. The number of replicates was 10 with five CM discs per replicate.

Microscopic analysis

Epidermal cells in the proximal, mid and distal regions of the neck were observed at ×300 using a digital microscope (VHX-7000; Keyence, Osaka, Japan). Calibrated images were taken from normal-shaped (7.4 ± 0.4 g) and necked (7.9 ± 0.4 g) 'Clery' fruit of approximately the same mass. The number of fruits per category was 10. The cell width and length, and the angle between the longitudinal axis of the cell and that of the fruit were quantified by image analysis. The number of replicates was 50.

Microcracks were studied using the procedure by *Peschel & Knoche (2005)*. Briefly, normal and necked fruit were incubated in 0.1% acridine orange (Carl Roth, Karlsruhe, Germany) for 5 min and rinsed with deionized water. The proximal, mid and distal regions of the neck surface were inspected using a binocular microscope (Leica MZ10F; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a GFP plus filter (480–440 nm excitation wavelength, ≥510 nm emission). Calibrated micrographs were taken.
The orientations of the microcracks relative to the longitudinal axis of the fruit was

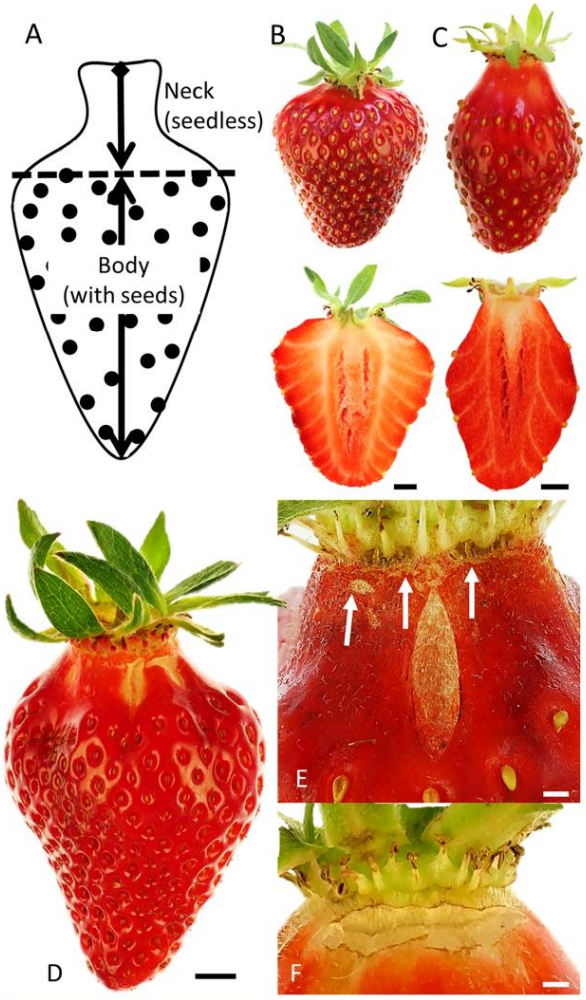

Figure 1 Strawberry fruit without and with neck and macroscopic cracks. (A) Sketch illustrating the nomenclature used to distinguish the neck (seedless) from the body (with seeds) of a mature strawberry fruit. For simplicity we refer to the strawberry pseudocarp as a 'fruit' and its achenes as 'seeds' even though *sensu stricto* they are not. Top view and longitudinal section of (B) normal-shaped or (C) necked strawberries. (D) Strawberry fruit with macrocracks. Detailed view of (E) a longitudinal macrocrack in the mid region of the seedless zone or 'neck' and (F) of a latitudinal macrocrack in the proximal region of the neck. White arrows in E indicate latitudinal cracks in the proximal region of the neck. Scale bars in B, C, D = 5 mm E, F = 1 mm. Full-size DOI: 10.7717/peerj.15402/fig-1

measured on necked fruit. There were no (or only very few) microcracks on normal-shaped fruit. The total number of replications per region was 40.

Cracking induction

The tissue tension in different regions of the fruit surface was evaluated using gaping assays (*Skene, 1980*; *Grimm et al., 2012*). Following incisions of the fruit surface, the resulting wound gapes as the tissue strain is gradually released. In a mature strawberry fruit, the flesh is under compression, whereas the skin is under tension. As a result, an incision in the fruit surface gradually gapes and the extent of gaping is a measure of the tissue tension released. The following experiments were conducted: First, a time course of gaping was determined on the distal portion of necked fruit by making a latitudinal or a longitudinal standard cut of 5 mm length and 1 mm depth using a razor blade.

Calibrated images of the resulting wound were taken using a binocular (MZ6 microscope; Leica Mikrosysteme GmbH, Bensheim, Germany) equipped with a video camera (Hitachi Denshi Europa GmbH, Rodgau, Germany) 1 min and 4, 8, 24 and 48 h after making the incision. Fruit were held at 100% RH to minimize transpiration. Gape width was measured by image analysis. Second, tissue tension was investigated in different regions of the fruit surface of normal and necked fruit. Latitudinal and longitudinal standard cuts were made in the proximal, mid and distal regions of the neck and in the region of maximum diameter of the body. Fruit were then incubated in deionized water for 4 h. Calibrated images were taken, and the gape widths and gape lengths measured. The number of replicates per treatment was 10.

Cracking was induced by incubation in deionized water. In the first experiment, a time course of cracking was established by incubating necked fruit and counting the number of fruit that had cracked in the longitudinal and latitudinal directions in the neck region after 0, 2, 4, 6 and 24 h. The total number of fruits used was 50. In the second experiment, cracking was compared between normal and necked fruit. Both categories of fruit were incubated in deionized water to induce cracking. The directions and positions of cracks were recorded after 4 h of incubation. The number of replicates was 40.

Terminology

Although in botanical terms, the 'fruit' of the strawberry is a pseudocarp; achenes are the real fruit containing the seeds and the 'calyx' is a calyculus formed by sepals in the inner ring and bracts or epi-sepals in the outer ring (*Darrow, 1966*; *Hollender et al., 2012*), for conventional terms in this manuscript we will refer to these strawberry parts as the 'fruit' and 'seeds' and 'calyx', respectively.

Data analyses

Results from counting and ratings were subjected to Chi-square tests and the main effects were determined by analysis of deviance (like ANOVA-tables) on generalized linear models (*Pekár & Brabec, 2016*). Continuous data were analyzed by analysis of variance (ANOVA). Means were compared using Tukey's test at $p = 0.05$. All statistical analyses

Table 1 Frequency of cracking on small, medium size and large normal-shaped or necked strawberry cultivars.

Cultivar	Shape	Frequency of fruit (%)	Frequency of cracked fruit (%)			
			Small[c]	Medium	Large	Mean
Flair	Normal	57.3	5.0	22.1	45.0	24.0
	Necked	42.7	11.1	43.3	64.0	39.5
Lambada	Normal	34.7	10.0	10.7	6.7	9.1
	Necked	65.3	13.2	24.6	48.9	28.9
Joly	Normal	10.0	11.7	20.0	10.0	13.9
	Necked	90.0	69.1	74.0	83.3	75.5
Dream	Normal	30.0	32.7	45.0	75.0	50.9
	Necked	70.0	79.0	86.9	95.6	87.2
Overall mean	Normal	33.0a[b]	14.8	24.5	34.2	24.5a[a]
	Necked	67.0b	43.1	57.2	72.9	57.7b
	Mean	50.0	29.0a[a]	40.8b	53.5c	41.1

Notes:
[a] Means followed by the same letter are not significantly different according to Tukey's studentized range test at $p = 0.05$.
[b] Means followed by the same letter are not significantly different according to the Chi-square-test at $p = 0.05$.
[c] Small fruit <11 g, medium 11–22 g, large >22 g.

were performed using R (version 3.5.1; *R Core Team, 2018*). Unless frequencies of counting are shown, data are presented as means ± standard errors.

RESULTS

Most cracking occurred in the necked fruit (those with pronounced necks) as compared to normal-shaped fruit (Fig. 1D, Table 1). Macrocracks were observed mainly in the seedless zone of the necked fruit (Figs. 1D–1F). Cross-sections of normal-shaped and necked fruit indicate the absence of seeds in the neck region. Vascular bundles in the neck were parallel to the longitudinal axis of the fruit. The fruit body region was characterized by the presence of seeds and of vascular bundles which were branched and connected radially with the seeds on the fruit surface (Figs. 1B and 1C).

Field ratings of fruit of the different cultivars confirmed that the frequency of cracking was consistently greater in necked fruit as compared to normal-shaped fruit. Also, large fruit was clearly more susceptible to cracking than medium-sized or small fruit (Table 1). There was no significant interaction between fruit size and cultivar.

To distinguish necked from normal-shaped fruit in subsequent analyses, objective criteria for characterizing both categories of shape were determined. There was no consistent difference in the length/width ratio between normal and necked fruit (Fig. 2A). However, necked fruit had longer necks than normal fruit (Fig. 2B). This distinction was consistent for small fruit but less so for larger fruit. We determined that the most useful criterion for differentiating between necked and normal-shaped fruit was to determine the relative (%) length of the neck, *i.e.*, neck length/total fruit length ×100. Fruit with necks longer than 16% of total fruit length were classified as 'necked'. Fruit with relative lengths below 16% were categorized as 'normal-shaped' (Fig. 2C).

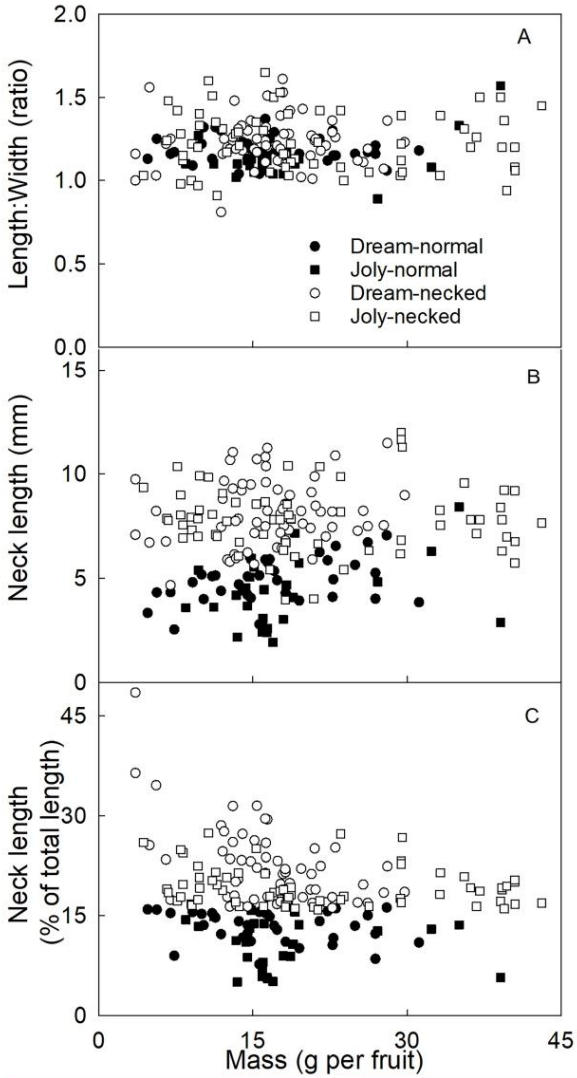

Figure 2 Characterizing strawberries without and with neck. Effect of fruit size on (A) the ratio between maximum length and width of the fruit, (B) the absolute and (C) relative length of the neck.
Full-size DOI: 10.7717/peerj.15402/fig-2

The orientation of macrocracks in the neck was predominantly in the latitudinal direction (at ±90° to the fruit's long axis) and the longitudinal direction (at 0°) (Figs. 1E, 1F and 3A). In the proximal portion of the neck, near the calyx-receptacle junction, the macrocracks were most frequently latitudinal in orientation (Figs. 1E, 1F and 3B). In contrast, in the mid and distal regions of the neck, the macrocracks were mainly longitudinal (Figs. 1E and 3C).

Cuticle thickness, as indexed by cuticular mass per unit area, was greater in the neck region than in the body for both normal-shaped and necked fruit. In addition, the cuticle of the fruit body was thicker in necked fruit than in normal-shaped fruit (Table 2).

Surface scans of the neck region revealed that epidermal cells were polygonal in shape (Figs. 4A and 4C). In both normal-shaped and necked fruit, the longer axes of the epidermal cells were consistently parallel to the longitudinal axis of the fruit. This is indicative of skin stretching along the longitudinal fruit axis (Fig. 5). The epidermal cells of necked fruit were markedly more stretched longitudinally than those of normal-shaped fruit, particularly in the proximal and mid regions of the neck as indexed by higher aspect ratios (epidermal cell length/width) (Figs. 4A and 4C; Table 3).

Fluorescence microscopy indicates a large number of microcracks in the proximal, mid and distal regions of the necks of necked fruit as compared to the less pronounced necks of normal-shaped fruit (Figs. 4B and 4D). Microcrack orientation in the proximal region was mainly latitudinal (Fig. 6A) while that in the mid and distal regions was mainly longitudinal (Figs. 6B and 6C). Due to the lack of microcracks in normal-shaped fruit, this analysis could only be performed in the necked fruit.

The time course of the gaping test revealed a rapid increase in gape width that slowed with time as gape width approached an asymptote. There was little difference in gaping behavior between incisions in the longitudinal and latitudinal directions (Fig. 7A). Comparing gaping in different regions between fruit without and with necks, indicated significantly higher strain relaxation in necked fruit than in normal-shaped fruit (Table 4). Markedly more gaping was observed after the incision when fruit were incubated in water than when held at 100% RH. Latitudinal incisions resulted in less gaping in the proximal region of the neck than in the mid region (Table 4). No significant differences were found in the gape length between normal and necked fruit (Data S1).

Incubating fruit in deionized water induced macrocracks in about 75% of necked fruit (Fig. 7B). Cracking was rapid, as indexed by half of the fruit cracked within about 4.4 h of starting incubation. At this time the fruit had taken up about 506 mg of water (5% of fruit mass). Necked fruit cracked with a markedly higher frequency than normal-shaped fruit (Table 4). Most of the macrocracks were oriented latitudinally in the proximal region of the neck and longitudinally in the distal region of the neck (Table 4).

DISCUSSION

Our main findings are: (1) growth strain is the principal driver of cracking of strawberries and (2) necked fruit are more susceptible than normal-shaped fruit and (3) water uptake greatly exacerbates cracking.

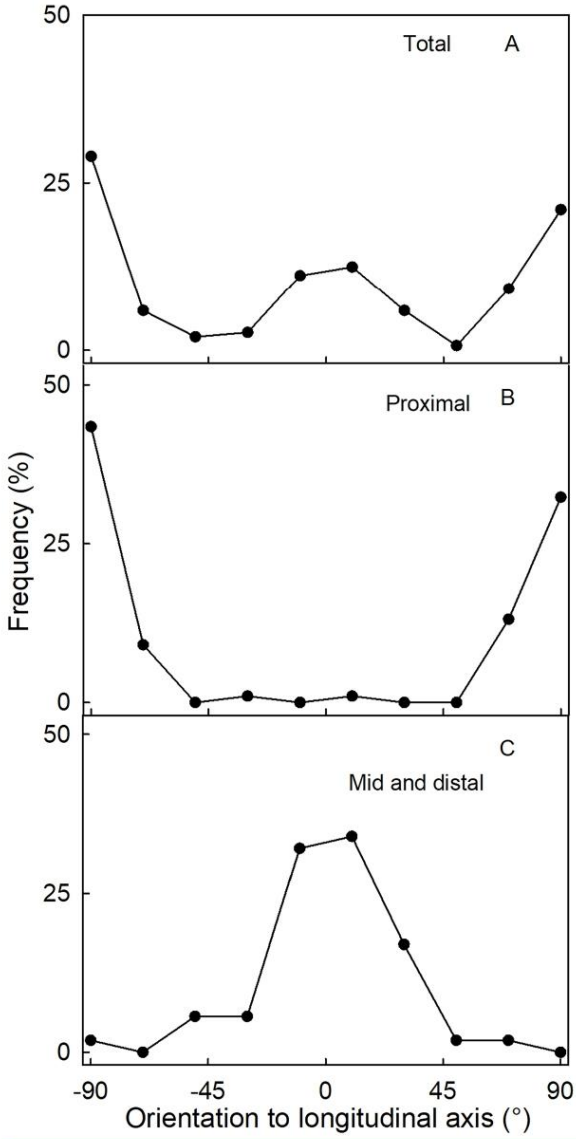

Figure 3 Frequency distribution of the orientation of macrocracks on a strawberry fruit with neck. (A) All macrocracks of the neck. (B) Macrocracks of the proximal region of the neck. (C) Macrocracks in the mid and distal regions of the neck of 'Dream and Joly' strawberries. The macrocrack orientation was measured relative to the fruit's longitudinal axis. Full-size DOI: 10.7717/peerj.15402/fig-3

Table 2 Mass of the cuticular membrane (CM) per unit area in the neck and the body regions of normal-shaped or necked 'Joly' strawberries.

	CM (g m^{-2})		
Fruit zone	**Normal**	**Necked**	**Mean**
Neck	0.88 ± 0.06a[a]	0.88 ± 0.05a	0.88 ± 0.03
Body	0.42 ± 0.02c	0.55 ± 0.02b	0.48 ± 0.02
Mean	0.65 ± 0.02	0.71 ± 0.02	0.68 ± 0.02

Note:
[a] ANOVA results indicated a significant interaction between main factors. Means followed by the same letter are not significantly different according to Tukey's studentized range test, $p = 0.05$.

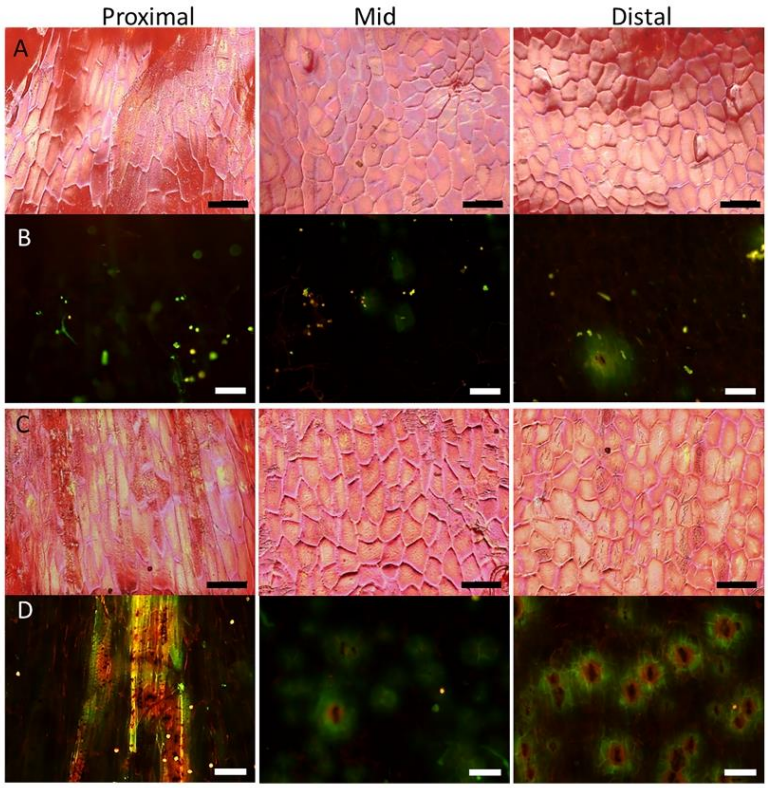

Figure 4 Micrographs of strawberry fruit without and with neck. Surface scans taken on a digital microscope (A, C) and fluorescence micrographs taken on a fluorescence binocular (B, D) of the seedless neck of (A, B) normal-shaped and (C, D) necked 'Clery' strawberries. Left column: Proximal neck. Centre column: Mid neck region. Right column: Distal neck region. Bars in A, C = 150 µm; B, D = 200 µm.
Full-size DOI: 10.7717/peerj.15402/fig-4

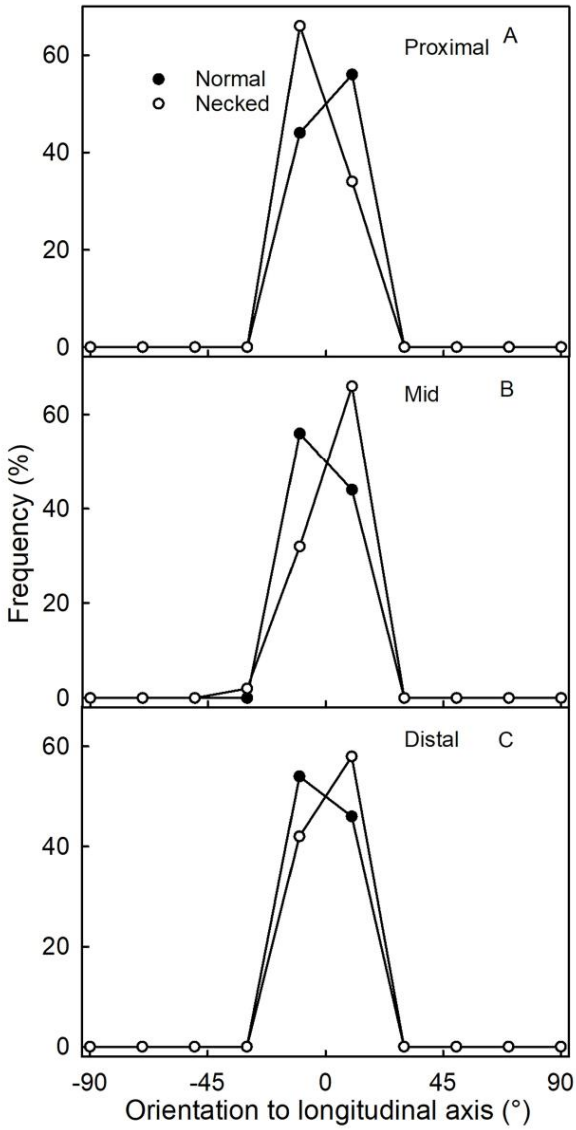

Figure 5 Frequency distribution of the orientations of epidermal cells on a strawberry fruit. (A) Proximal neck. (B) Mid neck. (C) Distal neck. The cell orientations were measured relative to the fruit's longitudinal axis of normal-shaped and necked 'Clery' strawberries.

Full-size DOI: 10.7717/peerj.15402/fig-5

Table 3 Ratio of length to width ('aspect ratio') of epidermal cells in the proximal, mid and distal regions of necks of normal-shaped and necked 'Clery' strawberries.

Zone	Aspect ratio (length: width)		
	Normal	Necked	Mean
Proximal	3.3 ± 0.1b	4.6 ± 0.2a[a]	4.0 ± 0.1
Mid	2.7 ± 0.1d	3.2 ± 0.2c	2.9 ± 0.1
Distal	2.0 ± 0.1e	2.1 ± 0.1e	2.1 ± 0.1
Mean	2.7 ± 0.1	3.3 ± 0.1	3.0 ± 0.1

Note:
[a] ANOVA results indicated a significant interaction between main factors. Means followed by the same letter are not significantly different according to Tukey's studentized range test, $p = 0.05$.

Growth strain—the driver for cracking

A strawberry differs from a true fruit in *sensu stricta* in several respects. First, the body of the strawberry is comprised mostly of receptacle tissue and the actual fruits are the small pips (achenes) embedded in and on this receptacle. As such, the strawberry is a pseudocarp or a false fruit. Second, a strawberry fruit develops over a very short period of time compared to other soft fruits (*i.e.*, 20–40 days, not 15–20 weeks). Thus, the skin of the receptacle (*i.e.*, the 'fruit' surface) is subject to very rapid rates of strain compared to the skins of other soft fruits (*Darrow, 1966*; *Batal, Weigle & Foley, 1970*; *Considine, 2015*; *Khanal, Grimm & Knoche, 2011*; *Grimm et al., 2012*).

Several observations indicate the key role of growth strain in strawberry fruit cracking. (1) The percentage of cracking was depended on fruit size. The number of cracked fruit increased as fruit size increased. Interestingly, fruit with an extended neck always cracked more than normal-shaped fruit. (2) The skin 'gaped' following artificial incisions. Gaping indicates that the flesh is held under compression by a strained skin. (3) The extent of gaping and the distribution of macrocracks across the fruit surface were closely related. Necked fruit gaped more and cracked more than normal-shaped fruit. Furthermore, for longitudinal gapes, the width and the number of longitudinal macrocracks were largest in the zone of the maximum body diameter and the distal neck in its close vicinity, as compared to other regions or to latitudinal macrocracks. (4) There was marked elongation of cells as indexed by higher length/width aspect ratios in regions of higher incidence of cracking. Again, necked fruit that had the higher percentage of cracking also had higher aspect ratios than normal-shaped fruit. In addition, cells were the most extended in the proximal neck region which also had the highest number of macrocracks. (5) Cuticle thickness was lower in the body than in the neck, which is consistent with the larger surface area of the body *vs* the neck. As in other fruit crops, cuticle deposition in strawberries is likely to be limited to the early stages of development and any expansion occurring after cessation of cuticle deposition simply 'dilutes' the cuticle (*i.e.*, the same amount of cuticular material becomes spread over an increasing area of skin) resulting in a steady decrease in cuticular thickness (*Knoche et al., 2004*; *Becker & Knoche, 2012*). Whether the resulting strain in the cuticle is elastic or plastic is not known.

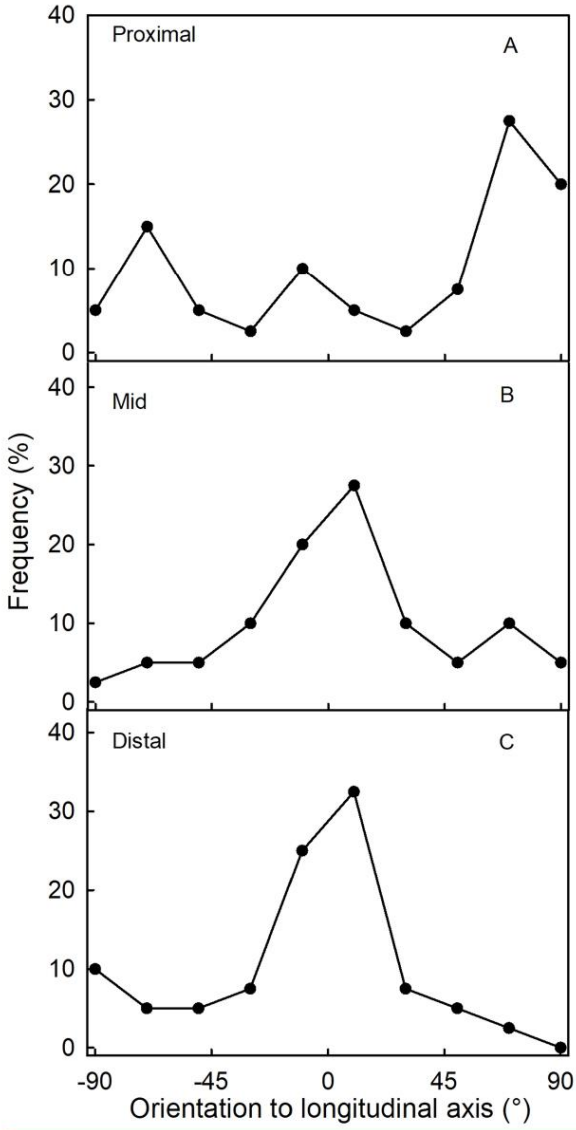

Figure 6 Frequency distribution of the orientations of microcracks on a strawberry fruit with neck. (A) Proximal neck. (B) Mid neck. (C) Distal neck. The orientations of microcracks were measured relative to the fruit's longitudinal axis of necked 'Clery' strawberries.

Full-size DOI: 10.7717/peerj.15402/fig-6

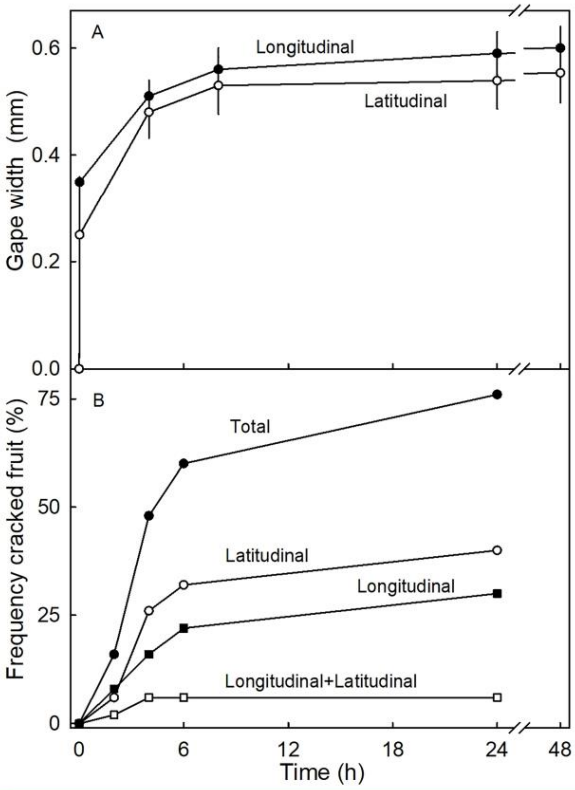

Figure 7 Time courses of strain release and crack formation in strawberry fruit. (A) Strain release following incisions of the fruit surface with longitudinal or latitudinal orientations. The strain release was indexed by measuring the gape size of the cut. (B) Formation of macrocracks of 'Joly' strawberry fruit incubated in deionized water. Fruits that cracked longitudinally *or* latitudinally, or longitudinally *and* latitudinally were counted separately. Full-size DOI: 10.7717/peerj.15402/fig-7

These arguments indicate that growth strain provides a conclusive explanation for the behavior of cracking in strawberries and for the higher susceptibility of the necked *vs* the normal-shaped fruit.

Role of water uptake in cracking

Water uptake through the fruit surface exacerbates rain-cracking in strawberry, but also in a range of other soft fruit including sweet cherry (*Christensen, 1996*; *Knoche & Winkler, 2017*) and grape (*Considine, 2015*). Evidence for water uptake exacerbating cracking is based on the following observations: First, surface wetness increases microcracking of the cuticle (*Hurtado & Knoche, 2021*). Second, water-induced macrocracks did not differ in

Table 4 Gaping following artificial incisions and frequency of cracking of strawberries.

Crack or cut direction	Zone		Gape width (mm)			Frequency of cracks (%)	
			Normal	Necked	Mean	Normal	Necked
Latitudinal	Neck	Proximal	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1a[a]	3.4	25.4
Latitudinal	Neck	Center	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1bc	1.7	1.7
Latitudinal	Neck	Distal	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1abc	3.4	3.4
Latitudinal	Body	Max. diameter	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1ab	1.7	3.4
Longitudinal	Neck	Distal	1.1 ± 0.1	1.5 ± 0.1	1.3 ± 0.1c	8.5	25.4
Longitudinal	Body	Max. diameter	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1abc	5.1	11.9
Other	Body	–	–	–	–	1.7	3.4
Total/Mean			0.9 ± 0.1a	1.2 ± 0.1b	1.1 ± 0.1	25.4a[b]	74.6b

Notes:
[a] Means followed by the same letter are not significantly different according to Tukey's studentized range test, $p = 0.05$.
[b] Means followed by the same letter are not significantly different according to the Chi-square-test at $p = 0.05$.
Fruit were immersed in water. Gaping and frequency of cracking were assessed in the proximal, mid and distal neck regions and in the bodies of normal-shaped and necked 'Clery' strawberries.

size or orientation from 'natural' macrocracks that were not induced by water uptake, Third, water uptake increased growth strain in the skin, as indexed by more gaping after incubation in water as compared to holding fruit at 100% RH. Fourth, the abscission zones of petals and microcracks in the calyx-receptacle junction, around the base of stamina in the seedless neck are further openings, where the cuticle's barrier function in controlling water transfer is impaired. In these regions, rapid uptake of water occurs as demonstrated by infiltration with the fluorescent tracer acridine orange (*Hurtado & Knoche, 2023*). An opening in the cuticle acts in a way analogous to an aperture in an optical instrument. It 'focusses' water uptake into a highly localized region of the fruit skin. This uptake occurs by viscous flow (*Hurtado et al., 2021*), which is much more rapid than by diffusion through an intact cuticle (*Winkler et al., 2016*). The close proximity of these preferential pathways to the vascular bundles in the seedless neck, ensures rapid water uptake and subsequent movement which further contributes to the greater incidence of cracking in this region.

How do microcracks subsequently extend to macrocracks? An explanation for the mechanism of extension and the role of water uptake therein may be found in the 'Zipper' model. This model accounts for rain cracking in sweet cherries (*Winkler et al., 2016*). It also explains the water soaking disorder of strawberries (*Winkler et al., 2016*; *Hurtado & Knoche, 2021*). Briefly, water uptake through a microcrack in the cuticle causes individual underlying cells to burst. Their cell contents are released into the apoplast. Sweet cherries and strawberries are rich in organic acids (70 mM malic acid in sweet cherry *vs* 23 mM malic acid and 39 mM citric acid in strawberry (*Herrmann, 2001*)). These organic acids increase the membrane permeability of the adjacent cells, causing further leakage, a weakening of the cell walls, and triggering a chain reaction that extends radially into the flesh and longitudinally into the skin like a "zipper" (*Winkler et al., 2016*). This chain reaction extends a (shallow) microcrack into a (much deeper) macrocrack or—as the busting of skin cells proceeds—leads to water-soaking (*Hurtado & Knoche, 2021*). The latter was also observed in our time course of cracking induction in this study.

A simple model to explain macrocracking of strawberry

In this section, we focus on macrocracks. Macrocracks arise from microcracks. Macrocracks on strawberries are highly orientated. The orientation is consistent within regions but differs between regions. Two different orientations dominate: In the fruit body and the distal neck region, macrocracks are orientated longitudinally. In contrast, in the proximal neck region latitudinally-orientated macrocracks dominate. These observations are in agreement with *Herrington et al. (2011)*. Furthermore, the orientations of macrocracks and microcracks are identical, this implies that macrocracks arise from the extension of microcracks. Our study established that fruits with extended necks are particularly prone to both micro- and macrocracking. To explain these observations, we propose a model that relates the frequency and the orientation of micro- and macrocracks to the growth strains in the strawberry fruit (Fig. 8).

A longitudinal orientation is favored by one or several of the following factors. (1) The cone shape of a strawberry favors cracking in a longitudinal direction. (2) Most longitudinal cracks occur in the regions where the diameter of the fruit is largest and in the distal region of the neck. In the physicist's mathematical analysis of a 'thin-walled, cylindrical, pressure vessel', the tangential stress in the wall (σ, N m^{-2}) at a given pressure (P, N m^{-2}) is directly proportional to the radius (R, m) of the vessel, divided by its wall thickness (t, m) according to the equation (*Nobel, 1999*):

$$\sigma = \frac{P \times R}{t}$$

This physical model holds for the strawberry within the body and within the neck. However, it does not explain why the neck cracks more frequently than the body, despite the neck having a smaller diameter (Fig. 8). Based on the difference in diameter, we would expect the neck to have fewer macrocracks, not more microcracks. Two explanations may be offered. The first explanation relates to the absence of seeds (achenes) in the neck region. In a strawberry, every seed is supplied by a vascular bundle. In mechanical terms, a fruit's vascular bundles represent 'structural reinforcements' (c.f., steel-reinforced concrete) embedded in the soft and extensible parenchyma of its receptacle. In the neck region (no seeds), the vascular bundles run parallel to the fruits' long axis. In contrast, beyond the neck in the fruit's body region (with seeds), the vascular bundles also branch off in the radial direction to supply the seeds on the fruit surface. The radial orientation of this vasculature now provides structural reinforcement in the body of the fruit also in the radial direction—this radial reinforcement is lacking in the neck (Fig. 8). In consequence, the radial reinforcement restricts cracking in the body, but does not do so in the neck, which lacks radial reinforcement. This explanation also accounts for the 'corrugated' nature of the strawberry fruit surface and the location of the seeds in shallow depressions. Since expansion of the parenchyma is restricted by the radial vascular bundles, the seeds are 'pulled' down into the expanding parenchyma. That the strawberry forms a neck may simply be the result of greater longitudinal extension in this region where fruit diameter is lower. In this region, elongation per unit cross section will be largest. This explanation would account for (1) the higher aspect ratio of epidermal cells in the neck of a necked fruit

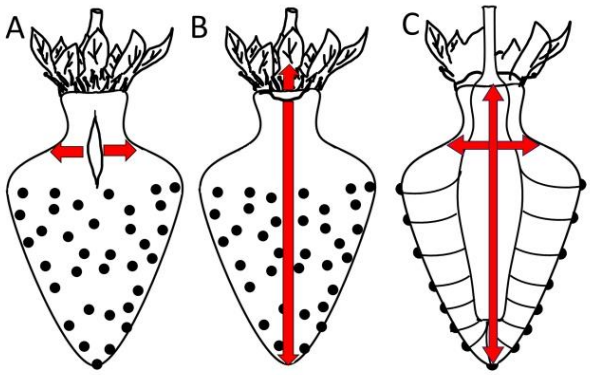

Figure 8 Sketch of growth strains and crack formation in strawberry fruit. (A, B) Direction of growth strains and the resulting directions of crack formation in the neck region of necked fruit. (C) Vascular bundles connecting peduncle to seeds. Full-size DOI: 10.7717/peerj.15402/fig-8

vs in the neck of a normal-shaped fruit and (2) the decrease in frequency of longitudinal macrocracks within the neck region from the proximal to the distal end as the diameter of the neck increases.

The second explanation relates to the corrugated nature of the strawberry surface. The seeded region of a strawberry has an enlarged surface area due to the tiny ridges and valleys that form the multiple tiny indentations of the epidermis that accommodate the seeds. In contrast, the seedless neck is flat. So, as fruit volume expands rapidly, the corrugated fruit body has a relatively large surface that can accommodate a rapid area increase by a conformational change (it can extend by flattening out), rather than by stretching *per se*. The non-corrugated, seedless neck does not have this advantage. Hence, the seedless neck lacks this mode of area increase and so cracks more easily than the seeded body of the fruit.

Latitudinal cracks were limited to the proximal neck region. The following factors are likely to be causal: (1) The junction between the calyx and the receptacle is likely to be weakened by discontinuities in the skin (corky, stiff patches) associated with the petal and the stamen abscission zones. (2) The calyx, petal and stamen bundles are all linked into the shoot vasculature to secure water supply for transpiration. However, distal to the staminal whorl, the number of vascular bundles decreases, as those that continue connect only to the seeds. Since vascular bundles offer significant structural support to any tissue in which they are embedded, the abrupt decrease in bundle numbers (and thus structural support) beyond the staminal whorl represents a relative weakening of these distal tissues, especially in terms of longitudinal extension. This discontinuity may serve as a stress concentrator that focuses longitudinal growth to the proximal neck region. (3) The proximal neck region is also an area of preferential water uptake. Water uptake exacerbates cracking.

The above arguments seem to account fully for the observed differences in cracking susceptibility between necked and normal-shaped strawberry fruit and also for the consistent patterns of crack orientation in the neck region of necked fruit.

CONCLUSION

The model proposed here offers a satisfactory explanation for the failure patterns of cracking in strawberries and this pattern's close relationship to fruit shape and structure. Our results demonstrate that fruit with extended necks are especially susceptible to cracking. The frequency of necking in strawberry depends on genotype, but also on as-yet-unknown environmental factors. Further research is needed to unravel the mechanism of neck formation. Protected cultivation is a useful way for reducing cracking in necked fruit. As, in unprotected cultivation systems, the seedless neck region is likely to suffer extended periods of surface wetness, that will increase cuticular microcracking, the microcracking then soon progresses to macrocracking, that extends these cracks deep into the flesh.

ACKNOWLEDGEMENTS

We thank Bendix Meyer, Heike, Karl Walter and Martin Brüggenwirth for the gift of fruit, Hana Weiß, Marcel Pasta and Peter Grimm-Wetzel for technical support and Sandy Lang and Jannis Straube for helpful comments on an earlier version of this manuscript.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (KN 402/19-1). The publication of this article was funded by the Open Access fund of the Leibniz Universität Hannover. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Deutsche Forschungsgemeinschaft: KN 402/19-1.
Open Access fund of the Leibniz Universität Hannover.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Grecia Hurtado conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Moritz Knoche conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The data used to generate tables and figures are available in the Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.15402#supplemental-information.

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Supplementary material

Electronic File E4. Dataset. Excel file containing all data produced in figures and tables throughout the manuscript. (XLSX) (access through: https://doi.org/10.25835/5p308h3f)

4.5. Water soaking disorder in strawberries: triggers, factors, and mechanisms

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Figures 3.A, and 4 were part of Master thesis. Moritz Knoche: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing original draft, Writing review and editing.
Journal	Frontiers in Plant Science
Impact factor	6.627(2022)
Date of publication	20.07.2021
DOI	10.3389/fpls.2021.694123

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fpls.2021.694123

Water Soaking Disorder in Strawberries: Triggers, Factors, and Mechanisms

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Water soaking is an important surface disorder of strawberries that limits unprotected field production. The objective was to identify the mechanism(s) of water soaking. Symptomatic fruit show pale, deliquescent patches of skin. This damage extends into the flesh. Numerous cuticular microcracks occurred in water-soaked areas. Water soaking occurred only if the skin was exposed to liquid water. Water soaking was more rapid when the cuticle had been abraded. Water soaking, anthocyanin leakage, and water uptake all increased with incubation time. There was a lag phase for water soaking and anthocyanin leakage, but not for water uptake. Susceptibility to water soaking increased with fruit ripening and mass. Incubation in isotonic PEG 6000 increased cuticular microcracking but decreased water soaking and water uptake. Incubation in hypotonic fruit juice (natural and artificial) increased water soaking incidence and severity but reduced water uptake. Incubation in dilute citric and malic acids increased plasma membrane permeability as indexed by anthocyanin leakage and increased water soaking. Thus, water soaking involves cuticular microcracking, localized water uptake, bursting of cells, and the release of organic acids into the apoplast. The damage propagates from cell to cell.

Keywords: rain damage, water soaking, *Fragaria* × *ananassa* Duch, cracking, leakage, cuticle, microcrack

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *12 April 2021*
Accepted: *11 June 2021*
Published: *20 July 2021*

Citation:
Hurtado G and Knoche M (2021) Water Soaking Disorder in Strawberries: Triggers, Factors, and Mechanisms. Front. Plant Sci. 12:694123. doi: 10.3389/fpls.2021.694123

INTRODUCTION

Strawberry is a soft fleshy fruit of global importance (Hummer and Hancock, 2009). It is highly perishable, but nevertheless grown mainly in the open field where fruit quality is often severely compromised by rain. The main disorders arising from rain are water soaking and cracking (Herrington et al., 2009). With water soaking, the appearance of the fruit surface is compromised, with the skin looking lighter-colored and deliquescent (Herrington et al., 2009). With cracking, easily visible, gaping cracks occur that often extend deep into the flesh. Both disorders may be observed on different regions of the fruit surface, the calyx, neck, shoulder, or tip (Herrington et al., 2011). Both water soaking and cracking increase the incidence of fruit rots pre- and post-harvest. Together, these disorders result in significant economic loss due not only to the lost opportunity (reduced yield) but also to the additional labor involved in harvesting, grading, and packing a fruit population high in individuals suffering compromised quality (Herrington et al., 2009). In addition, the loss at the consumer level increases.

As a result of these limitations, strawberry production is slowly shifting from open field, to semi- or fully protected cultivation in plastic tunnels or greenhouses. This shift is occurring particularly in areas where the incidence of rainfall is high during the later stages of fruit growth and harvest. However, the additional costs of the protection structures and of energy increase the

production costs (Khoshnevisan et al., 2013). Furthermore, not all strawberry cultivars are suitable for production in tunnels where, in susceptible cultivars, protected cultivation sometimes increases the incidence of powdery mildew, spider mites, and calcium deficiency disorders (Grijalba et al., 2015).

The mechanistic bases of water soaking and cracking in strawberries have not yet been properly determined. However, such understanding is the obvious prerequisite to the development of effective countermeasures that mitigate water soaking and cracking through the introduction of innovative cultural methods and/or breeding. The objective of this study was to identify the triggers, factors, and mechanisms underlying the water soaking disorder in strawberry fruit.

MATERIALS AND METHODS

Plant Material

Strawberry fruit (*Fragaria* × *ananassa* Duch., cultivars Clery, Faith, Florentina, Malwina) were harvested from commercial plantings at Gleidingen (lat. 52°16′ N, long. 9°50′ E) and Bad Nenndorf (lat. 52°21′ N, long. 9°20′ E). The change in cultivars was necessary, because in strawberry a given cultivar is available at the optimum stage of ripeness only for a limited period of time. Unless otherwise specified, fruits were harvested randomly at commercial ripeness (>80% of the fruit surface red), placed in a foam tray, and selected for uniformity of size, shape, and color and for freedom from visual defects. Care was taken to not touch the fruit surface. The pedicel was cut to a maximum length of 5 mm. Fruits were processed fresh on the day of sampling or held at 2°C and 80% RH for no longer than 1 day. Previous studies showed that holding fruit for up to 2 days under these conditions had no effect on rates of water uptake or transpiration (data not shown). Unless otherwise specified, the calyx was removed from the fruit by carefully pulling the tip of the calyx toward the pedicel. This occasionally required the fruit to be held down by holding on to the pedicel. In most instances, gravity was sufficient for the fruit to remain in the foam tray during the removal of the calyx. The fruit surface was not touched by hand during the entire procedure. The pedicel stump and the attachment zones of the calyx represent openings that are accessible for water uptake. To exclude artifacts, these holes were sealed using a fast-curing, non-phytotoxic silicone rubber (SE 9186 Clear; Dow Corning Corp., Midland, United States). This procedure is illustrated in **Supplementary Figure 1**.

General Procedure

The water soaking damage was induced by incubating fruit in deionized water (one fruit per 100 mL) at room temperature. The fruits were forced under water using a soft plastic-foam plug. After a period of immersion, the fruits were carefully blotted using the soft tissue paper. The extent of water soaking was quantified using a 5-point rating scale or by direct measurement using image analysis. The 5-point rating scale was as follows: score 0 = no water soaking; score 1 = <10% of the surface water-soaked; score 2 = 10–35%; score 3 = 35–60%; and score 4 = >60% of the fruit surface area water-soaked.

The water-soaked fruit surface was inspected by light and fluorescence microscopy. Fruit were incubated in 0.1% acridine orange (Carl Roth, Karlsruhe, Germany) for 3 min, then rinsed with deionized water, and carefully blotted. The fluorescent tracer acridine orange penetrates any microscopic cracks in the cuticle, but not an intact cuticle (Peschel and Knoche, 2005; Becker and Knoche, 2012b; Khanal et al., 2021). The fruit surface was then inspected under incident white and incident fluorescent light using a binocular microscope (Leica MZ10F with filter GFP plus 480–440 nm excitation, ≥510 nm emission; Leica Microsystems GmbH, Wetzlar, Germany). Furthermore, surface scans of water-soaked regions and non-water-soaked control regions were prepared at ×500 using a digital microscope (VHX-7000; Keyence, Osaka, Japan) and coaxial illumination.

Water uptake into submerged fruit was quantified gravimetrically. Fruits were incubated individually in deionized water. Before each weighing, a fruit was carefully blotted dry using a soft paper tissue, then weighed (CPA225D; Sartorius, Göttingen, Germany), and thereafter immediately returned to the incubation solution for a further period. Unless specified otherwise, the number of replicates was 15.

Experiments

The **effect of partial immersion in water on water soaking** was investigated in "Florentina" fruit. Fruits were incubated for 24 h in deionized water such that (a) one half of the fruit was submerged (longitudinal axis horizontal), or (b) just the fruit tip (longitudinal axis vertical), or (c) just the calyx end (longitudinal axis vertical).

The **effect of wounding on water soaking** was investigated in "Florentina" fruit. The treatments were (a) an incision 5 mm long, 3 mm deep using a razor blade; (b) a hole 1.6 mm diameter and 5 mm deep; and (c) abrasion of the cuticle with carborundum powder (grain size 1,200; Schriever, Hamburg, Germany) of about 10 mm^2. Treated fruits were then incubated in deionized water for 3 h, and calibrated photographs were taken immediately thereafter. The soaked areas around the wounds were quantified by image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions, Münster, Germany). The numbers of replicates were 10.

Different procedures to quantify water soaking were compared using "Florentina" fruit. These procedures included the comparison of the rating scheme (above) with direct measurement of water-soaked areas using image analysis. In designs involving repeated measures, to exclude artifacts resulting from repeated blotting, the experiment was run twice: First, using independent measurements and ratings (destructive sampling), and second, using repeated observations (measurements and ratings) on the same fruit (non-destructive sampling). For the repeated measurements, the fruit was rotated and photographed from opposite sides at each time interval. Only when the area of water soaking exceeded 60% of the total fruit surface was the fruit peeled and the peel flattened on a glass plate. A calibrated photograph was taken (Canon DS126271; Canon Inc., Tokyo, Japan). The water-soaked area was quantified using image analysis (cellSens Dimension 1.7.1; Olympus Soft

Imaging Solutions, Münster, Germany). The minimum number of replicates was 60.

The **time courses** of change in the water-soaked area, in the absorbance of the incubation solution, and in the mass of water uptake were determined over a 10-h incubation period. The water-soaked area was quantified using image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions), and the water uptake was measured gravimetrically as described above. The leakage of anthocyanin into the incubation medium was determined by measuring the absorbance of the incubation medium at 520 nm using a spectrophotometer (Specord 210; Analytik Jena, Jena, Germany). Since the absorbance of an anthocyanin solution depends on pH, the pH was first adjusted to pH 2.3 using citric acid at a final concentration of 37 mM.

Relationships between water soaking, microcracking, and water uptake were studied by incubating "Clery" fruit for 0, 2, 4, 8, 16, and 24 h in deionized water. Water soaking was quantified using the rating scheme described above. Microcracking of the cuticle was indexed by quantifying the area infiltrated with acridine orange using fluorescence microscopy. The fruits were dipped in 0.1% (w/w) aqueous acridine orange (Carl Roth, Karlsruhe, Germany) for 5 min. Thereafter, fruits were rinsed, blotted dry, and viewed at ×6.3 under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Four calibrated images within randomly selected microscope "windows" were taken (Camera DP71; GFP-plus filter, 480–440 nm excitation, ≥510 nm emission wavelength) per fruit on a total of 10 fruit. The area infiltrated by acridine orange was quantified using image analysis (cellSens Dimension 1.7.1; Olympus Soft Imaging Solutions, Münster, Germany). A tissue infiltrated with acridine orange exhibits orange, yellow, and green fluorescence (Peschel and Knoche, 2005). To quantify the infiltrated areas, the appropriate color thresholds were selected, and all images were batch-processed using the same settings for these thresholds. The infiltrated areas were expressed as a percentage of the area of the microscope window. Water uptake was determined gravimetrically.

The **site on the fruit surface** where water soaking first appeared was identified in "Florentina" strawberries. Fruits were immersed in deionized water and continually inspected for symptoms of water soaking. When a fruit exhibited water soaking, it was cut into four slices of equal thickness and perpendicular to its longitudinal axis. The slice in which symptoms first appeared was recorded. Two batches of fruit were inspected: (a) one with the calyx present and (b) one with the calyx removed. The total number of replicates was 92 per treatment. The osmotic potentials of the juice expressed from the different slices from the same batch were measured. A fruit was cut into two halves along its longitudinal axis. One half was used to express the juice to determine the mean osmotic potential of the fruit. The other half was cut transversally into four slices of equal thickness. The osmotic potential of the expressed juices was determined by water vapor pressure osmometry (VAPRO 5600; Wescor, Utah, United States).

A "gaping assay" was carried out to **evaluate the strain relaxation** in the different regions of the fruit surface. A cut 5 mm long and 2 mm deep was made using a razor blade. Calibrated photographs (Lumix DMC-G80; Panasonic Corporation, Osaka, Japan) were taken on a macrostand immediately after the cut had been made and again 24 h later. Gape width was measured by image analysis. Fruits was maintained at 100% RH during the assay to minimize transpiration. The experiments were carried out using "Florentina" fruit. The number of replicates was 10.

The **effect of ripeness on water soaking** was studied in the fruit of "Florentina." Fruit were selected at six stages of ripeness as indexed by color, ranging from white to dark red (CM-2600 d, orifice 3 mm diameter; Konica Minolta, Tokyo, Japan). All fruit were incubated for 6 h to induce water soaking. Water soaking was quantified using the rating scheme described above. Water uptake was measured gravimetrically (CPA225D; Sartorius, Göttingen, Germany). The osmotic potentials (VAPRO 5600; Wescor, Utah, United States) of the expressed juices were analyzed from the fruit of the same batch.

The **effect of fruit size on water soaking** was investigated by incubating fruit of five different size classes in water for 6 h. The size classes were <15, 15–20, 20–25, 25–30, and 30–40 g. Water soaking was rated as indicated above. Color (CM-2600 d, orifice 3 mm diameter; Konica Minolta, Tokyo, Japan) and soluble solids (°Brix) using a refractometer (DR6200-T; A. Kruess Optronic, Hamburg, Germany) were determined. This experiment was carried out using "Malwina" fruit. The total number of replicates was 92.

The **effect of water uptake on water soaking** was studied by incubating "Clery" strawberries in solutions of polyethylene glycol 6000 (PEG 6000) or in deionized water. The PEG 6000 solution was prepared to be isotonic to the juice expressed from the fruit of the same batch. The time courses of change in water-soaked area and in water uptake were established by sampling fruit at 0, 1, 2, 4, 6, 8, 11, and 24 h. In addition, the fruit surface was inspected for microcracks as described above.

The role of the osmolytes contained in expressed strawberry juice in the phenomenon of water soaking was addressed in two different experiments.

We analyzed the **effect on water soaking of expressed strawberry juice (natural) and of a synthetic juice** prepared by combining pure solutions of the five major strawberry osmolytes (artificial). Natural juice was expressed from the fruit of the same batch as used in the experiment. The osmolytes in the artificial juice and their relative amounts were glucose (30.3%), fructose (33.2%), sucrose (7.6%), citric acid (9.7%), malic acid (5.7%), and potassium applied as KOH (9.6%) (Herrmann, 2001). Natural and artificial juice was used at "full" (isotonic with the fruit) or "half strength" (half isotonic). Osmotic potentials of the solutions were measured by water vapor pressure osmometry (VAPRO 5600; Wescor, Utah, United States). Deionized water served as control. Fruits were incubated for 4 h, and the water-soaked area was then quantified. The rates of water uptake were determined gravimetrically on fruit from the same batch at 0.5-h intervals for up to 1.5 h. The rates of water uptake were calculated (mg h^{-1}) on an individual fruit basis, from the slope of a linear regression fitted through a plot of increasing fruit mass vs. time. The experiment was carried out using "Faith" fruit.

In the second experiment, the **effect of the individual major osmolytes** of "Florentina" strawberry, i.e., of glucose (121.0 mM),

fructose (132.7 mM), sucrose (30.4 mM), citric acid (38.9 mM), and malic acid (22.6 mM), on water soaking and the rate of water uptake was established. The solution's concentrations were derived from the composition of the isotonic artificial juice of fruit from the same batch. Deionized water and the isotonic artificial juice were used as controls. The water-soaked area and the rate of water uptake were determined as described above.

The **role of malic and citric acid** in water soaking was studied using a leakage assay and anthocyanin as an indicator of cell membrane damage in "Florentina" (Winkler et al., 2015). Cell walls were stressed to varying extents by incubating fruit in solutions of PEG 6000 of different osmotic potentials, and a time course of anthocyanin leakage was established. Cylinders of the outer flesh and fruit skin were excised using a biopsy punch (8 mm diameter), and these were cut to 2 mm length, rejecting the skin, using parallel razor blades. Flesh disks were blotted and rinsed, and then incubated in isotonic PEG 6000 solution, with and without (control) 39 mM citric and 23 mM malic acid. Disks were removed from solution after 1, 2, 4, 8, and 24 h. The absorbance of the incubation medium was quantified at 520 nm using a spectrophotometer (Specord 210; Analytik Jena, Jena, Germany). Before measuring, the pH of the solution used as a control was adjusted by adding the same volume of acids (after incubation was terminated) as that present in the treatment solution. In this way, the control and treatment solutions had the same pH (pH 2.5), and there was no confounding in anthocyanin detection due to variable pH. In a subsequent experiment, disks were incubated in PEG 6000 solutions of osmotic potential 0, −0.6, −1.2, −1.8, or −2.4 MPa with and without the two acids. Disks were removed from the solutions after 4 h, and the anthocyanin content of the incubation medium was measured as described above. Six disks were excised per fruit and used as paired observations. Three disks represent one replicate. The experiment was carried out using 10 replicates.

Data Analyses

All experiments were conducted and analyzed using completely randomized designs. Data were analyzed by analysis of variance and linear regression. Means were compared using Tukey's studentized range tests ($p < 0.05$) and the statistical software R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Unless individual observations are shown (e.g., **Figure 3C**-inset, **Figures 4A,B**-insets, and **Figure 4D**), data are presented as means ± standard errors.

RESULTS

Water soaking in strawberries appeared as irregular patches of skin that are pale, deliquescent, and sometimes pinkish. At times, they looked slightly translucent compared to the shiny, dark-red controls (**Figure 1A**). The symptoms usually started on the margin between the depressions of adjacent achenes. When severe, the patches covered a major portion of the fruit surface (**Figures 1A,B**). Water soaking was not limited to the fruit skin but extended several mm below the surface into the flesh (**Figure 1C**). Scans of the fruit surface and fluorescence microscopy of water-soaked fruit revealed the presence of numerous microcracks in the water-soaked areas (**Figures 1E–G**). There were no or only few microcracks in non-treated control fruit (no water soaking) (**Figure 1D**). Water soaking was never associated with fungal development during the short incubation periods of our experiments.

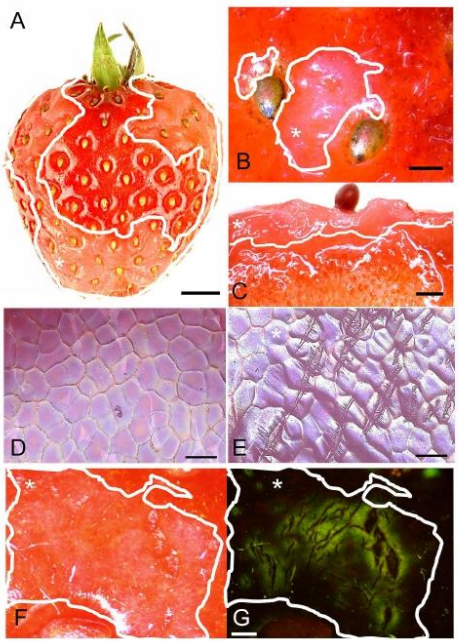

FIGURE 1 | (A) Macroscopic view of fruit with water soaking symptoms; **(B)** Detail of achenes with surrounding water-soaked area viewed under a light microscope; **(C)** Micrograph of cross-section of tissue with water soaking. **(D,E)** Scans of the surface in a digital microscope of non-treated and water-soaked fruit with numerous microcracks. **(F,G)** Micrographs of developing symptoms of water soaking viewed under incident **(F)** and fluorescent light **(G)**. Microcracks were infiltrated with acridine orange. The penetrated fluorescent tracer appears as a green fluorescence around a microcrack in the cuticle. Water soaking symptoms are marked by a solid white line. White asterisks in **A**, **B**, **C**, **E**, **F** and **G** identify the water-soaked area. Scale bar in **(A)** = 5 mm, **(B,C)** = 1 mm, **(D,E)** = 0.1 mm and in **(F,G)** = 0.5 mm.

Water soaking was always localized and limited to the regions in direct contact with the incubation solution. It never occurred in regions above the water surface when fruit was partially submerged (**Figures 2A–C**). Water soaking rapidly developed at a wound. Abrading the cuticle using carborundum powder was highly effective in inducing water soaking (**Figures 2E–I**). Incisions made using a razor blade or puncturing the fruit skin were markedly less effective in inducing water soaking.

When completely submerging fruit, the surface area affected by water soaking increased primarily because the water-soaked area expanded, and not because the number of water-soaked

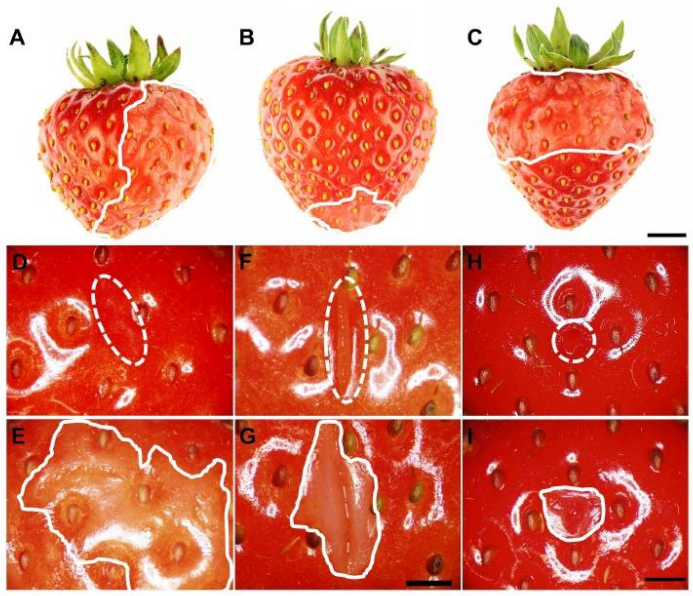

FIGURE 2 | (A–C) Induction of local water soaking symptoms by partial immersion of a strawberry fruit in water. **(A)** Half of the fruit (long axis horizontal), **(B)** tip or **(C)** calyx end of the fruit (long axis vertical). Water soaking following abrasion of the cuticle (~10 mm^2), **(D,E)** incision of the skin using a razor blade (5 mm length × 3 mm depth), **(F,G)** and puncture of the skin using a dissection needle (1.6 mm diameter and 5 mm depth) **(H,I)**. **(D,F,H)** images taken immediately after wounding ($t = 0$ h); **(E,G,I)** images of same area taken after a 3-h incubation in deionized water. The fruit was submerged completely. The water-soaked area around the wound averaged 179.4 ± 48.8 mm^2 **(E)**, 37.5 ± 5.7 mm^2 **(G)**, and 20.7 ± 4.2 mm^2 **(H)**. Scale bar in **(A–C)** = 5 mm and **(D–I)** = 2.5 mm. The dashed white line indicates the wound area, and the solid white line indicates the margin of the water-soaked area. The area outside of the solid white line was also in contact with deionized water and serves as control for a non-water-soaked surface.

patches increased (**Figure 3A**). The surface area affected, increased sigmoidally with time until a major portion of the skin was affected (**Figure 3B**). There was no difference in the area affected or the rating score for water soaking between fruit that was sampled destructively and fruit that was monitored in a repeated-measures design (**Figures 3B,C**). Water soaking as indexed by the rating scheme was closely and significantly correlated with the water-soaked area as measured directly by image analysis (**Figure 3C**, inset).

The sigmoidal time course of the increase in water-soaked area had an initial lag phase where no symptoms appeared before water soaking began. Thereafter, the affected area increased (**Figure 4**). Similarly, following an initial lag phase, the leakage of anthocyanin increased. The leakage increased at a constant rate. However, for water uptake, there was no lag phase—water uptake began at the time of submersion and also accumulated at a constant rate (**Figure 4C**). When the water-soaked area or the leakage of anthocyanin (relative absorbance) was plotted vs. water uptake, a lag phase without water soaking and without anthocyanin leakage was present (**Figures 4A,B**, insets). These results indicate that water uptake increases up to some critical threshold beyond which water soaking and anthocyanin leakage begin. Water-soaked area and anthocyanin leakage were positively related until about 60% of the area was water-soaked. Above this value, anthocyanin leakage continued to increase, but water-soaked area remained largely constant (**Figure 4D**).

The water-soaked area, the infiltrated area, and the water uptake all increased nearly linearly with time (**Figure 5**). The water-soaked area and the area infiltrated by the fluorescence tracer were significantly and positively related (r^2= 0.95***; **Figure 5D**). It is worth noting that the rate of water uptake and the rate of water soaking were both markedly lower in "Clery" than in the cultivars used in other experiments.

Most water soaking began at the tip of the fruit, followed by the calyx end, the calyx equator zone, and the equator tip zone (**Figure 6A**). There was no difference between fruit with or without the calyx. Within the fruit, osmotic potential decreased and became more negative from the calyx, toward the tip of the fruit (**Figure 6B**). There was no significant difference in stress relaxation between different regions of the fruit as indexed by a lack of difference in gaping following skin incision (data not shown). In all regions, the gap produced by the incision averaged about 0.95 ± 0.03 mm ($n = 78$) (Hurtado, unpublished data).

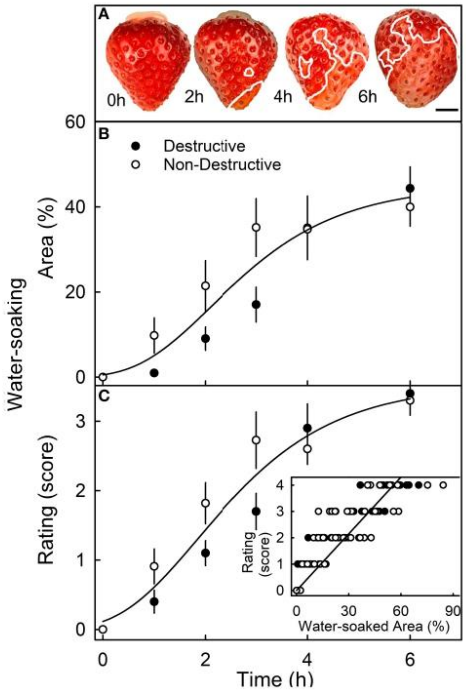

FIGURE 3 | Time course of water soaking. **(A)** Macroscopic view of change in water-soaked area with time. The symptomatic area is indicated by a solid white line. Scale bar, 5 mm. **(B,C)** Change in water soaking as indexed by the measurement of the water-soaked area **(B)** or as assessed by a 5-point rating scale **(C)**. (**C**, inset): Relationship between the water-soaked score and the water-soaked area. For the destructive sampling depicted in **(B)**, an independent set of fruit was assessed at any one time. For the non-destructive sampling in **(B)**, repeated observations were made on the same fruit that was photographed repeatedly. The water-soaked area was quantified by digital analysis. The 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%.

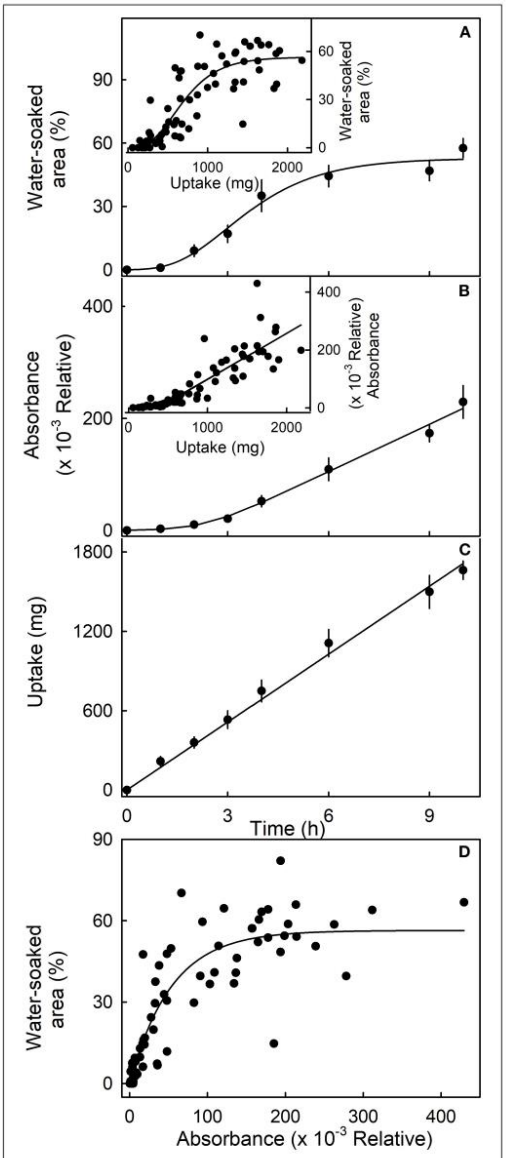

FIGURE 4 | Time course of **(A)** change in water-soaked area, **(B)** change in anthocyanin leakage as indexed by absorbance of the incubation solution at 520 nm, and **(C)** water uptake of strawberries incubated in deionized water. Insets. Relationship between the water-soaked area (inset in **A**), the change in absorbance (inset in **B**), and water uptake. **(D)** Relationship between the water-soaked area and the leakage of anthocyanins as indexed by the change in absorbance.

The susceptibility to water soaking increased sigmoidally with ripening as indexed by the change in fruit color from white to dark red (**Figure 7A**). Across the different ripeness stages, the water-soaked area and water uptake rate were linearly related (**Figure 7B**, main graph). Unripe fruit (white) had the lowest values of both uptake and water soaking, while fully ripe fruit (dark red) showed the highest values. Accordingly, the relationship between osmotic potential and water uptake was linear and positive. The fully ripe, dark-red fruit also had the most negative osmotic potentials and the highest rates of water uptake (**Figure 7B**, inset).

Water soaking increased as fruit mass increased (**Figure 8**, main graph). The largest fruit also had the highest rating

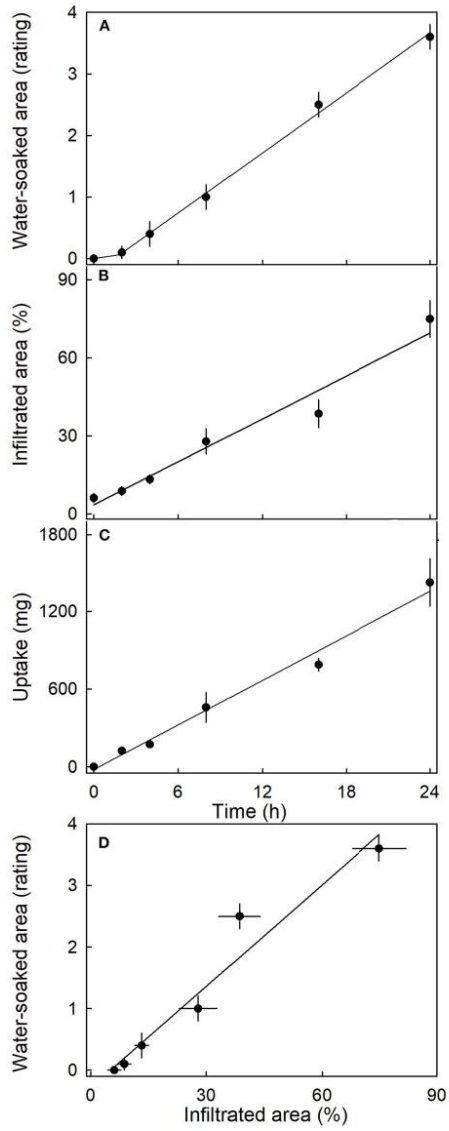

FIGURE 5 | Time course of **(A)** change in water-soaked area, **(B)** change in the area infiltrated by acridine orange, and **(C)** water uptake of strawberries incubated in deionized water. **(D)** Relationship between the water-soaked area and the area infiltrated by acridine orange. Acridine orange penetrates the strawberry fruit skin *via* microcracks in the cuticle. Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%.

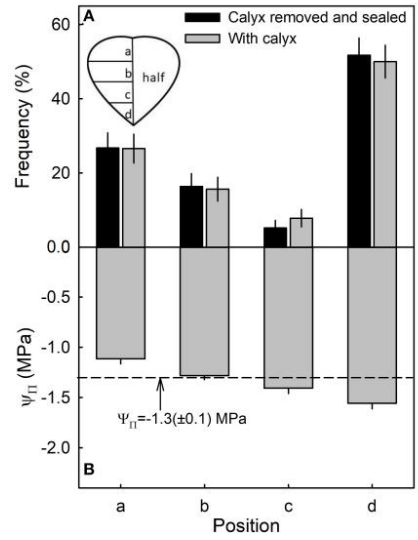

FIGURE 6 | Frequency of symptoms of water soaking **(A)** and the osmotic potential (Ψ_Π) of the juice **(B)** in different regions of a strawberry. The dashed line represents the mean osmotic potential of the fruit.

scores and hence the largest portions of their surfaces water-soaked. Fruit mass was independent of the soluble solids content (**Figure 8**, inset).

Incubating strawberries in isotonic PEG 6000 resulted in less water soaking and negligible water uptake compared to the control incubated in water (**Figure 9**). Interestingly, there was little difference in microcracking between fruit incubated in isotonic PEG 6000 or in water as indexed by the areas infiltrated by acridine orange (**Figure 9B**). The water-soaked area and the area infiltrated by acridine orange were positively related (**Figure 9D**).

Incubating strawberries in natural juice or artificial juice prepared at hypotonic, but not at isotonic concentrations, significantly increased water soaking. In contrast, water uptake decreased in both juices at hypotonic and even more so at isotonic concentrations as compared to the water control (**Table 1**). There were no differences either in water-soaked area or in water uptake between natural and artificial juice (data not shown). Interestingly, the rates of water uptake were consistently greater than zero, even though the incubation solution was isotonic to the fruit's expressed juice.

Studying the effects of the individual components of strawberry juice on water soaking and water uptake revealed that citric and malic acids markedly increased water soaking compared to the water control. The rates of water uptake were higher compared to the other osmolytes or the water control.

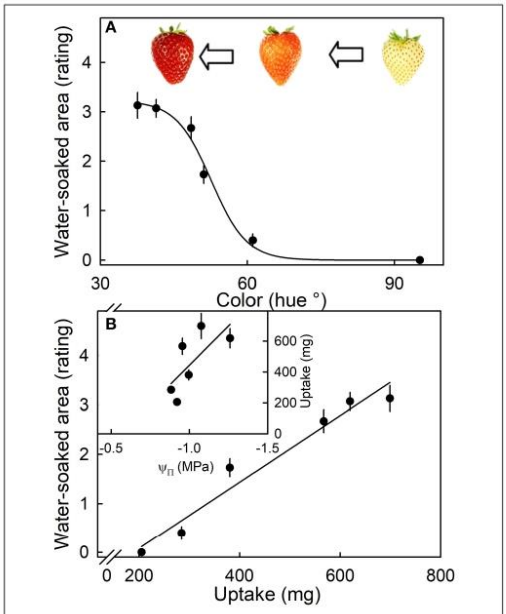

FIGURE 7 | (A) Effect of ripening stage (as indexed by hue angle) on the water-soaked area when strawberries are incubated in deionized water. **(B)** Relationship between the water-soaked area and water uptake. Inset: Uptake vs. osmotic potential. Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%.

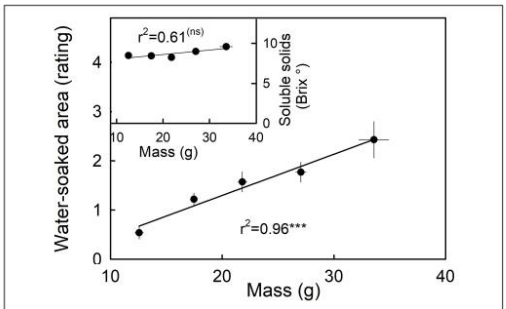

FIGURE 8 | Relationship between water soaking and fruit mass. Inset: Relationship between the soluble solids as indicated by °Brix and water soaking. Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%.

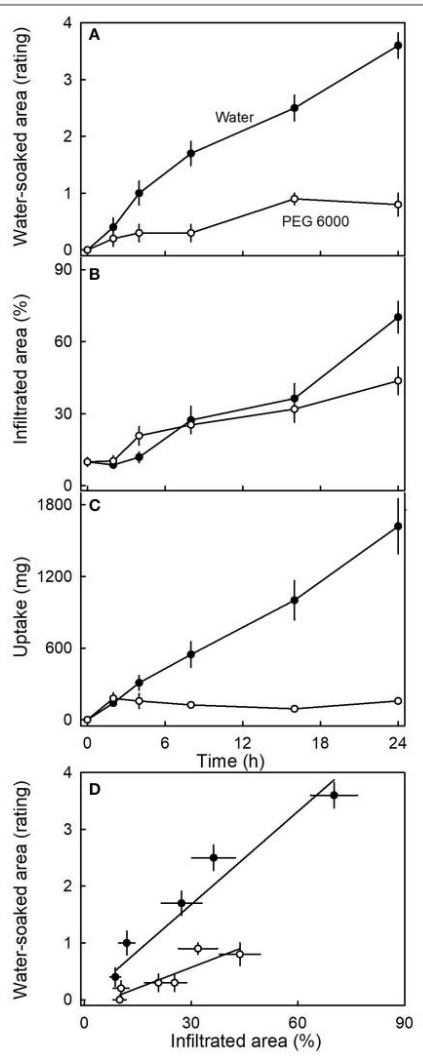

FIGURE 9 | Time course of **(A)** change in water-soaked area, **(B)** change in the area infiltrated by acridine orange, and **(C)** water uptake of strawberries incubated in deionized water or in isotonic polyethylene glycol 6000 (PEG 6000). **(D)** Relationship between the water-soaked area and the area infiltrated by acridine orange. Acridine orange penetrates the strawberry fruit skin *via* microcracks in the cuticle. Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%.

The carbohydrates that accounted for most of the osmolarity in strawberry juice decreased both water soaking and rates of water uptake to levels below the water control (**Table 2**).

Anthocyanin leakage from flesh disks increased with time and was higher in the presence of citric and malic acid, than in the control (deionized water) (**Figure 10A**). The amounts of leakage

were also higher from hypotonic than from hypertonic solutions. Again, leakage in the presence of the acids exceeded that in their absence. A two-factorial analysis of variance revealed the significant main effects for osmotic potential and the acids; there was no significant interaction term between these two factors (**Figure 10B**).

DISCUSSION

The main findings are as follows:

1) Water soaking involves microcracking of the cuticle, water uptake, bursting of cells, and leakage of cell contents
2) The behavior of water soaking in strawberry bears many similarities to the zipper model used to explain rain cracking in sweet cherries.

Water Soaking Requires Microcracking of the Cuticle, Water Uptake, and the Bursting of Cells

This hypothesis is based on the following evidence.

(1) Surface wetness induced microcracking in the cuticle of the strawberry fruit when incubated in water in this and our earlier study (Hurtado et al., 2021) or in isotonic solutions (PEG 6000) (**Figure 9**). This is consistent with the literature reports of other fleshy fruit crops, including sweet cherry (Knoche and Peschel, 2006), apples (Knoche and Grimm, 2008; Khanal et al., 2021), *Ribes* berries (Khanal et al., 2011), and grapes (Becker and Knoche, 2012a). Microcracks impair the barrier properties of the cuticle. They represent a pathway for a rapid and localized water uptake by viscous flow through the strawberry fruit surface (Hurtado et al., 2021). Microcracking is an essential, but not the only requirement, in water soaking. For example, incubation in isotonic PEG 6000 induced microcracking, but markedly reduced water uptake and hence water soaking. Simulating microcracking by abrading the cuticle from the fruit surface induced water soaking like symptoms. After puncturing the fruit skin, the water-soaked area was much smaller. This is also consistent with the above hypothesis. It is important to note that microcracking of the cuticle due to moisture exposure also accounts for the localized nature of water soaking. Microcracks impair the cuticle's barrier function and allow a rapid, localized water uptake (Hurtado et al., 2021). This also explains why partial incubation of fruit resulted in water soaking only in the exposed regions.

(2) Water uptake markedly increased water soaking. Manipulations such as incubation in isotonic PEG 6000 that resulted in reduced water uptake also reduced water soaking. The only exceptions were treatments where the incubation solutions contained organic acids. The effect of organic acids is addressed in detail below. Also, more mature fruit, with more negative osmotic potentials and hence, higher rates of water uptake, also showed more water soaking. A more negative osmotic potential and hence, a higher rate of water uptake, is also consistent with a higher incidence of water soaking at the tip of the fruit. These results indicate that mature, ripe fruit is more susceptible to water soaking, particularly at the tip. The relatively high frequency of water soaking in the calyx end of the fruit (regions a, b) may have resulted from growth stress and strain that will be maximal in these regions. Additional evidence for a water uptake requirement comes from experiments where the fruit was incubated in artificial or natural juices. Here, water soaking was more severe in the hypotonic juices treatments, which had higher rates of water uptake, compared with the isotonic juices.

(3) Finally, bursting of cells is involved in water soaking. Evidence for this comes from the experiment on anthocyanin leakage. Here, up to some critical threshold, water uptake did not induce water soaking. However, beyond this threshold, water soaking began. The timing of the critical threshold for water uptake coincided with that for the onset of leakage. The latter indicates the onset of cell bursting. It is interesting to note that the release of organic acids that accompanies cell bursting seems to exacerbate water soaking. Experiments using natural or artificial juices and the major components thereof clearly identified citric and malic acids as the critical constituents that accelerate the development of water soaking. Both acids are released into the apoplast when a cell bursts. Here, they increase the permeability of the plasma membrane of neighboring cells. This causes cell leakage to propagate. Similar observations have been made for malic acid in sweet cherry, and these led to the development of the "Zipper model" where the acids "unzip" the skin (Winkler et al., 2015). In contrast to sweet cherry, in strawberry, citric and malic acids had no effect on the strength of the cell walls. This conclusion is inferred from the lack of a significant interaction between the osmotic potential of the incubation solution and the presence of the acids. At low (more negative) osmotic potentials, the incubation solutions were isotonic or hypertonic. A gradient for osmotic water uptake into the cells, and hence increased stress on the cell walls, must be absent. In contrast, at higher (less negative) osmotic potentials, the incubation solution was hypotonic, and hence, osmotic water uptake by the cells occurred. This imposes increased stress on the cell walls. However, the effect of the acids was independent of the applied stress on the cell walls. Hence, the acid effect must have been on the plasma membrane and not on the cell walls. This conclusion is also consistent with the wounding effect. Cuticle abrasion was more effective in inducing water soaking than either incisions or punctures. With abrasion, relatively large areas of the skin are damaged. It is the skin cells that contain most citric and malic acids (in the skin, the summed masses of both acids was 10.6 mg g^{-1}) compared with about half this amount in the flesh (in the flesh, the summed masses of both acids were 5.5 mg g^{-1}) (Holcroft and Kader, 1999).

The bursting of cells also offers a convincing explanation for the characteristic translucency of a water-soaked tissue. Water-soaked tissue typically results from the flooding of the gas-filled intercellular spaces (Sideris and Krauss, 1933). In addition, the leakage of both the tonoplast and the plasma membranes results in a general mixing of apoplastic and symplastic components (the anthocyanins are naturally contained within the tonoplast—vacuole). The apoplast pH is likely to increase, compared to that of the vacuole. As a result, the anthocyanins will likely react with water, to form colorless pseudobases (Brouillard et al., 1997; Holcroft and Kader, 1999). This explains the lighter color of a water-soaked tissue.

TABLE 1 | Effect of natural (expressed) juice and artificial (compounded) juice on development of water soaking in "Faith" strawberry.

Treatment	Water uptake (rate, mg h^{-1}) Osmotic potential (MPa)			Water soaking (score, arbitrary) Osmotic potential (MPa)		
	0.5	1.0	Mean	0.5	1.0	Mean
Artificial juice	104.3 ± 18.6	53.8 ± 12.4	78.9 ± 12.2$^{(ns)}$	2.2 ± 0.2	1.6 ± 0.3	1.9 ± 0.2$^{(ns)}$
Natural juice	83.2 ± 31.9	58.1 ± 13.0	70.7 ± 17.0	1.8 ± 0.2	1.5 ± 0.2	1.7 ± 0.1
Mean	93.8 ± 18.2a^{a}	55.8 ± 8.8 b	74.8 ± 10.4	2.0 ± 0.1 a	1.5 ± 0 b	1.8 ± 0.1

Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, <10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%. In the deionized water control, the rate of water uptake was 122.8 ± 18.7 mg h^{-1}, and the score for water soaking was 1.6 ± 0.2.
Analysis of variance revealed no significant interaction term but a significant main effect for osmotic potential. $^{(ns)}$Non-significant effect for treatment.
^{a}Means followed by the same letter are not significantly different, Tukey's test at p = 0.05. ns, non-significant effect.

TABLE 2 | Effect of major osmolytes on the rate of water uptake and the development of water soaking in strawberries of cultivar "Florentina."

Component	Concentration (mM)	$-\psi_{\pi}$ (Mpa)	pH	Rate of uptake (mg h^{-1})	Rating (score)
Water	0.0	0.0	6.4	117.3 ± 13.8 b^{a}	1.9 ± 0.2 b
Malic acid	25.2	0.1	2.6	207.6 ± 27.1 a	3.7 ± 0.1 a
Citric acid	43.4	0.1	2.3	176.4 ± 16.8 a	3.5 ± 0.2 a
Carbohydrates	316.6	0.8	6.5	63.0 ± 7.4 c	1.0 ± 0.2 c
Artificial juice	428	1.1	3.2	50.2 ± 5.2 c	1.6 ± 0.2 b

Water soaking was indexed using a 5-point rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10–35%; score 3, 35–60%; score 4 > 60%. The artificial juice was compounded from the major osmolytes found in strawberries (together these account for 96.1% of the osmolarity of a strawberry) (Herrmann, 2001). The artificial juice was isotonic with the expressed natural juice.
^{a}Means followed by the same letter are not significantly different, Tukey's test at p = 0.05.

The Mechanism of Water Soaking Is Similar to That of Cracking in Sweet Cherry Fruit

Water soaking in strawberry can be substantially explained in terms of the zipper model that accounts for sweet cherry fruit cracking during/following rainfall (Winkler et al., 2016). Based on this model, a series of sequential events leads to water soaking. First, the barrier function of the cuticle is impaired by the formation of microcracks in response to surface wetness (**Figure 2**). Because the cuticle of a strawberry is very thin (Hurtado et al., 2021), microcracks allow a rapid, localized water uptake into the subtending tissues. This uptake occurs by viscous flow and is more rapid than that by diffusion through an intact cuticle (Hurtado et al., 2021). Unlike in apple, there is no evidence of any repair processes in strawberry: wax deposition in a microcrack (Curry, 2009) or the formation of a periderm (Evert, 2006; Macnee et al., 2020). Second, water uptake causes cells to burst, as indicated by the leakage of anthocyanin. Because strawberries contain large amounts of citric and malic acid, particularly in the skin cells, and because these acids increase the permeability of plasma membranes, the neighboring cells also begin to leak. This propagation process causes the water-soaked area to spread both laterally and down into the fruit.

Unlike in sweet cherry, there is no evidence in strawberry that these organic acids cause a weakening of cell walls. Nevertheless, future evidence for a weakening would not be surprising, as both citric and malic acids are likely to extract Ca from the cell walls, and this loss will decrease cell-to-cell adhesion (Winkler et al., 2015). However, at this stage, there is no support for such effects in strawberry.

CONCLUSIONS

Our results demonstrate that water soaking in strawberry results from a series of events that involves microcracking of the cuticle, water uptake, a cell bursting, and the release of organic acids into the apoplast. In many ways, it is similar to rain cracking in fleshy fruit, such as sweet cherry. Based on our findings, a reliable strategy to prevent water soaking would be to limit a direct contact between water and the fruit surface. This may be achieved preharvest by protected cultivation, either in a greenhouse or in a plastic tunnel. Important measures postharvest include the use of appropriate packaging materials and handling practices that avoid the occurrence of liquid water on the surface, for example, due to condensation of water vapor. In the long run, the susceptibility of commercial strawberry genotypes to water soaking may be decreased by breeding. Our results and those by Herrington et al. (2009) demonstrate that susceptibility to water soaking differs among cultivars. Water soaking occurred in all cultivars used in our study, but at a markedly lower rate in "Clery" as compared to "Faith" and "Florentina." The basis of these differences has not yet been identified. Among the three cultivars, "Clery" also had the lowest rate of water uptake, which probably contributed to the lower rate of water soaking. Based on the findings presented herein and their similarity to the

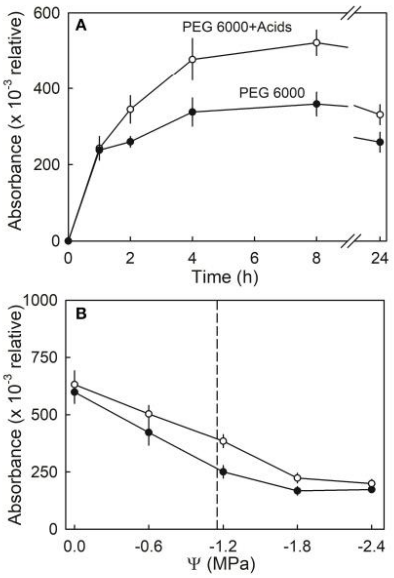

FIGURE 10 | (A) Time course of anthocyanin leakage from flesh disks excised from ripe strawberries in the presence or absence of citric or malic acids. Flesh disks were incubated in isotonic polyethylene 6000 (PEG 6000) solutions. **(B)** Effect of osmotic potential (Ψ) of the incubation solution on anthocyanin leakage in the presence or absence of citric or malic acids. The vertical dashed line indicates the osmotic potential of juice expressed from the fruit of the same batch.

phenomenon of rain cracking in sweet cherry, potentially useful aims for further research in water soaking are to determine (a) the pattern of cuticle deposition in developing strawberry fruit, (b) the water uptake characteristics, (c) the mechanical strength of their cell walls, and (d) the strength of their cell-to-cell adhesion. A better understanding of the phenomenon of microcracking would also be beneficial in reducing the incidence of fruit rots. Pathogens like *Botrytis cinerea* benefit from microcracks in the cuticle because of the impaired barrier functions (Jarvis, 1962).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MK obtained the funds to support the study. GH and MK planned the experiments, analyzed the data, wrote, revised, and edited the manuscript. GH conducted the experiments. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by a grant from the Deutsche Forschungsgemeinschaft (KN 402/19-1).

ACKNOWLEDGMENTS

We thank Dr. Alexander Lang and Dr. Andreas Winkler for helpful comments on an earlier version of this manuscript, and Heike, Karl Walter, and Martin Brüggenwirth and Bendix Meyer for the gift of fruit.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.694123/full#supplementary-material

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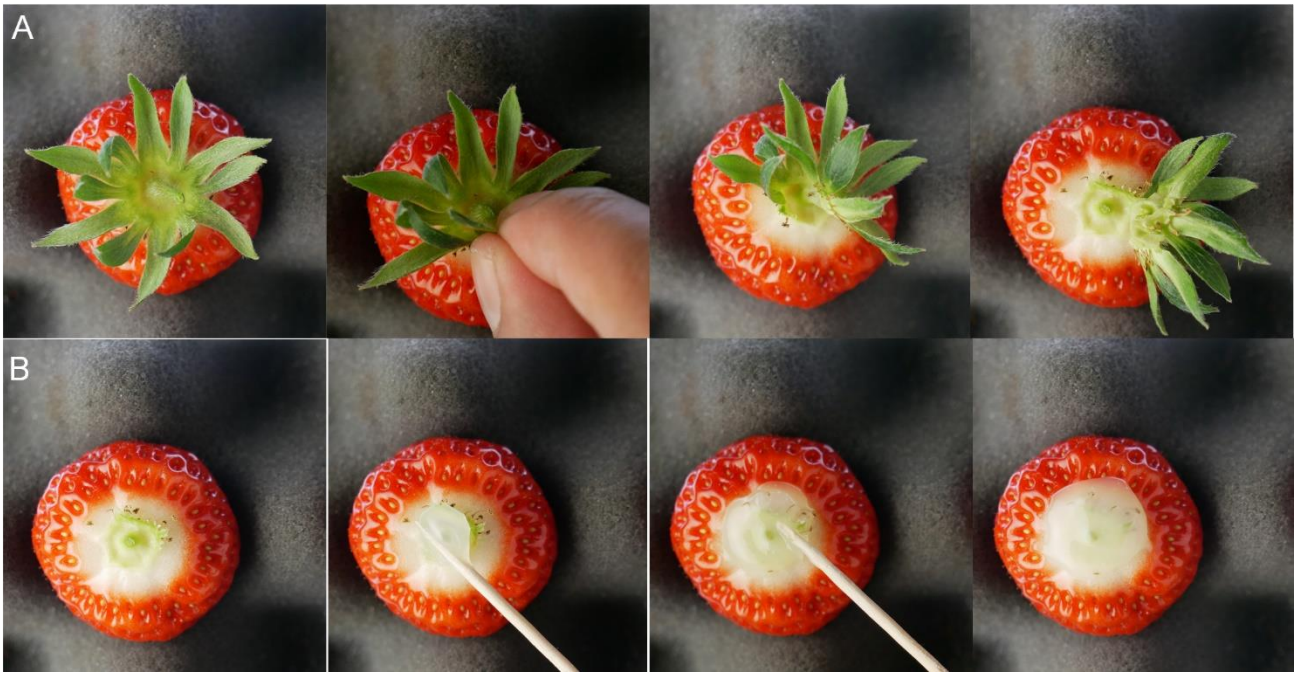
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material



Supplementary Figure S1. Procedure of calyx extractions and sealing. **(A)** Manual extraction of calyx. **(B)** sealing of the remaining.

4.6. Calcium ions decrease water-soaking in strawberries

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Type of authorship	First author
Type of article	Research article
Author contributions	Grecia Hurtado: Methodology, Investigation, Visualization, Formal analysis, Validation, Writing original draft, Writing review and editing. Moritz Knoche: Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Writing original draft, Writing review and editing.
Journal	PloS One
Impact factor	3.752 (2022)
Date of publication	15.08.2022
DOI	10.1371/journal.pone.0273180

PLOS ONE

RESEARCH ARTICLE

Calcium ions decrease water-soaking in strawberries

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OPEN ACCESS

Citation: Hurtado G, Knoche M (2022) Calcium ions decrease water-soaking in strawberries. PLoS ONE 17(8): e0273180. https://doi.org/10.1371/journal.pone.0273180

Editor: Hernâni Gerós, Universidade do Minho, PORTUGAL

Received: May 27, 2022

Accepted: August 3, 2022

Published: August 15, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting information files.

Funding: The funding of this study was provided by grant nr. KN402/19-1 (to MK) from the German Science Foundation (DFG). The publication of this article was funded by the Open Access fund of the Leibniz Universität Hannover. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

Abstract

Water soaking is a common disorder of field-grown strawberries (*Fragaria × ananassa* Duch.). It develops when ripe fruit is exposed to rain. Here we investigate the effects of Ca on water soaking. Fruit was incubated in solutions of various Ca salts and the extent of water soaking quantified using a simple rating scheme. Exposure to $CaCl_2$ (10 mM) decreased water soaking and anthocyanin leakage but had no effect on water uptake. The decrease in water soaking due to $CaCl_2$ was not limited to a single cultivar but occurred in all cultivars examined. Incubating fruit in a chelating agent (EGTA) increased water soaking compared to the water control. Calcium salts of different acids varied in their effects on water soaking. Only $CaCl_2$ reduced water soaking significantly. The chlorides of different cations, also varied in their effects on water soaking. Those of the monovalent cations had no effects on water soaking, while those of the divalent cations ($CaCl_2$, $BaCl_2$ and $SrCl_2$) and of the trivalent cations ($FeCl_3$ and $AlCl_3$) were all effective in decreasing water soaking. Overall, $CaCl_2$ decreased microcracking of the strawberry cuticle as compared to deionized water. Furthermore, $CaCl_2$ also reduced the leakage of anthocyanins from flesh discs, irrespective of the osmotic potential of the incubation solution. Our results indicate that $CaCl_2$ reduced water soaking by decreasing cuticular microcracking, by decreasing leakage of plasma membranes and, possibly, by increasing the crosslinking of cell wall constituents.

Introduction

Water soaking is an economically important disorder of field grown strawberries [1]. It develops when fruit is exposed to rain during ripening. Protected cultivation in greenhouses or tunnels covered with plastic film generally avoids water soaking [2] but it markedly increases the cost of production. Other countermeasures for water soaking are not known.

Recently, the physiological background of water soaking has been identified [2]. In many ways the process of water soaking in strawberry resembles the process of rain cracking in sweet cherry. Both, water soaking and rain cracking are multistep processes that can be described using the analogy of a 'zipper', that unzips the fruit skin [3]. Based on the 'Zipper' model [3], water soaking is initiated by the formation of microscopic cracks ('microcracks') in the cuticle [2]. Microcracking impairs the barrier function of the cuticle thereby permitting localized water uptake. When water uptake exceeds a critical limit, cells begin to burst [4]. Cell

Competing interests: The authors have declared that no competing interests exist.

contents, including anthocyanins and organic acids, are thus released from the vacuole and move into the cell wall space. Strawberry is particularly rich in malic and citric acids [5]. Exposure to these acids increases the permeability of the membranes of adjacent cells. This causes water soaking to spread tangentially over the skin and also radially down into the flesh [2].

The above sequence of events is largely identical to the early steps of rain cracking in cherry. Also, the countermeasures taken against rain cracking in sweet cherry are similar to those taken in strawberry, i.e., production in protected environments using rain shelters or tunnels [3]. In addition, in sweet cherry, whole canopy sprays of Ca salts are reported to decrease susceptibility to cracking [6]. For strawberry, there is no published information on the possible benefits of whole canopy Ca sprays on water soaking of the fruit. However, given the hypothetical mode of action of Ca in decreasing rain cracking susceptibility in cherry, such beneficial effects are not unlikely with water soaking in strawberry.

The objective of our study was to establish the effects of Ca on water soaking in strawberry. The effects of Ca were compared to those of other monovalent, divalent and trivalent cations. Water soaking was induced using a laboratory based immersion assay [2].

Materials and methods

Plant material

Strawberry fruit were harvested from commercial plantings at Gleidingen (lat. 52˚16´ N, long. 9˚50´E), Ohndorf (lat. 52˚21´ N, long. 9˚21´E) and from a greenhouse and growth chamber on the Herrenhausen Campus of the Leibniz University, Hannover, Germany. Temperature and relative humidity (RH) of the growth chamber were set at 20/16˚C and 60/80% RH during a 16 h day/night photoperiod. The cultivars used in our study were 'Clery', 'Sonsation', 'Malwina', and 'Faith'. These cultivars were selected based on uniformity of fruit and availability at the optimum stage of ripening. The change of cultivars ensures that the observed effects are valid for strawberry in general and not limited to a specific cultivar. Fruit were harvested randomly at commercial ripeness (>80% of the fruit surface red) and selected for uniformity of size, shape, color, and freedom from visual defects. Earlier studies established that at this stage of development ripeness has no effect on the rate of water uptake [4] or on water soaking [2]. Fruit were processed fresh on the day of harvesting or held at 2˚C and 80% RH for no longer than 1 d.

General procedure

Unless otherwise specified, the so-called 'calyx' was removed from the fruit (strictly a false fruit) by carefully tearing away individual bracts. Fruit mass was sufficiently high that there was no need to hold down (and potentially damage) the fruit skin during this process. The remains of the bract whorl including the cut peduncle end, were sealed using a fast-curing, non-phytotoxic silicone rubber (Dowsil SE 9186 Sealant; Dow, Midland, MI, USA).

Water soaking was induced by incubating fruit individually in deionized water (one fruit per 100 mL). The fruit was forced underwater using a soft plastic-foam plug. After a pre-specified interval, a fruit was removed from the water, carefully blotted using soft tissue paper and weighed (ME235P-OCE; Sartorius, Goettingen, Germany). Water soaking was quantified using a five-point rating scale [2]. The rating scale was: score 0 = no water soaking; score 1 = <10% of the surface area water-soaked; score 2 = 10 to 35%; score 3 = 35 to 60% and score 4 = >60% of the surface area water-soaked. Earlier studies established that the water soaked area (a continuous variable) and the rating scores (a discontinuous score) were about linearly related [2].

All experiments were carried out in a temperature-controlled laboratory at 22˚C. The number of individual fruit replicates per treatment was 15, unless otherwise specified.

Experiments

Establishing the effect of $CaCl_2$. The time course of water soaking was established in the presence and absence of 10 mM $CaCl_2$. Deionized water served as control. Fruit were incubated for 0, 2, 4, 8, 16, 24, 36 or 48 h. Water uptake, water soaking and leakage of anthocyanin into the incubation solution were determined. The leakage of anthocyanin was quantified by measuring the absorbance of the incubation medium at 520 nm using a spectrophotometer (Specord 210; Analytik Jena, Jena, Germany). The pH was adjusted to 2.3 using citric acid at a final concentration of 37 mM before measuring absorbance.

The effect of the $CaCl_2$ concentration on water soaking was studied by incubating fruit in solutions of 0, 1, 3, 10, 30 or 100 mM $CaCl_2$. Water uptake and water soaking were quantified as described above. The amount of Ca taken up during incubation was also established. Fruit were freeze-dried for 3 to 4 d, followed by drying at 103˚C for 15 d. Individual fruit dry mass was also determined. The dried samples were ground in a ball mill at 30 Hz for 10 s (MM 400 mill; Retsch, Haan, Germany). The powder was re-dried for 3 d at 103˚C before an aliquot of 100 mg was taken and ashed in a muffle furnace (L24/11/B180; Nabertherm, Lilienthal, Germany) at 500˚C (heating phase: from 20 to 500˚C 2 h, holding phase: 4 h at 500˚C). When ashing was incomplete as indexed by dark black ash, the samples were again taken up in 200 μl of 1 N HCl and re-ashed using the same settings. The ash was taken up in 2 ml of 1 N HCl plus 8 ml of deionized water and filtered (MN 640 M; Macherey-Nagel, Dueren, Germany). To eliminate interference from P in the Ca analyses, $LaCl_3$ was added to the solutions at a final concentration of 1% [7]. The solution was diluted with deionized water as required to obtain a Ca concentration within the measuring window (range 0 to 4 mg l^{-1} of Ca). Samples were analyzed using an atomic absorption spectrometer (AAS) (Analyst 300; Perkin Elmer, Waltham, MA, USA) equipped with a Ca lumina hollow cathode lamp (wavelength 422.7 nm, slit 0.7 nm) using an air–acetylene flame.

Whether the effect of $CaCl_2$ was specific to 'Florentina' strawberry was investigated by comparing different cultivars, i.e., Clery, Sonsation, Malwina, Faith. In each case fruit were incubated with or without $CaCl_2$ at 10 mM for 24 h. Water uptake and water-soaking were quantified.

The mode of action of Ca. The effect of the chelating agent EGTA (Ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic acid, CAS Nr. 67-42-5) on water soaking was established by incubating fruit in 10 mM EGTA and 10 mM $CaCl_2$, or deionized water for 4 h. The chelating agent EGTA was selected because it has a high affinity for Ca and, therefore, can extract Ca from the cell wall. Water uptake and water soaking were quantified.

To establish whether a putative decrease in water soaking due to $CaCl_2$ was related to the cation or the anion, two experiments were conducted. The first focused on the anions. Different organic (Ca-acetate, Ca-formate, Ca-propionate, Ca-lactate, Ca-heptagluconate) and inorganic ($CaSO_4$, $Ca(NO_3)_2$, $CaCl_2$) Ca-salts were compared, each at 10 mM. The second experiment focused on the cations. Here, we compared the effects of monovalent (Na^+, K^+, NH_4^+, Li^+), divalent (Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}) and trivalent (Fe^{3+}, Al^{3+}) cations on water uptake and water soaking. All these cations were partnered with chloride due to their high water solubility, concentrations were all 10 mM. Deionized water served as control. The incubation period was 8 h. Water uptake and water soaking were quantified.

The effect of $CaCl_2$ on microcracking was determined after incubating fruit in deionized water or in isotonic polyethylene glycol 6000 (PEG 6000) with or without 10 mM $CaCl_2$ for 4

h. Isotonic PEG 6000 was used to effectively eliminate water uptake as a potential factor in microcracking. This allowed separation of the effects of surface wetness from those of water uptake. Following incubation, fruit were immersed in 0.1% of the fluorescent tracer acridine orange (Carl Roth, Karlsruhe, Germany) for 5 min, rinsed with deionized water and carefully blotted. The fruit surface was then inspected at ×3.2 under incident fluorescent light using a binocular microscope (MZ10F with filter GFP plus excitation wavelength 480–440 nm, emission wavelength ≥510 nm; Leica Microsystems GmbH, Wetzlar, Germany). Four randomly selected calibrated images were taken (Camera DP71; Olympus, Hamburg, Germany) in the region of the maximum diameter of the fruit. A total of 20 fruit per treatment were inspected. The fluorescent tracer acridine orange penetrates any microscopic cracks in the cuticle. Tissue infiltrated with acridine orange emits orange, yellow and green fluorescence [8]. The area infiltrated by acridine orange was quantified using image analysis (cellSens Dimension 2.3.1; Olympus). The infiltrated area was expressed as a percentage of the area of the microscope window (2.2 x 1.7 mm). The total number of replicates per treatment was 80.

The effects of $CaCl_2$ on the integrity of the cell wall and plasma membrane were studied using anthocyanin leakage from flesh discs as an indicator [9]. A time course of anthocyanin leakage was established. Tissue cylinders were excised from the outer flesh using a biopsy punch (8 mm diameter). The skin was removed. Using parallel-mounted razor blades, cylinders were cut transversely to form 2 mm thick discs. The discs were then blotted, rinsed and incubated in isotonic PEG 6000 solution with or without 10 mM $CaCl_2$ for 0, 2, 4, 8, 16 or 24 h. For sampling, discs were removed from incubation medium, the pH of the medium adjusted to pH 2.5 and the absorbance quantified at 520 nm (Specord 210; Analytik Jena, Jena, Germany). Based on these results a 24 h time interval was selected for the subsequent experiment. Here, the effects of $CaCl_2$ on the bursting pressure of the cell wall were examined. Cell walls were stressed to varying extents by incubating discs in solutions of PEG 6000 at osmotic potentials of 0, -0.5, -1.0, -1.5 or -2.0 MPa with or without 10 mM $CaCl_2$. Absorbance of the incubation medium was measured as described above. Six discs were excised per fruit and used as paired observations with three discs representing one replicate. The experiment was carried out using ten replicates.

Data analyses

All experiments were conducted and analyzed using completely randomized designs. Data were analyzed by analysis of variance. Means were compared using the Dunnett test or Tukey's studentized range tests (all $p < 0.05$) using R (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria), and regressions were carried out using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). Data are presented as means ± standard errors. All data shown in the Figures and Tables are available in the S1 File.

Results

Water soaking appeared as irregular pale patches of deliquescent skin. These were watery, slightly translucent and dull, compared with the dark-red and shiny appearance of an adjacent intact surface on the same fruit or on a control fruit. The symptoms induced by incubation in deionized water or in 10 mM $CaCl_2$ did not differ significantly, except that the affected surface areas, were markedly smaller in fruit incubated in $CaCl_2$ (Fig 1).

Water uptake increased linearly with time. There were no significant differences in rates of water uptake, with or without $CaCl_2$ (Fig 2A). However, the leakage of anthocyanin, as indexed by the absorbance of the incubation medium, increased with time in an expo-linear pattern (Fig 2B). Following an initial lag phase of about 16 h, medium absorbance increased rapidly

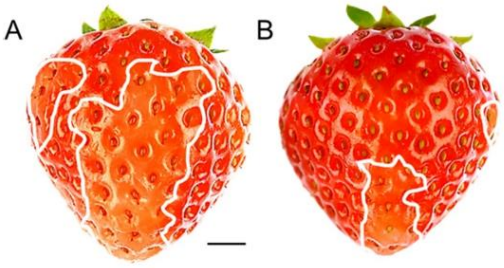

Fig 1. Typical symptoms of water soaking in strawberry 'Florentina' after incubation in (A) water (score 3) and in (B) 10 mM of $CaCl_2$ (score 2) for 8 h. Water soaking was indexed using a five-point rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%. The white line indicates the extent of the water-soaked area. Scale bar in A = 5 mm. The images are representative of 15 individual fruit replicates per treatment.

https://doi.org/10.1371/journal.pone.0273180.g001

for fruit incubated in water, but the increase was markedly less in fruit incubated in $CaCl_2$. During the lag phase, leakage occurred at a low rate for fruit incubated in water, but leakage did not occur for fruit incubated in $CaCl_2$ (Fig 2B). The relationship between uptake and leakage had a breakpoint that corresponded to the onset of cell bursting. At about 364 mg uptake, anthocyanin leakage began to increase for fruit incubated in water (Table 1). In contrast, in the presence of $CaCl_2$, the breakpoint was increased markedly to 712 mg. Furthermore, the amount of leakage per unit of water uptake was higher for fruit incubated in water, than for fruit in $CaCl_2$ (Fig 2B, inset; Table 1). Water soaking increased with time up to 8 h for fruit incubated in deionized water or in $CaCl_2$. Beyond 8 h, water soaking increased at a higher rate for fruit incubated in water as compared to fruit incubated in $CaCl_2$ (Fig 2C). At low levels of water soaking, the rating scores and anthocyanin leakage were positively related (Fig 2C, inset). However, as the rating scores approached a maximum, the rating became less dependent on anthocyanin leakage (Fig 2C, inset).

Water uptake decreased linearly with increasing concentrations of $CaCl_2$ (Fig 3A). There was no significant change in water permeance, indicating that the decrease in uptake was most likely an osmotic effect, and resulting from a decrease in driving force (Fig 3A, inset). Increasing $CaCl_2$ concentrations decreased the water-soaked area, particularly at concentrations up to 10 mM $CaCl_2$ (Fig 3B). As $CaCl_2$ concentration increased, fruit calcium content increased asymptotically (Fig 3C). Water soaking and fruit calcium content were negatively and linearly related (Fig 3C, inset).

The decrease in water soaking with $CaCl_2$ was not specific to just one cultivar but occurred in all cultivars investigated. In contrast, the effect of $CaCl_2$ on water uptake was variable. Compared with in water, water uptake decreased in the presence of $CaCl_2$ in 'Clery' and 'Malwina', whereas in 'Sonsation', 'Faith' and 'Florentina' water uptake increased in the presence of $CaCl_2$. Interestingly, the three cultivars that had the greater water uptake in the presence of $CaCl_2$ were also the ones more susceptible to water-soaking (Table 2).

Incubating fruit in EGTA markedly increased water soaking compared to in the control (water), whereas incubation in $CaCl_2$ deceased water soaking (Table 3). Incubation in EGTA resulted in the highest water uptake, and that incubated in $CaCl_2$ the lowest water uptake.

The Ca salts differed in their effects on water soaking. Only $CaCl_2$ significantly reduced water soaking, as compared to the water control. The effects of all other anions were not significant. None of the salts had a significant effect on water uptake (Table 4).

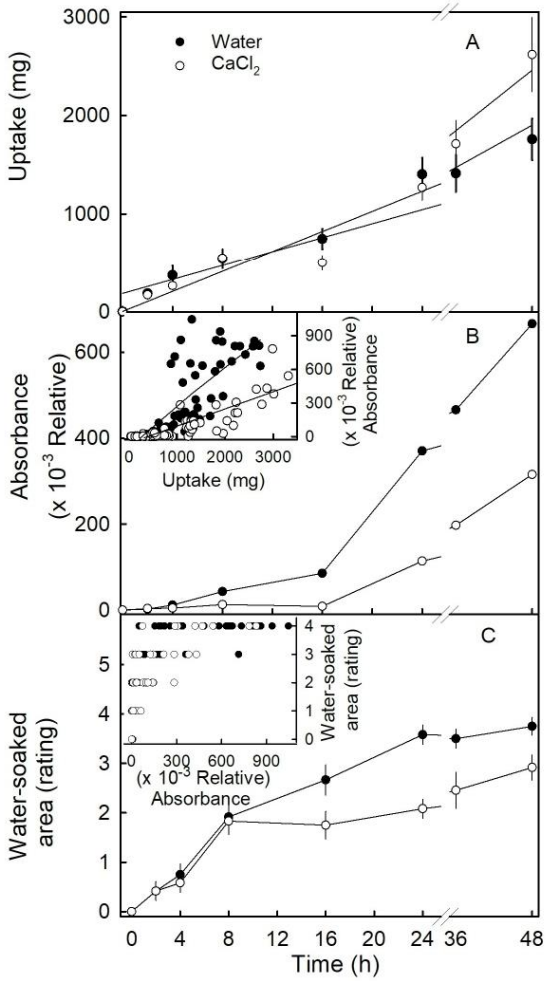

Fig 2. Time course of (A) water uptake, (B) leakage of anthocyanins as indexed by the absorbance (520 nm) of the incubation solution; and (C) change in area affected by water soaking. Inset (B): Relationship between anthocyanin leakage and water uptake, Inset (C): Relationship between water-soaked area and anthocyanin leakage. Strawberry fruit 'Clery' was incubated in deionized water or in 10 mM $CaCl_2$.

https://doi.org/10.1371/journal.pone.0273180.g002

Comparison of the effects of the cations as chlorides, revealed that the monovalent cations had no effects, either on water soaking or on water uptake. Three out of the six divalent cations (Ca, Ba, Sr) and the trivalent cations (Fe, Al) all decreased water soaking compared to the water control. Only the trivalent cations (Fe, Al) significantly decreased water uptake compared to the control (Table 5; Fig 4).

Table 1. Parameter estimates of the Goudriaan and Monteith [10] expo-linear regression model used to describe the relationship between anthocyanin leakage (absorbance) and water uptake.

Treatment	Parameter			Root mean squared error (RMSE)	P-value
	A x 10^{-4} (Relative absorbance mg^{-1})	B x 10^{-2} (mg^{-1})	K (mg)		
Control	3.8 ± 0.4	3.3 ± 32.6	364 ± 107	0.17	<0.0001
$CaCl_2$	1.7 ± 0.2	0.4 ± 0.3	712 ± 168	0.08	<0.0001

The regression equation was: $Absorbance = \frac{A}{B} \times \ln(1 + e^{B \times (Uptake - K)})$ where A is the slope of the linear phase representing the amount of leakage per unit water uptake, B is the maximum relative rate of the non-linear phase that represents the increase in leakage per unit of absorbance per unit water uptake, and K is the x-axis intercept the linear phase that the amount of water taken up when the bursting of cells begins.

https://doi.org/10.1371/journal.pone.0273180.t001

It is interesting that the appearance of symptoms of water soaking changed when incubating strawberries in $CuCl_2$ or $FeCl_3$, but not in $CaCl_2$ (Fig 5A and 5B). After incubation in $CuCl_2$, the water soaked tissue was opaque and reddish (Fig 5C). Fruit incubated in $FeCl_3$ had numerous localized black-colored precipitates particularly in the depressions around the achenes (Fig 5D).

Aqueous $CaCl_2$ also decreased microcracking of the strawberry cuticle as compared to incubation without $CaCl_2$. However, when fruit was incubated in isotonic PEG 6000 in the presence of $CaCl_2$, water uptake was markedly reduced, and there was no effect of $CaCl_2$ on water soaking or on microcracking (Table 6).

Incubating flesh discs in isotonic PEG 6000 solution, with or without 10 mM $CaCl_2$, rapidly increased leakage of anthocyanin up to about 4 h, thereafter leakage was at a lower rate (Fig 6A). Discs incubated in $CaCl_2$ had lower anthocyanin leakage than the control. When varying the osmotic potential of the PEG 6000 solution in the presence or absence of $CaCl_2$, anthocyanin leakage increased as osmotic concentration increased, particularly beyond isotonicity, when the osmotic concentration of the incubation solutions exceeded that of the fruit's juice (i.e., the solution osmotic potential became more negative) (Fig 6B). Irrespective of osmotic potential, $CaCl_2$ reduced the leakage of anthocyanins. There were no significant interactions between the two factors.

Discussion

In our discussion we will focus on (1) the decrease in water soaking due to the presence of Ca salts and (2) the effects of the Ca salts on water uptake.

Calcium decreases water soaking

Calcium, and some of the other divalent and trivalent cations decreased water soaking. Accordingly, extraction of Ca by incubation in the chelating agent EGTA increased water soaking. Due to the complex nature of water soaking according to the Zipper model, one or several of the component processes leading to water soaking must have been affected by Ca.

First, Ca decreased microcracking of the cuticle. This effect was significant only when the Ca solution was not osmotically buffered using PEG. This observation indicates that Ca most likely increased the crosslinking of the underlying cell walls that are probably causal in cracking of the cuticle. This is consistent with (1) the extremely thin and fragile cuticle of the strawberry fruit [4] and (2) numerous studies reporting increased crosslinking of cell walls by Ca and other divalent or trivalent cations [6, 11]. Pectins are major constituents of cell walls and carry a negative charge at physiological values of pH. Cations bind to these negative charges and di- and trivalent cations thereby increase crosslinking. Calcium also decreases pectin

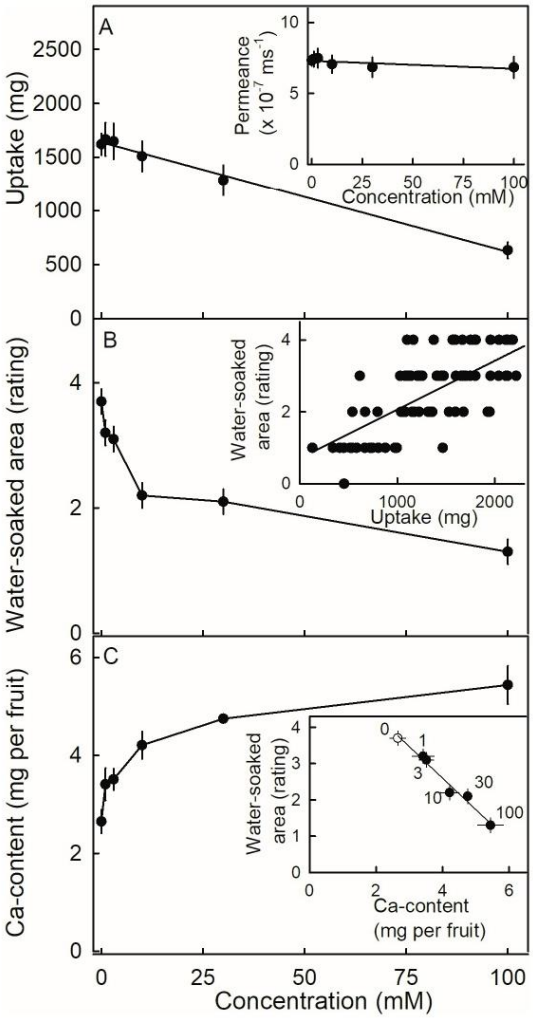

Fig 3. Effect of the concentration of $CaCl_2$ on (A) water uptake, (B) water soaking, and (C) Ca content of 'Clery' strawberry. Inset in (A): Relationship between permeance and $CaCl_2$ concentration. Inset in (B): Relationship between water soaking and water uptake. Inset in (C): Relationship between water soaking and fruit Ca content. The numbers on the graphs represent the corresponding $CaCl_2$ concentration (mM).

https://doi.org/10.1371/journal.pone.0273180.g003

solubilization [6, 12]. Similar effects on crosslinking have been reported for $FeCl_3$ in cracking of sweet cherry fruit [13]. The effect of Ca on crosslinking of cell walls in strawberry was statistically significant, but not very large. In the presence of Ca, the amount of water uptake tolerated without bursting of cells and leakage of anthocyanins was higher compared to the amount

Table 2. Effect of $CaCl_2$ (10 mM) on water soaking of ripe strawberries of different cultivars. Fruit was incubated for 24 h.

Cultivar	Uptake (mg)			Water-soaked area (rating)		
	Control	$CaCl_2$	Mean	Control	$CaCl_2$	Mean
Clery	1069 ± 93 a[a]	721 ± 105 b	895 ± 76	3.2 ± 0.2	1.6 ± 0.2	2.4 ± 0.2 b
Sonsation	1537 ± 104 a	2463 ± 261b	2000 ± 162	3.7 ± 0.2	2.9 ± 0.3	3.3 ± 0.2 a
Malwina	1122 ± 134 a	713 ± 134 b	917 ± 100	1.7 ± 0.2	0.7 ± 0.2	1.2 ± 0.2 c
Faith	1511 ± 153 a	2339 ± 306 b	1925 ± 185	3.3 ± 0.3	2.9 ± 0.3	3.1 ± 0.2 a
Florentina	889 ± 49 a	1480 ± 129 b	1185 ± 87	3.7 ± 0.1	2.9 ± 0.2	3.3 ± 0.2 a
Mean	1226 ± 57	1543 ± 125		3.1 ± 0.1 a	2.2 ± 0.1b	

[a] Two factorial AOV revealed significant interaction for water uptake, but not significant main effects for water soaking. Mean separation for water uptake within cultivars, mean separation for water soaking within main effects by Tukey's studentized range test, p = 0.05. Deionized water served as control. Water soaking was indexed using a five-score rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%.

https://doi.org/10.1371/journal.pone.0273180.t002

Table 3. The effect of $CaCl_2$ (10 mM) and the chelating agent EGTA (10 mM) on water soaking of 'Clery' strawberries. The fruit was incubated for 4 h.

Treatment	pH	Uptake (mg)	Water-soaked area (rating)
Water	6.3	797 ± 89 ab	2.0 ± 0.2 b
$CaCl_2$	6.7	553 ± 63 b	1.4 ± 0.2 c
EGTA	8.2	889 ± 101 a	2.9 ± 0.2 a

[a]Mean separation within columns by Tukey's studentized range test at $p = 0.05$. Water soaking was indexed using a five-score rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%.

https://doi.org/10.1371/journal.pone.0273180.t003

Table 4. Comparison of Ca salts all at 10 mM on water soaking of 'Florentina' strawberries incubated for 8 h.

Salt	pH	Uptake (mg)	Water-soaked area (rating)
Water	6.9	689 ± 74 [(ns)]	2.8 ± 0.2 a[a]
$CaSO_4$	6.9	445 ± 62	2.0 ± 0.2 a
$Ca(NO_3)_2$	6.8	592 ± 108	2.4 ± 0.3 a
$CaCl_2$	6.7	550 ± 57	1.8 ± 0.1 b
Calcium heptagluconate	6.8	574 ± 89	2.2 ± 0.2 a
Calcium lactate	6.9	467 ± 48	2.0 ± 0.2 a
Calcium propionate	7.2	425 ± 56	2.4 ± 0.3 a
Calcium formate	6.8	485 ± 63	2.8 ± 0.2 a
Calcium acetate	7.2	520 ± 89	2.5 ± 0.2 a

[(ns)]Means do not differ significantly from the control.

[a]Mean followed by the same letter do not differ significantly from the control, Dunnett's test at $p = 0.05$. Water soaking was indexed using a five-score rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%.

https://doi.org/10.1371/journal.pone.0273180.t004

taken up in the water control. Moreover, the amount of leakage per unit uptake was markedly reduced in the presence of $CaCl_2$. Meanwhile, when incubating flesh discs in hypertonic and hypotonic solutions with and without Ca, the Ca effect was not affected by the tonicity of the incubation solution. If Ca had a pronounced effect on crosslinking of cell walls, we would expect the Ca effect to be larger in hypotonic than in hypertonic solutions. When incubated in

Table 5. Comparison of chloride salts with different cations all at 10 mM on water soaking in 'Florentina' strawberry incubated for 8 h.

Salt	pH	Uptake (mg)	Water-soaked area (rating)
Water	6.9	817 ± 74 a[a]	3.1 ± 0.2 a
NaCl	7.4	703 ± 95 a	2.7 ± 0.3 a
KCl	6.8	601 ± 70 a	2.5 ± 0.3 a
NH_4Cl	6.8	860 ± 85 a	3.1 ± 0.2 a
LiCl	7.7	865 ± 83 a	2.9 ± 0.3 a
$MgCl_2$	7.2	837 ± 99 a	2.7 ± 0.3 a
$CaCl_2$	6.5	668 ± 102 a	1.9 ± 0.3 b
$CuCl_2$	5.1	560 ± 58 a	2.3 ± 0.2 a
$MnCl_2$	6.4	779 ± 81 a	2.6 ± 0.3 a
$SrCl_2$	6.4	664 ± 58 a	2.1 ± 0.2 b
$BaCl_2$	5.5	633 ± 80 a	1.7 ± 0.2 b
$FeCl_3$	2.4	404 ± 72 b	1.9 ± 0.2 b
$AlCl_3$	4.1	360 ± 49 b	1.7 ± 0.2 b

[a]Means followed by the same letter do not differ significantly from the control, Dunnett 's test at $p = 0.05$. Water soaking was indexed using a five-score rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%.

https://doi.org/10.1371/journal.pone.0273180.t005

hypotonic solutions, we would expect water uptake to strain the cell walls, and eventually lead to cell bursting. This would not be the case during incubation in hypertonic solutions.

Second, Ca reduced the amount of leakage of anthocyanin, indicating that the semipermeability of plasma membrane and tonoplast were better maintained in the presence of Ca, than in its absence [14]. Similar effects have been reported for the membrane permeabilities in

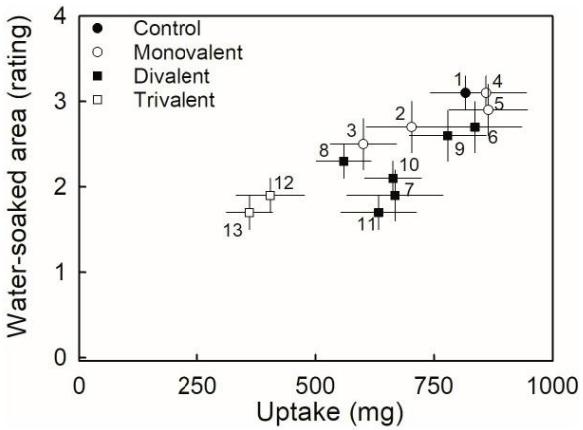

Fig 4. Relationship between water soaking and water uptake of 'Florentina' strawberry as affected by the chlorides of various cations. Fruit were incubated in chlorides of monovalent (NaCl (2), KCl (3), NH_4Cl (4), LiCl (5)), divalent ($MgCl_2$ (6), $CaCl_2$ (7), $CuCl_2$ (8), $MnCl_2$ (9), $SrCl_2$ (10), $BaCl_2$ (11)) or trivalent cations ($FeCl_3$ (12), $AlCl_3$ (13)). Deionized water (1) served as control. The coefficient of correlation was r = 0.80***.

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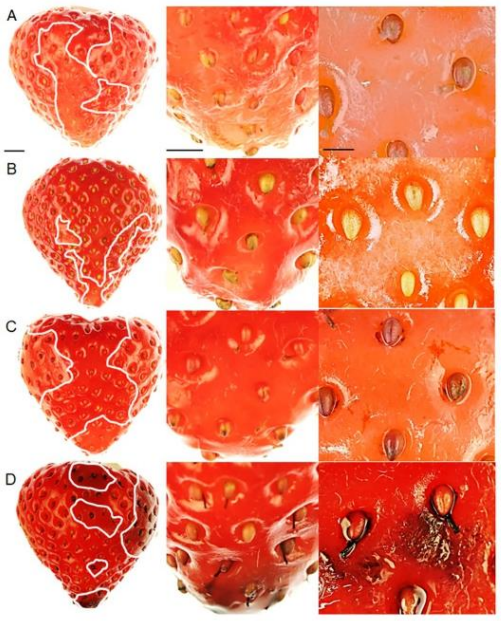

Fig 5. Water soaking of fruit incubated in (A) deionized water, (B) $CaCl_2$, (C) $CuCl_2$, and (D) $FeCl_3$. The white line indicates the extent of the water-soaked area. Scale bar in A = 5 mm (top row), 2 mm (center row), 1 mm (bottom row).

https://doi.org/10.1371/journal.pone.0273180.g005

apples and tomatoes [15–17]. Since anthocyanin leakage was reduced in the presence of $CaCl_2$, we also expect less leakage of citric and malic acids. The leakage of these organic acids triggers the chain reaction that causes neighboring cells to collapse and thus also to release their organic acids [18]. In strawberries, this reaction results in a tangential spreading of the water-soaked area over the fruit surface and also radially, deeper into the flesh [2]. Less leakage in presence of Ca therefore implies less water soaking.

Table 6. Effect of $CaCl_2$ (10 mM) on water uptake, water soaking and microcracking of the cuticle of 'Florentina' strawberry.

Incubation	Uptake (mg)	Water-soaked area (rating)	Microcracking area (%)
None	-	0.0 ± 0.0 c	6.7 ± 0.8 a
Water	554 ± 79 a[a]	2.0 ± 0.2 a	31.7 ± 3.7 c
Water+ $CaCl_2$	434 ± 41a	1.4 ± 0.2 b	17.3 ± 2.8 b
Isotonic PEG 6000	165 ± 23 b	0.2 ± 0.1 c	12.3 ± 1.6 b
Isotonic PEG 6000+ $CaCl_2$	135 ± 18 b	0.2 ± 0.1 c	12.5 ± 1.2 b

[a]Mean separation within columns by Tukey's studentized range test at $p = 0.05$. Microcracking area (%) was indexed as the area infiltrated by aqueous acridine orange. Acridine orange penetrates the strawberry fruit skin via microcracks in the cuticle. Non-incubated fruit served as control. Water soaking was indexed using a five-point rating scale: score 0, no water soaking; score 1, < 10% of the surface area water-soaked; score 2, 10 to 35%; score 3, 35 to 60%; score 4 > 60%.

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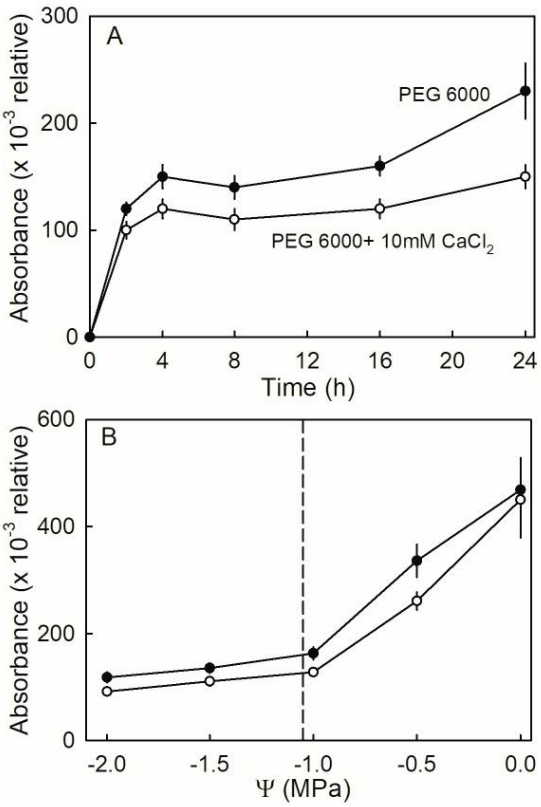

Fig 6. (A) Time course of anthocyanin leakage from flesh discs excised from ripe 'Clery' strawberries in the presence or absence of $CaCl_2$. Flesh discs were incubated in isotonic polyethylene glycol 6000 (PEG 6000) solutions. (B) Effect of osmotic potential (Ψ) of the incubation solution on anthocyanin leakage in the presence or absence of $CaCl_2$. The vertical dashed line indicates the osmotic potential of juice expressed from fruit of the same batch.

https://doi.org/10.1371/journal.pone.0273180.g006

Effects of Ca and other cations on water uptake

Calcium had no consistent or specific effects on water uptake. The effect of Ca was purely osmotic and, hence, the result of a decrease in driving force for osmotic water uptake. Assuming an osmotic potential of strawberry juice of -1.06 MPa, a 10 mM $CaCl_2$ solution (osmotic potential -0.04 MPa) would account for about a 4% reduction in water uptake rate due to a decreased osmotic driving force. This increases to a 58% reduction in driving force for 100 mM $CaCl_2$ (osmotic potential -0.62 MPa). Correcting for the change in driving force due to $CaCl_2$ reveals the permeance of the cuticle to water remained constant and independent of $CaCl_2$ concentration. This is consistent with earlier findings for water uptake into sweet cherry fruit [13]. This explanation also applies to the chlorides of other divalent cations. For monovalent cations, the reduction in driving force will be even smaller due to their less negative osmotic potentials [13]. It is important to note that Ca may indirectly affect the change in fruit

mass associated with water uptake due to effects on microcracking and on leakage of cell contents, and particularly so after the longer incubation periods.

The effects of the trivalent chloride salts, $FeCl_3$ and $AlCl_3$, on water uptake must have a different basis. Both markedly reduced water uptake at 10 mM. Although their osmotic potentials were more negative than those of the divalent chlorides, the decrease in water uptake cannot be accounted for by the decrease in driving force. Both salts must instead have had their effects via a change (a decrease) in the permeance of the fruit skin. This conclusion is consistent with findings reported for sweet cherry [19, 20]. These authors attributed the decrease in permeance of sweet cherry fruit skin to a plugging of the 'rapid-penetration' pathways that bypass the cuticle barrier [19, 20]. This plugging is due to a precipitation reaction as a result of the increase in pH encountered during penetration [20]. Aqueous solutions of both salts are highly acidic (their pHs are very low, in the range of pH 1.7 to pH 3.6 [19]. When encountering the higher pH of the apoplast, viscous oxides and hydroxides are formed that precipitate and plug the rapid-penetration pathways across the cuticle. As a consequence, the permeance of the fruit skin to water decreases. Support for this interpretation comes also from the black (ferrous) precipitates that formed when strawberries were incubated in the $FeCl_3$ solutions. Thus, water soaking decreased due to the decreased water uptake. Unfortunately, the effects of $FeCl_3$, or of other ferric salts, or of $AlCl_3$ are of no practical value in horticulture due to their unacceptable ecotoxicological profiles.

Conclusion

Our results indicate that Ca and, among the Ca salts investigated, $CaCl_2$ was most effective in reducing water soaking. The mechanism through which Ca reduces water soaking is via a decrease in cuticular microcracking, hence, a decrease in leakage through the plasma membrane and tonoplast and an increase in the crosslinking of cell wall constituents.

Due to the high economic importance of water soaking in field grown strawberry production, the effects reported here warrant further study. In particular, it would seem worthwhile to explore cultural ways through which strawberry Ca content could be increased. There being no Ca translocation via the phloem [21], Ca must be imported into developing strawberries only via the xylem. Furthermore, recent investigations show that, like many other fruit species, strawberries suffer a decrease in xylem conductance during development [22]. This renders increased Ca fertilization via the soil unlikely to be effective in increasing the supply to the fruit. Consequently, Ca spray applications directly to the developing fruit remains as the only alternative. To our knowledge, this avenue for mitigating water soaking in strawberry has not been studied in greater detail. Also, uptake of Ca salts into strawberry fruit following spray application has only been addressed in a limited number of studies [23–25]. Potential benefits of an increase in fruit Ca will not be limited to a decrease in water soaking, but will also improve shelf life and fruit quality [26, 27].

Supporting information

S1 File. This is the excel file containing the data in Figs 2–4, 6 and Tables 1–6.
(XLSX)

Acknowledgments

We thank Heike, Karl Walter and Martin Brüggenwirth and Bendix Meyer for the gift of fruit, Marcel Pasta and Peter Grimm-Wetzel for technical support and Drs. Sandy Lang and Andreas Winkler for helpful comments on an earlier version of this manuscript.

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Formal analysis: Grecia Hurtado.

Investigation: Grecia Hurtado.

Methodology: Grecia Hurtado.

Supervision: Moritz Knoche.

Writing – original draft: Grecia Hurtado, Moritz Knoche.

Writing – review & editing: Grecia Hurtado, Moritz Knoche.

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Supplementary material

Electronic File E5. Dataset. Excel file containing all data produced in figures and tables throughout the manuscript. (XLSX) (access through: https://doi.org/10.25835/5p308h3f)

5. General Discussion

The main findings of this dissertation were the following:

(1) The strawberry fruit skin had the highest permeance of all fruit skins investigated so far. The permanence for osmotic uptake was two orders of magnitude higher than the permeance for transpiration. The high osmotic uptake permeance can be attributed to an exceptionally thin cuticle. In addition, viscous flow through microcracks and, possibly, polar pathways contributed to water movement. The high permeance of strawberry fruit skins is responsible for the high susceptibility to water loss and shrivel and the limited resistance against rainfall (Chapter 4.1).

(2) The junction region between the calyx and receptacle, the abscission zones of the petals and stamina, and microcracks of the calyx region and receptacle were identified as preferential pathways for osmotic water uptake in strawberry fruit. These pathways facilitate water movement through viscous flow, which is notably quicker compared to diffusion through an intact cuticle (Chapter 4.2).

(3) The strawberry cuticle is subject to significant growth stress and strain during fruit development. This can be attributed to two factors: (i) A low rate of cuticle deposition in strawberries, that is limited to the early stage of fruit development; (ii) rapid growth within a relatively short time. Consequently, cuticle thickness decreases and cuticular strain increases resulting in the formation of microcracks. Microcracks in the achene depressions and on the rims between achenes were either parallel or transversely oriented. Both orientations are consistent with growth strain as the dominant cause of cuticular microcracking. Additionally, exposure of the strained cuticle to liquid-phase or vapor-phase water exacerbates microcracking (Chapter 4.3).

(4) Cracking in strawberry fruit appears principally in the neck zone that lacks achenes. Hence, strawberries with neck are more susceptible than normal-shaped fruit. Growth strain is the main driver of cracking. Cracking is further increased by water uptake through the fruit surface. Macrocracks occur mainly in longitudinal orientation in the distal neck and in latitudinal orientation in the proximal neck. Furthermore, the orientation of macrocracks and microcracks is identical, this implies that macrocracks arise from the extension of microcracks. A model that considers the fruit's shape, the corrugated nature of the surface, the structural reinforcement due to the configuration

of the vascular bundles, the presence of discontinuities, and the distribution of growth strains was proposed to explain the crack pattern of strawberry fruit (Chapter 4.4).

(5) Water-soaked fruit showed pale, deliquescent patches of skin. Water soaking results from a series of events involving microcracking of the cuticle, subsequent water uptake, cell bursting, and the release of organic acids into the apoplast. This mechanism is similar to the Zipper model developed for rain cracking in sweet cherries (Chapter 4.5).

(6) Calcium, particularly $CaCl_2$, was effective in mitigating water soaking. The mechanism through which Ca mitigates water soaking involves a decrease in cuticular microcracks, decreased leakage of plasma membranes, and possibly increased cross-linking of cell wall constituents. (Chapter 4.6).

These findings are discussed in detail with the pertinent literature in the corresponding chapter. The subsequent general discussion will therefore focus on the implications of this dissertation for possible future research and for the development of counter-measurements.

5.1. Potential implication for further research.

5.1.1. Unknown aspects of the cuticle and trichomes

Microcracking in the cuticle was the initial step towards surface disorders after rainfall. Thus, maintaining cuticle integrity is key to mitigating skin disorders, keeping fruit quality, and preventing fungal infections (Khanal and Knoche, 2017; Jarvis, 1962). In this study, it was shown that the syntheses of cutin and wax cease during early fruit development resulting in strain accumulation as the fruit increases in surface area. For technical reasons, the strain could not be quantified yet. The fragility of the thin cuticle and the presence of the achenes makes an evaluation challenging. Moreover, the exact developmental stage when cuticle deposition ceases and the genes involved in cuticular biosynthesis in strawberries have not been identified. Also, at present it is not known, whether there is genotypic variability in this trait. A better understanding of cuticle formation in strawberries and its relationship to microcracking would be helpful in selecting strawberry genotypes that are able to maintain an intact fruit surface throughout development.

The base of trichomes is a site of preferential **microcracking** as indexed by fluorescent microscopy. The role of these structures in microcracking and water uptake is unknown. Generally, such hair-like structures have functions in protection from pests and UV radiation, water loss and temperature regulation, and secretion of secondary metabolites (Werker, 2000;

Xiao et al., 2016). There are only few studies on strawberry trichomes and these focused on leaf trichomes (Biswas et al., 2019; Benatto et al., 2018). To our knowledge, none addressed the role of fruit trichomes. Recently, Fich et al. (2020) reported that transpiration in tomato fruits occurs primarily through trichome-associated transcuticular polar pores. These pores are situated at the base of trichomes on the fruit's surface. They serve as hydrophilic channels enabling the potential diffusion or flow of water and aqueous solutes. While the existence of transcuticular polar pores at the base of trichomes in strawberries would offer a potential explanation for the reported polar pathways in the skin, it's important to note that conclusive evidence is currently lacking.

5.1.2. Properties of skin dermal tissues.

Based on the physiological similarity between the phenomena of water soaking in strawberries and cracking in sweet cherries, further studies in the following aspects are warranted.

Mechanical properties of skin of strawberry have not been studied in detail. Due to the thin cuticle of strawberries, it would be expected that the mechanical backbone of the skin may be dominated by the dermal tissue, as is generally the case in other soft fruits (Brüggenwirth et al., 2014; Bargel and Neinhuis, 2004; Khanal and Knoche, 2017). Studies on the mechanical properties of the strawberry fruit have not quantified the contribution of the cuticle to the mechanical properties of the skin (An et al., 2023; An et al., 2020; Contigiani et al., 2018). This is likely to be technically challenging because of the fragility of the fruit tissues.

Cell wall properties accounted for the variations in skin mechanical characteristics among different sweet cherry cultivars, and consequently to differences in susceptibility to cracking (Brüggenwirth and Knoche, 2016). Moreover, Brüggenwirth and Knoche (2017) determined that swelling of the cell wall and the mechanical properties of the sweet cherry skin are closely related. Cell wall swelling particularly weakens cell to cell adhesion causing dermal cells to separate schizogenously, i.e. neighbouring cells part from one another along their anticlinal middle lamellae (Schumann et al., 2019). Cell wall swelling is not exclusive to sweet cherries; it is a common occurrence in the ripening process of various fruit species, including strawberries (Redgwell et al., 1997); hence similar skin failure pattern may be likely however evidence is lacking. Therefore, research on the mechanical strength of the strawberries' cell walls, and their cell-to-cell adhesion, and skin failure mode using immunolabeling of cell wall epitopes would be meaningful.

5.2. Potential implication for developing countermeasures.

5.2.1. Breading efforts.

Susceptibility to water soaking differs among cultivars (Herrington et al., 2009; Hurtado and Knoche, 2021). We also noted differences in water-soaking susceptibility between cultivation systems for the same cultivar. The susceptibility to water soaking could potentially depend on various factors, including the permeance of the skin to water movement, the physical properties of the dermal tissue such as their mechanical strength and properties of the cuticle and its tendency to microcrack. The physiological, genetic, and molecular mechanisms underlying these differences remain unidentified. Unraveling the underlying mechanisms could help to obtain molecular markers, that would enable the breeding of more resistant cultivars (Gupta et al., 1999).

Susceptibility to cracking was greater in fruit with an extended neck. The frequency of necked fruit differed among cultivars suggesting that neck shape is a genetic trait. However, the frequency of necked fruit varied and also depends on other yet unknown factors (e.g., environmental conditions). Therefore, further research is warranted to determine the mechanism of neck formation. Our observations indicated that the neck formed during the expansion of the receptacle. There were no visible differences in flower shape. Moreover, the frequency of necked fruit varies with the hierarchy of the flower in an inflorescence of a given cultivar (Hurtado, Unpublished). It may be speculated that this observation is related to an uneven distribution or an insufficient number of fertilized carpels. Indeed, the number of anthers and carpels present on the flower declines with position on the inflorescence (category). Moreover, differences between basal and apical carpels in terms of maturation rates and stigma receptivity have been reported for some cultivars (Ariza et al., 2009; Yoshida et al., 1991). Unfertilized carpels fail to produce achenes and hence, fail to induce growth hormone synthesis, especially auxin (Nitsch, 1950; Archbold and Dennis, 1985). Whether this is related to neck formation is still unknown.

5.2.1. Horticultural practices.

Limiting surface wetness or exposure to high humidity of the fruit surface is key to preventing rain damage disorders. This may be attained preharvest through protected cultivation, either within a greenhouse or a plastic tunnel. However, occasionally overhead sprinklers and nozzles are used to irrigate or control humidity (Lieten, 2002; Kroggel and

Kubota, 2017; Xiao et al., 2001) in protected cultivation. These systems may facilitate the exposure of the fruit surface to wetness or excessively high humidity. Therefore, the use of this system needs to be properly managed to avoid wetness, particularly in the course of ripening. During the postharvest phase effective measures may include the design and use of adequate packaging materials (Bovi et al., 2019) and the implementation of handling practices aimed at preventing the accumulation of liquid water on the surface. This prevention extends to scenarios where water vapor condensation occurs.

Calcium mitigates water soaking. Considering the significant economic implications of water soaking in field-grown strawberry production, the effects reported here warrant further studies. These investigations need to focus on two main aspects: (1) the physiology of Ca and the (2) increase of calcium concentration in the fruit.

1. Little information about the physiology of Ca in strawberry fruit is available. Mahmood et al. (2012) reported a decrease in Ca content during ripening, and Makus and Morris (1998) established a gradient in Ca content between the fruit's outer and inner tissues (Makus and Morris, 1998). However, important aspects such as the fruit-fruit variability, effects of flower categories, fruit size and sections, development stages, differences among tissues, and the effect of transpiration are still unknown. It is essential to note that calcium is only imported into the fruit through the xylem (Hocking et al., 2016) and a recent study has identified a decline in xylem conductance during strawberry development (Winkler et al., 2021). Hence, considering the limited effectiveness of increasing Ca supply through soil fertilization, the more viable alternative is the direct application of Ca through spraying onto the developing fruit.

2. Some studies have established the effect of Ca spray on strawberry fruit (Dunn and Able, 2006; Cieniawska et al., 2023; Chéour et al., 1990). However, some inconsistency has been reported regarding the effectiveness of these treatments (Makus and Morris, 1998). Moreover, to our knowledge, there is no complete information regarding the mechanism and factors affecting Ca uptake, nor the presence of a preferential pathway for Ca uptake on the surface of strawberries. Potential benefits of increasing Ca content in fruits could extend beyond mitigating water soaking, including enhanced shelf life and improved fruit quality (Lara et al., 2004; Martín-Diana et al., 2007).

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Acknowledgements

I would like to express my profound gratitude to Prof. Dr. Moritz Knoche for the guidance, support, and encouragement I have received throughout this academic journey. I deeply appreciate his invaluable mentorship, patience, and expertise.

I would like to extend my gratitude to Dr. Andreas Winkler, Dr. Sandy Lang Dr. Eckhard Grimm, Dr. Martin Brüggenwirth, Jannis Straube for their time and expertise that contributed to the development of the manuscripts in this thesis.

I want to express my gratitude to Heike, and Karl Walter Brüggenwirth, Dr. Ludger Linnemannstöns, and Bendix Meyer who generously shared their resources to make this dissertation possible. A special appreciation to the Deutsche Forschungsgemeinschaft (KN 402/19-1) for providing the funds for this study.

I also wish to acknowledge the Fruit Science department, Mr. Simon Sitzenstock, Mrs. Sylvia Janning, Mrs. Friederike Schröder, Mrs. Germaine Malon, Mr. Peter Grimm-Wetzel, Mrs. Hana Weiß, Dr. Bishnu Khanal and, Mr. Marcel Pastwa. I am grateful for their generous support and assistance.

Furthermore, I would also like to extend my sincere gratitude to Prof. Dr. Traud Winkelmann and PD Dr. Klaus Olbricht for undertaking the role of co-referees and Prof. Dr. Jutta Papenbrock for undertaking the role of examiner, of my dissertation and disputation.

Finally, I would like to express heartfelt gratitude to my husband, David Bonilla. Without his unconditional help and support, I would not have been able to complete this journey. Thank you for believing in me and inspiring me. My deepest gratitude also goes to my family and friends; their love and support are the fuel in my life.

This endeavor would not have been possible without the contribution of each person mentioned above, and for that, I am deeply thankful.

"For from Him and through Him and to Him are all things. To Him be the glory forever"

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