Mechanical Properties of Apple (*Malus* × *domestica* Borkh.) Fruit Skin and Their Potential Role in Fruit Russeting

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Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 3 und 8.

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B.P. Khanal, E. Grimm, and M. Knoche planned the experiments. B.P. Khanal conducted all the mechanical studies and the data analysis. B.P. Khanal and A. Blume conducted DSC measurements. B.P. Khanal, S. Finger, and A. Blume conducted ATR FT-IR measurements. B.P. Khanal analyzed most of the data. A. Blume helped to analyze and interpret DSC and ATR FT-IR data. B.P. Khanal and M. Knoche wrote the manuscript.

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B.P. Khanal and M. Knoche planned the experiments. B.P. Khanal and L. Hückstädt conducted CM isolation, dewaxing and mass determination, and mechanical tests. B.P. Khanal and R. Shrestha conducted the measurement of biaxial strain release. This test was originally developed by B.P. Khanal. B.P. Khanal and M. Knoche analyzed the data, wrote the manuscript and edited the paper.

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1. Zusammenfassung

Berostung ist ein Defekt der Apfelfruchthaut (*Malus* × *domestica* Borkh.) von großer wirtschaftlicher Bedeutung. Es wird angenommen, dass Berostung die Folge mikroskopischer Risse (‘Mikrorisse’) in der Kutikula (CM) mit anschließender Periderm (PM)-Bildung ist. Um Erkenntnisse über die Entstehung von Berostung zu gewinnen, wurden die mechanischen Eigenschaften von Fruchthautsegmenten (excised segment, ES) sowie enzymatisch isolierten CM und PM mit Hilfe von Entspannungs- und Zugversuchen untersucht. Die Untersuchungen konzentrierten sich auf 1) die Bedeutung der Wachse der Kutikula für die mechanischen Eigenschaften, 2) die mechanische Rolle der Kutikula als äußerer Teil des Fruchthaut-Komposits und 3) den Vergleich der mechanischen Eigenschaften von enzymatisch isolierter Kutikula und Periderm.

Nach Extraktion der Wachse nahm die Fläche der entwachsten CM (DCM) Scheiben ab, so dass die biaxiale Dehnung der CM als Verringerung der Fläche quantifiziert werden konnte. Steifigkeit (*S*), Dehnungsmaximum (ε_{max}) und Maximalkraft (F_{max}) wurden durch uniaxiale Zugversuche an ES-Streifen, hydratisierten CM-sowie DCM-Streifen untersucht.

Durch die Wachsextraktion reduzierte sich die Fläche der CM um 24.5±1.3%. Der uniaxiale Zugversuch zeigte eine Verringerung von *S* und F_{max}, sowie eine Zunahme von ε_{max} der CM nach Wachsextraktion. Es bestand eine lineare Beziehung zwischen der biaxialen Entspannung der CM von Früchten und Blättern verschiedener Spezies, *S*, ε_{max} und der Menge extrahierter Wachse. F_{max} dagegen war unabhängig von der extrahierten Wachsmenge. Die größten Änderungen der mechanischen Eigenschaften traten bei Apfel-CMs auf, die den höchsten Wachsgehalt aufwiesen. Allerdings wurde bei Zugversuchen an CMs, die von reifen Äpfeln von 22 Sorten mit unterschiedlicher Berostungsanfälligkeit isoliert wurden, kein signifikanter Zusammenhang zwischen den oben erwähnten Eigenschaften der CM und der Berostungsanfälligkeit festgestellt.

Die Untersuchung der mechanischen Eigenschaften von Fruchthautsegmenten (ES) und der CM von sich entwickelnden Früchten der Sorte Elstar zeigte, dass zwischen *S* und F_{max} im Zeitraum vor (51 bis 141 Tage nach Vollblüte; days after full bloom, DAFB) und nach (141 bis 259 DAFB, Kühllagerung bei 4.5°C und 95% relativer Feuchte) der Ernte und der Dicke der ES ein positiver Zusammenhang bestand, wohingegen ε_{max} unabhängig davon war. Die *S* von ES mit einer Standarddicke von 0.5 mm und die der CM nahm im Zeitraum vor der Ernte zu und während der Lagerung leicht ab. Es bestand kein Unterschied in *S* zwischen isolierten CM und ES. F_{max} und ε_{max} nahmen sowohl zur Ernte als auch während der Lagerung, allerdings mit geringerer Rate, kontinuierlich ab. Sowohl F_{max} wie auch ε_{max} der CM waren erheblich geringer als in den ES. Das erhöhte Auftreten von Mikrorissen in der Kutikula während des Zugversuchs von ES belegt, dass Defekte in der CM vor Defekten in der ES auftreten.

Der Vergleich von enzymatisch isolierten CM und PM in uniaxialen Zug- und Relaxationsversuchen zeigte, dass *S* und F_{max} in der CM höher sind als in PM. Gesamtdehnung, sowie elastische, viskoelastische und viskose Dehnung der PM Prüfkörper waren stets größer derjenigen der CM.

Die Ergebnisse zeigen, dass 1) kutikuläre Wachse wirkungsvoll die reversible elastische Dehnung der CM durch Umwandlung in irreversible plastische Dehnung fixieren. Dieser Effekt wurde bei allen untersuchten Apfelsorten beobachtet; 2) es bestand jedoch kein signifikanter Zusammenhang zwischen den mechanischen Eigenschaften der CM und der Berostungsanfälligkeit innerhalb der Apfelsorten; 3) die epidermalen und hypodermalen Zellschichten bilden das strukturelle ‘Rückgrat’ der Apfelfruchthaut, während der Beitrag der CM zu den mechanischen Eigenschaften der Fruchthaut marginal ist; und 4) bei der Berostung von Apfelfrüchten ein plastisches Periderm eine steife Kutikula ersetzt.

Schlagwörter: Dehnung, Kutikula, Periderm, Epidermis, Hypodermis.

2. Abstract

Russeting is a commercially important disorder of the apple (*Malus* × *domestica* Borkh.) fruit skin and thought to result from microscopic cracking of the cuticle (cuticular membrane; CM) and subsequent periderm (PM) formation. The CM is a natural lipoidal polymer composed out of cutin, wax, and polysaccharides. The PM has a cellular structure comprising phellem (cork), phellogen, and phelloderm. To develop a better understanding of the russeting disorder, mechanical properties of excised skin segments of apple fruit (ES) and enzymatically isolated CM and PM were studied using strain relaxation and tensile tests.

Biaxial strain release was quantified as the decrease in area of CM discs following wax extraction. Stiffness (*S*), maximum strain (ε_{max}), and maximum force (F_{max}) were determined in uniaxial tensile tests using strips of ES, hydrated CM and hydrated dewaxed CM (DCM).

Upon wax extraction the area of CM discs decreased indicating a release of biaxial strain (24.5±1.3%). Further, uniaxial tensile tests resulted in decreased *S* and F_{max}, whereas the ε_{max} of the CM increased. Across fruit and leaf CM of a range of different species biaxial strain release, *S* and ε_{max} but not F_{max}, were all linearly related to the amount of wax extracted. The largest changes occurred in apple CM that had the highest wax content. However, when performing tensile tests using CM isolated from mature fruit of 22 apple cultivars of differing susceptibility to russeting, there was no significant relationship between any of the above properties of the CM and russeting susceptibility.

Determining mechanical properties of skin segment (ES) and CM of developing 'Elstar' fruit revealed that *S* and F_{max} were positively related to ES thickness during the preharvest (51 to 141 days after full bloom; DAFB) and postharvest periods (141 to 259 DAFB, cold storage at 4.5°C, 95% RH,) whereas ε_{max} was independent of ES thickness. The *S* of an ES of 0.5 mm standard thickness and that of the CM both increased during preharvest period and then slightly declined during storage. There were no differences

in *S* recorded for isolated CM and ES. F_{max} and ε_{max} decreased steadily towards harvest and continued to decrease in storage but at a lower rate. The F_{max} and ε_{max} were markedly lower for CM than for ES. The increased incidence of microcracking of the cuticle when subjecting ES to tensile tests indicated that CM failure preceded ES failure.

Comparing enzymatically isolated CM and PM in standard uniaxial, creep/relaxation, and stepwise creep tests revealed that the *S* and the F_{max} were higher in CM than in PM. Total strain, elastic strain, viscoelastic strain and viscous strain were always greater in PM than in CM.

The data demonstrate that 1) cuticular wax effectively fixes reversible elastic strain of the CM by converting into irreversible plastic strain resulting in to less extensible CM. This effect was consistent among the CM of various apple cultivars differing in russeting susceptibility; 2) there was no significant relation between mechanical properties of the CM and russeting susceptibility across the apple cultivars; 3) the epidermal and hypodermal cell layers represent the structural 'backbone' of an apple skin whereas the contribution of the CM to the mechanical performance of the fruit skin is marginal; and 4) in apple fruit russeting a plastic periderm replaces a stiff cuticle.

Keywords: strain, cuticle, periderm, epidermis, hypodermis

3. General Introduction

Peel appearance is an important quality criterion in most fruit crops. Visible defects of the surface impair the fresh market quality of the fruit and cause significant economic loss. Russeting is a well-known surface defect in many fruit crops including apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.). It is a common problem in production worldwide.

In partly russeted apple the surface comprises two anatomically different surfaces, i.e. the primary and secondary fruit skin. The primary fruit skin consist out of a polymeric component, the outermost layer of cuticle and a cellular component, a single layer of epidermal cells and 5-8 layers of collenchymatous hypodermal cells (Evert 2006). The cuticle is a lipoidal polymer composed mainly of cutin, wax, and polysaccharides. Cutin is a polyester of oxygenated C16- and C18-fatty acids, cross-linked by ester bonds (Heredia 2003). It forms a matrix that contains cuticular wax (Petracek and Bukovac 1995). Cuticular wax deposited in the cutin matrix is termed 'intracuticular wax', that deposited on the surface of the matrix 'epicuticular wax'. Cuticular wax comprises a complex mixture of long-chain (C20–C40) alcohols, aldehydes, fatty acids, and alkanes (Dominguez et al. 2011a, Kunst and Samuels 2003). In the apple fruit cuticle triterpenoids (Ursolic acid) is the most dominating wax component whereas alkanes are the second largest component (Belding et al. 1998, Kolattukudy 1996). Polysaccharides from the epidermal cell walls are localized at the inner side of the cuticle and comprise cellulose, hemicelluloses, and pectins (Jeffree 1996). Other CM constituents in some species are cutan (Bargel et al. 2006, Jeffree 1996), sterol and flavonoids (Kunst and Samuels 2003, Samuels et al. 2008). The cuticle is the major protective barrier in water movement into and out of the fruit, and in pathogen defense (Kerstiens 1996, Kunst and Samuels 2003, Riederer and Schreiber 2001). Therefore, failure of the cuticle impairs the barrier function of the cuticle.

The secondary fruit skin has periderm on the outer surface. A periderm is composed out of 3 distinct tissues of different cell types. Phellem or cork cells are the outermost, suberized dead cells. These cells form a multilayered (~6 layers) structure which is a protective barrier in secondary fruit skin. The phellogen is the next layer underlying the

phellem cells. It is a single layer of meristematic cells (cork cambium) that divides and produces phellem cells on the outer side and phelloderm cells on the inner side. Phelloderm cells forms the innermost tier of the periderm. They are living cells and resemble the cortical parenchyma cells (Evert 2006, Lulai and Freeman 2001). The periderm causes the brown, corky, and dull appearance of a russeted fruit surface.

Study on the etiology of russeting indicated that russeting is preceded by the formation of microscopic cracks in the cuticle (Faust and Shear 1972a, b). These microcracks may be limited to the outer portion of the cuticle, but sometimes traverse the cuticle. Those that further extend into the epidermal and hypodermal cell layers apparently trigger the formation of a periderm that then forms in the hypodermal cell layers (Meyer 1944, Verner 1938). The nature of this signal is currently unknown. Russeting therefore is considered to be a repair process of a cracked surface where the periderm replaces the primary surface comprising a fractured cuticle and an epidermis. Subsequently, cuticle and the epidermis overlying the periderm dry and are shed before the periderm becomes visible.

Three categories of factors are involved in the formation of microcracks in the cuticle. First, fruit growth stress results in formation of microcracks in the cuticle (Maguire 1998, Skene 1982). During fruit growth, fruit volume and surface area increase. The expanding surface exerts a tangential stress on the fruit skin and on the cuticle. To cope with the expansion and increasing stress, either the fruit has to continuously build up and deposit new layer of polymer or the existing polymer layer on the surface is stretched. If the later process is dominating over the first, stress and strain will develop in the polymer. When stress and strain exceed the extensibility limits of the polymer, failure occurs. Apple fruit follows a sigmoid pattern of growth, where growth rates increase rapidly and peak at about 3 weeks after full bloom. During this phase fruit surface is exposed to the highest growth stress. This period also coincides with the highest susceptibility to russeting (Knoche et al. 2011, Wertheim 1982).

Second, in apple the distribution of growth stresses on the expanding surface is affected by the pattern of cell division in dermal tissue and this might contribute to increased russeting (Faust and Shear 1972a). Periclinal cell division in the epidermis and

hypodermis produce irregular shaped cells with cuticular pegs of variable thickness (Eccher 1975, Le 2013, Meyer 1944). Occasionally, epidermal cells become completely encased in the cuticle (Meyer 1944, Miller 1982). Therefore, the epidermis becomes irregular and uneven which causes inhomogenous stress/strain distribution and localized stress concentration in the cuticle. This phenomenon may differ among cultivars and could in part account for differential russet susceptibility.

Third, external biotic and abiotic factors may induce or suppress apple fruit russeting. For example, insects and micro-organisms (Gildemacher 2006), agrochemicals (Creasy and Swartz 1981), freezing temperatures (Faust and Shear 1972a), extended periods of high humidity and surface wetness (Fogelman et al. 2009, Knoche and Grimm 2008, Tukey 1969) induce russeting. In contrast, warm and dry climate (Fogelman et al. 2009), application of few agrochemicals (Gildemacher 2006, Reuveni et al. 2001) and plant growth regulators such as giberellins (GA_4 and GA_7, Fogelman et al. 2009, Knoche et al. 2011, Reuveni et al. 2001) suppress incidence of russeting in many apple cultivars.

Despite, largely established knowledge in apple fruit russeting, several aspects still remain unclear.
(1) Cuticular wax constitutes about 50% mass of apple fruit cuticle which is much higher than in cuticles of fruit of other crops or their leafy counterparts. What role does the wax play in terms of cuticle mechanics and possibly in russeting?
(2) Susceptibility to russeting differs among apple cultivars (Faust and Shear 1972a). For example, in a given climate ‘Karmijn de Sonnaville’ and ‘Egremont russet’ are highly susceptible, ‘Golden Delicious’ and ‘Elstar’ are medium susceptible, and ‘Idared’ and ‘Granny Smith’ are insensitive. Is susceptibility to russeting related to the cuticular mechanics?
(3) Collenchymatous dermal tissue of the plant is considered as structural tissue which gives mechanical strength to the plant organs (Evert 2006). There is also some believe that the cuticle is the load bearing structure (Bargel et al. 2006). These two statements are contradictory and raise the question about the contribution of cuticle, epidermal and hypodermal cell layers to the mechanical properties of the skin composite?

(4) The surface of a partly russeted fruit is a compound surface in that it comprises regions covered by primary and by secondary dermal tissue. Often the fruit remains regular in shape which is surprising considering the polymeric structure of the cuticle and the cellular structure of the cork produced by the periderm. Are these two types of dermal tissue similar in mechanical properties?

The objectives of this research therefore were to (1) identify the role of waxes in cuticular mechanics (2) quantify physical properties of cuticle of various apple cultivars and their relation to susceptibility to russeting, (3) determine the relative contributions of the different components of the fruit skin composite such as cuticle, epidermis and hypodermis to the mechanical properties of the composite , and, (4) compare the mechanical properties of primary and secondary apple fruit skins and compound fruit skins and potential relationship to the spread of russet.

4. Intracuticular Wax Fixes and Restricts Strain in Leaf and Fruit Cuticles

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Research

New Phytologist

Intracuticular wax fixes and restricts strain in leaf and fruit cuticles

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Key words: crystallinity, cuticle, fracture, rheology, stiffness, stress.

Summary

- This paper investigates the effects of cuticular wax on the release of strain and on the tensile properties of enzymatically isolated cuticular membranes (CMs) taken from leaves of agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) and from fruit of apple (*Malus* × *domestica*), pear (*Pyrus communis*), and tomato (*Lycopersicon esculentum*).
- Biaxial strain release was quantified as the decrease in CM disc area following wax extraction. Stiffness, maximum strain and maximum force were determined in uniaxial tensile tests using strips of CM and dewaxed CMs (DCMs).
- Biaxial strain release, stiffness, and maximum strain, but not maximum force, were linearly related to the amount of wax extracted. Apple CM has the most wax and here the effect of wax extraction was substantially accounted for by the embedded cuticular wax. Heating apple CM to 80°C melted some wax constituents and produced an effect similar to, but smaller than, that resulting from wax extraction.
- Our results indicate that wax 'fixes' strain, effectively converting reversible elastic into irreversible plastic strain. A consequence of 'fixation' is increased cuticular stiffness.

Introduction

The primary surface of all terrestrial plants is covered by a lipoid cuticular membrane (CM), which forms the critical interface between the plant and its environment (Jeffree, 1996). Cutin, wax and polysaccharides are the major constituents of the CM. Cutin is a polyester formed by oxygenated C16- and C18-fatty acids, cross-linked by ester bonds (Heredia, 2003). In some species, cutan, a polymethylene polymer, may also be present as part of the matrix (Jeffree, 1996; Bargel *et al.*, 2006). Waxes occur both epicuticularly as surface deposits on the cutin matrix and as intracuticular waxes embedded within it. They comprise a complex mixture mostly of long-chain C20–C40 alcohols, aldehydes, fatty acids, and alkanes (Kunst & Samuels, 2003; Dominguez *et al.*, 2011a). In some species, significant amounts of triterpenoids and flavonoids are also present (Kolattukudy, 1996; Kunst & Samuels, 2003; Samuels *et al.*, 2008). Polysaccharides are located on the cell wall side of the cuticle and represent epidermal cell wall constituents, such as cellulose, hemicelluloses, and pectins (Jeffree, 1996).

The cuticle functions as a barrier against water loss and uptake, and invasion by pathogens (Kerstiens, 1996; Riederer & Schreiber, 2001; Kunst & Samuels, 2003). Cuticles may also have mechanical functions (Matas *et al.*, 2004; Bargel & Neinhuis, 2005; Dominguez *et al.*, 2011a). Maintenance of these barrier functions requires an intact CM. This is challenging for a nonliving biopolymer deposited on an enlarging leaf surface (2–3 wk) and is even more of a challenge on a fruit surface where rapid and continuing expansion occurs throughout development (3–5 months). Nevertheless, cuticle deposition in fruit is often limited to their early development (e.g. grape, Becker & Knoche, 2012; plum, Knoche & Peschel, 2007 and sweet cherry Knoche *et al.*, 2004). Failure of the CM to cope with excessive expansion (strain) results in a build-up of stress (Knoche *et al.*, 2004), the formation of microscopic cracks (Peschel & Knoche, 2005), and ultimately in periderm formation in the surfaces of apple and pear ('russeting'; Faust & Shear, 1972), increased rain cracking in sweet cherry (Peschel & Knoche, 2005) and grape (Becker & Knoche, 2012), and infections with pathogens (Borve *et al.*, 2000). The situation in fruit contrasts with that in leaves where comparable phenomena are unknown. Presumably, this is because leaf expansion is usually limited to the early phase of development when the rate of cuticle deposition is high.

Interestingly, the development of fruit cuticles is sometimes very substantial and they often contain large amounts of wax compared with their leafy counterparts. As wax acts as a supporting filler (Petracek & Bukovac, 1995) that also increases the rigidity of the cutin matrix (Zlotnik-Mazori & Stark, 1988; Bargel *et al.*, 2006; Dominguez *et al.*, 2011a,b; Takahashi *et al.*, 2012), it is plausible that the high wax content of fruit CM may have a function in mitigating cuticular failure.

The objective of our study was to test this hypothesis by recording the relationship between the amounts of wax present in different cuticles and their rheological properties. We did this in isolated leaf and fruit cuticles of a number of species, quantifying the effect of wax extraction from the cuticle on the release of strain and on mechanical properties such as the stiffness, the maximum force, and the maximum strain. We include the leaf cuticle of the xeromorph, agave, in our study as its CM is thick and contains a large amount of wax. In this respect, it is similar to a fruit cuticle.

Materials and Methods

Plant material

Fully expanded leaves of wild agave (*Agave americana* L.) were collected in Tenerife, Spain, and those of bush lily (*Clivia miniata* L.), holly (*Ilex aquifolium* L.), and ivy (*Hedera helix* Reg.) were collected from the glasshouses and campus gardens of Leibniz University Hannover, Germany. Fruit of apple (*Malus* × *domestica* Borkh. cv Pinova), pear (*Pyrus communis* L. cv Conference), and tomato (*Lycopersicon esculentum* L. cv Encore RZ) were obtained locally at commercial maturity.

Isolation of CM

Leaf discs (24 mm diameter) were excised from the central portions of fully expanded leaves of agave, bush lily, holly, and ivy using a sharp, circular cutter. The midrib and major veins were avoided. The equatorial regions of apple, pear, and tomato fruit served as sources of similar epidermal segments (ESs), comprising cuticle, epidermis, adhering hypodermal cell layers and some parenchyma. The leaf and fruit ESs were incubated in 50 mM citric acid buffer solution (pH 4.0) 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); (Orgell, 1955; Yamada *et al.*, 1964)). Sodium azide (NaN_3) was added at a final concentration of 30 mM to prevent microbial growth. The enzyme solution was refreshed periodically until the CMs separated from the adhering tissue. Isolated CMs were rinsed thoroughly in deionized water and dried at room temperature (22°C, 50% relative humidity (RH)). From leaves, only the astomatous CMs from adaxial surfaces were used for further experimentation. Preliminary experiments established that the area of CM discs following excision and isolation remained essentially constant (B. P. Khanal, unpublished).

Pretreatments

For all species, DCMs were prepared from CMs by soxhlet extraction using chloroform : methanol (1 : 1 v/v) for 2.5 h. Subsequently, the DCMs were dried and stored under ambient laboratory conditions until further use.

To establish the relationship between the amount of wax extracted from apple CMs and the release of biaxial strain, apple CMs were extracted for 1, 10, 100, and 1000 min at 22°C and for 1000 min at 22°C followed by 150 min at 50°C using chloroform : methanol (1 : 1 v/v). The amount of wax extracted was determined gravimetrically.

Apple CMs without epicuticular wax (CM – ECW) were prepared by excising ESs from fruit previously treated by cellulose acetate stripping (Silcox & Holloway, 1986). A viscous solution of cellulose acetate (15% w/v) in acetone was applied to the fruit surface. After the acetone had evaporated (*c.* 2 h), the hardened cellulose acetate film was peeled from the fruit surface with forceps.

The effect of heating CMs before mechanical testing was studied in apple. Unless specified otherwise, CMs were heated in an oven to 80°C for 16 h overnight, then removed and held at ambient conditions (22°C, 50% RH). A heated CM is referred to as a CM80. Preliminary experiments established that the mass of apple CMs decreased and eventually discoloured at temperatures above 80°C.

Experiments

Release of biaxial strain To quantify the release of biaxial strain ($\varepsilon_{biaxial}$, %) following heating or wax extraction, a square pattern of four holes (*c.* 3.25 mm × 3.25 mm, hole diameter *c.* 0.5 mm) was made in untreated CM discs (8 mm diameter) using a custom punch. Next, the CM discs were photographed (×1.25) under a dissecting microscope (MZ10F; Leica Microsysteme GmbH, Wetzlar, Germany; camera DP71, Olympus, Hamburg, Germany; Software Cell^P, Olympus Soft Imaging Solution, Münster, Germany). They were then dried and either heat treated or solvent extracted to remove wax. Next they were rehydrated in deionized water at 22°C for 16 h and photographed again. The areas enclosed by the square patterns of the four holes were quantified before (A_{CM}) and after heating (A_{CM80}) and before (A_{CM}) and after wax extraction (A_{DCM}; $n = 13$–15). The strain releases upon heating ($\varepsilon_{biaxial}^{CM80}$) and upon wax extraction ($\varepsilon_{biaxial}^{DCM}$) were calculated as:

$$\varepsilon_{biaxial}^{CM80} = \frac{A_{CM} - A_{CM80}}{A_{CM80}} \times 100 \text{ and}$$
$$\varepsilon_{biaxial}^{DCM} = \frac{A_{CM} - A_{DCM}}{A_{DCM}} \times 100. \quad \text{Eqn 1}$$

A possible effect of hydrating CM and DCM discs on the change in disc area was quantified. For this experiment, apple was selected because apple CMs had the highest amount of wax among the species investigated. Digital photographs were prepared before and after a 16 h hydration period and the area of the entire CM and DCM discs were quantified on an individual disc basis ($n = 15$).

Tensile tests Cuticular membranes discs (24 mm diameter) were trimmed using parallel-mounted razor blades to obtain CM strips (5 mm wide). These strips were then mounted in a frame of paper and masking tape (TesaKrepp; tesaWerk Hamburg GmbH, Hamburg, Germany). Unless specified otherwise, all specimens were hydrated by incubating in deionized water at 22°C for a minimum of 16 h before testing. Subsequently,

frames were mounted between the clamps of a universal material testing machine (Z 0.5; ZwickRoell, Ulm, Germany; clamping distance $L_0 = 10$ mm) equipped with a 10 N standard force transducer (KAP-Z; Zwick/Roell). The frames were cut open and uniaxial tensile forces were applied. Specimens were strained at a rate of 1 mm min^{-1} until failure. Applied forces (F, in N) and corresponding specimen lengths (L) were continually recorded. The stiffness (S, in N) was calculated as the maximum slope of a linear regression line fitted through a plot of force (N) vs strain (%/100). This S differs from the commonly used modulus of elasticity in material science (E; in MPa) in that it reflects the properties of the specimens as present in different thicknesses on the respective leaf and fruit surfaces because it is not corrected for differences in cross-sectional area and, hence, in thickness between species. The *S value* allows an analysis of the change in stiffness (ΔS) upon extracting the CM simply by subtracting the S determined for DCMs from that of CMs. The strain at maximum force (ε_{max}, %) was obtained from

$$\varepsilon_{max} = \frac{L - L_0}{L_0} \times 100. \qquad \text{Eqn 2}$$

For a representative data set, this ε_{max} was within 98% of the ε at fracture. The maximum force (F_{max}) corresponds to the maximum force recorded, usually just before fracture. Data for specimens that failed in, or adjacent to, clamps and/or that had irregular force–displacement curves were excluded from the analyses as these may have been damaged during handling or mounting. The remaining specimens represented 91% of the total population ($n = 802$) of strips investigated. Using this procedure, the stiffness (S) and failure thresholds (F_{max}, ε_{max}) were quantified for CMs and DCMs taken from all species. Replication (n) ranged from 10 to 19.

In apple, where the effect of wax on CM rheology was most pronounced, S, F_{max}, and ε_{max} were also recorded for CM – ECW. Untreated apple CM (with ECW) and extracted CM (DCM) served as controls ($n = 11$–12).

The effects of temperature were quantified in apple by oven-heating CMs and DCMs to temperatures between 20 and 140°C for 16 h. Tensile properties were quantified at 22°C and 50% RH on dry specimens within 12 h of removal from the oven ($n = 9$–14).

To address the possibility that wax recrystallization occurred after heating (16 h at 80°C), apple CMs were held at ambient conditions (22°C, 50% RH) for 0.04, 0.5, 2, 7, 14, 28, and 56 d. Thereafter, tensile properties were quantified as described earlier. Unheated CMs served as controls ($n = 14$–15).

Differential scanning calorimetry (DSC) Unheated apple CMs, heated CMs (CM80) (80°C for 16 h), DCMs, and isolated wax were investigated by DSC. After heat treatment, CM80 were held at 22°C and 50% RH for up to 204 d to allow for possible wax recrystallization. Samples were weighed on aluminium pans (each *c.* 2.5 mg) and the pans crimped. Wax samples for DSC were prepared from a chloroform : methanol extract of apple CMs. Aliquots were taken to dryness in the aluminum pans and the pans crimped. Samples were loaded into the DSC system (Pyris 1 DSC; Perkin-Elmer Instruments, Waltham, MA, USA) and scanned at a heating rate of 10°C min^{-1} from 20 to 140°C and a cooling rate of −10°C min^{-1} from 140 to 20°C. Thereafter, the entire cycle was repeated on the same sample using the same settings. Empty pans were used as reference ($n = 3$).

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR) measurements Attenuated total reflectance Fourier transform infrared spectroscopy spectra were recorded using an IFS 66 spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a liquid N_2-cooled mercury cadmium telluride (MCT) detector. The CMs were placed with their outer surfaces facing a trapezoidal germanium crystal (50.7 mm × 10 mm × 3.9 mm). This geometry allowed five internal reflections at the sample surface with an incidence angle of 45°. The crystal was mounted in a custom-built aluminum sample holder that was held at a constant temperature by a computer-controlled circulating water bath (Haake C25P Phoenix II, Karlsruhe, Germany). For single-beam spectra, averages were taken of 128 scans with a spectral resolution of 4 cm^{-1}. Spectra were recorded at 2°C intervals between 28 and 74°C, with an equilibration time of 15 min at each temperature. Temperatures were measured inside the cover plate of the sample holder using a Pt100 resistor (Omega Newport, Deckenpfronn, Germany). Final absorbance spectra were calculated from the single beam spectra using the spectra of the Germanium crystal without CM (Ge–air interface) at each temperature as a reference. All absorbance spectra were baseline-shifted to zero in a spectral region where no vibrational peak occurred. To determine the peak position of the CH_2-vibrational bands in a certain wavenumber interval, the second derivatives of the spectra were calculated and the 'peak picking' function included in the Bruker OPUS software was used.

Data analysis and presentation

Data in tables and in Figs 1 and 3–5 are presented as means and SEMs. Where error bars are not shown in figures, they were smaller than data symbols. Data were subjected to ANOVA (Proc GLM) or linear regression analysis (Proc REG) using SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Percentage strain data were arcsine-transformed before ANOVA. Means were compared using Tukey's studentized range test ($P < 0.05$). Significance of coefficients of determination (R^2) and of coefficients of correlation (R) at the probability levels 0.05, 0.01 and 0.001 are indicated by *, ** and ***, respectively.

Results

Mass per unit area of leaf CMs ranged from the bush lily (lowest) to the xeromorphic agave (highest). Values for the fruit CMs of apple were just below those of agave leaves (Table 1). Apple fruit CMs had the highest masses of wax per unit area both in absolute terms and in percentage wax content. Wax mass per unit area and wax content were lowest in

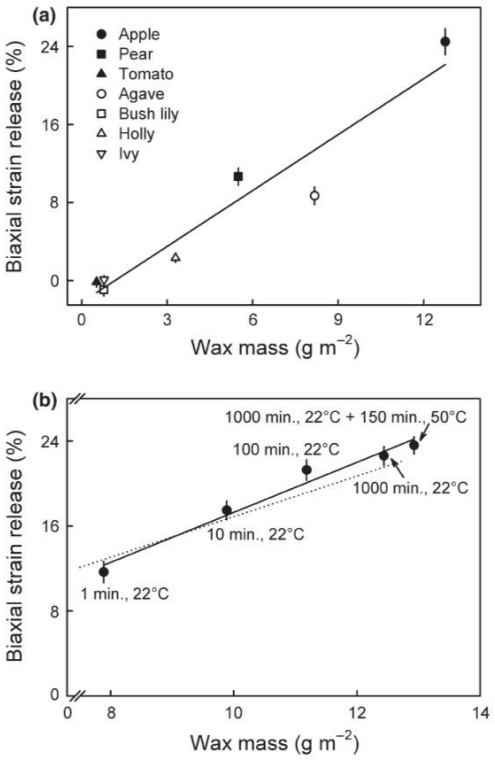

Fig. 1 Effects of wax extraction on the release of biaxial strain from cuticular membranes (CMs) isolated from the fruit of apple (*Malus* × *domestica*), pear (*Pyrus communis*), and tomato (*Lycopersicon esculentum*) (closed symbols) or leaves of agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) (open symbols) (a) and from apple fruit CMs extracted for various periods of time at 22°C and at 50°C (b). The dotted line in (b) is the regression line redrawn from (a). Values represent means ± SEM (n = 13–15).

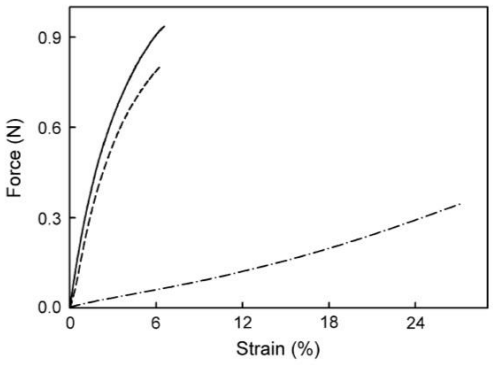

Fig. 2 A typical force–strain diagram of enzymatically isolated cuticular membranes (CMs, solid line) of apple (*Malus* × *domestica*) fruit equilibrated at 22°C and 50% relative humidity before tensile testing. Dashed line, CM with epicuticular wax removed (CM – ECW) by stripping with cellulose acetate before excision of epidermal segments (ESs); dashed and dotted line, CM with all wax removed (DCM) by extraction with chloroform : methanol (1 : 1 v/v).

tomato fruit CMs (Table 1). Across all species, CM and wax mass per unit area were positively and significantly related (R^2 = 0.72; P = 0.016).

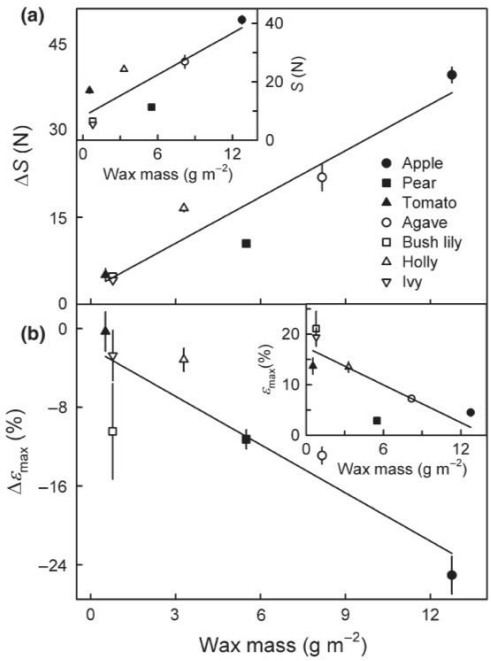

Fig. 3 The relationship between the rheological properties and wax mass per unit area of isolated cuticular membranes (CMs) taken from apple (*Malus* × *domestica*), pear (*Pyrus communis*), and tomato (*Lycopersicon esculentum*) fruit (closed symbols) and agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) leaves (open symbols). Change in stiffness (ΔS; a) and in maximum strain (Δ ε_{max}; b) of selected fruit and leaf CMs upon wax extraction in relation to wax mass per unit area. Insets: effect of wax mass per unit area on S (a; inset) and on ε_{max} (b; inset). Values represent mean ± SEM (n = 10–19).

Extracting the wax from CMs caused a significant decrease in area of the DCM discs of apple, pear, agave, and holly, indicating that a release of biaxial strain was associated with wax removal. There was little or no release of biaxial strain in ivy or bush lily (leaves), or tomato (fruit) (Table 1). Across both organs and all species, strain release was a positive, linear and highly significant function of the amount of wax extracted (R^2 = 0.92, P = 0.001; Fig. 1a). In apple, where the release of strain was largest, an essentially identical linear and positive relationship was obtained between the amount of wax extracted and the release of strain (R^2 = 0.97, P = 0.002; Fig. 1b). Heating apple CM to 80°C released a small but significant amount of strain (2.8% ± 0.4%). Hydrating apple CMs and DCMs increased the surface area, on average, by 2.6 (± 0.1) and 5.7 (± 0.2)%.

Uniaxial force–strain diagrams obtained in tensile tests of apple CMs and DCMs were approximately linear up to about half the maximum strains (Fig. 2). The stiffness S, as indexed by the slope of the force–strain diagrams, decreased upon wax extraction (Fig. 2). Furthermore, F_{max} decreased and ε_{max} increased (Table 2). There was little or no difference in S, F_{max}, or ε_{max} between CMs with and without ECW, indicating that the effect of wax extraction on CM rheology resided largely with the embedded cuticular wax (Table 3; Fig. 2).

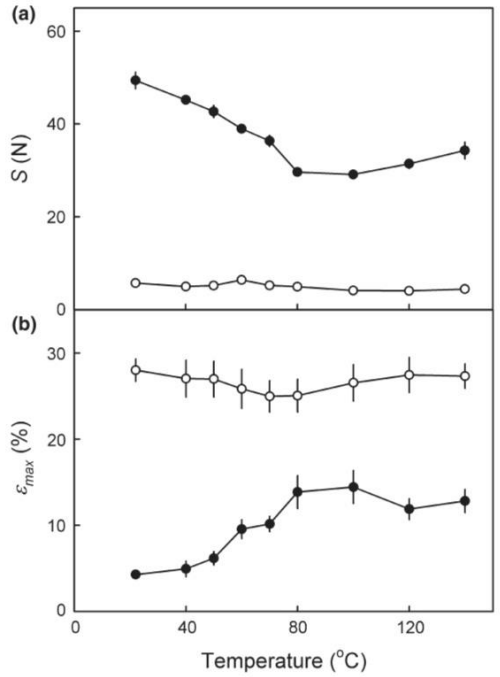

Fig. 4 Effect of heating enzymatically isolated cuticular membranes (CMs, closed circles) and dewaxed CMs (DCM, open circles) excised from apple (*Malus* × *domestica*) fruit on the stiffness (*S*; a) and on the maximum strain (ε_{max}; b). CMs and DCMs were pretreated by exposure to temperatures between 20 and 140°C for 16 h before tensile testing at 22°C and 50% relative humidity (RH). Values represent means ± SEM (n = 9–14).

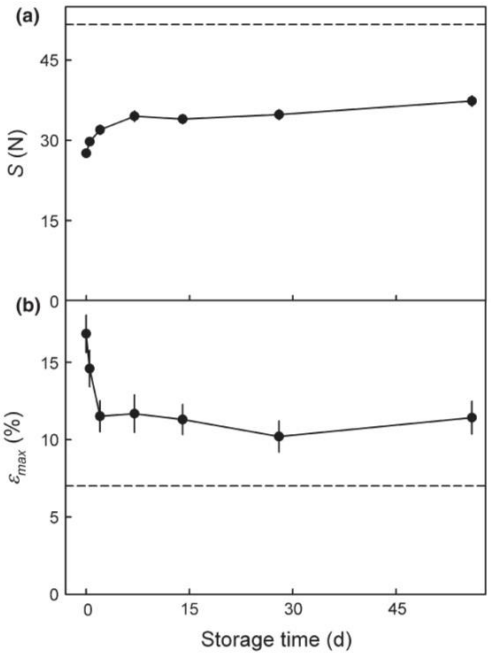

Fig. 5 Effect of storage time on the stiffness (*S*; a) and maximum strain (ε_{max}; b) of cuticular membranes (CMs) isolated from apple (*Malus* × *domestica*) fruit following 16 h of exposure to 80°C. Samples were held and tested at 22°C and 50% relative humidity. The horizontal dashed line indicates results for an unheated CM control. Values represent means ± SEM (n = 14–15).

Across all organs and species, the decrease in S upon wax removal was consistent (Table 2). Furthermore, the F_{max} of apple, pear, holly, and ivy decreased upon wax extraction. There was no significant change for tomato, bush lily or agave. The ε_{max} generally increased, the only exceptions being tomato (fruit) and ivy (leaves) (Table 2). Across all species and organs, linear and significant relationships were obtained between S or the change in S upon wax extraction (ΔS) and the amount of wax extracted (Table 4, Fig. 3a). Further, ε_{max} and the change thereof upon wax extraction ($\Delta\varepsilon_{max}$) were closely related to the amount of wax extracted (Table 4, Fig. 3b). Coefficients of determination obtained with CM mass per unit area as the independent variable were consistently lower than those with wax mass (Table 4). There was no relationship between F_{max} or the change thereof and either CM, DCM or wax mass per unit area (data not shown). Interestingly, the release of biaxial strain ($\varepsilon_{biaxial}^{DCM}$; Table 1) and the increase in ε_{max} following wax extraction ($\Delta\varepsilon_{max}$; Table 2) were closely related ($\Delta\varepsilon_{max}$ (%) = −1.06 (± 0.18) × $\varepsilon_{biaxial}^{DCM}$ (%), R^2 = 0.85, P = 0.001).

To address a possible effect of wax crystallization, apple CMs were subjected to heat treatments before the tensile tests. Heating of apple CMs significantly decreased S and increased ε_{max} (Fig. 4), but had no effect on F_{max} (data not shown). The effect of heating increased with rising temperature up to 80°C and remained essentially constant between 80 and 140°C (Fig. 4).

Table 1 Mass per unit area of apple (*Malus* × *domestica*), pear (*Pyrus communis*), and tomato (*Lycopersicon esculentum*) fruit and agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) leaf cuticular membranes (CMs), dewaxed CMs (DCMs), wax content of CMs, and release of biaxial strain following wax extraction

	Mass (g m^{-2})			Wax content (%)	Biaxial strain release (%)
Species	CM	DCM	Wax		
Fruit CMs					
Apple	28.2 ± 0.6	15.4 ± 0.4	12.8 ± 0.6	45.3 ± 0.4	24.5 ± 1.3
Pear	16.6 ± 0.3	11.1 ± 0.2	5.5 ± 0.2	33.1 ± 0.6	10.7 ± 0.9
Tomato	15.5 ± 0.3	15.0 ± 0.2	0.5 ± 0.1	3.4 ± 0.3	−0.1 ± 0.5
Leaf CMs					
Agave	32.8 ± 1.2	24.6 ± 1.1	8.2 ± 0.4	25.0 ± 1.0	8.7 ± 0.9
Bush lily	4.4 ± 0.01	3.7 ± 0.1	0.8 ± 0.1	17.5 ± 1.3	−0.9 ± 0.6
Holly	14.6 ± 0.4	11.3 ± 0.3	3.3 ± 0.1	22.5 ± 0.2	2.3 ± 0.5
Ivy	4.7 ± 0.2	4.0 ± 0.2	0.8 ± 0.04	16.5 ± 0.6	0.1 ± 0.4

Values are means ± SEM; n = 5 (mass) and 13–15 (biaxial strain release).

Higher temperatures discoloured the apple CM and caused significant mass loss (B. P. Khanal, unpublished). There was no effect of heating to 80°C on either S or ε_{max}, or on F_{max} of the DCMs (Fig. 4; F_{max} data not shown).

Holding apple CM80 samples at ambient temperature and humidity for several days gradually and partially restored their

Table 2 Rheological properties of cuticular membranes (CMs) and dewaxed CMs (DCMs) isolated enzymatically from apple (*Malus* × *domestica*), pear (*Pyrus communis*) and tomato (*Lycopersicon esculentum*) fruit and from adaxial surfaces of agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) leaves

	S (N)		F_{max} (N)		ε_{max} (%)	
Species	CM	DCM	CM	DCM	CM	DCM
Apple	41.3 ± 1.3 a[a]	1.7 ± 0.08 b	1.05 ± 0.06 a	0.38 ± 0.04 b	4.5 ± 0.7 b	29.5 ± 1.8 a
Pear	11.3 ± 0.4 a	0.9 ± 0.04 b	0.27 ± 0.01 a	0.14 ± 0.01 b	2.9 ± 0.1 b	14.2 ± 1.0 a
Tomato	17.0 ± 0.9 a	12.0 ± 0.6 b	1.59 ± 0.13 a	1.31 ± 0.10 a	13.7 ± 1.6 a	14.0 ± 1.2 a
Bush lily	6.5 ± 0.3 a	1.7 ± 0.1 b	0.33 ± 0.02 a	0.30 ± 0.02 a	21.1 ± 3.4 b	31.5 ± 3.4 a
Holly	24.3 ± 0.6 a	7.7 ± 0.2 b	1.63 ± 0.09 a	1.20 ± 0.06 b	13.5 ± 1.0 b	16.7 ± 0.5 a
Agave	26.9 ± 2.2 a	5.0 ± 0.6 b	1.11 ± 0.10 a	0.87 ± 0.09 a	7.3 ± 0.4 b	20.2 ± 0.8 a
Ivy	5.4 ± 0.3 a	1.2 ± 0.1 b	0.30 ± 0.01 a	0.25 ± 0.01 b	19.4 ± 1.8 a	22.2 ± 1.8 a

The stiffness (S), maximum force (F_{max}), and maximum strain (ε_{max}) were determined in uniaxial tensile tests on fully hydrated specimens. The S (in N) represented slopes of the F–ε relationships for CMs and DCMs.
[a]Means within columns followed by the same letter are not significantly different, Tukey studentized range test. $P < 0.05$. Values are means ± SEM; $n = 10$–19.

Table 3 Rheological properties of cuticular membrane (CM), CM without epicuticular wax (CM – ECW) and dewaxed CM (DCM) obtained from apple (*Malus* × *domestica*) fruit

	S (N)	F_{max} (N)	ε_{max} (%)
CM	29.2 ± 1.4 a[a]	0.83 ± 0.04 a	6.4 ± 0.8 b
CM – ECW	24.7 ± 0.6 b	0.82 ± 0.05 a	6.8 ± 0.8 b
DCM	1.6 ± 0.04 c	0.33 ± 0.01 b	26.8 ± 0.9 a

Epicuticular wax was removed by cellulose acetate stripping before excision and isolation of the CM. The stiffness (S), maximum force (F_{max}), and maximum strain (ε_{max}) were determined under fully hydrated conditions. The S (in N) represented slopes of the F–ε relationships.
[a]Means within columns followed by the same letter are not significantly different, Tukey's studentized range test. $P \leq 0.05$. Values are means ± SEM; $n = 11$–12.

Table 4 Parameters of linear regression lines for the relationships between the stiffness (S), the change of S upon wax extraction (ΔS), the maximum strain (ε_{max}) and its change upon wax extraction ($\Delta\varepsilon_{max}$) and the mass per unit area of the cuticular membrane (CM) or wax in apple (*Malus* × *domestica*), pear (*Pyrus communis*), and tomato (*Lycopersicon esculentum*) fruit CM and agave (*Agave americana*), bush lily (*Clivia miniata*), holly (*Ilex aquifolium*), and ivy (*Hedera helix*) leaves CM of adaxial surfaces

Dependent variable	Independent variable Mass (g m^{-2})	Regression parameters a ± SE	b ± SE	R^2	P-value
S (N)	CM	1.00 ± 0.29	2.24 ± 5.71	0.70	0.019
	Wax	2.38 ± 0.65	8.15 ± 4.03	0.73	0.014
ΔS (N)	CM	0.95 ± 0.33	−1.27 ± 6.34	0.63	0.033
	Wax	2.66 ± 0.37	2.54 ± 2.33	0.91	0.001
ε_{max} (%)	CM	−0.53 ± 0.18	20.65 ± 3.45	0.64	0.030
	Wax	−1.24 ± 0.41	17.40 ± 2.56	0.65	0.029
$\Delta\varepsilon_{max}$ (%)	CM	−0.47 ± 0.28	−1.51 ± 5.46	0.36	0.153
	Wax	−1.63 ± 0.37	−2.00 ± 2.30	0.80	0.007

The ΔS values were calculated as the differences in S between CM and dewaxed CM (DCM), and the $\Delta\varepsilon_{max}$ values as the differences in ε_{max} between CM and DCM.

extensibility as indicated by an increase in S and a decrease in ε_{max} within 2–7 d (Fig. 5). Holding them beyond a 7 d period produced little additional effect. There was no significant effect on F_{max} (data not shown).

Differential scanning calorimetry thermograms of apple fruit CM showed endothermic peaks at 53.2 and 62.3°C during a first heating cycle. After cooling, a second heating cycle was imposed. Although the two critical peak temperatures were unchanged, the peak heights were markedly reduced (Fig. 6a). The peaks are reasonably ascribed to the melting of cuticular wax and the differences in peak areas between heating cycles to the presence of differing amounts of crystalline wax (Fig. 6a). The area beneath the melting peaks decreased when heat-treated CMs (80°C for 16 h) were subjected to DSC after 0.2 d of heating (Fig. 6b). However, the peak height of heat-treated CM recovered if held at laboratory temperature for a period of 14 or 204 d (Fig. 6c,d). The melting peaks became even more pronounced after extracted wax had been subjected to DSC (Fig. 6e). There were no endothermic peaks in thermograms of DCM (Fig. 6f).

The wavenumber (cm^{-1}) of the maximum absorbance of CH_2 symmetric (ν_s) and asymmetric (ν_{as}) stretching bands increased with increasing temperature (Fig. 7a). The maxima of the absorption bands of the ν_s (CH_2) and ν_{as} (CH_2) stretching vibrations showed sigmoidal increases in wavenumber at *c.* 50 and 58°C, respectively. These indicate melting of the long-chain aliphatic fraction of cuticular wax (Fig. 7a; Merk *et al.*, 1998). In the subsequent cooling cycle, the absorption maxima shifted downwards in wavenumber, indicating recrystallization upon cooling (Fig. 7b). The temperatures determined from the sigmoidal increase in wavenumber coincided with the peak maxima in the DSC thermograms (Fig. 6). The peak of the CH_2 scissoring absorption band was split into two components at low temperature (*c.* 30°C). The splitting disappeared as the temperature increased to *c.* 50°C when the two absorption bands merged to a single band. This temperature corresponded to the first sigmoidal increase in wavenumber of the ν_s (CH_2) and ν_{as} (CH_2) stretching bands and the first DSC peak. A further sigmoidal decrease in the wavenumber of the CH_2 scissoring band was observed at *c.* 58°C, which is consistent with the second endothermic peak of the DSC and the largest increase in wavenumber for the CH_2 stretching bands. In the subsequent cooling cycle, the scissoring

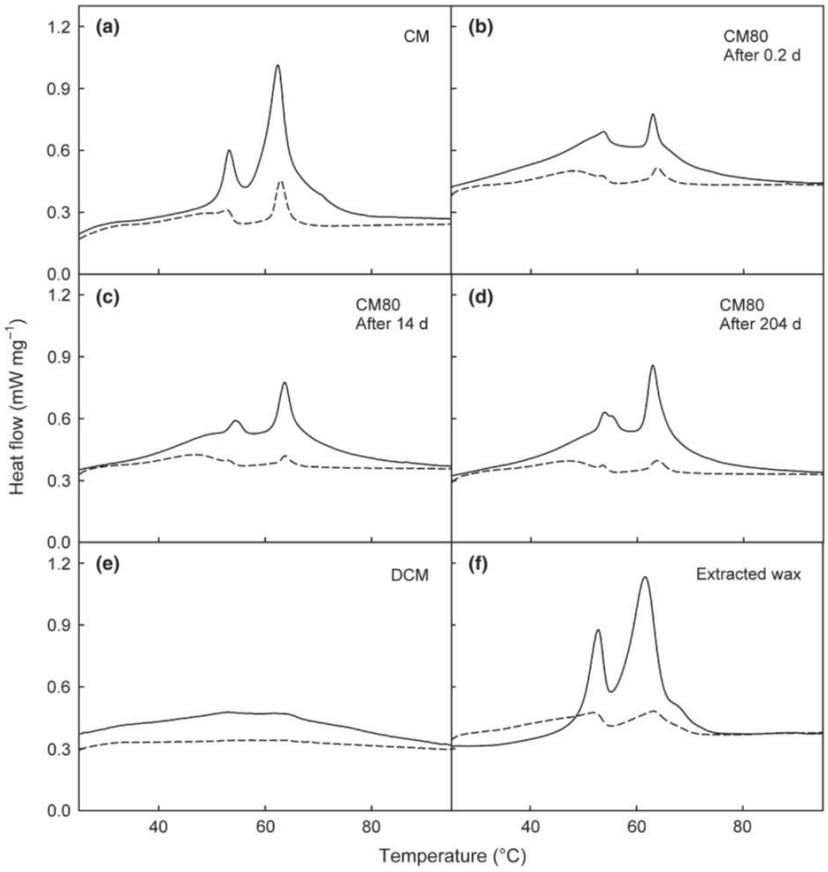

Fig. 6 Differential scanning calorimetry (DSC) thermograms of apple (*Malus* × *domestica*) fruit cuticular membranes (CMs). (a) An untreated CM; (b) a CM heated to 80°C for 16 h (CM80) then held at ambient temperature and humidity for 0.2 d before testing; (c) a CM80 held at ambient temperature and humidity for 14 d; (d) a CM80 held at ambient temperature and humidity for 204 d; (e) a CM following solvent extraction of cuticular wax (DCM); and (f) a thermogram of extracted cuticular wax. All samples were exposed to two heating–cooling cycles within 1 h. The solid line represents the first heating cycle and the dashed line the second heating cycle. Heat flows during the two cooling cycles are omitted. See text for further details.

peak reappeared (Fig. 8b), but disappeared again when CMs were reheated (data not shown). The higher absorption intensity in the cooling cycle (Fig. 8b) or the second heating cycle (data not shown) as compared with the first heating cycle (Fig. 8a) was the result of improved contact between the CM and ATR crystal as a result of the heating. The increase in absorption intensity occurred during the first heating cycle exactly at the melting temperature of the wax, indicating that heating must have improved the contact between CM and the crystal.

Discussion

Our results demonstrate that wax fixes the strain in CMs and decreases their extensibility. They also show that the magnitude of this effect is a linear function of the amount of wax in the cuticle per unit area. In other words, expansion growth of the leaf or fruit epidermis creates a reversible strain in the overlying cutin matrix. The presence of wax in this matrix effectively 'fixes' the strain, converting a reversible strain into a strain that, under *in vivo* conditions in the plant, is irreversible and, hence, plastic. This conclusion is based on the following arguments. First, the release of biaxial strain in the CMs upon wax extraction was a highly significant, linear function of the amount of wax present across a wide range of leaf and fruit cuticles (Fig. 1a). Furthermore, when varying the amount of wax extracted from apple CMs by varying extraction time (1–1000 min) and temperature (22 vs 50°C), strain release was again a linear function of the amount of wax extracted (Fig. 1b). In fact, the regression lines fitted in both figures were essentially superimposed (Fig. 1b). Secondly, the decrease in S (ΔS) obtained in tensile tests was linearly related to the amount of wax extracted (Fig. 3a). Thirdly, the release of biaxial strain from CM discs and the increase in ε_{max} that occurred upon wax extraction bore a 1 : 1 relationship that was highly significant across the seven CM sources investigated. Fourthly, the ε_{max} increased following wax extraction (Table 3), with the increase being positively and linearly related to wax mass per unit area (Fig. 3b). Fifthly, heating CMs beyond the melting point of their low-melting wax constituents yielded effects on strain release and on the tensile properties S and ε_{max} that were qualitatively identical to those of wax extraction (Fig. 4). Artefacts arising from differences in hydration between CMs and DCMs because of a change in polarity of the specimens as a result of extraction of wax can be excluded. The change in surface area as a result of swelling of apple CMs and DCMs upon hydration was similar and small compared with the amount of strain released after extraction. Thus, it is reasonable to suppose that the stresses and strains that occur in the CMs during fruit development as the surface expands, and which are fixed by the

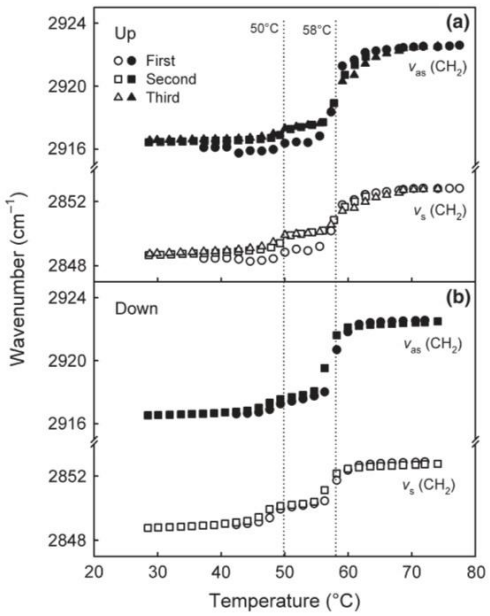

Fig. 7 Results of Fourier transform infrared (FT-IR) spectroscopic investigations of cuticular membranes (CM) enzymatically isolated from apple (*Malus* × *domestica*) fruit. Wavenumbers of the maximum of the CH_2 symmetric (v_s) and asymmetric (v_{as}) stretching bands as a function of temperature. The CMs were scanned repeatedly during the heating (Up; a) and cooling (Down; b) parts of complete temperature cycle from 28 to 74 to 28°C. For details see the Materials and Methods section.

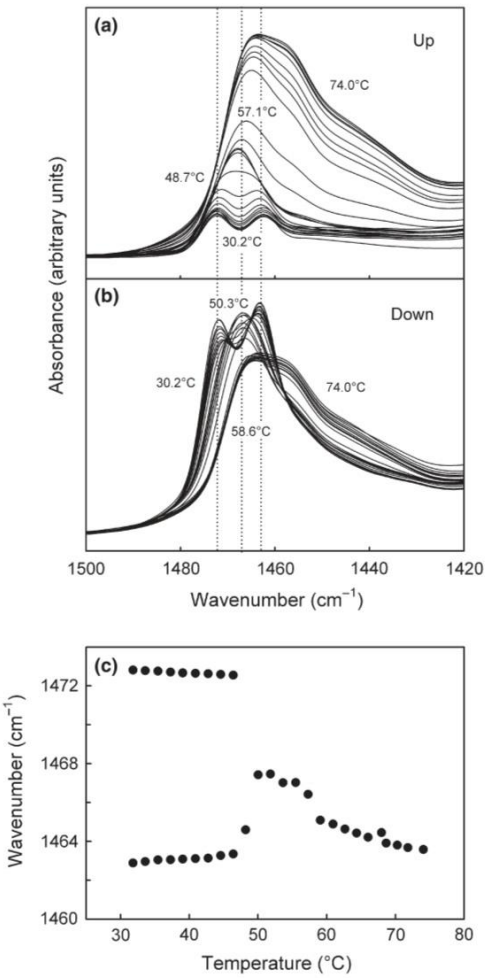

Fig. 8 (a, b) Fourier transform infrared (FT-IR) spectra in the 1500–1420 cm^{-1} wavenumber region of cuticular membranes (CMs) enzymatically isolated from apple (*Malus* × *domestica*) fruit. Absorption is a result of the scissoring CH_2 mode of the alkane chain of the wax (Merk *et al.*, 1998). CMs were scanned using heating (first up scan, a) and cooling (first down scan, b) cycles within a temperature range of 28–74–28°C. (c) The wavenumber of maximum absorbance and the splitting of the CH_2 scissoring band as affected by temperature. The doublet peak of the scissoring absorption band at low temperature (*c.* 30°C) disappeared as temperature increased to *c.* 50°C and the two absorption bands merged to a single band.

concurrent deposition of wax in the cutin matrix, are released suddenly upon either the melting or the extraction of the wax. These data are consistent with a role for wax as a filler in the flexible network of the cutin matrix (Zlotnik-Mazori & Stark, 1988; Petracek & Bukovac, 1995; Dominguez *et al.*, 2011a,b).

Because the removal of epicuticular wax had little or no effect on biaxial strain release, or on the tensile characteristics of the CM, we can infer that the CM's decreased extensibility is wholly the result of the wax embedded within the cutin matrix – the epicuticular wax is not involved.

Cuticular wax occurs in both amorphous and crystalline states (Reynhardt & Riederer, 1994; Bargel *et al.*, 2006). The observation that heating the CM to 80°C produced an effect qualitatively identical to, but quantitatively smaller than, that of wax extraction on the release of biaxial stain and on tensile properties leads to the conclusion that the crystalline wax fraction accounts, in part, for the effect on tensile properties. Apparently, the component of elastic strain in the CM fixed by the cuticular wax was released either when the wax was melted or when it was completely extracted. Consistent with this conclusion is the observation that extending the heating period beyond 2 h did not produce any additional effects (data not shown). Also, the gradual recrystallization that occurred when holding previously heated CMs at ambient temperature for up to 56 d partly restored the effect of heating on CM rheology. This is also consistent with a decrease in water permeability of citrus cuticles during storage that has been attributed to a time-dependent recrystallization of cuticular wax (Geyer & Schönherr, 1990). Direct support for recrystallization comes from the increase with storage time in the endothermic peak of heat-treated CMs (Fig. 6b–d). Further, the disappearance upon heating and reappearance upon cooling of the splitting of the CH_2 scissoring bands observed in the FT-IR spectra may be attributed to a breakdown and re-formation of an orthorhombic chain packing (Merk *et al.*, 1998; Fig. 8a–c). This change in crystal structure occurs at *c.* 50°C, which corresponds to the first endothermic peak in the DSC thermograms. The second endothermic peak and the shift of the absorption maxima of the v_s (CH_2) and v_{as}

(CH_2) stretching bands in the FT-IR spectra at *c.* 58°C must be attributed to the melting of a major constituent of wax. The most likely candidate for this is the alkane fraction that constitutes *c.* 30% of total apple wax (Belding *et al.*, 1998). The dominant alkane representative is nonacosane which, in the pure form, has transition and melting temperatures of 58.2 and 63.4°C, respectively (Schaerer *et al.*, 1955; Belding *et al.*, 1998).

That heating the CM did not produce the same quantitative effect on biaxial strain release as wax extraction is probably a result of the restricted temperature range we investigated. We would expect the heating effect to be equivalent to the wax extraction effect if all wax constituents had been melted during the heat treatment. Under these conditions, the elastic (and reversible) strain of the cutin matrix would have been released quantitatively. Unfortunately, exposing apple CMs to the melting temperature range of the ursolic acids (290–300°C; Ritter *et al.*, 2001), which are another major (up to 70%) component of apple fruit wax (Belding *et al.*, 1998), results in severe mass loss and discoloration (charring) of the cuticle (B. P. Khanal, unpublished).

The effect of heating on the increase in ε_{max} of CM is surprisingly large compared with the release of biaxial strain upon heating. Although this result was consistent among several experiments, the reason for it is unknown. It is speculated that an embedded wax crystal deforms the cutin network in its immediate vicinity, but less so further away. In this way it causes a localized stress and strain in the matrix. This phenomenon is recognized in filled rubbers where it is referred to as 'strain amplification' (Vincent, 1990; Westermann *et al.*, 1997). Strain amplification by embedded crystalline wax is consistent with the observation that unheated CM fails at lower strains than heated CM. In this case, the melting wax crystals will partly release the strain in their vicinity.

Our results provide the first clear evidence that cuticular wax 'fixes' strain in the cutin matrix of leaf and fruit cuticles. This strain results from the expansion of the underlying surface during growth. The strain is 'fixed' because neither excision nor isolation of the CM results in significant strain release. However, upon wax extraction, this strain is released. Therefore, wax deposition in the cutin polymer of the cuticle on the expanding surface has effectively converted a reversible elastic strain into a strain that, *in vivo* in the plant, is irreversible and plastic. From an ecological point of view, this modification of the mechanical properties of the cuticle should be seen as a mechanism that operates to reduce cuticular failure in the expanding surface of a leaf or a fruit. However, a negative-going consequence of such 'strain fixation' by wax deposition is an increase in the stiffness of the cutin matrix that could well restrict further expansion.

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5. Russeting in Apple Seems Unrelated to the Mechanical Properties of the Cuticle at Maturity

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Russeting in Apple Seems Unrelated to the Mechanical Properties of the Cuticle at Maturity

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Abstract. **Russeting is a commercially important disorder of the fruit skin of apples (*Malus ×domestica* Borkh.). It is thought to result from microscopic cracking of the cuticle on the fruit surface and the subsequent formation of a periderm just below. The study investigates 22 apples cultivars having widely different russeting susceptibilities to determine if susceptibility could be related to the mechanical characteristics of the cuticles at maturity. The mass per unit area of the cuticular membrane (CM), the dewaxed cuticular membrane (DCM), and the cuticle's wax content all varied significantly among the cultivars examined but no simple correlative relationships with russeting susceptibility could be found. Across all cultivars, the mass of wax per unit area was linearly related to CM mass per unit area ($R = 0.77$, $P < 0.0001$). The cuticle of all cultivars was markedly strained as indexed by the release of biaxial strain in the CM on extraction of the wax. The release of biaxial strain was linearly and positively related to wax mass per unit area. Maximum force (F_{max}) in uniaxial tensile tests, strain at maximum force (ε_{max}), and the stiffness (S) differed widely among the cultivars tested, but, again, there were no relationships between these mechanical properties and russeting susceptibility. Wax extraction from the CM decreased the F_{max} in uniaxial tensile tests, increased ε_{max}, and decreased the S. Our results show that none of the cuticle variables measured at maturity nor any of the isolated-cuticle mechanical properties contributes significantly to russeting susceptibility.**

Russeting is an important disorder of the fruit surface of many fruit crops including apple (Faust and Shear, 1972a, 1972b). In anatomical terms, a russeted area represents an area of periderm comprising phellogen and phellem. The periderm is thought to form in the hypodermal cell layer of developing apple fruit (Meyer, 1944; Verner, 1938). The phellem appears at the fruit surface after the epidermis and cuticle are shed. The phellem's suberized cell walls are responsible for the brownish, dull appearance.

Susceptibility to russeting differs widely among commercial apple cultivars (Faust and Shear, 1972a). Thus, the fruits of some cultivars (e.g., Braeburn) remain essentially russet-free under all growing conditions, whereas in other cultivars (e.g., Egremont Russet), the fruit are almost completely russetted and this is seen as an acceptable cultivar characteristic. However, a large number of cultivars (e.g., Elstar) exhibits russeting only under unfavorable growth conditions. As a result, any russeted fruit of this intermediate cultivar group are usually of reduced market value with potentially serious economic consequences for the grower.

The mechanistic basis for russeting is not clear. Fine, cuticular cracks are considered the first detectable symptom of russeting (Faust and Shear, 1972a). These are thought to result from mechanical failure caused by excessive rates of growth strain, probably during early fruit development (Maguire, 1998; Skene, 1982) and/or from extended exposure to surface moisture (Knoche and Grimm, 2008). Russeting can also be a response to some chemical sprays (Sanchez et al., 2001).

Wax affects the mechanical properties of the cuticle (Dominguez et al., 2011; Petracek and Bukovac, 1995) and this, in turn, may affect susceptibility to russeting in various ways. First, wax prevents the release of biaxial elastic strain by converting reversible elastic into irreversible plastic strain (Khanal et al., 2013a). During growth, the fruit surface enlarges and the cuticle is strained. Because wax acts as a filler in the strained polymer network of the cutin matrix (Petracek and Bukovac, 1995), the deposition of wax essentially "fixes" strain (Khanal et al., 2013a). Second, wax decreases the extensibility of cuticles by increasing their stiffness (Khanal et al., 2013a; Petracek and Bukovac, 1995). Third, scanning electron microscopy (SEM) studies suggest that wax deposition occurs in microscopic cracks in the cuticle and this may have a healing effect (Curry, 2008; Roy et al., 1999).

Based on these observations, it seems reasonable to hypothesize that differential susceptibility to russeting could be related to certain mechanical properties of the fruit cuticle and the effects of wax thereon. The aim of this study, therefore, was to measure a number of physical properties of the fruit cuticle and also its key mechanical properties (indexed by the release of biaxial strain and the tensile properties of isolated cuticles) in a large number (22) of apple cultivars selected to represent a maximum range in russet susceptibility.

Materials and Methods

Mature apple (*Malus ×domestica* Borkh.) fruit were obtained from the following sites: 'Granny Smith' from South Tirol (lat. 46°41′ N, long. 11°32′ E), Italy; 'Pinova', 'Karmijn de Sonnaville' (hereafter referred to as 'Karmijn'), 'Gala', 'Elstar', and 'Braeburn' from the experimental orchards of the Horticultural Research Station of the Leibniz University at Ruthe (lat. 52°14′ N, long. 9°49′ E); and 'Elshof', 'Elstar Boerekamp', 'Golden Delicious Klon B', 'Golden Delicious Reinders', 'Boskoop Green', 'Holsteiner Cox', 'Idared', 'Inacox', 'Jonagold', 'Marina', 'Nicoter', 'Boskoop Red', 'Rubinette', 'Starking', 'Superkalfs', and 'Egremont Russet' from orchards of the Federal Fruit Variety Office at Wurzen (lat. 51°22′ N, long. 12°45′ E), Germany. These cultivars were chosen because they are considered to differ markedly in their susceptibility to russet. To enable quantitative analysis, the russet susceptibility of each cultivar was assessed independently on a discrete, 3-point scale (0 = low, 1 = intermediate, 2 = high) by three industry experts. Where rating scores were available for different regions of the fruit surface, they were all averaged. The mean of these scores was used in the subsequent correlative analyses (Table 1).

Cuticle isolation and wax extraction. Cuticular membranes were isolated enzymatically (Orgell, 1955; Yamada et al., 1964). An epidermal disc (24 mm diameter) comprising cuticle, epidermis, hypodermis, and adhering parenchyma was excised from a russet-free, equatorial region of each fruit. The discs were incubated in 50 mM citric acid buffer solution (pH 4.0) containing pectinase [90 $mL \cdot L^{-1}$ (Panzym Super E flüssig; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark)] and cellulase [5 $mL \cdot L^{-1}$ (Cellubrix L.; Novozymes A/S)]. Sodium azide (30 mM) was added to prevent microbial growth. Enzyme solutions were refreshed periodically over a period of 30 d until CMs separated from the underlying tissues. Isolated CMs were rinsed thoroughly with deionized water, dried on Teflon sheets, and held at room temperature (22 °C and 50% relative humidity). Dewaxed CMs were prepared by Soxhlet extraction of cuticular wax using chloroform/methanol (1:1 v/v, 50 °C)

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Table 1. Russeting susceptibility of 22 apple cultivars, mass of cuticular membrane (CM), dewaxed cuticular membrane (DCM) and wax, calculated wax content of CM, and the release of biaxial strain in the CM on wax extraction.[z]

Cultivars	Russet susceptibility score	Mass per unit area ($g \cdot m^{-2}$)			Wax content	Biaxial strain release
		CM	DCM	Wax	(%)	(%)
Boskoop Green	1.9	29.8 ± 0.6	17.1 ± 0.3	12.7 ± 0.2	42.6 ± 0.2	13.8 ± 1.0
Boskoop Red	1.8	29.9 ± 0.6	17.1 ± 0.3	12.7 ± 0.2	42.6 ± 0.2	18.3 ± 0.7
Braeburn	0.0	23.1 ± 0.5	12.0 ± 0.2	11.1 ± 0.2	47.9 ± 0.2	19.1 ± 0.9
Egremont Russet	2.0	29.2 ± 1.6	19.2 ± 1.2	10.0 ± 0.4	34.5 ± 0.6	14.5 ± 1.0
Elshof	0.9	25.2 ± 0.3	14.2 ± 0.2	11.0 ± 0.1	43.5 ± 0.2	14.4 ± 0.9
Elstar	0.9	25.8 ± 0.1	14.6 ± 0.1	11.2 ± 0.1	43.3 ± 0.3	13.6 ± 0.6
Elstar Boerekamp	0.8	25.9 ± 0.5	14.8 ± 0.4	11.1 ± 0.2	42.8 ± 0.5	15.4 ± 0.7
Gala	0.7	22.6 ± 0.4	11.7 ± 0.2	10.9 ± 0.2	48.2 ± 0.2	18.7 ± 0.9
Golden D. Klon B	1.2	30.1 ± 0.4	17.9 ± 0.3	12.2 ± 0.3	40.4 ± 0.7	12.7 ± 0.6
Golden D. Reinders	0.8	36.2 ± 0.9	21.4 ± 0.5	14.8 ± 0.4	40.8 ± 0.2	15.2 ± 0.8
Granny Smith	0.3	22.1 ± 0.4	11.1 ± 0.2	11.0 ± 0.3	49.7 ± 0.3	11.9 ± 0.4
Holsteiner Cox	1.6	24.8 ± 0.2	12.8 ± 0.1	12.0 ± 0.04	48.4 ± 0.2	15.0 ± 0.5
Idared	0.4	20.3 ± 0.3	11.3 ± 0.1	8.9 ± 0.2	44.1 ± 0.2	9.6 ± 0.9
Inacox	0.9	25.9 ± 0.3	13.5 ± 0.2	12.4 ± 0.2	47.8 ± 0.5	19.1 ± 0.8
Jonagold	0.4	28.2 ± 0.4	15.2 ± 0.2	13.1 ± 0.2	46.3 ± 0.2	15.2 ± 0.9
Karmijn	1.8	25.0 ± 0.1	13.0 ± 0.2	12.0 ± 0.1	48.0 ± 0.6	20.4 ± 1.1
Marina	0.1	23.2 ± 0.2	12.9 ± 0.1	10.3 ± 0.1	44.4 ± 0.3	13.6 ± 0.7
Nicoter	0.4	28.2 ± 0.5	14.8 ± 0.3	13.4 ± 0.2	47.4 ± 0.2	15.2 ± 0.9
Pinova	1.1	25.8 ± 0.5	12.8 ± 0.3	13.1 ± 0.2	50.6 ± 0.3	23.4 ± 1.2
Rubinette	0.8	30.0 ± 0.3	15.9 ± 0.2	14.1 ± 0.1	47.0 ± 0.2	20.5 ± 0.8
Starking	0.1	30.9 ± 0.8	17.8 ± 0.5	13.1 ± 0.3	42.5 ± 0.2	16.1 ± 1.0
Superkalfs	1.6	28.4 ± 0.5	16.2 ± 0.3	12.2 ± 0.2	42.9 ± 0.2	15.4 ± 1.0
Grand mean		26.7 ± 0.3	14.9 ± 0.2	11.9 ± 0.1	44.6 ± 1.8	16.0 ± 0.2

[z]Russet susceptibility was indexed by averaging three, independent scores (0 = not susceptible, 1 = intermediate, 2 = susceptible) offered by three industry specialists. The release of biaxial strain was indexed by the fractional decrease in area of the CM disc on wax extraction. For further details, see text.

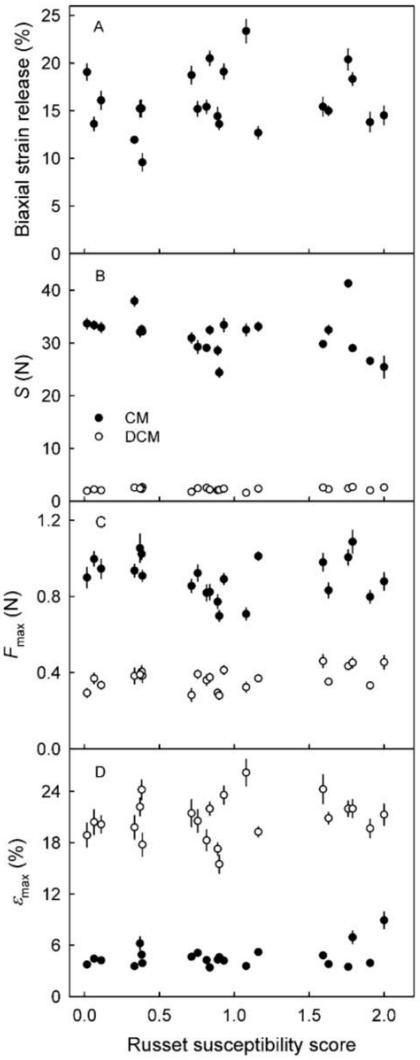

Fig. 1. Mechanical properties of isolated cuticular membranes (CMs) of 22 apple cultivars in relation to their russet susceptibility. Russet susceptibility was indexed by averaging three, independent scores (0 = not susceptible, 1 = intermediate, 2 = susceptible) offered by three industry specialists. Release of biaxial strain in the CM on wax extraction vs. russet susceptibility score (**A**). Stiffness (*S*; **B**), maximum force (F_{max}; **C**), and strain at maximum force (ε_{max}; **D**) of CM and dewaxed CM (DCM) of 22 apple cultivars vs. russet susceptibility score.

for 2.5 h. Cuticle mass and wax mass per unit area were established gravimetrically on an analytical balance (CPA2P Sartorius AG Germany) with five replications, where one replicate comprised five pooled CM or DCM discs (24 mm diameter).

Strain relaxation assay. The release of biaxial strain ($\varepsilon_{biaxial}$) from CMs after wax extraction was quantified as described previously (Khanal et al., 2013a). Briefly, a square pattern of four holes (≈3.25 mm × 3.25 mm, hole diameter ≈0.5 mm) was punched in untreated CM discs (8 mm diameter). The area enclosed by the square pattern was quantified both before (A_{CM}) and after (A_{DCM}) wax extraction using calibrated digital images taken at ×1.25 magnification using a dissecting microscope (MZ10F; Leica Microsysteme GmbH, Wetzlar, Germany; camera DP71; Olympus) and image analysis (Software Cell^P; Olympus). The release of biaxial strain ($\varepsilon_{biaxial}$; %) was calculated as:

$$\varepsilon_{biaxial} = \frac{A_{CM} - A_{DCM}}{A_{DCM}} \times 100 \qquad (1)$$

Tensile test. Strips (5 mm wide) were excised from CM or DCM discs (24 mm diameter) using parallel razor blades. The strips were then stabilized by mounting in a frame made from paper and masking tape (Tesa Krepp[R]; tesa Werk Hamburg GmbH, Hamburg, Germany) to avoid imposing unintentional strain before the tensile tests were initiated. Specimens were fully hydrated by incubating in deionized water at 22 °C for 16 h. Subsequently, frames were mounted between the clamps of a universal material testing machine (Z 0.5; Zwick Roell, Ulm, Germany). The distance between the clamps (L_0) was 10 mm. Paper frames were then cut open and the tensile tests initiated (crosshead speed 1 $mm \cdot min^{-1}$) until failure occurred. The forces applied (10 N, standard force transducer) and the corresponding specimen lengths (L) were recorded. The F_{max} (Newtons) that closely resembled the force at failure was obtained from the recorded data. Strain (%) was calculated as:

$$\varepsilon_{max} = \frac{L - L_0}{L_0} \times 100 \qquad (2)$$

This ε_{max} correspond to the strain at F_{max}. Stiffness (Newtons) was determined as the maximum slope of a linear regression fitted through a plot of force (Newtons) vs. fractional strain (percent/100). Data for specimens that failed in, or adjacent to, the clamps were excluded from the analyses. Also excluded were any specimens that exhibited irregular force-vs.-displacement curves. The excluded results were ≈17% of the total population. The number of replicates ranged from 10 to 15.

Data analyses. The *S* (Newtons) we refer to throughout this article reflects the properties of the CM with or without wax. It differs from the modulus of elasticity (*E*; MPa) commonly used in engineering in that it is not based on the sample cross-sectional area but on the sample width (sample thickness is undefined). Data were subjected to correlation (Proc Corr) and regression analysis (Proc Reg) using SAS (Version 9.1.3; SAS Institute, Cary, NC). The data in the figure and tables are presented as means and SEMs. Where not shown, the error bars are smaller than the symbols.

Results

Mass per unit area of CM, DCM, and wax varied significantly among the cultivars (Table 1). CM, DCM, and wax mass per unit area were lowest in 'Idared' and highest in 'Golden Delicious Reinders'. The wax content was lowest in 'Egremont Russet' and highest in 'Pinova'. Across all cultivars, wax mass per unit area was linearly related to CM mass per unit area ($R = 0.77$, $P < 0.0001$). In all cultivars, wax extraction released biaxial strain in the CM. The release of biaxial strain was lowest in 'Idared' and highest in 'Pinova'. There was no significant (linear) relationship between russet susceptibility and cuticle mass

Table 2. Relationships among selected physical properties of cuticular membrane (CM), dewaxed cuticular membrane (DCM), and the change in physical properties on wax extraction (CM-DCM) and the russet susceptibility of 22 selected apple cultivars.[z]

		Stiffness (S; N)			Maximum force (F_{max}; N)			Strain at maximum force (ε_{max}; %)		
	Biaxial strain release (%)	CM	DCM	CM-DCM	CM	DCM	CM-DCM	CM	DCM	CM-DCM
Russet score	0.15	–0.27	0.21	–0.29	–0.07	0.31	–0.32	0.33	0.12	0.03
CM mass (g·m^{-2})	0.11	–0.35	0.08	–0.36	0.19	0.17	0.12	0.39	0.11	0.07
DCM mass (g·m^{-2})	–0.09	–0.49*	0.21	–0.51*	0.18	0.22	0.07	0.55	–0.04	0.27
Wax mass (g·m^{-2})	0.45*	0.06	–0.20	0.07	0.14	0.01	0.18	–0.06	0.36	–0.36
Wax content (%)	0.45*	0.64**	–0.40	0.68***	–0.10	–0.25	0.06	–0.68	0.33	–0.59**

[z]Relationships were indexed by Pearson's coefficients of correlation. Russet susceptibility was indexed by averaging three, independent scores (0 = not susceptible, 1 = intermediate, 2 = susceptible) offered by three industry specialists.
Significance of Pearson's correlation coefficient (R) for *P < 0.05, **< 0.01, and ***< 0.001.

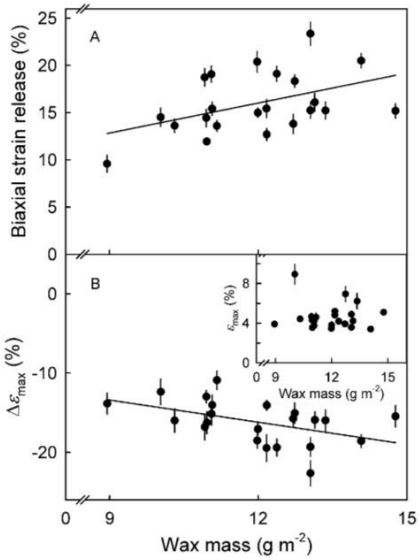

Fig. 2. Effect of wax on the mechanical properties of cuticular membranes (CMs) isolated from a number of apple cultivars selected for their wide-ranging susceptibilities to russet. Release of biaxial strain in CM on wax extraction as a function of the mass of wax extracted (**A**). Change in maximum strain ($\Delta\varepsilon_{max}$; **B**) of the CM on wax extraction in relation to the mass of wax extracted per unit area. ε_{max} of the CM as a function of the mass of wax extracted (*B*; inset).

Table 3. Mechanical properties of fruit cuticular membrane (CM) and dewaxed cuticular membrane (DCM) isolated from various apple cultivars selected for a maximum range of russet sensitivity.[z]

	S (N)		F_{max}(N)		ε_{max}(%)	
Cultivars	CM	DCM	CM	DCM	CM	DCM
Boskoop Green	26.6 ± 0.7	2.1 ± 0.1	0.80 ± 0.03	0.33 ± 0.02	3.9 ± 0.2	19.7 ± 1.1
Boskoop Red	29.0 ± 0.6	2.7 ± 0.1	1.09 ± 0.06	0.45 ± 0.03	6.9 ± 0.7	22.0 ± 1.1
Braeburn	33.7 ± 1.0	2.0 ± 0.1	0.90 ± 0.05	0.29 ± 0.02	3.8 ± 0.3	18.9 ± 1.4
Egremont Russet	25.5 ± 2.1	2.6 ± 0.2	0.88 ± 0.05	0.45 ± 0.04	8.9 ± 1.0	21.3 ± 1.3
Elshof	28.6 ± 0.9	2.1 ± 0.1	0.77 ± 0.04	0.29 ± 0.02	4.3 ± 0.3	17.3 ± 0.7
Elstar	24.4 ± 0.9	2.1 ± 0.1	0.70 ± 0.03	0.28 ± 0.02	4.6 ± 0.4	15.5 ± 1.1
Elstar Boerekamp	29.1 ± 0.7	2.5 ± 0.1	0.82 ± 0.04	0.36 ± 0.03	4.3 ± 0.4	18.3 ± 1.2
Gala	30.9 ± 1.0	1.8 ± 0.2	0.86 ± 0.03	0.28 ± 0.03	4.7 ± 0.4	21.5 ± 1.6
Golden D. Klon B	33.1 ± 0.9	2.4 ± 0.1	1.01 ± 0.02	0.37 ± 0.02	5.2 ± 0.2	19.3 ± 0.6
Golden D. Reinders	29.3 ± 1.3	2.5 ± 0.1	0.92 ± 0.04	0.39 ± 0.02	5.1 ± 0.2	20.6 ± 1.3
Granny Smith	38.0 ± 0.9	2.6 ± 0.2	0.94 ± 0.03	0.38 ± 0.04	3.6 ± 0.2	19.8 ± 1.4
Holsteiner Cox	32.5 ± 0.9	2.3 ± 0.1	0.83 ± 0.04	0.35 ± 0.02	3.8 ± 0.4	20.9 ± 0.7
Idared	32.2 ± 0.4	2.7 ± 0.1	0.91 ± 0.03	0.38 ± 0.04	3.9 ± 0.2	17.8 ± 1.3
Inacox	33.4 ± 1.3	2.4 ± 0.1	0.89 ± 0.03	0.41 ± 0.02	4.2 ± 0.3	23.6 ± 1.1
Jonagold	32.6 ± 0.8	2.3 ± 0.1	1.02 ± 0.03	0.41 ± 0.03	4.9 ± 0.2	24.2 ± 1.2
Karmijn	41.3 ± 0.7	2.4 ± 0.1	1.01 ± 0.04	0.43 ± 0.02	3.5 ± 0.2	22.0 ± 0.9
Marina	33.4 ± 0.8	2.3 ± 0.1	1.00 ± 0.04	0.37 ± 0.03	4.4 ± 0.3	20.4 ± 1.5
Nicoter	32.1 ± 0.9	2.4 ± 0.1	1.05 ± 0.08	0.39 ± 0.03	6.2 ± 0.8	22.2 ± 1.1
Pinova	32.5 ± 1.1	1.6 ± 0.1	0.71 ± 0.03	0.32 ± 0.03	3.6 ± 0.3	26.2 ± 1.6
Rubinette	32.5 ± 0.8	2.2 ± 0.1	0.82 ± 0.04	0.37 ± 0.02	3.4 ± 0.2	22.0 ± 0.8
Starking	32.9 ± 1.0	2.1 ± 0.1	0.94 ± 0.05	0.33 ± 0.02	4.2 ± 0.3	20.1 ± 1.0
Superkalfs	29.8 ± 0.5	2.6 ± 0.1	0.98 ± 0.05	0.46 ± 0.04	4.8 ± 0.3	24.3 ± 1.7
Grand mean	31.6 ± 0.3	2.3 ± 0.03	0.90 ± 0.01	0.37 ± 0.01	4.6 ± 0.1	20.8 ± 0.3

[z]Maximum force (F_{max}), strain at maximum force (ε_{max}), and stiffness (S) were quantified from uniaxial tensile test using fully hydrated specimens.

(R = 0.29; P = 0.18) or wax mass (R = 0.09; P = 0.70) or the release of biaxial strain (Fig. 1A; Table 2). The release of biaxial strain was linearly and positively related to wax mass per unit area (Fig. 2A; R^2 = 0.21, P = 0.033).

The S established in uniaxial tensile tests ranged from a low of 24.4 ± 0.9 N for 'Elstar' CM to a high of 41.3 ± 0.7 N for 'Karmijn' CM. It ranged from a low of 1.6 ± 0.1 N for 'Pinova' DCM to a high of 2.7 ± 0.1 N for 'Idared' DCM (Table 3). Extraction of cuticular wax markedly decreases the S of the cuticle in all cultivars (Table 3). However, neither the value taken by S nor the decrease in S on wax extraction was significantly related to russet susceptibility (Fig. 1B; Table 2).

The F_{max} values of the CMs and DCMs ranged from 0.70 ± 0.03 N (CM; 'Elstar') to 1.09 ± 0.06 N (CM; 'Boskoop Red') and from 0.28 ± 0.02 N (DCM; 'Elstar') to 0.46 ± 0.04 N (DCM; 'Superkalfs'). Wax extraction usually decreased F_{max} by approximately half (Table 3). There was no significant relationship between F_{max} or the change in F_{max} on wax extraction and russet susceptibility (Fig. 1C; Table 2).

The ε_{max} values ranged from 3.4% ± 0.2% (CM; 'Rubinette') to 8.9% ± 1.0% (CM; 'Egremont Russet') and from 15.5% ± 1.1% (DCM; 'Elstar') to 26.2% ± 1.6% (DCM; 'Pinova'). On average, wax extraction increased ε_{max} ≈5-fold (Table 3). Neither ε_{max} nor the change in ε_{max} on wax extraction were significantly related to russet susceptibility (Fig. 1D; Table 2). The increase in ε_{max} on wax extraction was significantly greater for those cultivars that had more wax per unit area in their CM (Fig. 2B) (R^2 = 0.23; P = 0.026). However, there was no significant relationship between ε_{max} and the amount of wax (Fig. 2B, inset).

Discussion

There were no significant relationships between a cultivar's russet susceptibility and any of the characteristics of their CM's investigated at maturity. It is therefore reasonable to infer that factors other than the CM's mechanical properties reported here dominate in determining russeting susceptibility among the 22 apple cultivars examined here. Alternatively, changes in CM properties that have occurred between near bloom and fruit maturity may have masked their relation to russet formation. However, at present there is no evidence for this being the case. Several conclusions emerge.

First, it would seem reasonable to infer that the cuticle is not the load-bearing structure of an apple fruit skin. Instead, load bearing would seem to be sustained primarily within the epidermal and hypodermal cell layers (Khanal et al., 2013b). It can be speculated that if there were any periclinal variations in the efficacy of the structural support of the CM afforded by the underlying epidermal and hypodermal layers, then this might cause stress concentration and thus premature failure of the CM in the less well-supported zones. This scenario might occur more frequently in the russet-susceptible cultivars. It may also be speculated that such variation could arise from changes in the orientation of the cellulose fibrils in the cell walls of the periclinal surfaces of the epidermal cell walls or from the presence of a viscous, pectic middle lamellae between abutting anticlinal epidermal cell walls. These are oriented

perpendicular to the direction of maximum tension. Also, epidermal and hypodermal cells are known to elongate during fruit growth (Meyer, 1944). Maguire (1998) hypothesized that during the periclinal elongation of apple's epidermal cells, abutting portions of two epidermal cells' anticlinal walls peel apart and reorientate to form extensions of their periclinal walls. This would likely focus strain in the CM at a point immediately above the anticlinal epidermal cell walls (Maguire, 1998). These observations are consistent with the characteristic pattern of cuticular cracks in apple fruit often observed in SEM studies (Curry, 2008; Roy et al., 1999). To our knowledge, there is no quantitative information available on any of these properties relating to apple cultivars of differing russet susceptibility.

Second, the apple cuticle may itself be mechanically non-homogeneous. For example, cuticle thickness is variable over the surface of a fruit and especially between cultivars. In some cultivars, cuticles even encase one or several cell layers (Meyer, 1944; Miller, 1982). Also, microcracks occur in the apple cuticle and these are likely to alter the mechanical properties of the isolated CM (Knoche and Grimm, 2008; Knoche et al., 2011; Maguire et al.; 1999).

Third, polysaccharides such as cellulose and pectins are also constituents of cuticles (Schreiber and Schönherr, 1990) and thus are potentially important covariables that may determine the rheological properties of the cuticle (Dominguez et al., 2011; Lopéz-Casado et al., 2007). If any of these factors varied among the apple cultivars investigated here, this might have weakened any relationships between the CM's mechanical properties and their russet susceptibility.

Fourth, in this study, we have focused on measurements made on CMs at fruit maturity, whereas it is during the first 4 weeks after full bloom that conditions are considered to be the most important for russeting (Knoche et al., 2011; Wertheim, 1982).

Finally, the effect of wax on russeting may, itself, be more complex and its influence not restricted to altered mechanical properties. For example, there is evidence that wax is deposited in the cracks of the cuticles (Curry, 2008; Roy et al., 1999). This is not considered unlikely, because the diffusion resistance experienced by wax constituents migrating from their site of synthesis (in the epidermal cells) to the outer surface of the cuticle would presumably be least over the shorter distance to the base of a crack. This being the case, the natural "filling" or "healing" of any cuticular cracks might keep pace with their rate of formation with the result that a periderm need not be formed and the temporary cuticular defect (microcrack) might pass unnoticed having no further consequence.

The effect of wax on the mechanical properties of the apple fruit CM was consistent among the cultivars investigated. In all 22 cultivars, wax extraction 1) released the biaxial strain in the CM; 2) it decreased S; 3) it decreased F_{max}; and 4) it increased ε_{max}. These changes are also consistent with previous observations (Khanal et al., 2013a), in which the release of biaxial strain in the CM and the change in ε_{max} on wax extraction were both related to the amount of wax. Compared with the CM of tomato fruit (Lopéz-Casado et al., 2007; Matas et al., 2004; Petracek and Bukovac, 1995), that of apple was thicker and stiffer, and fracture force was lower.

In summary, from our results, there is no indication of a simple relationship between an apple cultivar's russet susceptibility and the mechanical properties of its fruit cuticle at maturity and the effects of wax thereon.

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6. Mechanical Properties of Apple Skin Are Determined by Epidermis and Hypodermis

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Mechanical Properties of Apple Skin Are Determined by Epidermis and Hypodermis

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Additional index words. cracking, cuticle, extensibility, fracture, *Malus ×domestica*, stiffness, strain, stress

Abstract. Mechanical failure of the fruit skin is an early event in the etiology of the disorders russeting and skin spots in a number of apple cultivars including 'Elstar' (*Malus ×domestica* Borkh.). The objective was to quantify the mechanical properties of excised epidermal segments (ES) of fruit skin and of enzymatically isolated cuticular membranes (CM) using uniaxial tensile tests. ES thickness ranged from 0.25 to 1.8 mm because thin ES samples of more uniform thickness are difficult to prepare. Sample values for stiffness (S), maximum force (F_{max}) and strain at F_{max} (ε_{max}) were recorded. Measured values were adjusted by regression to refer to a hypothetical standard ES of 0.5 mm thickness. Generally, S and F_{max} values were positively related to ES thickness during the preharvest period from 51 to 141 days after full bloom (DAFB) and during the postharvest period from 141 to 259 DAFB in cold storage (1.7 °C, 92% relative humidity). The ε_{max} recorded were independent of ES thickness. The S of a standardized ES decreased slightly from 51 to 90 DAFB, then increased up to 161 DAFB, and then declined. There were essentially no differences in S recorded for isolated CM and ES. The F_{max} and ε_{max} were highest in young fruit at 51 DAFB but decreased steadily toward harvest and continued to decrease in cold storage after harvest but at a lower rate. The F_{max} and ε_{max} were markedly lower for CM samples than for ES ones. Monitoring the increased incidence of CM microcracking during a tensile test performed on an ES revealed that CM failure preceded ES failure. The decrease in the F_{max} for ES during fruit development was accounted for in part by a decrease in the mass of cell wall per unit surface area. Our results show that the epidermal and hypodermal cell layers represent the structural backbone of an apple skin during pre- and postharvest development. Furthermore, CM microcracking has limited relevance to the overall mechanical properties of the skin.

Appearance is an important quality attribute in fresh fruit with cosmetic blemishes significantly reducing their value, especially in the premium export market. In apple, commercially relevant examples include the skin disorders russeting (Khanal et al., 2013a) and skin spots (Grimm et al., 2012a). The etiology of both of these disorders involves mechanical failure of the fruit skin with cuticular microcracking being the first detectable symptom (Faust and Shear, 1972a, 1972b; Grimm et al., 2012a). Apples are considered to be susceptible to russeting early in their development (Knoche et al., 2011; Wertheim, 1982), whereas the sensitive period for skin spots is late in development (Grimm et al., 2012a).

A fruit skin is a composite material comprising both cellular and polymeric components. The cuticle is the outermost layer. It is a lipophilic polymer film with cutin and wax as the major constituents (Dominguez et al., 2011a; Heredia, 2003). The cuticle functions as a protective barrier in water transport, gas exchange, and pathogen defense (Dominguez et al., 2011b; Jeffree, 1996; Kerstiens, 1996; Riederer and Schreiber, 2001). The cuticle is deposited on the outer cell wall of the epidermis and also impregnates inner epidermal and, possibly, hypodermal cell walls. In some cases, entire epidermal cells are encased (Meyer, 1944; Miller, 1982). The epidermis is supported by one or several layers of collenchymatous, hypodermal cells (Evert, 2006).

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During growth, the skin composite is subjected to marked strain and stress (Skene, 1982). The cellular components of the skin accommodate the strain by cell division and cell enlargement. In addition, the epidermal and/or hypodermal cells flatten as indicated by increases in their length (periclinal) to thickness (anticlinal) ratio (Maguire, 1998; Meyer, 1944; Tukey and Young, 1942). The situation is more complex for the cuticle polymer that is deposited on these cell layers. In the fruit of some species [e.g., sweet cherry (*Prunus avium* L.)], cuticle deposition is limited to the early developmental period so that the expansion of the fruit surface later in development causes significant reversible (elastic and viscoelastic) and irreversible (plastic) straining of the skin and even more so of the cuticle (Grimm et al., 2012b; Knoche et al., 2004; Knoche and Peschel, 2006). In apple, however, the pattern is different. Throughout fruit development, cuticle material is deposited as the area of the skin surface expands with no apparent buildup of reversible elastic strain (Khanal et al., 2013a, 2013b). Nevertheless, micrographs from scanning electron microscopy (SEM) of apple fruit surfaces often reveal a network of fine cuticular cracks, even in the absence of any russeting (Curry, 2012; Faust and Shear, 1972b; Maguire et al., 1999; Roy et al., 1999). Whether these cracks are mechanically relevant in the development of fruit surface disorders is not yet known. These observations suggest that 1) the appearance of a fruit is closely related to the mechanical properties of its skin; 2) surface area expansion subjects the fruit skin to marked strain; and 3) maintenance of skin integrity is essential if the fruit is to remain blemish-free. Considering the importance of these relationships, surprisingly little is known about the mechanical properties of apple skin.

The objective of this study was to quantify the mechanical properties of the skin (cuticle, epidermis, and hypodermis) of developing and cold-stored apple fruit. We used excised skin samples of developing and cold-stored 'Elstar' apples as a model system because 1) 'Elstar' is susceptible to both russeting and skin spots; 2) susceptibility to both disorders changes in the course of fruit development; and 3) mechanical failure of the skin is considered to be the first detectable symptom in both disorders (Faust and Shear, 1972a, 1972b; Grimm et al., 2012a).

Materials and Methods

Plant materials. Fruit of 'Elstar' apples were randomly sampled from the block of 50 trees (two to three fruit per tree per side of row) grafted on 'M.9' rootstocks growing in an experimental orchard of the Leibniz University at Sarstedt, Germany (lat. 52°14′ N, long. 9°49′ E) and managed according to the European Union regulations for integrated fruit production. Fruit were sampled periodically from 51 d after full bloom (DAFB) onward. At this stage, fruit diameter averaged ≈40 mm, which is the minimum size needed for preparation of epidermal segments (ES) of the fruit skin for tensile testing. At 141 DAFB when fruit were commercially mature, all fruit were harvested and put into cold storage [1.7 °C, 92% relative humidity (RH)] for up to 118 d (equivalent to 259 DAFB). At selected time intervals, fruit were picked in the field, transferred to the laboratory, and either processed fresh or held at 2 °C for no longer than 24 h. Fruit sampled from storage were always processed on the day of sampling.

Preparation of ES and isolation of CM. Preliminary experiments established that the mechanical properties of ES did not differ when excised in longitudinal {i.e., parallel to the calyx/pedicel axis [maximum force (F_{max}) 2.8 ± 0.1 N, strain at F_{max} (ε_{max}) 11.5% ± 0.6% (mean ± SE)]} or latitudinal direction [i.e., perpendicular to the calyx/pedicel axis (F_{max} 2.7 ± 0.2 N, ε_{max} 12.4% ± 0.5%)]. Thus, the ES were judged to be isotropic in the plane of the fruit surface (B.P. Khanal, unpublished data). The ε_{max} for ES from the blush surface were 11.0% ± 0.4% at an F_{max} of 2.6 ± 0.1 N, whereas the same values for the non-blush surface were 10.8% ± 0.5% (similar) and 2.1 ± 0.1 N (lower). To minimize variation, ES were always excised longitudinally from the non-blush surface. Because of its reduced curvature, ES were taken from the equatorial region using a custom-made punch that produces a biconcave (dumbbell-shaped) specimen with 4.25-mm waist width and 30- to 40-mm length. Because we found it impossible to reproducibly prepare thin ES of preset thickness, ES of varying thickness were cut using a razor blade. ES thickness was subsequently determined by image analysis (software package Cell-P; Olympus Europa, Hamburg, Germany) from calibrated images prepared under a binocular microscope. To minimize artifacts resulting from transpiration of the ES, tensile tests were performed and completed within 3 min of excision and preparation of the ES.

Cuticular membranes were isolated from ES excised as described previously by incubating in 50 mM citric acid buffer solution (pH 4.0) containing 90 mL·L^{-1} pectinase (Panzym Super E flüssig; Novozymes, Bagsvaerd, Denmark), 5 mL·L^{-1} cellulase (Cellubrix L; Novozymes), and NaN_3 at 30 mM (Orgell, 1955; Yamada et al., 1964). The isolation medium was refreshed periodically until the CMs separated from adhering cell layers. CMs were thoroughly rinsed with deionized water, dried at room temperature, and stored under laboratory conditions (≈22 °C, 50% RH).

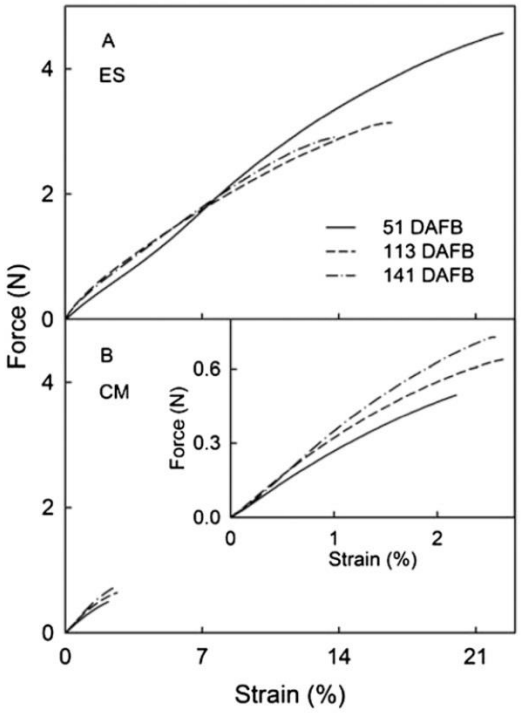

Fig. 1. Representative force/strain diagrams of excised epidermal segments (ES) of the fruit skin (**A**) and of enzymatically isolated cuticular membranes (CM) (**B**) subjected to uniaxial tensile tests. Force/strain diagram of CM redrawn at a different scale [**B** (inset)]. ES were excised in the longitudinal direction, parallel to the pedicel–calyx axis, from the equatorial region of 'Elstar' apple fruit at 51, 113, and 141 d after full bloom (DAFB).

Tensile test. Without further handling, ES were mounted between the clamps of a material testing machine (Z 0.5 and KAP-TC 50-N force transducer; Zwick/Roell, Ulm, Germany). The isolated CMs were mounted in a frame of paper and masking tape to prevent unintentional strain and damage during handling. The frames with CM strips were hydrated by incubating in deionized water for 16 h and thereafter mounted between the clamps of the material testing machine (Z 0.5). The distance between the clamps corresponding to the free length of the specimen was 16 mm with the position of the waist arranged in the center between the clamps. An uniaxial tensile force was applied at a constant strain rate of 3 mm·min^{-1} until failure of the ES or CM. Applied force and crosshead travel (in millimeters) were recorded and maximum force F_{max} (in Newtons) and travel at F_{max} (in millimeters) were extracted from the data and the strain at F_{max} (ε_{max}) was calculated (Khanal et al., 2013a). Stiffness (S in Newtons) was calculated as the maximum slope of the force (Newtons) vs. fractional strain (in percent per 100) relationship. Like the commonly used modulus of elasticity in material science [E (in megapascals)], this S is a measure of the elasticity of the sample. However, unlike E, S reflects the properties of the specimens as excised from the surface because it is not corrected for differences in sample thickness and hence sample cross-sectional area. Furthermore, a meaningful comparison of E would assume isotropic properties of the specimen in all three dimensions. Tensile tests were carried out at ≈22 °C

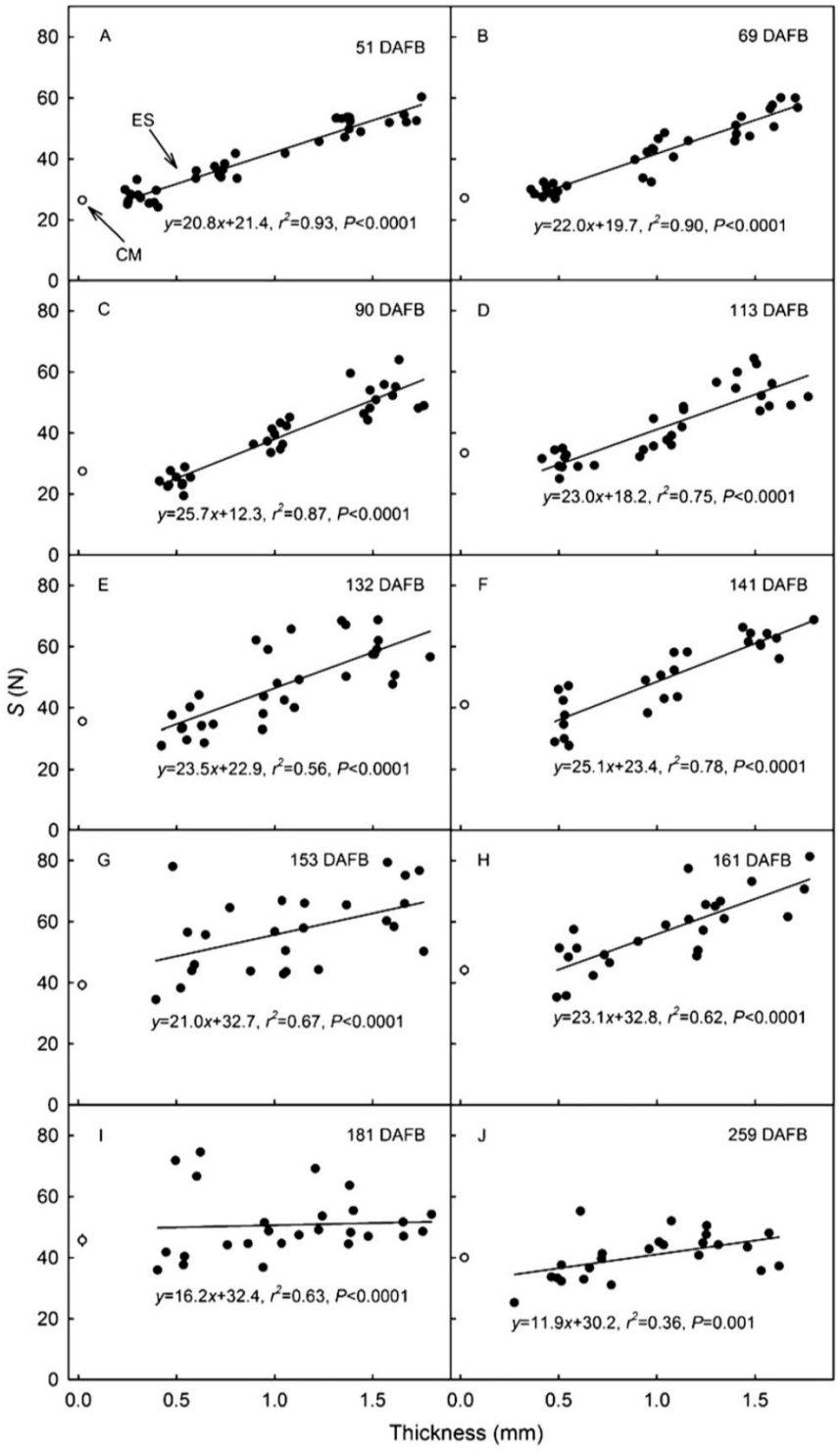

Fig. 2. Stiffness (*S*) of excised epidermal segments (ES) of the fruit skin as a function of their thickness (closed symbols) and of enzymatically isolated cuticular membrane [CM (open symbols)] at various developmental stages of 'Elstar' apple (**A–F**) and various durations of cold storage (1.7 °C, 92% relative humidity) after harvest (**G–J**). Fruit were harvested at 141 d after full bloom (DAFB). The values of *S* of the CM are the means (± SE, n = 10). For ES data point represent individual observations.

and 50% RH on a 23 to 36 ESs and 10 CMs per sampling date. For data analysis, the *S*, F_{max}, and ε_{max} derived for each ES on a given sampling date were plotted against thickness of the same ES and a regression line fitted (see Fig. 1 for an example for F_{max}). From these regression equations, the hypothetical *S*, F_{max}, and ε_{max} for a standard ES of constant thickness of 0.5 mm were calculated and compared through the course of fruit development and subsequent storage (see Fig. 3 for an example). This procedure avoids extrapolation beyond the thickness ranges investigated.

Monitoring of CM microcracking during tensile testing. ES samples of ≈0.5 mm thickness were prepared as described previously, mounted in the material testing machine, and subjected to increasing tensile force up to 2.3 N. This tensile force represents 0.75 × F_{max} of the specimens (F_{max} 3.1 N) determined earlier on samples from the same batch of fruit. When 0.75 × F_{max} was reached, the force was held constant ("hold period"), whereas the strain continued to increase. To avoid failure of the ES as a consequence of creep, strain was held constant when ε reached ≈11%, which represents approximately 0.85 × ε_{max} of the specimen (ε_{max} 13%). Two 2-µL droplets of 0.1% aqueous acridine orange fluorescent dye were placed on the outer surface of the strained ES immediately after the beginning of the hold period. Droplets were allowed to rest on the ES for 10 min and were then carefully removed by blotting with soft tissue paper. At this time, the stained ES were allowed to relax again, removed from the testing machine, positioned in a petri dish, and observed using a fluorescence microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Calibrated images were prepared (camera DP 71, Olympus Europa) and the dye-infiltrated area was quantified over a representative fraction (3 mm^2) of the dye droplet contact area using image analysis (software package Cell-P). ES prepared in the same manner, treated with dye solution, but not subjected to tensile forces served as control (n = 15).

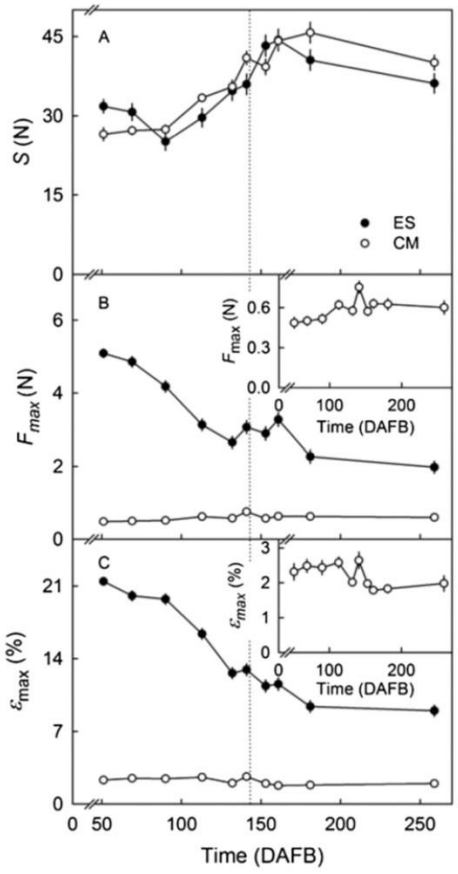

Fig. 3. Developmental time course of change in stiffness (S) (**A**), maximum force (F_{max}) (**B**) and strain at F_{max} (ε_{max}) (**C**) of excised epidermal segments (ES) of the fruit skin and enzymatically isolated cuticular membranes (CM) of developing, mature, and stored 'Elstar' apple fruit. F_{max} [**B** (inset)] and ε_{max} [**C** (inset)] of the CM as a function of time redrawn to a different scale. Fruit were harvested at 141 d after full bloom (DAFB), indicated by the vertical dotted line, and cold-stored (1.7 °C, 92% relative humidity). The values of S, F_{max}, and ε_{max} of the CM are the means (± SE, n = 10). For the ES, the values (means ± SE) of S, F_{max}, and ε_{max} were calculated for a hypothetical ES of 0.5 mm standard thickness using regression equations describing the relationships between S or F_{max} or ε_{max} and ES thickness (n = 23 to 36). For more detail see the "Materials and Methods" section.

Quantifying cell-wall mass. To establish potential relationships between the amount of cell wall adhering to an ES and its mechanical properties, the mass of cell wall per unit area of ES was quantified after the mechanical test. Briefly, the surface of the fractured ES strip was photographed (camera DP 71) under a dissecting microscope (MZ6; Leica Microsystems) and the area quantified by image analysis (software package Cell-P). ES were then dried at 70 °C, weighed, incubated in the enzyme solution used for CM isolation as described previously, and their CM isolated. The epidermis of 'Elstar' apple has irregular-shaped cells and some hypodermal development of the CM. However, epidermal cells are only occasionally encased in the CM (B.P. Khanal, unpublished data). Thus, these cells will be largely accessible for the enzyme solution. After isolation, CMs were then thoroughly rinsed, dried, and reweighed. The mass of cell wall per unit surface area was calculated as the difference between the dry weight of the ES and that of the CM divided by the area of the ES (n = 8 to12).

Compression test. To quantify the softening of fruit during cold storage, tissue cylinders of parenchyma (15 mm diameter, 10 mm height) were prepared at regular time intervals from the same batches of apples used in the tensile tests. The length axis of these cylinders was orientated tangentially relative to the fruit surface. Within 3 min of preparation, the cylinders were subjected to a compression test between two parallel plates using a material testing machine (Z 0.5 and KAF-TC 500-N force transducer; Zwick/Roell). The rate of compression was 3 mm·min^{-1}. Samples were compressed up to the failure point as indicated by a sudden drop in force. The force at failure [F_{max} (in Newtons)] was recorded and the failure stress [σ_{max} (in megapascals)] calculated by dividing F_{max} by the cross-sectional area of the tissue cylinder (n = 10).

Measurement of cell dimensions. Tissue blocks (5 × 5 mm, 1 mm thick) were excised from young (51 DAFB) and mature fruit (141 DAFB) with their 5 × 5-mm face oriented in anticlinal longitudinal (i.e., parallel to the calyx/pedicel axis) or in anticlinal latitudinal (i.e., perpendicular to the calyx/pedicel axis) directions using a razor blade. Cell walls were stained by incubating sections in 0.1% (w/v) aqueous calcofluor white (Fluorescent Brightener 28; Sigma-Aldrich, Steinheim, Germany) for 2 to 5 min. Skin sections were removed from the dye solution, rinsed with deionized water, positioned on a microscope slide, and viewed at ×10 under a fluorescence microscope (BX-60; Olympus Europa). Calibrated images of fruit skin cross-section were taken (camera DP 71) and dimensions of cells analyzed by image analysis (software package Cell-P). Periclinal (parallel to the surface) and anticlinal (perpendicular to the surface) diameters and the distance of the cells from the surface were quantified. Cross-sectional areas of cells were calculated assuming an elliptical shape, the aspect ratios were obtained by dividing the periclinal cell diameter by the respective anticlinal diameter. A minimum of 10 cells was measured on one image per section from a total of 10 fruit.

Data analysis and presentation. Values in Figures 3 and 7 and for the CM in all other figures are presented as means and SEMs. Where error bars are not shown in figures, they were smaller than data symbols. In all other figures the points represent individual ES. Data were subjected to analysis of variance [ANOVA (Proc ANOVA or GLM)] or linear regression analysis (Proc REG) using SAS (Version 9.1.3; SAS Institute, Cary, NC). Percentage strain data were arcsine-transformed before ANOVA. Means were compared using Tukey's Studentized range test ($P < 0.05$).

Results

The force vs. strain diagrams for ES and CM obtained in uniaxial tensile tests of specimens of all three sampling dates were essentially linear up to ≈70% of ε_{max} (Fig. 1A–B). At higher strains, slopes of the force strain diagrams slightly decreased. No discontinuities were apparent in the force vs. strain diagrams for fruit skin at any sampling time.

The values of S of the ES were positively, linearly, and significantly related to ES thickness throughout development (Fig. 2A–F) and (after harvest) during the early storage period

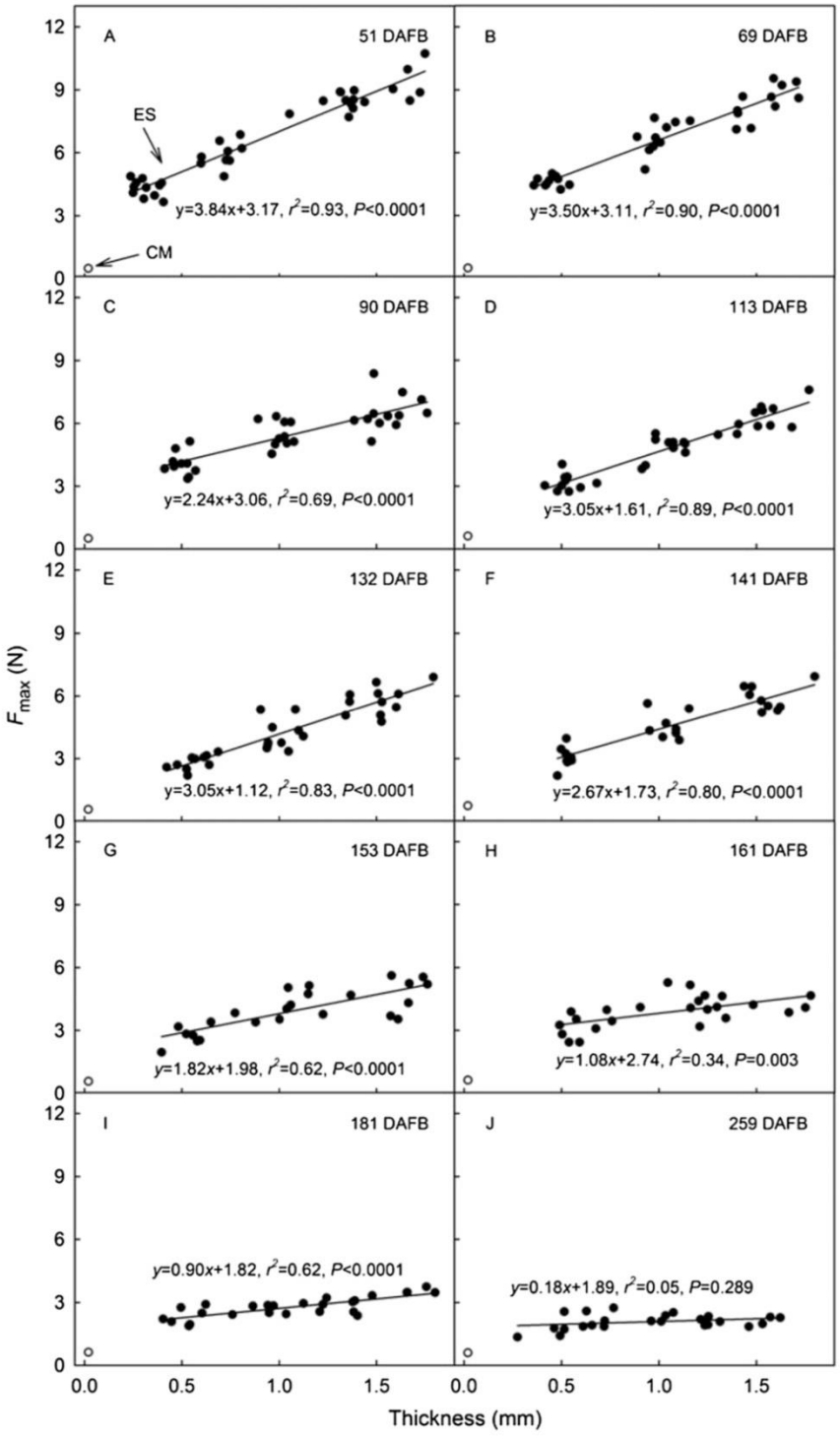

Fig. 4. Maximum force (F_{max}) of excised epidermal segments (ES) of the fruit skin as a function of their thickness (closed symbols) and enzymatically isolated cuticular membrane [CM (open symbols)] at various stages of development (**A–F**) and various durations of cold storage (1.7 °C, 92% relative humidity) of 'Elstar' apple after harvest (**G–J**). Fruit were harvested at 141 d after full bloom (DAFB). The values of F_{max} of the CM are the means (± SE, n = 10). For ES data point represent individual observations.

(Fig. 2G–H). There was little difference in slope among these relationships. In contrast, during the later phase of storage (i.e., at 181 and 259 DAFB), dependence of *S* on thickness decreased and essentially disappeared (Fig. 2I–J). Calculating the value of *S* for a standard ES of 0.5-mm thickness revealed that it decreased over the period 51 to 90 DAFB, then increased up to 161 DAFB, and reached a maximum around the time of harvest before decreasing again thereafter (Fig. 3A). The *S* of the isolated CM was similar to the *S* of the thinnest ES investigated (Fig. 2A–F). Furthermore, the value of *S* for the CM followed a similar temporal pattern as for the ES (Fig. 3A).

The F_{max} of ES increased linearly with ES thickness at all stages of development (Fig. 4A–F). The slope of the regression line of F_{max} vs. thickness remained essentially constant during the preharvest period except for at the 90 DAFB sampling, where the slope was somewhat smaller. However, during subsequent storage, the slope of the regression lines decreased consistently from 1.8 ± 0.3 at 153 DAFB to essentially zero at 259 DAFB (Fig. 4G–J). Calculating the F_{max} of a standard ES (0.5 mm thick) revealed that F_{max} decreased continuously up to approximately maturity. With some variation, the decrease in F_{max} continued in storage at a reduced rate until 259 DAFB (Fig. 3B). The F_{max} of the CM was always markedly lower than that of the standard ES. It increased slightly during development and remained about constant during cold storage [Fig. 3B (inset)].

The ε_{max} was largely independent of the thickness of the ES at all stages of development and throughout the postharvest period as indicated by the consistently low coefficients of determination that only occasionally were significant (Fig. 5A–J). The calculated ε_{max} of an ES of standard thickness (0.5 mm thick) decreased consistently during development, throughout maturation into the postharvest phase (up to 181 DAFB), and then remained constant (Fig. 3C). In contrast, the ε_{max} of the CM was always markedly lower than that of ES. It was

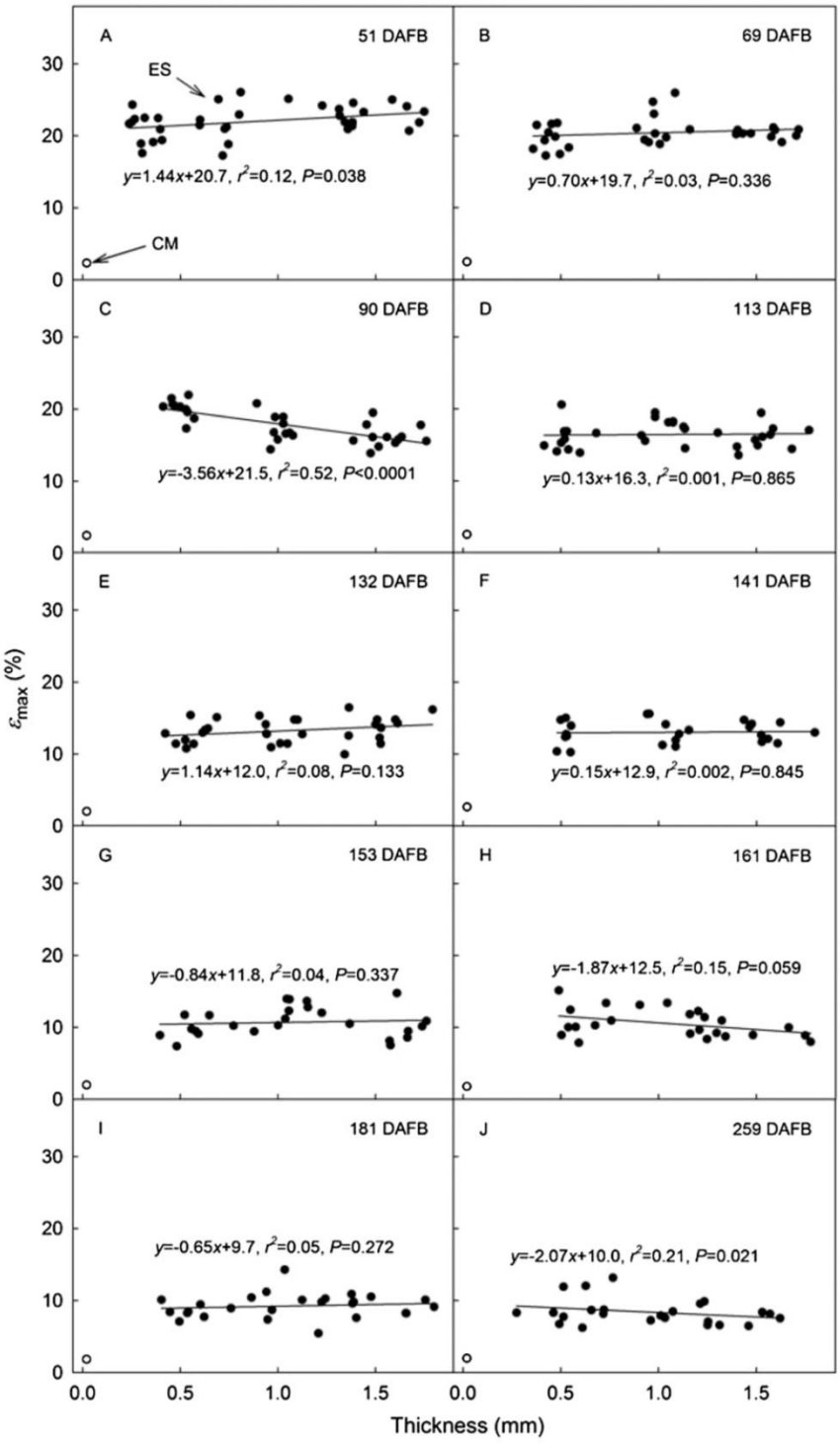

Fig. 5. Strain at maximum force (ε_{max}) of excised epidermal segments (ES) of the fruit skin as a function of thickness (closed symbols) and of enzymatically isolated cuticular membrane [CM (open symbols)] at various stages of development (**A**–F) and after various durations of cold storage (1.7 °C, 92% relative humidity) after harvest (**G**–**J**). 'Elstar' apple fruit were harvested at 141 d after full bloom (DAFB). The values of ε_{max} of the CM are the means (± SE, n = 10). For ES data point represent individual observations.

constant throughout development, decreased slightly at harvest, and remained constant again during storage [Fig. 3C (inset)].

Fluorescence microscopy of ES subjected to 0.75 × F_{max} revealed that the frequency of infiltrated microcracks in the CM of the strained ES increased markedly compared with the unstrained controls (Fig. 6). The percentages of area infiltrated by acridine orange averaged 33.1% ± 5.0% and 0.5% ± 0.2% for strained vs. non-strained ES, respectively. The microcracks on the strained ES were oriented perpendicular to the direction of the applied force (Fig. 6A).

The mass of cell wall material per unit fruit surface area of a standard ES of 0.5-mm thickness decreased linearly throughout development until harvest and remained approximately constant during storage (Fig. 7A). Plotting the mass of cell wall material vs. the F_{max} of the ES revealed a significant and positive relationship for developing fruit where the mass of cell wall material accounted for 57% of the variation in F_{max} (Fig. 7B). In contrast, the mass of CM per unit surface area increased up to approximately harvest and then remained constant [Fig. 7A (inset)]. There was no significant relationship between the mass of CM and its F_{max} [Fig. 7B (inset)]. The F_{max} of parenchyma decreased continuously during the storage period (Fig. 7C).

The cross-sectional area of the skin cells increased continuously from epidermis to hypodermis and into the parenchyma (Figs. 8A and 8C). There were no significant differences in cell cross-sectional areas when sectioned in longitudinal and latitudinal planes either in young or mature fruit. The hypodermal and parenchyma cells of mature fruit were somewhat larger than those of young fruit (Figs. 8A and 8C). The aspect ratios of cells of young fruit increased from epidermis into the hypodermis (down to approximately a 100-µm depth from the surface) but then decreased further into the hypodermis and in the underlying parenchyma tissue (Fig. 8B). In contrast, in mature fruit, aspect ratios of epidermal and hypodermal

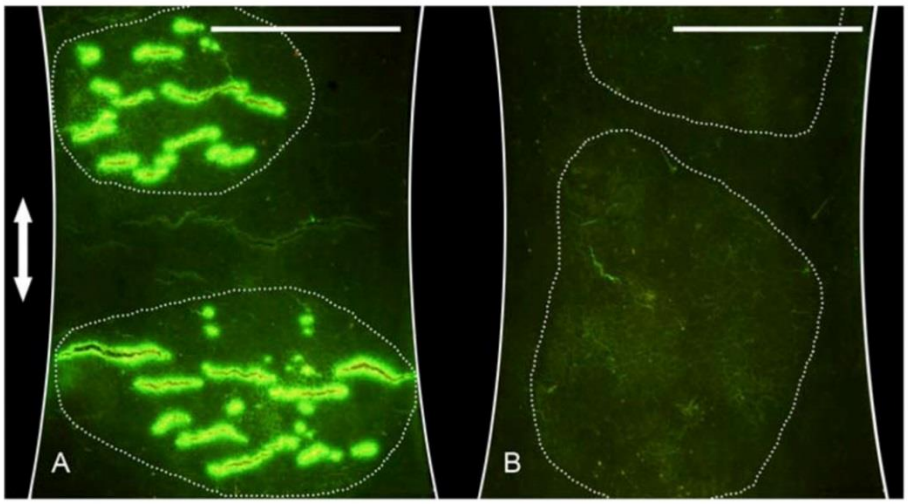

Fig. 6. Fluorescence micrographs of the surface of excised epidermal segments (ES) of the fruit skin of 'Elstar' apple. (**A**) ES subjected to a tensile force equivalent to 0.75 × F_{max} of the specimen. (**B**) Same as in **A**, but the ES not subjected to tensile forces. White lines show contour of the waist region of dumbbell-shaped specimens. The dotted lines show the footprints of the acridine orange droplets (2 µL) applied to the ES surface during the hold period of the tensile test. Arrow indicates the direction of the applied force (scale bars = 2 mm).

cells were highly variable and decreased as the distance from the surface increased (Figs. 8B and 8D). There was no consistent difference in the aspect ratios of cells in longitudinal and in latitudinal planes, either in young or mature fruit (Figs. 8B and 8D).

Discussion

The results presented provide several new and important findings: 1) epidermis and hypodermis represent the mechanical "backbone" of the skin of apple fruit, whereas the mechanical contribution made by the cuticle is small or negligible; 2) the mechanical properties of the skin (S, F_{max}, and ε_{max}) change markedly during preharvest development and, to a lesser extent, during postharvest cold storage; and 3) the ε_{max} of the CM is markedly lower than that of the ES and, therefore, the CM inevitably fails first in response to straining of the skin. This failure results in the formation of microcracks. However, CM failure has no significant mechanical relevance to the overall mechanical properties of the skin composite.

Epidermis and hypodermis as the structural backbone of the fruit skin. That the epidermal and hypodermal cell layers represent the structural backbone as indexed by the F_{max} of the fruit skin is inferred from the following observations: 1) the F_{max} of a standard 0.5-mm-thick ES was 3- to 10-fold higher than the F_{max} of the CM; 2) monitoring the formation of microcracks during a tensile test clearly indicates that the CM fails before the underlying cell layers (epidermis, hypodermis, and parenchyma); 3) the F_{max} of the fruit skin was significantly and positively related to the mass of the cell wall material of the ES. This would be as expected if the cellulosic cell walls were the primary load-bearing structures (Chanliaud et al., 2002; Vincent, 1990, 1999). In fact, the mechanical properties of the CM polymer are expected to be related to the amount of cellulose that remains in the CM after enzymatic isolation (Lopez-Casado et al., 2007); 4) microscopic analysis of cross-sections of the fruit skin revealed that the aspect ratios of the epidermal and hypodermal cells are larger than those of the parenchyma cells. This suggests that larger strains occurred in the epidermal and hypodermal cell layers than in the parenchyma; and 5) the epidermis and hypodermis are collenchymatous as indexed by their thick cell walls (Evert, 2006) suggesting that these layers are to some extent structured to resist mechanical stress. That the contribution of parenchyma to the mechanical characteristics of the whole is small compared with that of the epidermal and hypodermal cell layers is also inferred from the positive y-axis intercepts of the regressions lines relating F_{max} to ES thickness. Also, the slopes of the regression lines are low indicating that varying the thicknesses of the parenchyma in the ES have only small effects on F_{max}. The epidermal and hypodermal cell layers make up ≈300 µm of the skin thickness in a mature 'Elstar' apple (B.P. Khanal, unpublished data). Unfortunately, it is technically impossible to excise ES samples comprising intact epidermis and hypodermis but containing no parenchyma at all. However, from the relationships obtained between F_{max} and ES thickness, we can estimate the F_{max} of a hypothetical 300-µm-thick ES at 2.5 N. This is 83% of the measured F_{max} of a 500-µm-thick (0.5 mm) ES. It is reasonable to infer that the remaining 17% of the F_{max} value was accounted for by a 200-µm thickness of adhering parenchyma. This calculation also reveals that the F_{max} of a 300-µm-thick ES exceeds that of an isolated CM by a factor of ≈3.4.

Change in properties throughout development. During fruit development, the surface area of the skin expands, subjecting it to tangential tensile forces. These forces bring about a continuous increase in strain that becomes constant only at harvest. Skin properties are also subject to continuous change resulting from ongoing cell division and enlargement, the deposition of new cell-wall material, and changes that occur within the cell wall as a result of the activity of cell-wall-modifying enzymes such as expansins, cellulases, and pectinases. The latter are particularly active during the postharvest period. One or, more likely, several of these processes must account for the observed developmental changes in the rheological properties of apple fruit skins.

The developmental increases in S and decreases in ε_{max} and F_{max} may be consequences of increasing strain in the skin during growth. Because there was essentially no relaxation of the fruit skin on excision [grand mean 5% (B.P. Khanal, unpublished data)], any growth strain of the ES must have been irreversible and maintained when ES were excised and

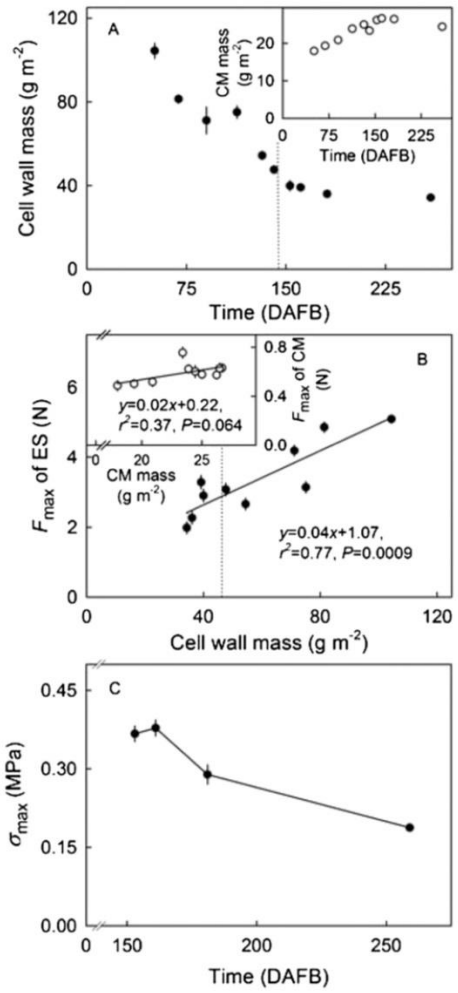

Fig. 7. (**A**) Time course of change in the mass of cell walls per unit surface area of excised epidermal segments (ES) of the fruit skin of 'Elstar' apple (**A**) and in the mass per unit surface area of cuticular membrane {CM [**A** (inset)]}. Cell wall mass was calculated for ES of standard 0.5-mm thickness. Maximum force (F_{max}) of ES was determined in uniaxial tensile tests as a function of cell wall mass per unit surface area (**B**). Maximum force (F_{max}) of the CM as a function of CM mass per unit surface area (**B**, inset). Values right of the dotted line (**A**) and left of the dotted line (**B**) represent samplings taken during cold storage. (**C**) Time course of change in maximum stress (σ_{max}) in the axial direction sustained by tissue cylinders (15 mm diameter, 10 mm high) excised from the fruit flesh of 'Elstar' apple during cold storage (1.7 °C, 92% relative humidity). Time scale in **C** is in days after full bloom (DAFB). Fruit were harvested at 141 DAFB. The value are means ± SE, n = 8 to 12.

subjected to the tensile tests. This "prestrain" may have contributed to the preharvest increase in S and decrease in ε_{max}. The presence of such "prestrain" is inferred from the shape of the epidermal and hypodermal cells that changes from anticlinally elongated to periclinally elongated (Meyer, 1944). The decrease in F_{max} may also be a result of this strain. During expansion, the cell wall material is distributed over an enlarging area. The effect of this (unless a compensatory synthesis of cell wall material occurs) will be a decrease in the mass of cell wall material per unit of skin surface area (Fig. 7A). During the storage period, when surface expansion ceases, the observed decrease in F_{max} as fruit softened was most likely accounted for by an increase in the activity of cell-wall-degrading enzymes (Fig. 7B). Furthermore, the decrease in slope of plots of S or F_{max} vs. ES thickness (Figs. 2F–J and 4F–J) was probably also related to the postharvest fruit softening (Fig. 7C).

Formation of microcracks in the CM. The lower ε_{max} of the CM compared with that of the ES causes the CM to fail when strained, long before the ES ruptures. This can be inferred from the appearance of microcracks in the CM of an ES subjected to a tensile force approaching 75% of its F_{max} (Fig. 6). The microcracks are highly oriented with a maximum incidence in a direction perpendicular to the applied force, which indicates a cause-and-effect relationship. Our observations are consistent with cuticular microcracks observed by SEM in the apple fruit surface (Curry, 2012; Faust and Shear, 1972b; Maguire et al., 1999; Roy et al., 1999). These microcracks are usually limited to the outer layers of the cuticle (Maguire et al., 1999) and do not extend deeply into the epidermal and hypodermal cell layers. As observed in the present study, they are often orientated in line with the anticlinal cell walls of groups of epidermal cells indicating that the pattern of the underlying cell walls affects the pattern of the cracks (Curry, 2012; Maguire et al., 1999). In fact, increased extensibility of the epidermal and, possibly, the hypodermal cell walls in the anticlinal regions might cause a concentration of the strain in these areas to produce this CM failure pattern (Maguire, 1998).

Conclusions

The fruit skin of a developing apple is subjected to continual growth strain in vivo. Because the ε_{max} exceeds the ε_{max} of the CM by almost an order of magnitude, failure of the polymeric CM precedes the failure of the cellular layers of the skin. As a result of the low F_{max} of the CM compared with that of the overall skin composite, the CM microcracking has a limited effect on the mechanical properties of the composite. This logic will not apply to the physiological consequences of an impairment of the CM's barrier properties. So long as the epidermal and hypodermal cells underlying the microcracks remain intact, continuing wax deposition in the CM microcracks will likely re-establish the skin's barrier function. This repair process is also referred to as the "stretching, shearing, and stitching mechanism" (Curry, 2009). When the rate of repair is insufficient and/or when the cracks extend more deeply into the underlying epidermal and/or hypodermal cell layers, we suggest that periderm formation is triggered by an as yet unknown mechanism causing the familiar symptoms referred to as russeting.

The findings presented in this article and the sequence of events outlined are consistent with the previously reported absence of significant relationships between the russeting susceptibilities of particular apple cultivars and the mechanical characteristics of their CM at maturity (Khanal et al., 2013a). Relationships between russet susceptibility and the rate of wax deposition and filling of cuticular cracks or russet susceptibility and the mechanical properties of epidermal and hypodermal cell layers would be more likely. In light of the economic importance of russeting and skin spot disorders, these topics merit more intense study.

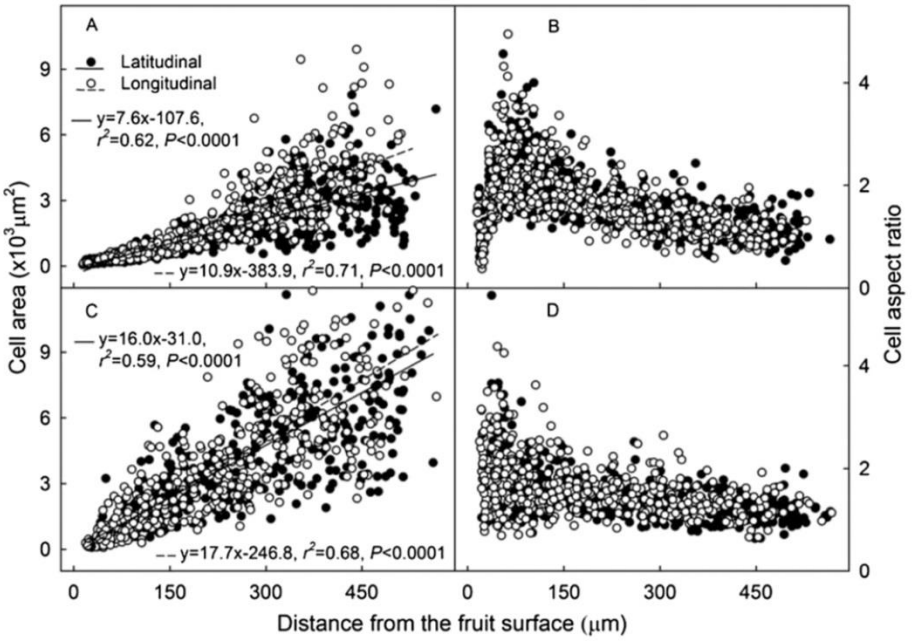

Fig. 8. Projected cross-sectional area (**A, C**) and aspect ratio (periclinal diameter/anticlinal diameter) (**B, D**) of the fruit skin cells of 'Elstar' apple at 51 d after full bloom (DAFB) (**A–B**) and at 141 DAFB (**C–D**) as a function of their depth below the skin surface. Data points represent individual cells.

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7. Russeting in Apple and Pear: A Plastic Periderm Replaces a Stiff Cuticle

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Russeting in apple and pear: a plastic periderm replaces a stiff cuticle

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Abstract

Background and aims Russeting in apples (*Malus* × *domestica* Borkh.) and pears (*Pyrus communis* L.) is a disorder of the fruit skin that results from microscopic cracks in the cuticle and the subsequent formation of a periderm. To better understand russeting, rheological properties of cuticular membranes (CM) and periderm membranes (PM) were studied from the russet-sensitive apple 'Karmijn de Sonnaville' and from 'Conference' pear.

Methodology The CM and PM were isolated enzymatically, investigated by microscopy and subjected to tensile tests, creep/relaxation tests and to stepwise creep tests using a material testing machine.

Principal results The isolated CM formed a continuous polymer, whereas the PM represented a cellular structure of stacked cork cells. Tensile tests revealed higher plasticity of the hydrated PM compared with the CM, as indicated by a higher strain at the maximum force (ε_{max}) and a lower modulus of elasticity (E). In apple, the maximum force (F_{max}) was higher in the CM than in the PM but in pear the higher F_{max} value was found for the PM. In specimens obtained from the CM : PM transition zone, the weak point in apple was found to be at the CM : PM borderline but in pear it was within the CM. In both apple and pear, creep/relaxation tests revealed elastic strain, creep strain, viscoelastic strain and viscous strain components in both the PM and CM. For any particular force, strains were always greater in the PM than in the CM and were also greater in pear than in apple. The ε_{max} and F_{max} values of the CM and PM were lower than those of non-russeted and russeted whole-fruit skin segments, which included adhering tissue.

Conclusions In russeting, stiff CM are replaced by more plastic PM. Further, the cell layers underlying the CM and PM represent the load-bearing structure in the fruit skin in apple and pear.

Keywords: cuticular membrane; fracture; fruit skin; mechanical properties; rheology; russet; strain

Introduction

Russeting is a commercially important surface defect in many fruit crops including apples and pears, with russeted fruit often having reduced market value (Faust and Shear 1972*a*; Wertheim 1982). In functional terms, russeting restores control of water loss through the skin by the formation of a waterproofing periderm (phellem, phellogen and phelloderm) just beneath the microcracked primary fruit skin (cuticle, epidermis and hypodermis).

Studies on the aetiology of russeting identify the formation of microscopic cracks in the primary fruit skin as the first visible sign of russeting (for reviews see

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Faust and Shear 1972*a*, *b*). These microcracks apparently trigger formation of a periderm (Faust and Shear 1972*a*). Microscopy reveals that the periderm is differentiated in the subepidermal cell layers underlying the cracks and the epidermis (Verner 1938; Meyer 1944). Later, the primary surface comprising cuticle and epidermis dries and is shed, and the phellem of the secondary surface becomes visible as the familiar 'russeting'. It is the phellem at the new surface that is responsible for the dull, rough, brown, corky appearance of a russeted fruit.

Growth stresses are considered to be the driving force for the formation of microcracks (Skene 1982). The increase in fruit volume during development subjects the skin to biaxial tangential strain and stress. Failure occurs when the extensibility limits of the skin are exceeded. Strain and stress are greatest in the outermost layers of a fruit. They peak when relative area growth rates are maximal. For a fruit such as the apple, which has a sigmoid growth pattern, the area growth peak occurs in the early phase of development in the period up to ~3 weeks after full bloom. Observations show that apples are most sensitive to russeting at this time (Wertheim 1982; Knoche *et al.* 2011). Additional factors contributing to microcracking are: (i) inhomogeneous surface expansion resulting from irregular cell division in the epidermal or hypodermal cell layers (Eccher 1975), (ii) increases in the turgor of dividing and expanding epidermal cells (Faust and Shear 1972*a*), (iii) extended periods of surface wetness or high humidity (Tukey 1969; Knoche *et al.* 2011) and (iv) a mismatch between surface expansion and cuticle deposition in many soft and fleshy fruit crops, such as sweet cherries (Knoche *et al.* 2004), various *Ribes* berries (Khanal *et al.* 2011) and grapes (Becker and Knoche 2012).

While the phenomenological sequence of events in russeting is largely established (Verner 1938; Faust and Shear 1972*a*), little is known about the mechanical properties of the load-bearing structures in primary and secondary fruit skins (Petracek and Bukovac 1995; Bargel and Neinhuis 2004; Dominguez *et al.* 2011). Of particular interest are the mechanical characteristics of the outermost layers of the fruit skin, because here strain and stress are maximal. These layers include the cuticle of the primary skin of non-russeted fruit and the periderm or the secondary skin of russeted fruit. For recent reviews on the chemistry of major constituents of cuticle and periderm, the reader is referred to Dominguez *et al.* (2011), Heredia (2003) and Franke and Schreiber (2007).

The objectives of the present study were to characterize the rheological properties of fruit cuticles and periderms, and to measure their failure thresholds. Also, we aimed to identify the site of failure of composite fruit-skin specimens comprising both cuticle and periderm to determine whether russeting increases due to (i) the 'spreading' of russeting as a result of failure within a russeted area or at the boundary between a russeted and a non-russeted area or (ii) the formation of new sites of russeting because of failure in a non-russeted area. For our studies, we employed uniaxial mechanical tests of the isolated cuticular membrane (CM) and periderm membrane (PM), and 'composite' fruit skins comprising CM and PM (CM/PM) from apple and pear as a model.

Methods

Plant material

Fruit of the apple (*Malus* × *domestica* Borkh.) 'Karmijn de Sonnaville' (hereafter referred to as 'Karmijn') and the pear (*Pyrus communis* L.) 'Conference' were obtained at commercial maturity from the experimental orchards (52°14′N, 9°49′E) of Leibniz University, Hannover, Germany. Fruit were grown according to the European Union regulations for integrated fruit production and harvested at commercial maturity. Unless otherwise specified, fruit that were used to supply CM and PM samples were stored in either conventional cold storage or controlled atmosphere storage for up to 8 months, and those serving as a source of epidermal segments (ES) and peridermal segments (PS) were taken from freshly harvested fruit.

Preparation of the ES and PS and isolation of the CM and PM

Segments of the fruit skin were excised from russeted, non-russeted or russeted/non-russeted transition regions (~50 % each) of apples and pears using a cork borer (24 mm inner diameter) or a custom-made punch that produces a biconcave (dumb-bell-shaped) specimen with a narrow waist (width 4.25 mm). To minimize natural curvature, samples were taken from the equatorial region of the fruit (minimum radius of curvature). Samples were used either fresh as ES when excised from non-russeted skins or as PS when excised from russeted skins. For the preparation of CM and PM, the samples were incubated in 50 mM citric acid buffer solution (pH 4.0) 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 NaN_3 at 30 mM (Orgell 1955; Yamada *et al.* 1964; Groh *et al.* 2002). The isolation medium was refreshed periodically until the CM, PM or CM/PM separated from the underlying tissue. Specimens were then rinsed with deionized water and dried at ambient temperature and humidity (22 °C and 50 % relative humidity (RH)). The

average mass per unit surface area was quantified using five replicates comprising six discs each.

Scanning electron and fluorescence light microscopy

Freeze-fractured CM and PM samples were prepared for scanning electron microscopy. Specimens were mounted on aluminium stubs using conducting (carbon) tape and viewed under a scanning electron microscope (Quanta 200; FEI Europe Main Office, Eindhoven, The Netherlands) at $\times 1200$, an acceleration potential of 10 kV and a pressure of 60 Pa.

Samples of the isolated CM and PM were also inspected using a fluorescence microscope (BX60 with filter U-MWU, 330−385 nm excitation, ≥420 nm emission; Olympus Europa Holding GmbH, Hamburg, Germany) and a dissecting microscope (MZ10F with filter GFP-plus, 440−480 nm excitation, ≥510 nm emission; Leica Microsysteme GmbH, Wetzlar, Germany). Micrographs were taken using a digital camera (DP71, Olympus; Software Cell^P, Olympus).

Strain of the CM and PM

The release of biaxial strain of the CM and PM following excision and isolation was quantified using epidermal and peridermal discs excised from freshly harvested apple and pear fruit. Before excision, a square pattern (7×7 mm) of four holes (0.55 mm diameter) was punched in the non-russeted and russeted surfaces in the equatorial region of the fruit using a custom-made punch. The area (A; mm^2) enclosed by the hole pattern in the ES (A_{ES}) and PS (A_{PS}) was quantified by light microscopy and image analysis (Cell^P). Subsequently, the epidermal and peridermal discs were excised and the CM and PM were isolated enzymatically as described above. The isolated discs were then mounted on microscope slides and re-photographed. The areas enclosed by the hole pattern on the CM (A_{CM}) and PM (A_{PM}) were re-quantified. The release of biaxial strain (ε, %) was calculated (Knoche *et al.* 2011) as

$$\varepsilon_{CM} = \frac{A_{ES} - A_{CM}}{A_{CM}} \times 100 \quad \text{and} \quad \varepsilon_{PM} = \frac{A_{PS} - A_{PM}}{A_{PM}} \times 100.$$

Mechanical tests

Strips (5 mm wide) were prepared from enzymatically isolated CM, PM and CM/PM discs using parallel razor blades. To facilitate handling during preparation and mounting, the strips were fixed in a frame made of paper and masking tape (Tesa Krepp®; Tesa Werk Hamburg GmbH, Hamburg, Germany). Unless specified otherwise, tensile tests were performed with both dry and hydrated specimens. Dry specimens were held at 50 % RH and 22 °C ('dry'), and hydrated specimens were preconditioned overnight by incubating in deionized water at 22 °C ('hydrated'). Thereafter, frames were mounted in a universal material testing machine (Z 0.5; Zwick/Roell, Ulm, Germany; clamping distance $l_0 = 10$ mm) equipped with a 10 N force transducer (KAP-Z; Zwick/Roell). Frames were cut open and the following experiments were conducted.

Uniaxial tensile tests were performed at a strain rate of 1 mm min^{-1} until failure of the specimen with tensile force (F; newton) and crosshead displacement (Δl; mm) being recorded. Uniaxial strains (ε; %) were calculated by dividing Δl by the initial length of the specimen (l_0; mm):

$$\varepsilon = \frac{\Delta l}{l_0} \times 100$$

The maximum force (F_{max}; newton) and the strain at the maximum force (ε_{max}; %) were recorded. The modulus of elasticity (E; newton) was calculated as the maximum slope of a linear regression line fitted through a plot of force (newton) vs. strain (ε/100). Following tensile tests, surface views of fractured apple CM were inspected by light microscopy (BX60) at $\times 10$. The lengths of the fracture were quantified in the two fracture-mode categories, 'fracture along cell walls' and 'fracture across cell walls', using image analysis (Cell^P). In apple, only ~10 % of the fracture length could not be assigned unambiguously to one or the other of these fracture modes.

A creep/relaxation test composed of a loading and an unloading cycle to monitor creep and creep relaxation, respectively, was performed on the hydrated CM and PM from apples and pears (Fig. 1A and B). During loading, a force equivalent to $0.5F_{max}$ of the respective specimen (F_{max} in apple: 1.00 ± 0.04 N for the CM and 0.59 ± 0.04 N for the PM; F_{max} in pear: 0.27 ± 0.01 N for the CM and 0.53 ± 0.03 N for the PM) was applied at a rate of 0.5 mm min^{-1} followed by a 500-s hold period (Fig. 1A). The instantaneous elastic strain during loading was calculated as the relative increase in specimen length during the period of force application (Fig. 1A and B). In the subsequent hold period (force constant), the specimen extended due to creep (Fig. 1A and B). The strain occurring during the hold period is referred to as the creep strain. This creep strain was calculated as the relative increase in specimen length during the hold period of constant force (Meyers and Chawla 2009; Fig. 1B). In the subsequent unloading cycle, the specimen contracted almost instantaneously upon force removal followed by a time-dependent viscoelastic contraction. The instantaneous elastic contraction

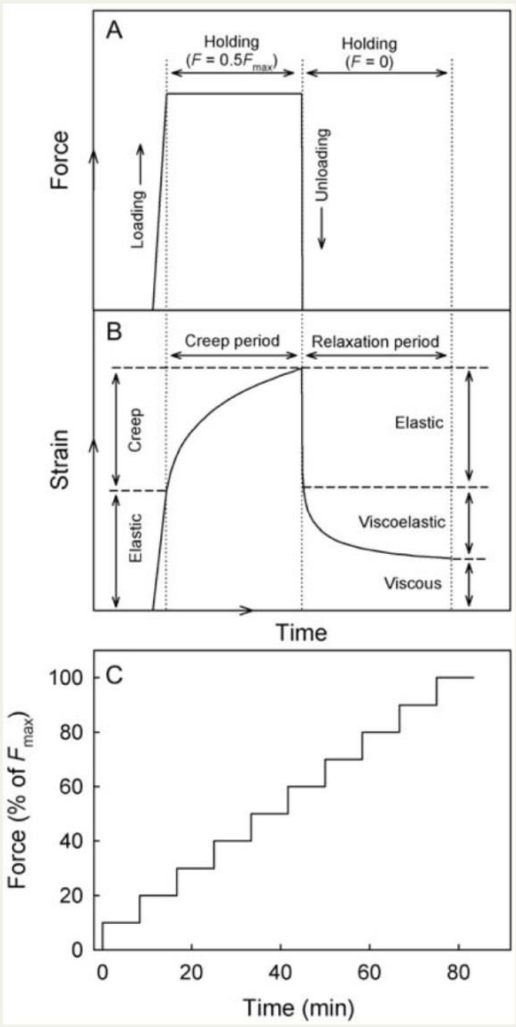

Fig. 1 (A and B) Schematic model of the creep/relaxation test used to quantify elastic strain and creep strain during the loading cycle, and elastic strain, viscoelastic strain and viscous strain during the unloading cycle of excised and enzymatically isolated CM and PM of apples and pears. During the loading cycle, specimens were subjected to tensile forces that equalled 50 % of the respective maximum forces (F_{max}). (C) Sketch of stepwise creep tests where CM and PM strips of apple and pear were loaded stepwise in increments of 10 % of their respective F_{max} values.

corresponds to the release of elastic strain, and the subsequent time-dependent viscoelastic contraction to the release of viscoelastic strain (Fig. 1A and B). Elastic and viscoelastic strains during unloading were calculated as the respective percentage decreases in length of the specimen (Δl) divided by the initial clamping distance (l_0). The extension of the specimen that remained after 1000 s from initiation of the experiment represents the plastic or viscous irreversible strain (Fig. 1B). During testing, the hydration state of the specimens was maintained by misting (Pariboy; Pari GmbH, Starnberg, Germany) and running deionized water over the specimen (Perfusor® Compact S, B BRAUN, Melsungen, Germany). The number of replications was 9 or 10.

The effect of stepwise force increases on creep strain was monitored in a stepwise creep test using fully hydrated apple and pear CM and PM (Fig. 1C). Preliminary experiments were conducted to quantify the F_{max} of the dry and hydrated apple CM (dry: 1.22 ± 0.05 N, hydrated: 0.83 ± 0.05 N) and pear CM (dry: 0.42 ± 0.03 N, hydrated: 0.27 ± 0.01 N) and dry and hydrated apple PM (dry: 1.27 ± 0.06 N, hydrated: 0.66 ± 0.02 N) and pear PM (dry: 0.81 ± 0.07 N, hydrated: 0.53 ± 0.03 N). Creep tests were performed by increasing the force stepwise in increments of $F_{max}/10$ each step followed by a 500-s holding period (Fig. 1C). Increase was continued until the specimen failed. The crosshead speed during force applications was constant at 0.5 mm min^{-1}. Specimens remained fully hydrated throughout the test. Force, crosshead travel and time were recorded. The elastic strain and the creep strain were quantified as described above.

To relate the rheological properties and fracture thresholds determined in the CM and PM to those of intact russeted and non-russeted fruit skins, uniaxial tensile tests were also performed on the ES and PS. Biconcave, dumb-bell-shaped specimens (waist width 4.25 mm) were excised from non-russeted or russeted regions of the cheek of fresh apple and pear fruit using the custom-made punch. The long axis of all specimens was oriented longitudinally (i.e. parallel to the calyx/pedicel axis). Preliminary experiments established that there was little difference in the mechanical properties of the ES excised in longitudinal and latitudinal orientations, implying that specimens were approximately isotropic (B. P. Khanal, unpubl. observ.). The ES and PS were carved by hand to varying thicknesses using a razor blade and a simple jig. The thickness of ES and PS samples was quantified by light microscopy. Uniaxial tensile tests were performed as described above (Z 0.5; Zwick/Roell; 50 N force transducer, Type: KAP-TC; Zwick/Roell). Clamping distance was $l_0 = 16$ mm and the crosshead speed 3 mm min^{-1}. There was no preconditioning of the specimen and all tests were completed within 3 min of excision. The F_{max} and ε_{max} values were measured. For comparison, enzymatically isolated CM and PM strips from the same batch of fruit were also investigated.

Data analysis and terminology

Occasionally, CM, PM or CM/PM specimens failed in, or adjacent to, the clamps. It is thought likely that such specimens may have been damaged during preparation and/or clamping and these data were therefore excluded from the analyses. The specimens not excluded in this manner represented 84, 81 and 91 % of the total population of CM, CM/PM and PM measurements, respectively. None of the ES or PS specimens failed in, or adjacent to, the clamps. Analysis of variance (ANOVA) and regression analyses were performed using SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Values expressed on a percentage basis were arcsine transformed before ANOVA. Unless individual observations are shown, values in the figures are presented as the means $\pm$ SE of means. Where not visible, the error bars are smaller than the plotting symbols.

Throughout the article, the fruit skin segments excised from non-russeted and russeted regions of the fruit surface are referred to as epidermal segments (ES) and peridermal segments (PS), respectively. The specimens obtained after enzymatic isolation of the ES and PS are referred to as the cuticular membranes (CM) or periderm membranes (PM).

Results

Microscopic structure of the CM and PM

Scanning electron microscopy of cross-sections of the CM revealed a continuous cuticular lamella above the former periclinal epidermal cell walls (Fig. 2A and B) and cuticular pegs of variable size above the former anticlinal epidermal cell walls (Fig. 2A, B, F and G). The larger and thicker pegs separated imprints of groups of

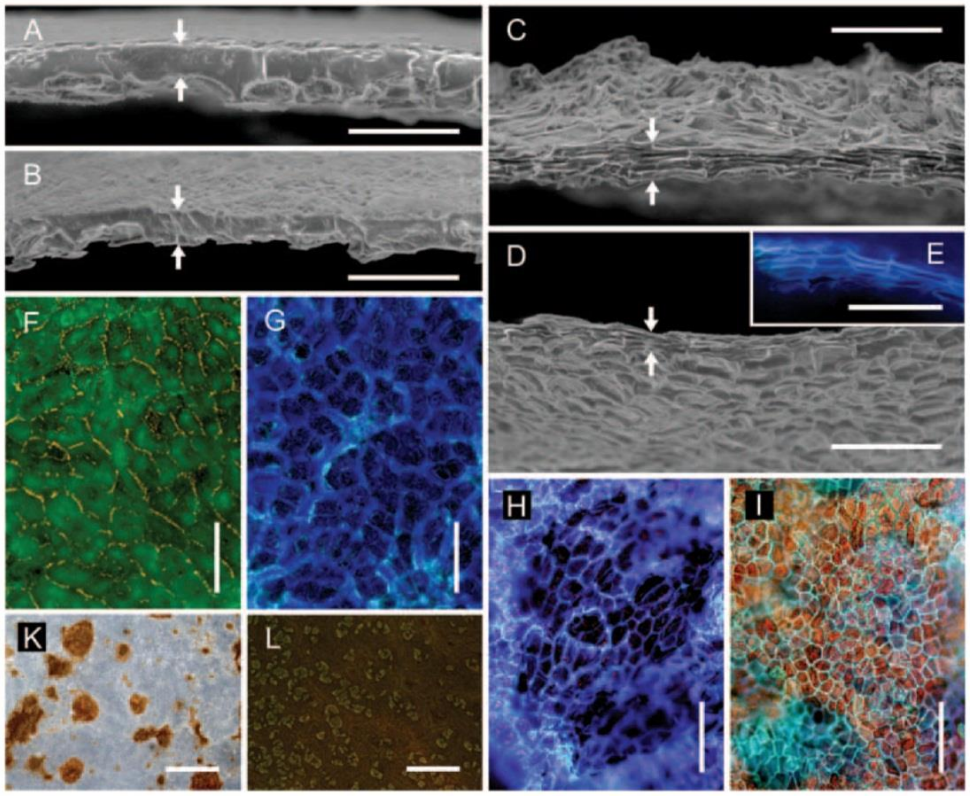

Fig. 2 Scanning electron (A–D) and fluorescence light micrographs (E–L) of the enzymatically isolated CM (A, B, F, G and K) and PM (C, D, E, H, I and L) excised and isolated from non-russeted and russeted regions of apple (A, C, F, G and L) and pear fruit surfaces (B, D, E, H, I and K). Cross-sections obtained by freeze fracture of the CM and PM (A–E), the CM and PM viewed from above (F, H, K and L) and below (G and I) under incident UV light (filter U-MWU; E, F, G, H and I; filter GFP-plus, L), and transmitted (F–I) and incident (K) white light. Upper and lower edges of the freeze-fractured surface are indicated by arrows. Scale bars: (A–E) 0.05 mm; (F–I) 0.1 mm; (K and L) 2 mm.

Table 1 Physical properties of CM and PM discs excised and isolated from mature 'Karmijn de Sonnaville' apples and 'Conference' pears. Biaxial strain release was calculated as a fractional (%) decrease in the area of the CM and PM upon excision and enzymatic isolation.

Specimen	Mass (g m^{-2})		Strain (%)	
	Apple	Pear	Apple	Pear
CM	23.7 ± 0.4a*	17.3 ± 0.4a	5.7 ± 0.4b	7.6 ± 0.5b
PM	17.4 ± 0.4b	11.7 ± 0.2b	11.0 ± 0.6a	21.8 ± 1.1a

*Data are the means and standard errors of five replicates (comprising six CM or PM discs each) for mass and of 30 replicates for strain. Mean separation within species by Tukey's Studentized range test, $P < 0.05$.

epidermal cells (Fig. 2F and G), while the smaller pegs provided a substructure within these groups, separating imprints of the individual epidermal cells (Fig. 2G). The CM of apple and pear often contained spots of periderm, as indicated by the brown colour and cellular structure when viewed from the morphological outer side (Fig. 2K). The percentage of surface area with periderm in the CM was somewhat higher in pear than in apple (E. Grimm, unpubl. observ.).

In contrast to the CM, the structure of the PM in apple and in pear was cellular (Fig. 2C, D, E, H, I and L). Cross-sections revealed stacked phellem cells, all elongated tangentially (Fig. 2C–E). When inspected from above or below, phellem cells were polygonal, isodiametric, with no preferential longitudinal or latitudinal orientation (Fig. 2C, D, H and I). Cell walls of the phellem cells were encrusted with suberin, as indicated by autofluorescence (Fig. 2E, H, I and L). In cross-sections, patches of the CM and epidermis overlaying the periderm were observed, which indicates that the PM was formed within the subepidermal tissue (Fig. 2L).

In both fruit types, the mass per unit area of the CM was significantly higher than that of the PM (apple, +26 %; pear, +34 %) (Table 1). Isolates obtained from transition zones between the CM and PM had mass per area values intermediate between those of the CM and PM (results not shown).

After excision and enzymatic isolation, the areas of both CM and PM discs were smaller than before processing, indicating that strain release had occurred. The release of strain from PM discs exceeded that from CM discs (1.9-fold in apple; 2.9-fold in pear) (Table 1).

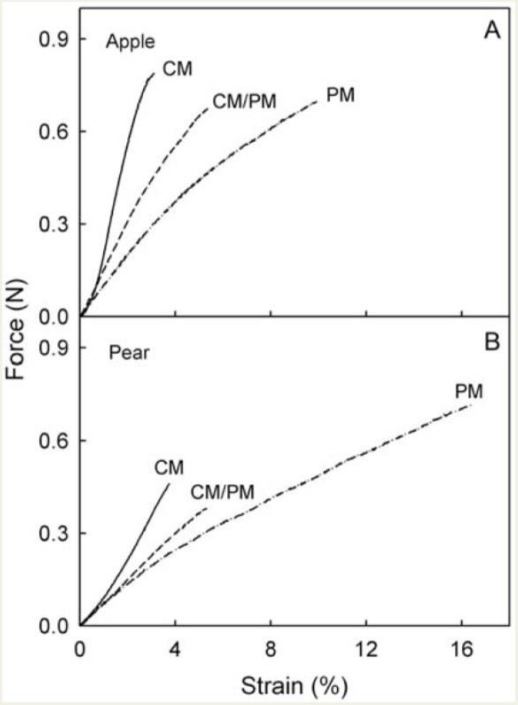

Fig. 3 Representative force/strain diagrams of hydrated CM, PM and membranes with CM/PM transition zones subjected to a uniaxial tensile test. Specimens were enzymatically isolated from non-russeted (CM), russeted (PM) and transition regions (CM/PM) of apple (A) and pear skins (B). For the maximum force, strain at the maximum force and modulus of elasticity, see Table 2.

Mechanical tests

Tensile tests of strips of hydrated CM, PM and of CM/PM transition zones revealed qualitatively similar force–strain relationships (Fig. 3; Table 2). The maximum force (F_{max}) was lower in the PM than in the CM in apple, but higher in the PM than in the CM in pear. The ε_{max} values were markedly higher in the PM than in the CM or the CM/PM (Table 2) and, hence, the E values were lower. When wet and dry specimens were compared, the hydrated CM, PM or CM/PM generally had lower F_{max} and E values than their dry counterparts

Table 2 Maximum force (F_{max}), strain at maximum force (ε_{max}) and modulus of elasticity (E) of CM and PM isolated enzymatically from the fruit skins of mature 'Karmijn de Sonnaville' apples and 'Conference' pears. CM/PM denotes specimens that were excised and isolated from a transition zone between the CM and PM. Approximately half of the specimen was composed of CM and the other half of PM. Specimens were subjected to uniaxial tensile tests in either a dry or a hydrated state.

Species	State	F_{max} (N)			ε_{max} (%)			E (N)		
		CM	CM/PM	PM	CM	CM/PM	PM	CM	CM/PM	PM
Apple	Dry	1.22 ± 0.05ab*	1.09 ± 0.04b	1.27 ± 0.06a	3.9 ± 0.3a	3.5 ± 0.1a	4.1 ± 0.2a	49.8 ± 0.5a	36.9 ± 0.8b	36.9 ± 1.0b
	Hydrated	0.83 ± 0.05a	0.66 ± 0.03b	0.66 ± 0.02b	2.9 ± 0.2c	5.5 ± 0.4b	9.6 ± 0.4a	39.1 ± 1.3a	17.0 ± 0.6b	10.6 ± 0.5c
Pear	Dry	0.41 ± 0.02b	0.33 ± 0.02b	1.09 ± 0.05a	3.5 ± 0.1b	3.2 ± 0.2b	7.4 ± 0.2a	13.9 ± 0.6b	12.1 ± 0.7b	17.8 ± 1.0a
	Hydrated	0.40 ± 0.02b	0.31 ± 0.03c	0.65 ± 0.02a	3.4 ± 0.2c	5.5 ± 0.4b	16.5 ± 0.5a	15.2 ± 0.9a	6.4 ± 0.5b	5.5 ± 0.4b

*Values represent the means and standard errors of a minimum of 14 replicates (apple) and 16 replicates (pear). Interaction between the state of hydration and type of specimen significant in two-factorial ANOVA for apple and pear ($P < 0.0001$ for F_{max}, ε_{max} and E in apple and pear except for F_{max} in apple where $P < 0.0663$). Therefore, one-factorial ANOVA was run and mean comparisons were made within species and state of hydration using Tukey's Studentized range test, $P < 0.05$.

(Table 2). Furthermore, the hydrated PM had higher ε_{max} values than the dry PM. However, there was little effect of hydration on the CM. Quantitatively similar results had been obtained in an earlier season (B. P. Khanal, unpubl. observ.) reinforcing this observation.

The site of failure of specimens from the CM/PM transition zone differed between apple and pear. In apple, failure was more frequent at the CM/PM transition, compared with failure at the PM or the CM (Table 3). In pear, however, segments failed more often at the CM portion, followed by the CM/PM transition zone or the PM portion (Table 3). The site of failure was not affected by the hydration state.

Microscopic inspection of the fracture surfaces obtained in tensile tests of the apple CM revealed that failure occurred mostly along the cuticular pegs and thus along the anticlinal cell walls (56 % of total fracture length; data not shown). Failure occurred less across the pegs and, hence, across the cell walls (34 % of the total fracture length). On average, the optical resolution was insufficient to assess the site of failure over 10 % of the total fracture length.

Creep/relaxation tests of the CM and PM for both apple and pear consistently yielded qualitatively similar but quantitatively different changes in strain with force and time (Fig. 4A). During loading, elastic strain and creep strain values in the PM exceeded those in the CM. Both sorts of strain were of similar magnitude in apple and pear (Fig. 4B). During unloading, the release of elastic strain and viscoelastic strain from the PM and the remaining viscous strain of the PM were all larger than those from the CM (Fig. 4C). There was little difference between apple and pear in this regard. A comparison of loading and unloading cycles revealed that the elastic strain of the CM and PM during loading exceeded the elastic strain released during unloading.

In the stepwise creep test, the strain in the CM increased with each force step for both apple and pear. This response was even more marked in the PM (Fig. 5). This increase is accounted for primarily by larger creep strains, which in each case exceeded the elastic strains several fold (Fig. 6). The F_{max} and ε_{max} values were lower in the CM of pear than in apple (Fig. 6A and C). There was little difference in F_{max} and ε_{max} between the PM of each species (Fig. 6B and D). Qualitatively similar results were obtained with the dry specimens (results not shown).

To relate the mechanical properties of the isolated CM and PM to those of the corresponding fruit skins, the excised ES and PS were subjected to tensile tests. It was found that F_{max} increased linearly with thickness and, hence, with the number of parenchyma cell layers in the ES and PS in both apple and pear (Fig. 7A and C). The extrapolated *y*-axis intercepts of these relationships

Table 3 Frequency of fracture in different regions of specimens excised from apple ('Karmijn de Sonnaville') and pear ('Conference') fruit surfaces. The specimens selected had a transition zone between the russeted portion with a PM and a non-russeted portion with a CM. Following enzymatic isolation, the specimens were subjected to uniaxial tensile tests, the position of failure (CM vs. CM/PM vs. PM) was noted, and the failure frequency (%) was calculated.

Species	State	*n*	Failure frequency (%)		
			CM	CM/PM	PM
Apple	Dry	27	14.8	51.9	33.3
	Hydrated	20	15.0	50.0	35.0
Pear	Dry	20	65.0	35.0	0
	Hydrated	17	82.4	11.8	5.9

predict F_{max} values that exceed those of the isolated CM and PM in both apple and pear. The ε_{max} values of the ES and PS did not depend on thickness in apple or in pear (Fig. 7B and D). There was little difference in ε_{max} of either the ES or PS between apple and pear. The ε_{max} values of the ES and PS generally exceeded those of the corresponding CM and PM.

Discussion

Our results demonstrate that: (i) the rheological properties of the CM and PM resemble those of viscoelastic polymers, (ii) the PM is a more plastic replacement for the stiffer CM in both apple and pear, (iii) the weak link in apple specimens obtained from russeted fruit surfaces is the borderline between the CM and PM, but in pear it is the CM, and (iv) the cell layers of the ES and PS underlying the CM and PM represent the principal load-bearing structure in both apple and pear.

The CM and PM are viscoelastic polymers

Deformation of viscoelastic polymers typically consists of reversible elastic, time-dependent reversible viscoelastic and irreversible residual viscous or plastic components. These were all observed in the CM and PM of both apple and pear. During the loading phase in the creep/relaxation experiment, as force increased the initial deformation was mostly elastic. Creep occurred in the subsequent hold phase. This creep strain comprised viscoelastic and viscous deformation components (Niklas 1992). Attempts to partition the creep strain into viscoelastic and viscous components are ambiguous because, in our experiments, clear transitions could not be identified during the creep period (Fig. 1B). However, during the subsequent relaxation period, the time-dependent reversible deformation represents the viscoelastic strain, while the viscous strain equalled the time-independent irreversible strain (Niklas 1992; Fig. 1B).

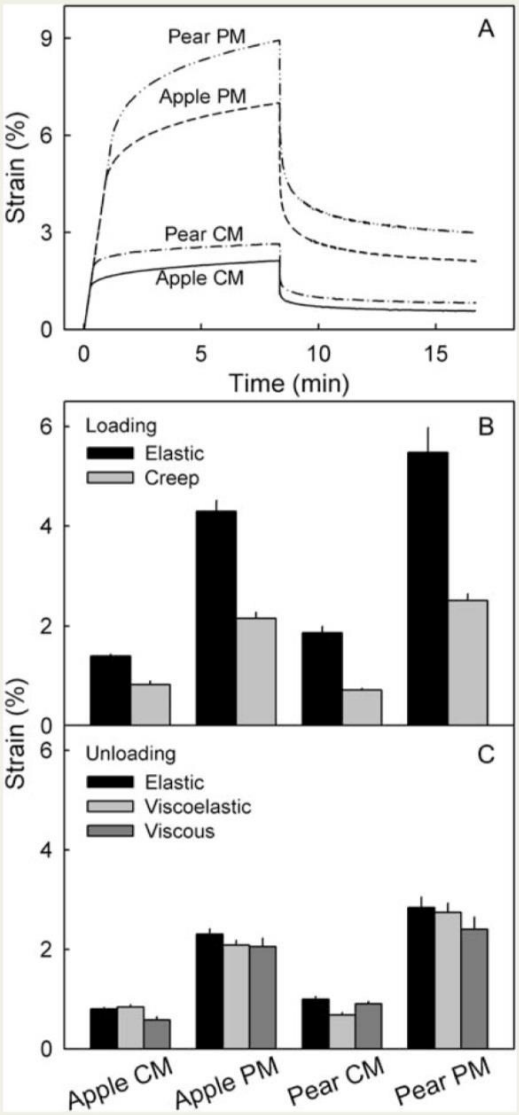

Fig. 4 Creep/relaxation test of CM and PM enzymatically isolated from non-russeted and russeted surfaces of mature 'Karmijn de Sonnaville' apple and 'Conference' pear fruit. (A) Representative time courses of strain during the loading and the unloading cycle. (B; $n = 9–10$) Elastic and creep strain during the loading cycle. (C; $n = 9–10$) Elastic strain, viscoelastic strain and viscous strain during the unloading cycle. For details see the text.

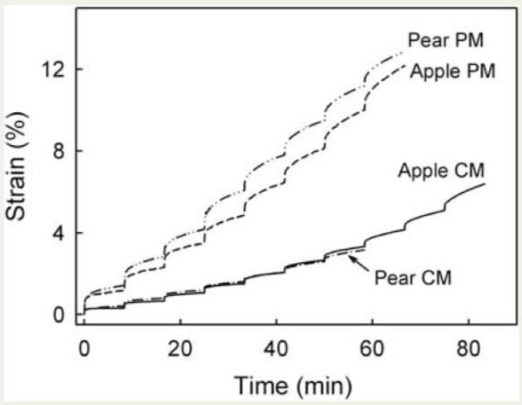

Fig. 5 Representative time courses of a stepwise creep test performed on CM and PM enzymatically isolated from non-russeted and russeted surfaces of mature 'Karmijn de Sonnaville' apple and 'Conference' pear fruit. All specimens were fully hydrated when tested.

Some differences between the force/strain behaviour of the viscoelastic CM and PM and that of the viscoelastic cell wall described by Niklas (1992) were observed. First, the release of elastic strain during unloading was only about half of the elastic strain occurring during loading (Fig. 4). Second, the sum of the viscoelastic and viscous strains during relaxation was about twice the creep strains during the hold period (Fig. 4). In the Niklas (1992) system, the sum of viscoelastic and viscous strains during unloading equalled the creep strain during loading. The reasons for these differences are unknown.

The PM is a plastic replacement for the stiffer CM

We now focus on the hydrated CM and PM because these reflect the *in vivo* condition in which the inner side of the CM and PM are in capillary contact with a water-saturated apoplast. A comparison of the mechanical properties of the CM and PM revealed that the hydrated PM was 3.3 times (apple) and 4.9 times (pear) more extensible than the CM, as indicated by the higher ε_{max} values (Table 2). This observation is consistent across experiments and across seasons (B. P. Khanal,

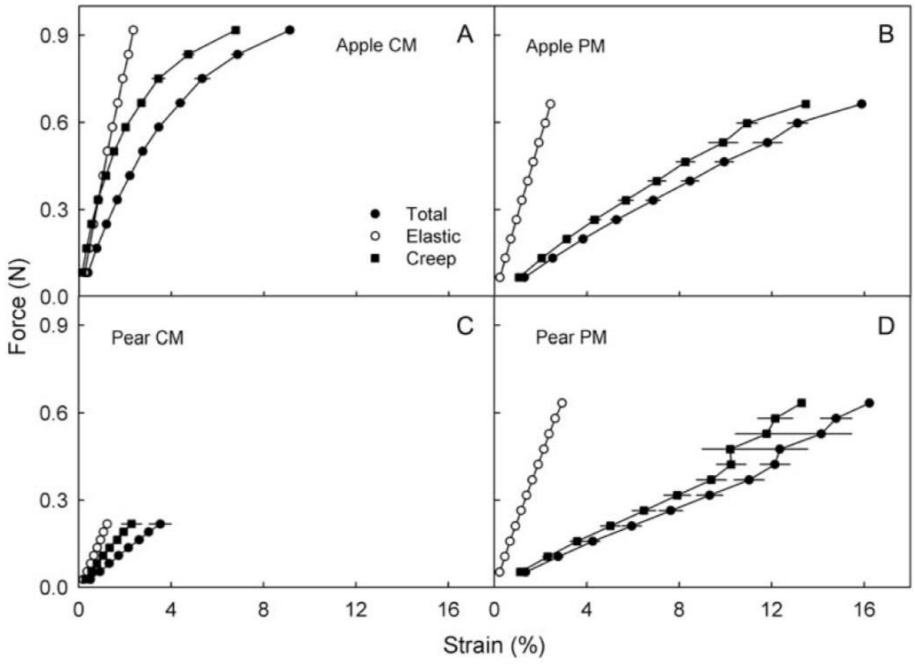

Fig. 6 Force/strain diagrams of CM and PM enzymatically isolated from non-russeted and russeted surfaces of mature 'Karmijn de Sonnaville' apple and 'Conference' pear fruit. The force was increased stepwise in increments of 10 % of the maximum force (F_{max}). Each increment was followed by a 500-s hold period. Total strain was partitioned into an elastic strain and a creep strain. For details see the text. $n = 14–16$ (apple) and $n = 10$ (pear).

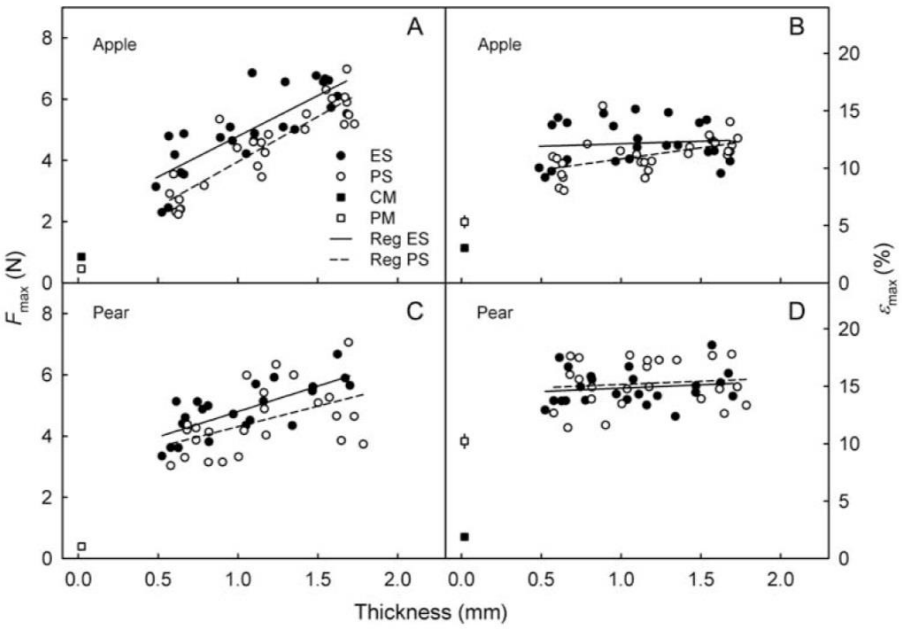

Fig. 7 Mechanical properties of skin segments excised from non-russeted (ES) and russeted (PS) regions of mature 'Karmijn de Sonnaville' apples (A and B; $n = 26$) and 'Conference' pears (C and D; $n = 24$). The maximum force (F_{max}) vs. thickness of the ES and PS (A and C), and strain at the maximum force (ε_{max}) vs. thickness of the ES and PS (B and D). For comparison, the results from tensile tests of enzymatically isolated CM and PM obtained from the same batches of fruit are included. Skin thickness was determined by microscopy prior to the test.

unpubl. observ.). It could be argued that the higher extensibility of the PM is related to its higher *in vivo* strain and that the tensile test merely re-imposed the *in vivo* strain upon the relaxed specimen. However, this interpretation is considered unlikely. Instead, converting the biaxial strain released from the CM and PM discs to a uniaxial strain (assuming, as a first approximation, isotropic and ideal behaviour; Knoche *et al.* 2004) yields uniaxial strains that are close to the ε_{max} of the CM in apple (release of uniaxial strain 2.8 ± 0.2 % vs. ε_{max} of 2.9 ± 0.2 %) and pear (release of uniaxial strain 3.7 ± 0.3 % vs. ε_{max} of 3.4 ± 0.2 %; Tables 1 and 2). However, the calculated uniaxial strains were lower than the ε_{max} of the PM in the two species (release of uniaxial strain in apple 5.4 ± 0.3 % vs. ε_{max} of 9.6 ± 0.4 %; release of uniaxial strain in pear 10.4 ± 0.6 % vs. ε_{max} of 16.5 ± 0.5 %; Tables 1 and 2). This calculation shows that for the CM, the *in vivo* strain was close to or even exceeded the ε_{max} in the tensile test and, hence, the strain at failure. In contrast, the strains of the PM on the fruit skins *in vivo* were still below their respective ε_{max} values.

Site of failure in a russetted fruit surface differs between apple and pear

The site of failure of a specimen composed of both the CM and PM differed between apple and pear. In apple the CM/PM borderline was the weakest point whereas in pear the CM itself failed more often. We do not fully understand the reason for this difference. Possible factors are: (i) the thinner CM in pear compared with apple, (ii) the more pronounced hypodermal development of the CM in pear compared with apple (B. P. Khanal, unpubl. observ.) and (iii) an uneven distribution of strain and, hence, the development of stress concentrations triggering an earlier CM failure.

Cell layers underlying the CM and PM represent the principal load-bearing structures

The load-bearing structures of both apple and pear skins were the ES and the PS rather than the CM and PM. This conclusion is based on the observation that the F_{max} values of the ES and PS always exceeded those of the CM and PM. This is not surprising, since the primary load-

bearing material of the ES and PS resides in the cellulosic cell walls of the underlying cell layers. Unfortunately, it was technically difficult to prepare ES or PS samples that were thinner than those investigated here, so we were unable to identify the relative contributions of the individual cell layer(s) to the mechanical properties of the whole ES and PS composites. However, the cells of the epidermal and hypodermal layers are the most likely candidates for a mechanical function because of their small size and thickened walls compared with the much larger, thin-walled cells of the parenchyma. The lower ε_{max} of the CM as compared with the ES is consistent with the observation that microscopic cracks in the CM are the first visible symptom of russeting (Faust and Shear 1972*a*).

Conclusions and forward look

The most striking and consistent difference in the mechanical properties between the CM of a non-russeted surface and the PM of a russeted surface is the higher plasticity of the hydrated PM compared with the CM. This is a desirable property in the PM if it is to function as a 'repair patch' for an overly strained surface having a cracked CM. It allows the PM to cope with ongoing area expansions during growth without excessive stress concentration arising (Considine and Brown 1982). Thus, growth stresses are also distributed uniformly when the surface is a composite of the CM and PM. This is an important prerequisite for a regular-shaped fruit. Our results further demonstrate that *in vivo* the CM of both apple and pear (but not the PM) are strained to near their failure limits. If these limits are exceeded, failure in apple will occur at the CM/PM transition, resulting in a continuous spread of russeting on the expanding surface. In pear, however, the CM appears to be the weakest point, as failures occur primarily within the CM rather than in the PM or at the CM/PM interface.

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Contributions by the authors

E.G. and M.K. obtained the funds to support the study. E.G., M.K. and B.P.K. planned the experiments. B.P.K. conducted the mechanical studies and E.G. and B.P.K. the microscopy work. B.P.K. and M.K. analysed the data and wrote the manuscript. B.P.K., E.G. and M.K. revised and edited the paper.

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Conflict of interest statement

None declared.

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8. General Discussion

The data presented provide several new and important findings:

1. Cuticular wax effectively fixes the elastic strain of the apple fruit cuticle by filling the strained cutin matrix as indicated by the release of significant strain on wax extraction. This effect is a linear function of the amount of wax present in the cuticle and also observed in leaf and fruit cuticles of other species (Chapter 4).
2. Physical and mechanical properties of the cuticle at maturity are not related to the difference in russet susceptibility across a range of 22 apple cultivars (Chapter 5).
3. Epidermis and hypodermis represent the mechanical backbone of the skin whereas the cuticle's contributions to the mechanical properties of the skin composite is small and negligible. At the same time the cuticle represents the outermost protective barrier of the fruit surface and here, the contributions of epidermal and hypodermal cell layers are marginal (Chapter 6).
4. In russeting of apple fruit a plastic periderm replaces a stiff cuticle (Chapter 7).

For discussions of the individual findings mentioned above the reader is referred to the respective chapters 4 to 7. In the general discussion below, these findings together with data from the literature are integrated into a unifying concept explaining how the apple surface copes with the increase in surface area during development.

Fruit growth stress is the major factor causing strain in the fruit skin (Maguire 1998, Skene 1982). This strain is inevitably associated with the increase in volume and mass of a growing spherical organ where the continuous increase in fruit volume the associated enlargement of the surface (Knoche et al. 2011) exerts a tensile stress in the skin. As the outermost layer of the skin, the cuticle is exposed to the highest growth stresses and strains. To cope with fruit enlargement, epidermal and hypodermal cells (1) increase in number by cell division and (2) elongate in periclinal direction (Meyer 1944). Cell elongation can occur either by stretching of cell walls and compensatory synthesis of cell wall material or by peeling apart the abutting portions of two epidermal cells' anticlinal walls (Maguire 1998). This phenomenon further increases and focuses

the stress in the overlying cuticle layer (Maguire 1998). Thus, it is challenging for the cuticle to maintain intact surface during fruit growth and development.

The fruit of apple has various mechanisms to accommodate increasing strain on the cuticle. (1) Continuous biosynthesis and deposition of cutin constituents (Knoche et al. 2011) into the strained cutin polymer matrix maintains or even increases cuticle thickness and is expected to fix the strain of the cuticle to some extent. This argument is consistent with the absence of strain release in apple upon isolation of the CM. Also, earlier findings indicate that in sweet cherry (Knoche et al. 2004), plum (Knoche and Peschel 2007), Ribes berries (Khanal et al. 2011) and grape (Becker and Knoche 2012) there is a mismatch of fruit surface expansion and cuticle deposition causing strain of the CM as indicated by the relaxation of the CM on isolation. The cuticles of apple and sweet cherry mark the extremes found in cuticle strain in fruit with continuous deposition of cuticle and essentially no release of strain on isolation of the cuticle in apple (Knoche et al. 2011), but the essential absence of cuticle deposition and a substantial release of strain upon isolation of the cuticle in sweet cherry (Knoche et al. 2004). This observation is consistent with the idea that deposition of cutin fixes strain.

(2) In apple, wax is continuously deposited in the cuticle on the expanding surface (Knoche et al. 2011). This filling of the cutin matrix with wax effectively converts reversible elastic strain of the cuticle into a strain that, in *vivo* in the plant, is irreversible and plastic (Khanal et al. 2013a). Further, this wax deposition increases the failure force and stiffness of the cuticle thus making the cuticle less extensible (Khanal et al. 2013a). The plastic deformation of the cuticle avoids localized stress concentration and therefore minimizes the risk of failure. By the same mechanism, further extension is limited (Khanal et al. 2013a).

If the deposition of materials (cutin and wax) and the fixing of strain of the cuticle is insufficient or does not keep pace with the enlargement of the fruit surface then failure of the fruit skin occurs. The data presented herein demonstrate conclusively, that failure (microcracking) of the cuticle precedes that of the skin.
Microcracks in the cuticle impair the barrier function of the CM but have limited relevance for the mechanical properties of the skin composite because the load bearing

structure of the fruit skin are epi- and hypodermal cell layers. Provided that underlying epi- and hypodermal cell layers underneath the microcracks remain intact crack healing mechanism may be induced.

There is substantial evidence in the literature for deposition of wax in microcracks in the cuticle (Curry 2008, Curry 2009, Roy et al. 1999). This may be attributed to 1) the shorter distance from the site of wax biosynthesis in the epidermal cells to the base of a microcrack as compared to the non-cracked surface of the cuticle and 2) decreased diffusive resistance of the microcrack. Both mechanism will result in preferential deposition of wax in the microcracks and a natural 'filling' or 'healing' of these cracks. Provided that the deposition of wax in microcracks re-establishes the barrier function of the cuticle, then these microcracks are expected to pass without causing any visible defects on the fruit surface. We expect the effect of wax synthesis and deposition to depend on genetic and environmental factors which could account for effects of cultivar and weather conditions on russet susceptibility (Faust and Shear 1972a). Unfortunately, direct evidence is not available.

If wax deposition and filling of microcracks was too slow or insufficient, microcracks are expected to extend into the epidermis and hypodermis where formation of a periderm will be triggered by as yet unknown factors (Faust and Shear 1972a, Meyer 1944). Periderm form in the hypodermal region underlying the microcracks (Meyer 1944, Verner 1938). The periderm then replaces the cuticle of the primary fruit skin causing the well known russeting symptoms of the fruit skin (Khanal et al. 2013b). The susceptibility to russeting differs among the various apple cultivars but there is no significant relationship between russeting susceptibility and the mechanical or physical properties of their cuticle (Khanal et al. 2013c). Therefore, other factors must be dominating in determining russeting susceptibility of the cultivars.

The cuticle (outermost layer of the primary fruit skin) and the periderm (outermost layer of the secondary fruit skin) differ in their mechanical properties. The periderm is more plastic than the cuticle (Khanal et al. 2013b). During fruit surface expansion the periderm can deform plastically more and faster than the cuticle. Thus the periderm can cope with the expanding fruit surface without stress concentration (Considine and

Brown 1982). A uniform distribution of growth stresses is an essential prerequisite for regular-shaped fruit even though the surface is a composite one comprising primary, i.e., cuticle with epidermis and hypodermis, and secondary, i.e. periderm, dermal tissue.

While the above sequence of events accounts for a number of genetic and environmental factors in russeting, it is difficult to explain the lack of russeting in late stages of development (Faust and Shear 1972a). The most likely explanation is that the hypodermal cells are unable of producing periderm in later stages. Then, microcracks traversing the cuticle are not filled with wax and would remain open. These unhealed microcracks enhance the transpirational water loss and fruit shriveling in storage (eg. cv 'Golden Delicious'). Furthermore, microcracks possibly result in death of epidermal cells in their immediate vicinity thereby leading to the development of the skin spot disorder as demonstrated for 'Elstar' apple. In this disorder a protective barrier is reestablished by lignin deposition in the cell wall that essentially seals off the collapsing cells from the remaining fruit (Grimm et al. 2012a).

From the above discussion the following conclusion are drawn. Maintaining an intact fruit surface is essential for high quality fruit. This may be achieved by 1) synchronized surface expansion and cutin deposition such that cuticle thickness remains constant or increases and 2) simultaneous wax deposition that decreases elastic strain of the cuticle. Both phenomena result in plastic deformation of the cuticle thereby minimizing stress concentration and microcracking. Once the microcracks formed in the cuticle, two possible crack repair mechanisms may be induced (1) sealing these cracks by wax production and deposition into the cracks or (2) formation of periderm underneath the cracks (russeting). This requires the underlying mechanically relevant cell layers to remain intact. Furthermore, the rate of wax deposition and filling of cuticular cracks, the mechanical properties of epi- and hypodermal cell layers of the developing fruit, and their morphological characteristic of epi- and hypodermal cells are likely variables that should be related to russet susceptibility of apple cultivars. Given the importance of peel appearance and the detrimental effects of cuticle damage thereon, this subject merits further study.

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10. Abbreviations

$\sigma_{\max}$	maximum stress at failure of the specimen
A_{CM}	area of CM
A_{CM80}	area of CM heated at 80^{o}C
A_{DCM}	area of dewaxed CM
A_{ES}	area of ES
A_{PM}	area of PM
A_{PS}	area of PS
$F_{\max}$	maximum force at failure of the specimen
$\Delta F_{\max}$	change in $F_{\max}$
$\Delta\varepsilon_{\max}$	change in $\varepsilon_{\max}$
$\varepsilon_{biaxial}$	biaxial strain
$\varepsilon_{biaxial}^{CM80}$	biaxial strain release of the CM on heating of CM at 80^{o}C
$\varepsilon_{biaxial}^{DCM}$	biaxial strain release of the CM on dewaxing
$\varepsilon_{\max}$	strain of the specimen at $F_{\max}$
ANOVA (statistics)	analysis of variance
AO	acridine orange (fluorescent dye)
ATR	attenuated total reflectance
CM	cuticular membrane
CM80	CM heated at 80^{o}C
Corr (statistics)	correlation
cv	cultivar
DAFB	days after full bloom
DCM	dewaxed CM
DSC	differential scanning calorimetry
E (location)	east (longitude)
ECW	epicuticular wax
ES	epidermal Segment or Fruit skin segment
Fig	figure
FT-IR	fourier transform infrared spectroscopy
L or *l*	total length of the test specimen at the end of test
L_0 or l_0	total length of the test specimen at the beginning of test

MCT	mercury cadmium telluride
n	number (replication)
N (location)	north (latitude)
P (statistics)	probability level
PM	periderm
PS	fruit skin segment obtained from the russeted (periderm) surface
R (statistics)	pearson's correlation coefficient
R^2 (statistics)	coefficient of determination
Reg (statistics)	regression
RH	relative humidity
S	stiffness
SE (statistics)	standard error
SEM	scanning electron microscopy
v/v (concentration)	volume by volume
ν_s	symmetric stretching of CH_2
ν_{as}	antisymmetric stretching of CH_2
w/v (concentration)	weight by volume
wk	week
ΔL or Δl	change in length of the test specimen after test
ΔS	change in *S*
ε	strain

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List of Publications

Khanal, B. P. and Knoche, M. (2014). Mechanical properties of apple skin are determined by epidermis and hypodermis. Journal of the American Society for Horticultural Science 139, 139-147. http://journal.ashspublications.org/content/139/2/139

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Oral presentation on 'Fruit growth, cuticle deposition, water uptake, and fruit cracking in jostaberry (*Ribes nidigrolaria*), gooseberry (*Ribes uva-crispa*), and black Currant (*Ribes nigrum*)'.

Organizer: Deutsche Gartenbauwissenschaftliche Gesellschaft e.V. (DGG) and Bundesverband Der Hochschul-Absolventen/Ingenieure Gartenbau und Landschaftsarchitektur e.V. (BHGL)

WeGa Workshop (21 to 22 February, 2013. Hannover)

Poster presentation on 'Russeting in apple : a plastic periderm replaces a stiff cuticle'.

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Poster presentation on 1) Skin spot in 'Elstar' apple is associated with microcracking of the cuticular membrane.

2) Russeting in apple is the replacement of a stiff cuticular membrane by a plastic periderm.

Organizer: WeGa – Kompetenznetz Gartenbau

47. Gartenbauwissenschaftliche Jahrestagung (23 to 26 February, 2011. Hannover)

Oral presentation on 'Russeting in apple and pear: A plastic periderm replaces a stiff cuticle'.

Organizer: Deutsche Gartenbauwissenschaftliche Gesellschaft e.V. (DGG) and Bundesverband Der Hochschul-Absolventen/Ingenieure Gartenbau und Landschaftsarchitektur e.V. (BHGL)

Declaration by Candidate

I hereby declare that my thesis, entitled 'Mechanical **Properties of Apple (*Malus* × *domestica* Borkh.) Fruit Skin and Their Potential Role in Fruit Russeting'** is an original research conducted by myself and has not been submitted for a degree in any other university.

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