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RESEARCH PAPER

Surface moisture increases microcracking and water vapour permeance of apple fruit skin

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ABSTRACT

- Surface moisture induces microcracking in the cuticle of fruit skins. Our objective was to study the effects of surface moisture on cuticular microcracking, the permeance to water vapour and russeting in developing 'Pinova' apple fruit.
- Surface moisture was applied by fixing to the fruit a plastic tube containing deionized water. Microcracking was quantified by fluorescence microscopy and image analysis following infiltration with acridine orange. Water vapour permeance was determined gravimetrically using skin segments (ES) mounted in diffusion cells.
- Cumulative water loss through the ES increased linearly with time. Throughout development, surface moisture significantly increased skin permeance. The effect was largest during early development and decreased towards maturity. Recovery time courses revealed that following moisture treatment of young fruit for 12 days, skin permeance continued to increase until about 14 days after terminating the moisture treatment. Thereafter, skin permeance decreased over the next 28 days, then approaching the control level. This behaviour indicates gradual healing of the impaired cuticular barrier. Nevertheless, permeance still remained significantly higher compared with the untreated control. Similar patterns of permeance change were observed following moisture treatments at later stages of development. The early moisture treatment beginning at 23 DAFB resulted in russeting of the exposed surfaces. There was no russet in control fruit without a tube or in control fruit with a tube mounted for 12 days without water.
- The data demonstrate that surface moisture increases microcracking and water vapour permeance. This may lead to the formation of a periderm and, hence, a russeted fruit surface.

INTRODUCTION

The cuticle is a biopolymer that envelopes all primary surfaces of terrestrial plants. It covers the fruits of most species and all leaf surfaces. The cuticle performs important functions as a barrier to pathogen invasion (Yeats & Rose 2013; Guan *et al.* 2015) and in regulating the passage of water and other substances across the surface. Depending on organ, circumstances and chemistry of the penetrant, the transcuticular movements can be either inwards or outwards (Kerstiens 1996; Schreiber & Schönherr 2009; Dominguez *et al.* 2011; Yeats & Rose 2013). Obviously, the maintenance of an appropriate level of regulatory function throughout fruit development requires the cuticle to remain intact. Compared with a leaf, maintenance of cuticular integrity in a fruit is particularly challenging. This is because fruits differ from leaves in that fruit expansion commonly occurs over a lengthy period – commonly around 5 months (Knoche & Lang 2017). The ongoing growth subjects the fruit cuticle and its subtending dermal layers (which together make up the skin) to continuous tangential strain (Skene 1982). The epidermal and hypodermal cell layers can accommodate this strain by ongoing anticlinal cell divisions and by gradual changes in cell anticlinal aspect ratio, from portrait to landscape (Tukey & Young 1942). The polymeric cuticle, however, is not 'alive' in the same sense and so must sustain the ongoing strain, which sometimes leads to thinning as the surface area increases (Lai *et al.* 2016). If critical thresholds in the rate of strain are exceeded, cuticular failure occurs; microcracks develop that compromise the cuticle's barrier function. Moreover, exposure of the strained cuticle to surface moisture, or even just to high humidity, can exacerbate microcracking in a number of fruit crop species, including apple (Knoche & Grimm 2008; Knoche *et al.* 2011) and sweet cherry (Knoche & Peschel 2006). Incidentally, extended periods of surface wetness or high humidity are also conducive to this russeting (Tukey 1959; Creasy 1980; Winkler *et al.* 2014).

Microcracking of the cuticle is the first step in the development of a number of fruit skin disorders, including shrivelling (Knoche *et al.* 2019), macrocracking (Schumann *et al.* 2019), russeting (Faust & Shear, 1972a,b; Winkler *et al.* 2014) and skin spotting (Grimm *et al.* 2012; Winkler *et al.* 2014). Taken together, these skin disorders are of considerable commercial importance. Although in most cases they do not affect the nutritional quality of the fruit or the taste, etc., they do affect fruit appearance and so compromise fruit value at the point of sale.

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Many fruit crop species are capable of repair processes that restore the functionality of the damaged cuticle barrier (Knoche & Lang 2017). For example, in russeting, a periderm is formed in the subtending hypodermal layer when the cuticular surface is breached by multiple microcracks (Meyer 1944; Faust & Shear, 1972a,b). The phellogen divides and produces stacks of cork cells that replace the barrier function of the primary surface. From a biological perspective, the formation of a periderm is beneficial as it restores (in part at least) the lost barrier functions of the primary surface in respect to the passage of water (Khanal *et al.* 2019). Unfortunately, the rough, brownish appearance of a russeted fruit usually leads to its downgrading and even rejection in high-end markets.

A second repair process is the deposition of wax in the microcracks. The filling of cracks with wax has been documented using scanning electron microscopy (SEM) for apple fruit surfaces on a number of occasions (Roy *et al.* 1999; Curry 2009; Curry & Arey 2010). In contrast to russeting, such wax deposition does not involve a morphological change in skin structure. Hence, this process is more rapid than the formation of a periderm. In addition, wax deposition in the strained cutin polymer alleviates stress by strain fixation (Khanal *et al.* 2013a). It is known that microcracks increase the water vapour permeance of the apple fruit surface (Maguire *et al.* 1999), but whether this secondary wax deposition and filling of microcracks completely restores the barrier properties of the fruit skin is not known. Also, it is not known, whether a filling of wax alters the subsequent susceptibility of the fruit surface to russeting.

The objectives of this study were: (a) to establish the effect of surface moisture on the formation of microcracks and the permeance of the skin to water vapour in developing apple fruit, and (b) to identify the effects of repair processes thereon. Because of the significance of russeting in commercial apple fruit production, (c) the relationship between microcracking and russeting was also quantified.

MATERIAL AND METHODS

Plant material

'Pinova' apple (*Malus* × *domestica* Borkh.) grafted on M9 rootstocks were grown in the experimental orchards of the Horticultural Research Station of Leibniz University in Ruthe, Germany (52° 14′ N, 09° 49′ E). Trees were cultivated according to the current regulations for integrated fruit production.

Fruit growth measurement

Fruits were sampled at 1- to 3-week intervals between full bloom and maturity (two fruits per tree, one from each side, for a total of 15 trees). Fruit mass was determined using a digital balance and fruit diameter was calculated from fruit mass, assuming a spherical shape and a density of 1. A sigmoidal regression model was fitted through the plot of fruit surface area *versus* time. Surface area growth rate ($cm^2 \cdot day^{-1}$) was calculated as the first derivative of this regression model. The relative growth rate at any time ($cm^2 \cdot cm^{-2} \cdot day^{-1}$) was obtained by dividing the growth rate at that time by the surface area at that time.

Moisture treatment

Fruits, free of visual defects, were selected and tagged at representative stages of development. For the moisture treatment, a polyethylene tube (8-mm inner diameter) was cut from the tip of a disposable Eppendorf reaction tube and glued to the fruit surface in the equatorial plane using fast-curing silicone rubber (Silicone RTV; Dow Toray, Tokyo, Japan).

After curing, tubes were filled with 1 ml deionized water using a disposable syringe. The hole in the tip of the tube was then sealed with silicone rubber. The tubes were inspected every 2 days and resealed when necessary. A untreated area in the equatorial region – usually opposite the tube – was left unprotected (without tube) on the same fruit and served as control.

To assure that water and not the tube was causal in inducing microcracking and subsequent russeting, an independent control experiment was conducted with three treatments: untreated control (no tube, no water), control with tube attached without water (with tube, no water), moisture treatment (with tube, with water). To prevent the accumulation of high humidity or rainwater inside the tube, the tube was cut in half and the cylindrical, non-tapered portion was glued to the equatorial surface of the fruit at 28 days after full blooming (DAFB). The tube was left open. After 12 days, the tubes were removed. Digital photographs of the surface of developing fruits were taken at 105 DAFB to document the presence or absence of a periderm.

The time course of moisture-induced microcracking was studied beginning at 29 DAFB. The duration of moisture exposure was 0, 2, 4, 8 or 12 days. Thereafter, the tubes were removed from the surface. The tubes detached very easily, there was no physical stress or damage to the fruit surface associated with tube removal. The effect of development stage on moisture-induced microcracking was studied beginning at 23, 44, 73 or 100 DAFB over 12-day periods of moisture exposure. Moisture-treated fruits were either harvested immediately after treatment or left on the tree to monitor the progress of any repair processes of the microcracked surfaces or to assess the extent of russeting at maturity. The fruits were processed immediately on the day of harvest or held overnight at 2 °C and 95% RH.

Water vapour permeance

The loss of water vapour through excised skin segments (ES) was quantified using stainless steel diffusion cells similar to those described by Geyer & Schönherr (1988). The ES (1.0- to 1.5-mm thick) were excised from the moisture-treated area or a untreated control area in the equatorial plane of the fruit. The cut surface of the ES was carefully blotted using soft tissue paper. The ES were then mounted on the diffusion cells using high-vacuum grease (Korasilon-Paste; Kurt Obermeier, Bad Berleburg, Germany). Diffusion cells were filled with deionized water through a port in the base and then sealed using clear transparent tape (Tesa film; Beiersdorf, Norderstedt, Germany). Following equilibration overnight, diffusion cells were incubated in a polyethylene box containing freshly dried silica gel at 24 °C. The diffusion cells in the polyethylene box were placed upside down on a metal grid such that the ES faced the silica gel. The amount of water loss from the diffusion cells was

quantified gravimetrically by weighing cells at regular intervals up to 4.5 h or 8.0 h. The rate of water loss (F in $g{\cdot}h^{-1}$) was obtained as the slope of a linear regression line fitted through a plot of cumulative transpiration *versus* time. The permeance (P; $m{\cdot}s^{-1}$) of the ES was calculated using the following equation:

$$\text{Permeance}\,(P) = \frac{F}{(\Delta C \times A)}.$$

In this equation, F represented the flow rate ($g{\cdot}h^{-1}/3600$) of water vapour, A the area of the transpiring surface of the ES (m^2) and ΔC the difference in water vapour concentration between the inside and the outside of the diffusion cells ($g{\cdot}m^{-3}$). Because the water vapour concentration above dry silica gel is close to zero (Geyer & Schönherr 1988), the water vapour concentration at saturation at 24 °C (21.8 $g{\cdot}m^{-3}$; Nobel 1999) represents the driving force for transpiration.

Microcracks

Microcracking of the cuticle was followed using the fluorescent tracer acridine orange. Fruits were dipped in a 0.1% (w/w) aqueous solution of acridine orange (Carl Roth, Karlsruhe, Germany) for 10 min. Subsequently, fruits were removed from the solution, rinsed with deionized water and blotted using soft tissue paper. Fruits were viewed under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Calibrated images of the moisture-exposed and of the untreated control regions were prepared under incident fluorescence light (Camera DP71; GFP-plus filter, 480–440 nm excitation, ≥510 nm emission wavelength). Three to four images per fruit and per treatment (control *versus* moisture treatment) were taken on a total of seven to ten fruits. The area infiltrated by the acridine orange solution was quantified using image analysis (Cell^P; Olympus Europa, Hamburg, Germany). Under the above-mentioned conditions, tissue infiltrated with acridine orange exhibits yellow and green fluorescence. Following setting of appropriate colour thresholds, all images were processed using the same thresholds. The areas exhibiting yellow and green fluorescence were quantified.

Using the experimental setup described above, the time course for different moisture exposure durations at 29 DAFB, the developmental time course of a 12-day moisture exposure period imposed at 23, 44, 73 or 100 DAFB and the recovery time courses following a 12-day moisture exposure that began at 23, 44, 73 or 100 DAFB were studied.

Russeting

Developing fruits exposed to moisture were tagged and harvested at 159 DAFB, when the fruit was fully mature. To identify the region treated with surface moisture through until harvest, the area of skin included within the tube was marked when the tube was removed by applying four dots on the fruit surface at approximately equal intervals around the perimeter using a black permanent marker. Calibrated images of the portion of the fruit surface that was exposed to moisture were taken (Canon EOS 550D, lens: EF-S 18-55 mm, Canon Germany, Krefeld, Germany). Images of the untreated surface on the same fruit served as control. The proportion of russeted area was quantified with image analysis (software package Cell^P; Olympus).

Statistical analysis

Data are presented as means ± SE. Where error bars are not visible, they were smaller than the data symbols. Pairwise t-tests and regression analyses were carried out using the statistical software package SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Significance of the coefficient of determination at 0.05, 0.01 and 0.001 is indicated by *, ** and ***, respectively.

RESULTS

Fruit mass and surface area increased in a sigmoidal pattern with time (Fig. 1). The growth rate in surface area reached a maximum of 1.6 $cm^2{\cdot}day^{-1}$ at about 77 DAFB (Fig. 1 upper left inset). The relative area growth rate (the rate of expansion per unit surface area) was maximal at the start of fruit development and decreased thereafter (Fig. 1 lower right inset).

The cumulative water loss through the ES exposed to moisture for up to 12 days increased linearly with time, indicating a constant rate of water loss (Fig. 2). The rate of water loss from an ES after 12 days of exposure to surface moisture was five-times higher than from a untreated control (Fig. 2).

When exposed to moisture at 29 DAFB, skin permeance increased rapidly, whereas the skin permeance of a untreated control surface on the same fruit decreased only slightly. After 2 days of moisture exposure (31 DAFB), the permeance increase was significant compared to the untreated control. After 8 days of moisture exposure (37 DAFB), the permeance reached a maximum and remained constant thereafter up to 12 days (41 DAFB), when the moisture treatment was terminated (Fig. 3a).

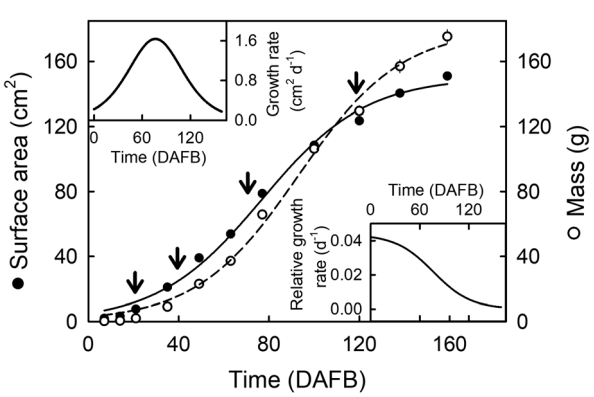

Fig. 1. Time course of changes in surface area and mass in developing 'Pinova' apple (main graph). The equations for the sigmoidal regression models were:Surface area (cm^2) = 180.26/(1 + exp(−(time(DAFB) − 93.19)/22.77; R^2 = 0.99,Mass (g) = 150.12/(1 + exp(−(time(DAFB) − 76.80)/22.96; R^2 = 0.99. Insets: Surface area growth rate (inset upper left corner) and relative surface area growth rate (inset lower right corner) in developing fruit. Arrows indicate the development stages when moisture treatments were imposed. Data represent mean ± SE, n = 30, x-axis scale in days after full bloom (DAFB).

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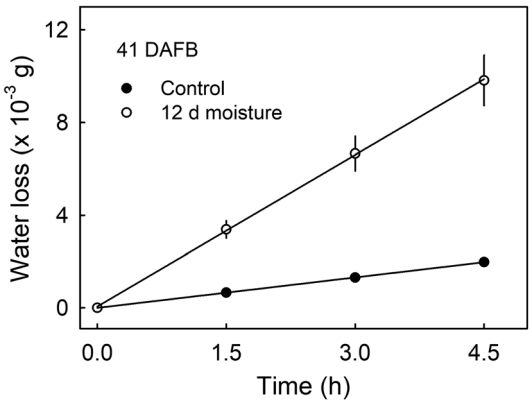

Fig. 2. Time course of water loss through excised skin segments (ES) of apple fruit exposed to moisture for 12 days, beginning at 29 days after full bloom (DAFB) until 41 DAFB. ES from the untreated surface of the same fruit served as control. Data represent mean ± SE of 15 fruits.

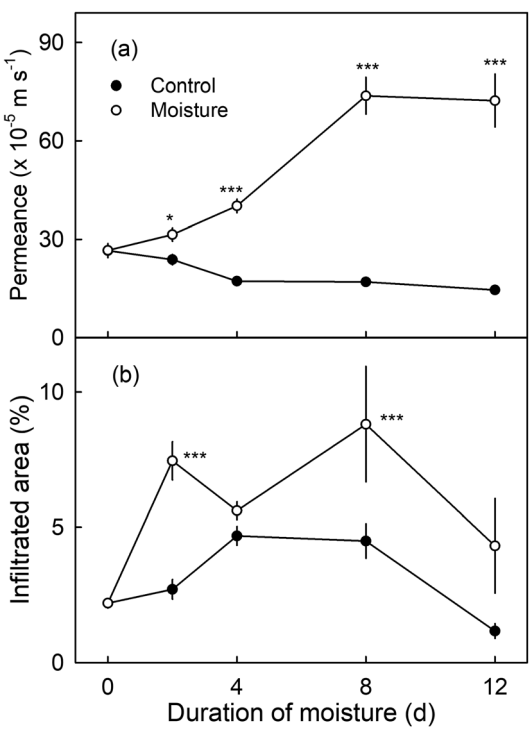

Fig. 3. Permeance (a) and acridine orange infiltrated area (b) as affected by the duration of exposure of the fruit surface to moisture. The surface was exposed to moisture beginning at 29 days after full bloom (DAFB) until 41 DAFB. Untreated surface of the same fruits served as control. Values represent mean ± SE, n = 12–15 (a) or 7–10 (b). * and *** indicate significant difference between control and moisture treatment at $P < 0.05$ and 0.001, respectively.

Moisture treatment increased the area infiltrated by acridine orange, indicating increased microcracking of the fruit surface. After 2 days of moisture treatment (31 DAFB), numerous, small, spot-like microcracks appeared (Fig. 4a–d). After 8 days, networks of long, wide microcracks had formed which were all infiltrated by the acridine orange (Fig. 4e,f). After 12 days, the area of infiltration of microcracks with acridine orange was reduced; many microcracks were visible, but they were not infiltrated by acridine orange (Fig. 4g–j). Quantifying the areas infiltrated by acridine orange indicates that the extent of infiltration varied markedly with time. At all times, the infiltrated areas were larger for moisture-treated fruit than for untreated control fruit (Fig. 3b).

When fruits were treated with moisture for 12 days at later stages of development (44 to 56 DAFB, 73 to 85 DAFB and 100 to 112 DAFB), the increases in permeance due to moisture treatment were markedly smaller, but they were still significant relative to the controls, even between 100 and 112 DAFB (Fig. 5a). Also, the area infiltrated by acridine orange was largest when young fruits (from 23 to 35 DAFB) were treated with moisture. At later stages of development (44 to 56 DAFB, 73 to 85 DAFB or 100 to 112 DAFB), the effect of moisture was smaller and not significant (Fig. 5b).

Interestingly, following the moisture treatment of young fruit from 23 to 35 DAFB, skin permeance continued to increase and peaked at about 49 DAFB; this was 14 days after termination of the moisture treatment. Thereafter, permeance decreased rapidly within 28 days, but remained significantly higher than the untreated controls (Fig. 6a). The change in area infiltrated by acridine orange essentially mirrored the change in permeance (Fig. 6b).

Performing the same experiment, but at later stages of fruit development, resulted in similar qualitative changes, *i.e.* decreases in permeance, but at markedly reduced levels following termination of the moisture treatment (Fig. 6a inset, b inset). Recovery of permeance was complete when microcracks were induced by moisture treatments between 73 to 85 DAFB and 100 to 112 DAFB, but not between 44 and 56 DAFB. As during early microcrack induction, the permeance remained higher in the moisture-treated fruits than in the untreated controls.

Monitoring infiltration of the ES with acridine orange revealed the same general trends – a transient increase in the infiltrated area up to about 49 DAFB (Fig. 7a,b). At this time, a dense network of open cracks had formed (Fig. 7c,d); the infiltrated area then decreased (Fig. 6b). The microcracks remained visible but they were not infiltrated by acridine orange (Fig. 7e–h). The fruits which were treated with moisture at 23 to 35 DAFB developed a significant amount of russet (Table 1, Fig. 8). There was no russet in the two control treatments regardless of the presence of the tube on the fruit surface, indicating that water exposure and not the tube was causal in russet formation (Fig. 8). Fruits which were treated at later stages of development (44 to 56 DAFB, 73 to 85 DAFB and 100 to 112 DAFB) did not produce russet at maturity (Table 1).

Across all development stages, permeances of fruit skins and the areas infiltrated by the fluorescent tracer acridine orange were positively related (Fig. 9). The regression equation for the relationships was:

$$\text{Permeance}\,(\times 10^{-5}\text{m}\cdot\text{s}^{-1}) = 8.3\,(\pm 0.7) \times \text{Area}\,(\%) - 4.6\,(\pm 2.4);\ R^2 = 0.78***,\ \text{n} = 40.$$

DISCUSSION

The most important findings of our study were:

1 A rapid increase in apple fruit skin microcracking and a corresponding increase in water vapour permeance in response to surface moisture.

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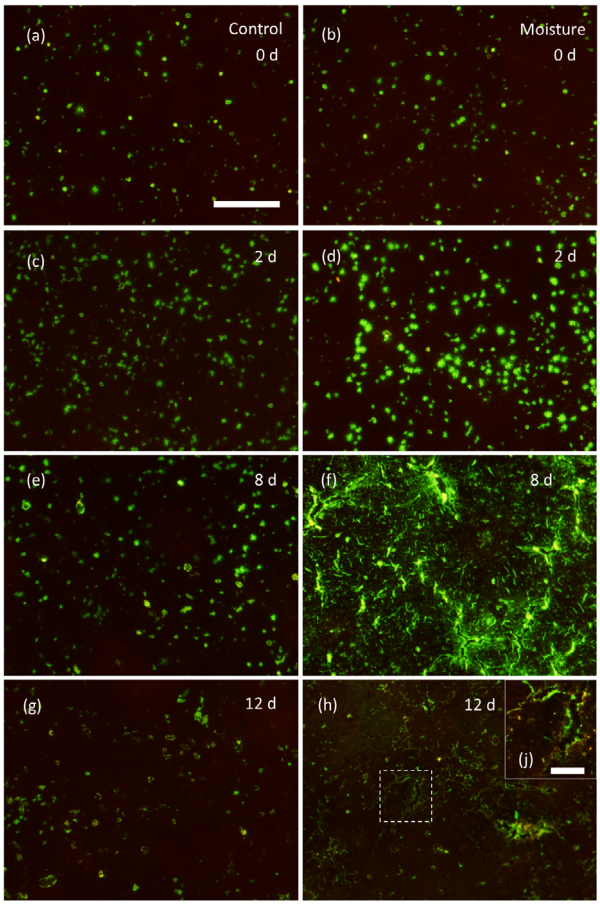

Fig. 4. Microscope images of fruit surfaces prepared after 10 min infiltration with a 0.1% aqueous solution of acridine orange. The surface was exposed to moisture beginning at 29 days after full bloom (DAFB) for 0 (b), 2 (d), 8 (f) or 12 (h) days. An untreated surface of the same fruit served as control (a, c, e, g). The image in (j) represents the magnified view of the area in (h) enclosed by the dotted rectangle. The scale bar (400 μm) in (a) is representative of images (b) to (f) of the composite. Scale bar in (j) = 100 μm.

2 A marked decrease (with some delay) in both microcracking and permeance following the termination of a moisture treatment; both values gradually approaching the control values.
3 A consistent effect of development stage on skin responses to exposure to moisture in terms of microcracking, of water vapour permeance and of russeting.

Microcracking and permeance to water vapour increase rapidly during and beyond the period of exposure to surface moisture

The effect of surface moisture observed in our *in vivo* study confirms earlier reports obtained *in vitro* using excised skin segments (Knoche & Grimm 2008; Knoche *et al.* 2011). As in earlier studies, the extent of moisture-induced microcracking depended markedly on the stage of fruit development (Knoche *et al.* 2011). Whole fruits and ES were most sensitive during early development (Wertheim 1982). During this stage, the growth strains are high as determined by the high relative area growth rates (Skene 1980; Lai *et al.* 2016).

Further indirect evidence for a relationship between russet and growth strain comes from studies in European pear (*Pyrus communis*), where a higher incidence of russet on the cheek as compared to the neck has been attributed to higher growth rates (Scharwies *et al.* 2014). Earlier studies established that the cuticle suffers from lower fracture strains compared to the underlying cellular layers of the dermis (Khanal & Knoche 2014), and that the fracture pattern of the cuticle is determined by the underlying cellular layers (Knoche *et al.* 2018). This is because the epidermal and hypodermal cell layers, and not the cuticle, represent the structural backbone of the apple fruit skin (Khanal & Knoche 2014). These arguments further suggest that microcracking, and the effect of surface moisture thereon, are also affected by the underlying cellular layers. It may be speculated that a swelling of anticlinal cell walls facilitates cell-to-cell separation along the abutting anticlinal walls as cell shape changes during growth from 'portrait' to 'landscape' (Meyer 1944; Maguire *et al.* 1999; Knoche *et al.* 2018). In sweet cherry, the swelling of cell walls reduces cell-to-cell adhesion, causing epidermal cells to partially separate at low rates of strain (Brüggenwirth & Knoche 2017). Whether this also applies for moisture-induced microcracking of apple fruit skin remains to be shown. The effect of moisture may be further exacerbated by decreases in the cuticle's fracture force and fracture strain due to hydration; this has often been reported for isolated cuticles (Knoche & Peschel, 2006; Khanal *et al.*, 2013b). In addition, surface wetness and high RH both decrease the biosynthesis and deposition of wax (Shepherd & Griffiths, 2006) and possibly also of cutin; this may lead to a thinner and mechanically weaker cuticle. However, direct evidence for effects of surface wetness and/or humidity on cutin and/or wax deposition in apple is lacking.

The changes in permeance observed in skins exposed to surface moisture throughout our study were a linear function of the extent of microcracking, as recorded by the areas infiltrated by acridine orange. This confirms an earlier report for Braeburn apples (Maguire *et al.* 1999).

It is interesting to note that the increase in microcracking and in water vapour permeance induced by surface moisture extended, and even increased further, well beyond the time when the surface moisture treatment was terminated. This observation is probably due to the ongoing growth strains causing gaping of the microcracks, before the cuticular repair processes were sufficiently active.

It could be argued that the moisture-induced russet is an artefact caused by the silicone and/or the Eppendorf tube. However, the following considerations make this possibility highly unlikely. First, when developing this technique, we also applied surface moisture using wet paper towels or wet tissue paper, or medical patches soaked and filled with water. All these rested loosely onto the fruit surface. These techniques were all equally effective in inducing russeting. However, these approaches were abandoned here because they were unreliable under field conditions. Second, natural moisture-induced microcracking and russeting can be seen in the stem cavity of most apple cultivars. During rain, the stem cavity fills with water. The area of skin beneath the 'puddle' so formed, remains wet for an extended period after the rain has stopped. Third, moisture-induced russeting has often been observed under field conditions (Tukey 1959; Creasy 1980); this is consistent with the findings reported herein. Fourth, we also

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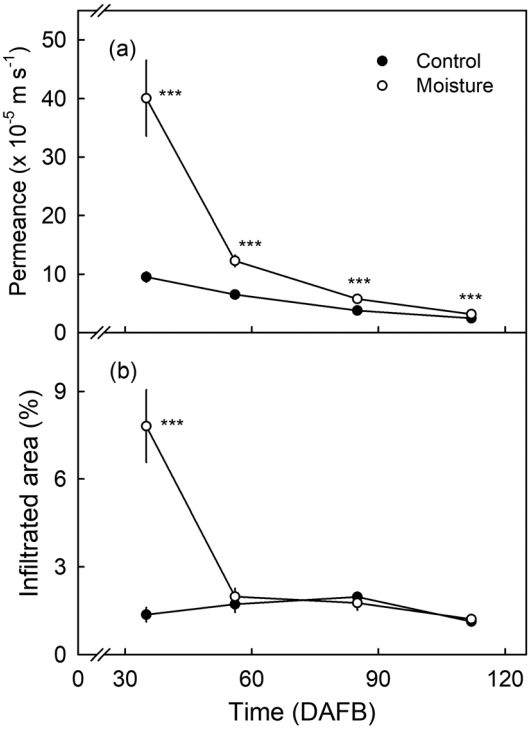

Fig. 5. Effect of surface moisture on permeance (a) and microcracking as shown by the area infiltrated by acridine orange (b) during fruit development. A selected area of the surface of a developing fruit was exposed to moisture for 12 days at four different stages of fruit development (from 23 to 35 days after full bloom (DAFB), 44 to 56 DAFB, 73 to 85 DAFB or 100 to 112 DAFB). The water vapour permeances and the surface areas infiltrated by acridine orange were quantified immediately after termination of the moisture treatment. Values represent mean ± SE of 18–20 (a) and 7–10 fruits (b). *** indicates significant difference between control and moisture treatment at $P < 0.001$.

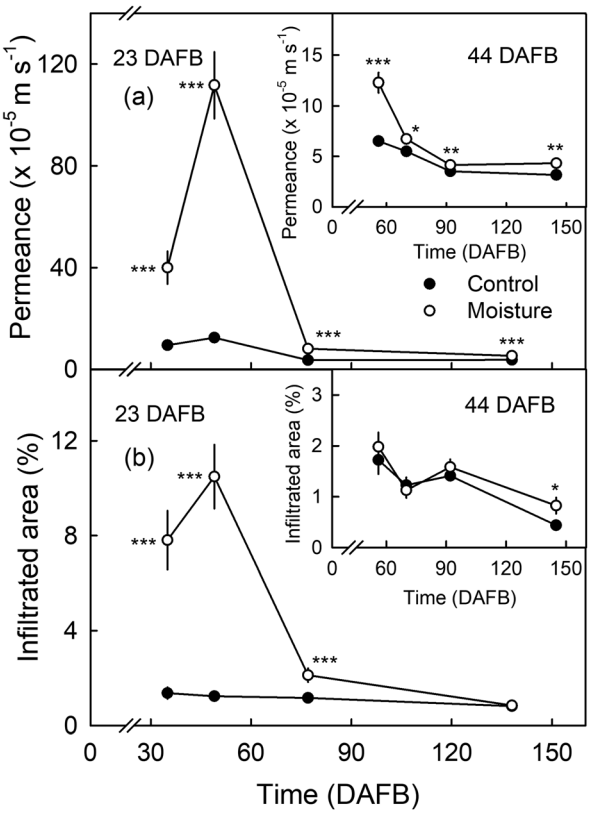

Fig. 6. Change in the permeances (a) main and inset) and acridine orange infiltrated areas (b) main and inset) of moisture-treated surfaces of developing fruits with time after termination of the moisture treatment. A selected portion of the fruit surface was exposed to moisture for 12 days, from 23 days after full bloom (DAFB) to 35 DAFB (main graphs) and from 44 DAFB to 56 DAFB (insets). Fruits were sampled at various stages of fruit development and the permeances and acridine orange-infiltrated areas of the fruit surface were quantified. Values represent mean ± SE of 18–20 (a, a inset) and 7–10 fruits (b, b inset). *, **, *** indicate significant difference between control and moisture treatment at $P < 0.05$, 0.01 and 0.001, respectively.

observed moisture-induced microcracking of the cuticle in earlier studies using excised epidermal segments of the apple fruit skin (Knoche & Grimm 2008). Fifth, if the silicone and/or the Eppendorf tube restricted growth, the fruit would be visibly deformed – it was not. Also, it would not be necessary to repeatedly reseal the tube to maintain surface wetness. The silicone we used attaches only very loosely to the fruit surface; it is thus very easily removed, without physical stress or damage to the fruit skin. Sixth, an empty tube (cylindrical, cut to only half length and left open) glued on the fruit did not produce any russet. Last, neither the silicone used nor the polyethylene Eppendorf tube release any chemicals that are phytotoxic. These arguments exclude possible artefacts due either to the silicone or to the Eppendorf tube.

For routine experimentation, we preferred to not mount empty tubes as control treatments. An empty tube may result in elevated humidity inside the tube and this would likely have induced microcracking and russeting (Knoche & Grimm 2008). Furthermore, condensation would likely have formed on the enclosed skin area due to the widely fluctuating temperatures in the field. Thus, unprotected exposure to the atmosphere (no tube) was selected as the most appropriate control.

Microcracking and permeance to water vapour decrease after removal of surface moisture

Our results demonstrate that following microcracking, fruit surface integrity recovers as demonstrated by parallel decreases in acridine orange infiltration and in water vapour permeance. Within 4 weeks of exposure to surface moisture, the barrier function was largely restored. Nevertheless, water vapour permeance remained slightly and significantly higher than in control fruit. Some microcracks remained visible but were not infiltrated by acridine orange. The decrease in the area of skin infiltrated by acridine orange was proportional to the decrease in skin permeance. The basis of this recovery effect may be twofold, as described below.

First, a likely candidate process is the deposition of wax in the microcracks. Indirect evidence comes from SEM images that show microcracks filled with wax crystals (Roy *et al.* 1999; Curry 2009; Curry & Arey 2010; Konarska 2013). Unfortunately, an attempt to gain direct quantification of microcrack infilling by wax crystals using interferometry was not successful

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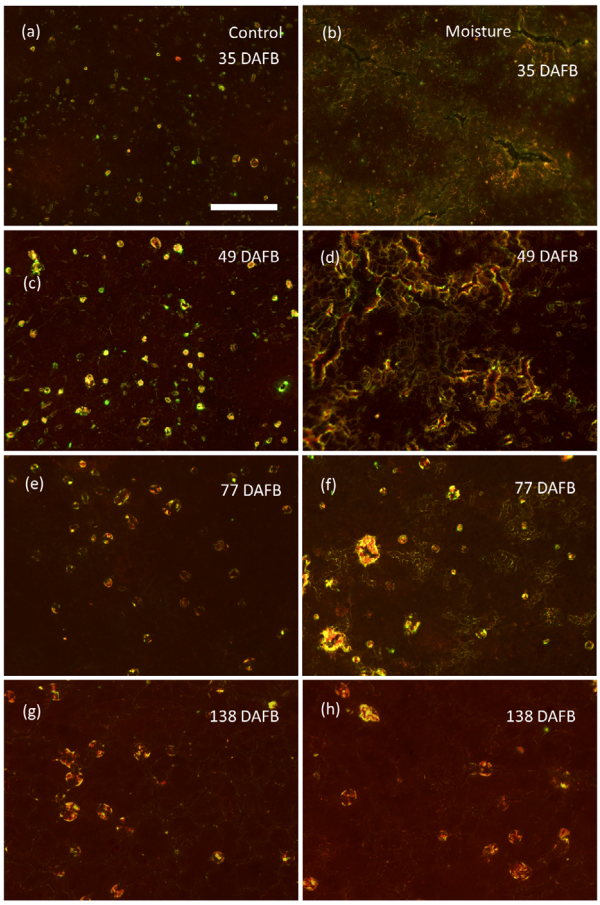

Fig. 7. Time course of changes in microcracking as recorded by acridine orange infiltration of the surface of developing apple fruit. Fruits were exposed to moisture for 12 days from 23 days after full bloom (DAFB) to 35 DAFB. Images were prepared from moisture-treated (b, d, f, h) and untreated (a, c, e, g) surfaces of the same fruit. The scale bar (400 μm) in (a) is representative of all images of the composite figure.

due to the high variability of microcracking over the apple fruit surface (B.P. Khanal, unpublished data). The wax that fills the microcracks in the cuticle surface is not necessarily derived from *de novo* synthesis in the epidermis and subsequent diffusion to the surface. Instead, wax deposition in microcracks is thought more likely derived from a redistribution of wax already within the cuticle. This view is based on the observation that wax is a highly dynamic structure that re-assembles itself if

Table 1. Effect of fruit development on surface moisture-induced russeting in 'Pinova' apple. Surface moisture was applied for 12 days at four stages of fruit development. The areas of russeting on the treated and untreated surfaces were quantified at harvest maturity. n = 21, DAFB = days after full bloom.

Stage of development (DAFB)	Frequency of fruit with russet (%)	Russeted area (% of treated area)	
		Moisture	Control
23 to 35	100	37.1 ± 7.3	0
44 to 56	0	0	0
73 to 85	0	0	0
100 to 112	0	0	0

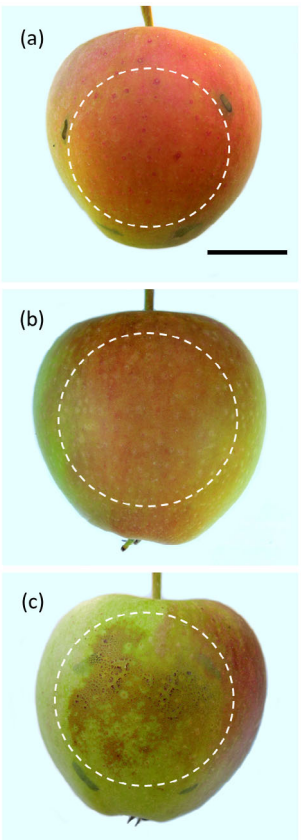

Fig. 8. Russet formation in 'Pinova' apple 105 days after full bloom (DAFB). (a) Untreated control fruit without tube and without water; (b) untreated control fruit with tube, but without water; (c) moisture-treated fruit with tube and with water. The tubes were mounted 28 DAFB, left on the fruit for 12 days and then removed. The dashed circle marks the original footprint of the tube. The moisture treatment, but neither of the two controls revealed marked russeting. The scale bar (2 cm) in (a) is representative of all images of the composite picture. For details see the Material and methods.

its structure is disturbed – either mechanically or by heat (Neinhuis *et al.* 2001; Koch *et al.* 2004). Also, the decrease in water vapour permeance of cuticles during storage has

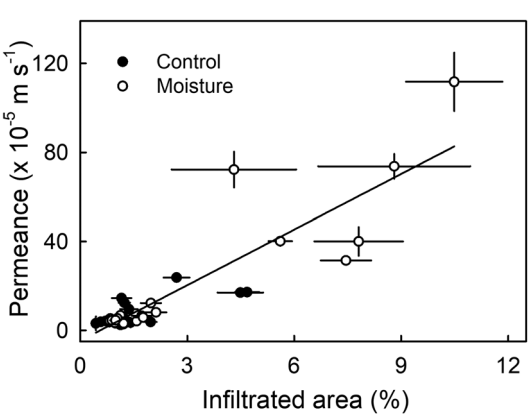

Fig. 9. Relationship between permeances and acridine orange infiltrations of the surface of apple fruits at various stages of development. Open circles are for moisture-treated skins, closed circles for untreated skins. Values represent mean ± SE of 12–20 (permeance) and 7–10 (infiltrated area) fruits.

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previously been attributed to a recrystallization of pre-existing wax (Geyer & Schönherr 1990). This behaviour is also consistent with its function during growth as a filler in the cutin polymer (Knoche *et al.* 2018).

Second, the formation of a subtending periderm in response to cuticular microcracking may also contribute to a decrease in microcracking and in water vapour permeance. However, the water vapour permeance of the periderm remains significantly higher than that of the cuticle on the primary surface (Khanal *et al.* 2019).

Effect of fruit development on microcracking, water vapour permeance and russeting

The effect of surface moisture on cuticular microcracking, skin permeance and russeting is consistent with the view that microcracking is the first visible symptom of cuticular damage, with increased permeance being the immediate consequence and this the probable trigger for russeting. Because surface moisture-induced microcracking is substantially limited to the early stages of fruit development, so susceptibility to russeting is also highest during the early stages of fruit development (Wertheim 1982). In the later stages of fruit development, apple fruit skin does not respond to the presence of surface moisture to nearly the same extent – in respect either to microcracking or to russeting.

The decrease in the response to surface moisture with increasing fruit maturity may be a characteristic of the cultivar 'Pinova' fruit investigated here. We note that in 'Elstar' apples, late-season exposure to surface moisture results in a skin spot disorder and this is also a consequence of surface-moisture-induced microcracking (Grimm *et al.* 2012; Winkler *et al.* 2014).

The relationship between exposure to surface moisture and microcracking of apple fruit skin is important from a practical point of view. Because of their high capital and maintenance costs, the provision of rain shelters for apples is uneconomic. Instead, the method of choice to decrease the duration of surface moisture and, hence, the incidence of microcracking is to train the apple orchard to an open canopy structure. This could be augmented by a typical gibberellin (GA_3 or GA_{4+7}) spray application programme that works to minimize cuticular microcracking (Knoche *et al.* 2011) and russeting (Wertheim 1982).

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AUTHOR CONTRIBUTIONS

M.K. and B.P.K. designed the research, Y.I., B.P.K., Y.H.C. and J.S. performed the experiments. B.P.K., Y.I. and M.K. analysed the data. B.P.K. and M.K. wrote the manuscript.

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