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Influence of Hydration and Temperature on the Rheological Properties of Plant Cuticles and Their Impact on Plant Organ Integrity

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ABSTRACT

The rheological properties of enzymatically isolated plant cuticular membranes (CM) of mature leaves of *Yucca aloifolia* L., *Hedera helix* L., *Nerium oleander* L., and *Lycopersicon esculentum* Mill. fruit were analyzed in a transient load-creep test. Cuticular membrane samples were tested dry and hydrated as submerged in distilled water. Apparent plastic extensibility turned out as delayed elastic extensibility, that is, CM showed viscoelastic behavior, if the system had sufficient time (up to 24 h) for relaxation. Both extensibility and the apparent plastic component increased in the hydrated state. In addition to hydration, different temperature regimes ranging from 7° to 30°C were established during testing to evaluate temperature sensitivity of cuticular rheology. Temperature-dependent changes of the rheological properties as small as 1°C could be detected. Extensibility was correlated with neither the thickness of the cuticles nor the specific structure of the cuticles as determined by scanning electron microscopy (SEM) and digital image analysis. For tomato fruit, no significant differences in extension behavior could be detected between CM and fruit skins, including the cell wall proper as analyzed in hydrated condition. The results demonstrate that the cuticle is a flexible biopolymer with rheological properties that can be dynamically modified by both hydration and temperature. The cuticle appears to have a pronounced impact on the overall mechanical behavior of the tomato fruit, implying a substantial contribution to the mechanical integrity of the whole organ. The described rheological properties of the tomato fruit CM are important features to accommodate growth processes without the loss of physiological integrity, but they may also help to understand fruit cracking as affected by hydration and temperature.

Key words: Plant cuticle; Plant biomechanics; Rheology; Load-creep test; Temperature; Hydration

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INDRODUCTION

The plant cuticle is an extracellular layer on the outer epidermal cell walls of primary plant organs such as leaves and shoots. Primarily, it protects the plant against uncontrolled water loss via evaporation (Schönherr 1982), while the membrane inherits a multitude of protective functions against the biotic and abiotic environment, as summarized by Bargel and others (2004). The plant cuticle represents a natural composite with a species-specific and organ-specific composition (Jeffree 1996; Marga and others 2001), but two main fractions are usually distinguished: an insoluble fraction composed of cutin, a biopolymer comprising C_{16} and C_{18} fatty acid monomers, and various soluble lipids collectively called *waxes* (Kolattukudy 1980). The latter are embedded in the cutin matrix as well as deposited onto the surface, where they often form complex three-dimensional crystals (reviews given by Jeffree 1986; Barthlott and others 1998). Cutinized cell wall polysaccharide fibers gradually incorporated into the matrix and a pectin layer link the plant cuticle to the underlying epidermal cell wall (Jeffree 1996).

A prominent feature of the outer polyester coverage is a cross-species variability in thickness (overview in Jeffree 1996). Extensive studies on water permeability have shown that cuticle thickness is not correlated with its water permeability (Schönherr 1982; Schreiber and Riederer 1996). Moreover, the mechanical properties of the biopolymer are independent of its thickness, as shown by Wiedemann and Neinhuis (1998). The mechanical and rheological properties of the cuticle are of significant importance for the plant. The physiologically important transport properties of the cuticle are influenced by its mechanical properties, being affected by factors like polymer density and the amount and type of fillers (Heredia 2003). In addition, the cuticle was proposed to mechanically stabilize plant organs, in particular in species that lack prominent internal reinforcing elements as in the berry fruit type (Wiedemann and Neinhuis 1998; Bargel and others 2004). Recently, it was demonstrated that the cuticle is a mechanically important component of the tomato fruit skin, as is the epidermal cell wall (Matas and others 2004a). The cuticle is of increasing importance for structural integrity during ripening, because, to a large extent, it mirrored the mechanical properties of the epidermis (Bargel and Neinhuis 2005). Material behavior of the cuticle from ripe tomato fruit was described as visco-elastic (Petracek and Bukovac 1995; Matas and others 2004a), whereas knowledge on other species is sparse. Clearly, the cuticle has to accommodate growth of epidermal cells and the entire organ without disturbing the physiological and mechanical integrity. Knowledge of the rheological properties of the cuticle could provide insights into adaptations to growth processes and the potential impact on plant organ integrity, as well as failure symptoms such as fruit cracking, for example, in ripe tomato fruit (Emmons and Scott 1997; Bertin and others 2000; Pascual and others 2000). Although it is unknown whether the biopolymer provides a feedback to modulate growth, lipid components or cutin monomers of the cuticle from *Arabidopsis thaliana* L. mutants were proposed to have a regulatory role in development (Jenks and others 1996; Lolle and others 1997; Pruitt and others 2000). Concerning abiotic factors, hydration of the cuticular membrane increases its elasticity, and as a consequence, water acts like a plasticizer, promoting flexibility of the polymer matrix on a molecular level (Petracek and Bukovac 1995; Wiedemann and Neinhuis 1998; Round and others 2000). Temperature has been shown to affect cuticular permeability (Schönherr and others 1979; Schreiber and others 2001), but temperature effects on the rheological properties of the cuticle have not been described to date. However, phase transitions have been reported in the cuticle of several species in the range between 16°–50°C, and in the isolated biopolyester cutin around 23°C (Eckl and Gruler 1980; Schreiber and Schönherr 1990; Luque and Heredia 1997; Matas and others 2004b). These phase transitions should provide conclusions on the rigidity of the biopolymer and its rheological properties.

The objectives of this study were to evaluate the mechanical behavior of the cuticle, and the influence of the abiotic factors water and temperature on the rheological properties of the biopolymer membrane. Enzymatically isolated leaf and fruit cuticular membranes (CM) from three species and from ripe tomato fruit were subjected to a load-creep test with high spatial resolution. The CM were tested dry, fully hydrated, and at different temperatures. A comparison between CM and corresponding epidermal strips of the tomato fruit was also carried out to analyze the contribution of the cuticle to the rheological properties of the fruit skin comprising cuticle, epidermis, and a few subdermal cell layers.

MATERIALS AND METHODS

Mature leaves of *Yucca aloifolia* L. (yucca, Spanish bayonet), *Hedera helix* L. (ivy), and *Nerium oleander*

L. (oleander) were collected from plants grown in the Botanical Gardens, University of Bonn, Germany. Commercially grown oblate-spherical tomato fruits (*Lycopersicon esculentum* Mill. cv. Seldeiro) at the mature-red ripening stage were obtained from a local fruit supplier. Average fruit size was between 4.0 and 4.5 cm. Epidermal strips parallel cut with a custom-built razor blade device (5 mm wide) were carefully peeled with forceps. Samples from leaves were cut in the axial direction from the adaxial leaf lamina, whereas samples from tomato fruit were derived from the cheeks with cutting direction from the peduncle insertion to the tip. The peels were transferred to a cellulase/pectinase solution containing cellulase (3.5 % [w/w], Roth, Darmstadt, Germany), pectinase (3.5 % [w/w], Fluka, Buchs, Switzerland), sodium azide (NaN_3, 2 mM; Merck Schuchardt, Hohenheim, Germany), polyvinyl pyrrolidone (0.2 % [w/w]) and citrate (20 mM; both Merck, Darmstadt, Germany) at 30°C for 10–30 days according to Wiedemann and Neinhuis (1998). A pH of 5.0 was established for optimum enzyme activity. Completely isolated CM were rinsed repeatedly with distilled water, air dried on Teflon mesh, and stored in Petri dishes at room temperature before testing. Tomato fruit skin samples comprising the cuticle, epidermis, and a few hypodermal cell layers were prepared from peeled strips by carefully removing adhering parenchyma tissue with a razor blade. These samples were incubated in ethanol (70%; Roth, Hamburg, Germany) for 5 min at room temperature to eliminate both the physiological stress reactions of living protoplasts and a possible effect of turgor during testing. The fruit skins were then repeatedly washed with distilled water, and stored in polyvinyl pyrrolidone/NaN_3 (0.2% [w/w]/2 mM) to prevent fungal growth until further processing.

Extensibility of the isolated CM as well as of tomato fruit skin was measured by means of a transient load-creep test with a custom-built extensometer according to the method of Edelmann and Köhler (1995) (Figure 1). In such a test, the material is placed under a constant stress level, and the deformation changes over time are measured. Visco-elastic material behavior is characterized by an instantaneous elastic deformation (De_1), followed by a relatively slow time-dependent deformation called *creep* (De_2). The rate at which the deformation occurs is governed by the material's viscosity (Niklas 1992). Total sample length of the CM was trimmed to 20 mm, and the free ends of the samples were fixed mechanically with vise-type clamps. The free sample length (l_0) between the clamps was 10 mm throughout all measurements. Dried samples most likely were slightly narrower than the 5 mm at the wet extraction stage, but this small shrinkage artifact was considered to be negligible for the comparative nature of this study. After mounting, the samples were allowed to equilibrate for 15 min prior to loading, because preliminary tests had shown that no further relaxation of the samples occurred from that time on. Two different constant forces were applied, that is, 0.79 N for yucca, oleander, and tomato (corresponding to a weight of 80 g) and 0.098 N for ivy (corresponding to a weight of 10 g), because CM samples from this species ruptured at the higher force of 0.79 N. The elastic and apparent plastic properties were characterized by determining the deformation of the strips after various time periods (up to 24 h) subsequent to stress removal. Deformation versus time-curves were recorded by a chart recorder at a high-resolution setting. Mechanical testing was either carried out on air-dried or fully hydrated CM samples submerged in distilled water employing a water reservoir around the clamps. The fruit skins were tested exclusively in the fully hydrated state. Different temperature regimes of hydrated tomato fruit CM ranging between 7°C and 30°C were adjusted in the water reservoir by a continuous-flow device connected to a temperature-controlled water bath. Temperature was measured close to the cuticles (1 mm) with a thermometer. Deformation versus time-curves were recorded and analyzed for each sample individually, with the number of samples n = 3 to 6 per species and experimental setup. Consistent results were obtained.

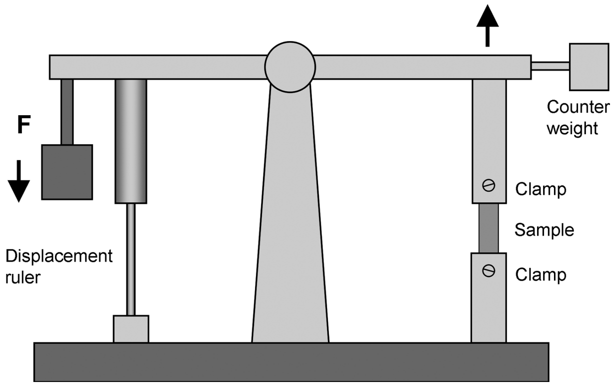

Figure 1. Schematic drawing of the custom-built extensometer used for rheological characterization. The displacement ruler was connected to a line-writer with a high-resolution setting. Free sample length (l_0) between the vise-type clamps was 10 mm throughout all measurements. The arrows indicate the direction of movement of the balance arm. *F* applied force, that is, 0.79 N and 0.098 N.

Ultrastructural examination of dry cross-sections of CM and of the fruit skins was carried out by Scanning Electronic Microscopy (SEM) and digital image analysis. Prior to analysis, fruit skin samples were critical-point-dried (CPD 020; Balzers Union, Wiesbaden, Germany). All samples were coated with gold in a sputter coater (SCD 040; Balzers Union, Wiesbaden, Germany, and K550; Emitech, Ashford, UK), mounted on a modified aluminum stub with a small amount of carbon cement, and subsequently examined at 15 kV (Cambridge Stereoscan 200; Leica, Bensheim, Germany, and 420i; LEO, Oberkochen, Germany). Digital image analysis was carried out with the free NIH Image software (http://rsb.info.nih.gov/nih-image/). Thickness measurements were carried out as double replicates ($n < 7$) and covered all cutinized layers of the isolated cuticular material including cuticular pegs, but excluded the areas of the former cells. Thicknesses of the cuticle and underlying epidermal cell wall of the tomato fruit skin were determined by scanning a selected range of each cross-section, excluding cell lumen as well as the hypodermal cell layers. To determine the actual stresses exerted on the CM samples during testing, the corresponding cross-sectional areas were calculated (mean thickness × width of the sample). Results are given as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Ultrastructural Analysis

Scanning electron microscopy analysis revealed a species-specific cross section and inner surface morphology of the isolated cuticles (Figure 2). Cuticular membranes of *Yucca* and *Lycopersicon* exhibited a cast of the arrangement and dimensions of the epidermal cells, and the cuticular layer extends into the anticlinal walls of the cells by cuticular ridges. Moreover, in tomato, the entire epidermal layer including the inner periclinal walls of the epidermal cells is cutinized (Figure 2b, 2d). In contrast, CM from *Hedera* and *Nerium* displays no pronounced cuticular ridges (Figure 2a, 2c), but typically low ridges tracing the undulate course of the anticlinal cell walls characterize the inner surface of ivy (Wiedemann and Neinhuis 1998). The fracture surfaces obtained from dry CM of all isolated CM except ivy are characterized by a more or less amorphous appearance and a mostly uniform texture. No special pattern or layers could be detected within the CM, and no distinction between cuticle proper and cuticle layer could be determined, even at high resolution. The fracture surface of ivy shows a distinctively granular structure. Thickness values were 12 ± 0.8 μm for *Yucca*, 10.2 ± 0.7 μm for *Nerium*, 3.7 ± 0.4 μm for *Hedera*, and 13.0 ± 0.9 μm for *Lycopersicon*. Thickness of the tomato fruit epidermal cell wall measured 1.2 ± 0.1 μm (results not shown). These thickness values are well within the range of published data, for example, 14.2 μm and 6.7–15.3 μm for ripe tomato fruit (Chaumat and Chamel 1991; Petracek and Bukovac 1995), and 4.25 μm for mature leaves of ivy (Viougeas and others 1996). Average cuticle thickness of oleander, as calculated on the basis of the reported cuticular mass/density (1170 kg m^{-3}), resulted in 11.7 μm (Schreiber and Schönherr 1990; Schreiber and Riederer 1996). For tomato fruit epidermal cell wall, a thickness range between 1.0 to 1.3 μm was recently reported (Bargel and Neinhuis 2005).

Load-Creep Tests with Isolated Dry Cuticles

Dry samples of isolated CMs were subjected to constant tensional stress in a transient load-creep test. In response to the load applied, the observed total extensibility in terms of strain (total deformation/l_0) differed between the species in the range of about 2%–5% (Figure 3). Extensibility of ivy CM was measured with a reduced force of 0.098 N. From the determination of the cross-sectional areas, these forces applied could be calculated as actual stresses exerted on the CMs, resulting in about 13 MPa for *Yucca*, 15.3 MPa for *Nerium*, 12 MPa for *Lycopersicon*, and 5.3 MPa for *Hedera*. The individual displacement versus time diagrams of isolated leaf and fruit CM showed a typical asymptotic time course behavior characterized by an instantaneous elastic deformation and a less steep creep phase, including delayed elastic and plastic deformation until no further extension could be measured (Niklas 1992). These diagrams were similar to earlier results for cell walls of rye (*Secale cereale* L.) coleoptiles (Edelmann 1995; Edelmann and Köhler 1995). Upon removal of stress, the CM contracted but showed residual extension or plastic deformation (Figure 3). Depending on the species, the irreversible stretching amounted to about 5%–15 % of the maximal deformation, even after up to 24 h of relaxation. To test if this portion of residual deformation was a systematic error introduced by the testing equipment, a similar load-creep test with a force of 0.78 N was carried out with strips of commercially available rubber, a material known for its exclusively elastic properties. Rubber also displayed an apparent plastic residue after removal of stress in the same order of magnitude (results not shown). Thus, the apparent plastic component detected for

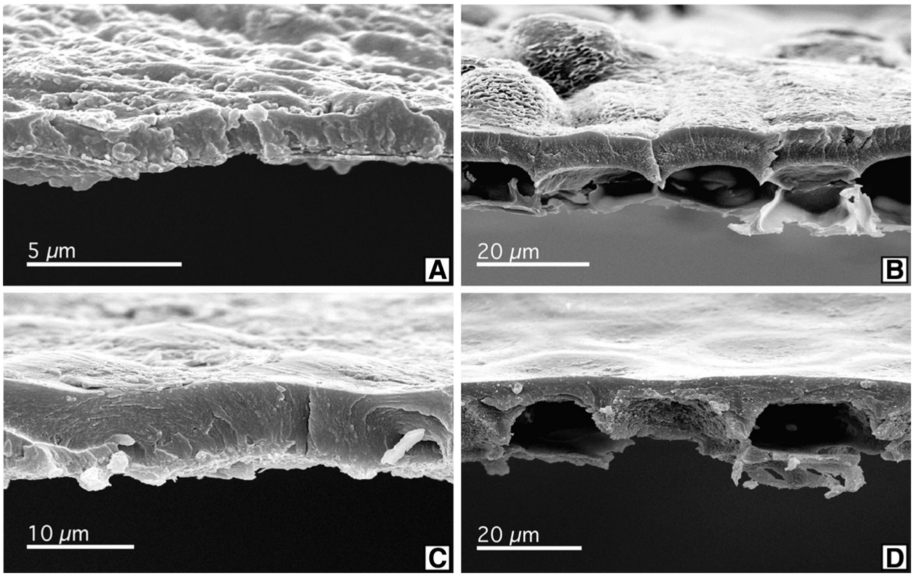

Figure 2. Scanning electron micrographs of cross-sections of isolated cuticles (CM) of *Hedera helix* **A.** *Yucca aloifolia*; **B.** *Nerium oleander*; **C.** *Lycopersicon esculentum* fruit; **D.** Note the granular fracture surface of the *H. helix* CM, as well as the cutinized inner periclinal wall of the tomato fruit.

the CM samples should be largely due to experimental setup and not to changes in the material. Therefore, if the system had sufficient time for relaxation after removal of stress, the material behavior of the CMs could be characterized as visco-elastic without a plastic component under the testing conditions described. As a consequence of this interpretation, the plastic component described for tomato cuticles from ripe fruits by Petracek and Bukovac (1995) could also be delayed fully elastic extensibility as outlined in this study. However, it is important to note that there might well occur plastic deformation that is masked by the testing equipment.

Obviously, the CM of all species tested, except ivy, was able to withstand the applied force of 0.78 N in the (visco-)elastic range. Viscoelasticity was also reported for tomato fruit skin and isolated CM of mature fruit (Matas and others 2004a), thistle staminal filaments (Hasenstein and others 1993), coleoptiles of maize (*Zea mays* L.) (Kutschera and Schopfer 1986), and rye (Edelmann and Köhler 1995). It was described to be a typical feature for young primary cell walls of actively growing cells (Niklas 1992). The cause of the complete reversibility of visco-elastic extension may depend to a certain extent on prestresses in, or preconditioning of, the cuticle that renders apparent plastic and elastic deformations entirely elastic (Hasenstein and others 1993; Wiedemann and Neinhuis 1998). Concerning the biological function, visco-elasticity of the cuticle could reduce the risk of mechanical failure owing to energy dissipation and reduction of stress peaks on naturally occurring dynamic loads. It may enable long leaves like that of *Yucca* to maintain their mechanical integrity, especially during swaying. For sclerophyllous leaves of *Nerium* it might be related to the leaf water status (Schreiber and Riederer 1996) by facilitating expansion/shrinkage cycles induced by the Mediterranean climate, as described for other species (Balsamo and others 2003). Expansion/shrinkage cycles may be particularly important for the rheological properties of the tomato fruit CM due to diurnal fruit expansion patterns (Ohta and others 1997; Bertin and others 2000).

The instantaneous elastic deformation (*De*$_1$) is most pronounced in CM of *Nerium* (Figure 3). Here, *De*$_1$ as determined after removal of stress measured about 67% (related to total deformation) compared to about 62% for *Yucca* and 61% for *Lycopersicon*, indicating a less rigid polymer arrangement within the cuticle of *Nerium*. A similar extension of about 58% could be measured for *Hedera*, with only 1/8 of the load implying a larger extensibility and an even less rigid cuticle than observed for *Nerium*. This exceptional mechanical property was also reflected by the unique texture of the fracture surface (Figure 2a). The delayed creep component (*De*$_2$) as determined after removal of stress was generally more than halftimes lower than the instantaneous elastic component; however, *De*$_2$ was highest with about 31% (related to total deformation) for *Yucca*, whereas it was about 25% for *Hedera* and *Nerium*, and 28% for *Lycopersicon*. Although *Hedera* had the thinnest CM, thickness was not correlated with load-creep behavior of the remaining species. Applying one-dimensional tension tests, similar trends have also been reported by Wiedemann and Neinhuis (1998). Their study provided the modulus

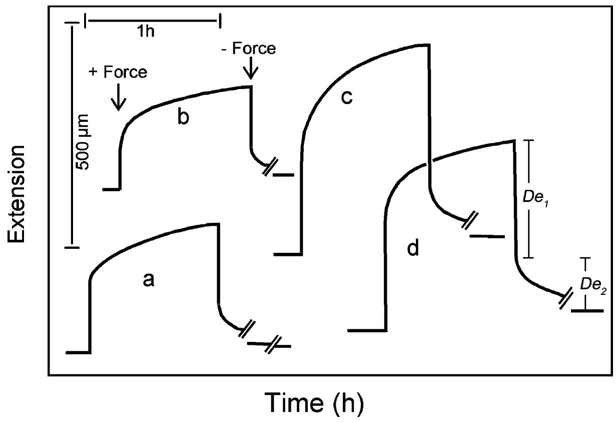

Figure 3. Representative displacement versus time diagrams of isolated cuticles of leaves of **a.** *Y. aloifolia*; **b.** *H. helix*; **c.** *N. oleander*, and of fruits of *L. esculentum* **(d)** subjected to a transient load-creep test. Constant tensional forces of 0.78 N in **a, c,** and **d,** and 0.098 N in **b** were applied and removed as indicated by arrows in **b.** Resulting total extensibility in terms of strain (extension/l_o) was in the range of about 2%–5%. The instantaneous elastic deformation (*De_1*) and creep component (*De_2*) were determined after stress removal as indicated in **d**.

of elasticity of the CM isolated from the same species, that is, 1360 MPa and 730 MPa for *Yucca*, 580 MPa and 370 MPa for *Nerium*, and 640 MPa and 290 MPa for *Hedera* in the dry and hydrated state, respectively. Generally, the force of 0.78 N applied to CM of *Yucca*, *Nerium*, and *Lycopersicon* was more than twice as high as the fracture force reported for ripe tomato fruit cuticle of cv. Pik Red by Petracek and Bukovac (1995). In a preliminary study, isolated cuticles of both maize and rye coleoptiles fractured far below a force of 0.294 N, which had been used earlier in creep measurements of frozen/thawed coleoptiles (Edelmann 1995). Wiedemann and Neinhuis (1998) reported breaking stresses close to the range of the stresses exerted on the CM samples in the load-creep tests reported here, for example, greater than 20 MPa for dry *Yucca* and *Nerium* CM, and greater than 15 MPa and 5 MPa for *Hedera* and *Lycopersicon*. For most of the hydrated CM, these authors reported stresses greater than 15 MPa. A reasonable explanation for the rather high stresses in this study might be the sample geometry that is, 5 × 10 mm. These dimensions result in an unfavorable length-to-width ratio of 1:2 and arguably evoked end effects caused by clamping that tend to stiffen the tested material (Vincent 1992; Niklas 1992). This might also have affected extensibility in absolute terms, but since sample geometry was the same for all measurements, relative comparison of the rheological properties of the CMs tested is not impaired.

Influence of Water on the Extensibility of Isolated Cuticles

The first set of experiments was performed with dry cuticles. However, plant cuticles *in situ* are more or less hydrated and may, under physiological conditions, be subject to a so far unknown complex cross-sectional hydration profile, both with respect to diurnal profiles and seasonal profiles. To evaluate the influence of water, we saturated tension-stressed dry cuticles with distilled water at 20°C to mimic the fully hydrated status. As shown in Figure 4, this operationally defined hydration resulted in an additional instantaneous deformation of more than 100% of the elastic deformation as compared to dry samples during an extension period of 1 h for all species tested, with species-specific differences between them. In addition, the creep component *De_2* of the hydrated samples was of a similar order of magnitude of the dry samples of *Yucca* and *Hedera*, and almost twofold higher for *Nerium* and *Lycopersicon* (related to the corresponding $\text{deformation}_{\text{dry}}$ and $\text{deformation}_{\text{hyd}}$). The delayed creep component of oleander observed in dry conditions is strongly enhanced by hydration (Figure 4c). Here, a short-term change from hydrated to dry conditions by air-drying of the samples resulted in a decrease of the temporarily enhanced cuticle extensibility. The apparent plastic component was more pronounced than in the dry state, that is, it was about 21% for *Yucca*, 35% for *Hedera*, 15% for *Nerium*, and 20% for *Lycopersicon* (as related to total deformation). These results indicate that the rheology of the plant cuticle of the species tested is considerably influenced by hydration, and that water enhances the viscous component, increasing the extensibility at a given stress as well as the plastic residue. This was also indicated during creep experiments on isolated tomato fruit cuticle (Petracek and Bukovac 1995) and rye coleoptiles, employing PEG solutions to establish different water potentials (Edelmann 1995). The modulus of elasticity of fully hydrated cuticles of the same species investigated in this study was about 35%–50% lower as compared to the dry condition (Wiedemann and Neinhuis 1998). A pronounced decrease of the surface elastic modulus of isolated tomato fruit cutin was also determined in nano-scale mechanical studies (Round and others 2000).

The chemical composition of mature tomato fruit cuticle has been characterized by predominant C_{16}

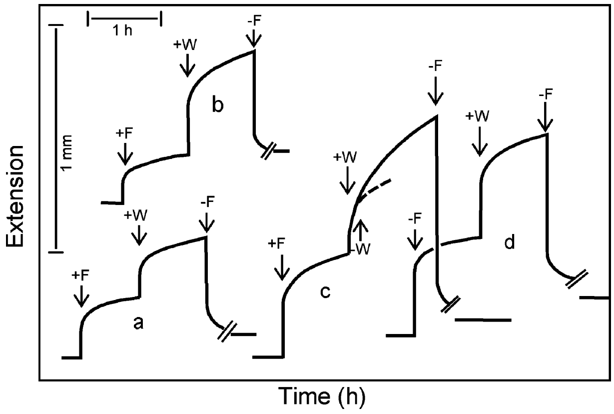

Figure 4. Representative displacement versus time diagrams of dry and subsequently hydrated isolated cuticles of leaves of **a.** *Y. aloifolia*; **b.** *H. helix*; **c.** *N. oleander*, and of fruits of *L. esculentum*; **d.** Pre-stressed dry cuticles were hydrated by addition of distilled water at 20°C (+w). The scattered line in **c** indicates the load-creep behavior of *Nerium* CM from hydrated to dehydrated state by subsequent air-drying (–w) of the sample at 20°C. Constant tensional forces of 0.78 N in **a, c,** and **d,** and 0.098 N in **b** were applied and removed as indicated by arrows.

monomers (Baker and others 1982; Graca and others 2002), whereas ivy cuticle has been classified as a C_{18} type (Holloway and Brown 1981; Graca and others 2002). The predominance of 10,16-dihydroxyhexadecanoic acid monomers in the cutin of ripe tomato fruit provides only limited hydrogen bonded cross-linking possibilities, allowing even small amounts of water absorption to exert a dramatic influence on flexibility and elasticity (Round and others 2000). The same may hold true for the CM of *Nerium*, explaining the rather instant rheological changes observed during hydration/drying. On the other hand, the reported high level of 9,10,18-trihydroxyoctadecanoic acid in ivy cuticles could account for the high extensibility at lower tension stresses. Analogous to unripe tomato fruit cutin, which was also characterized as having high levels of trihydroxy C_{18} fatty acids (Baker and others 1982), hydroxyl groups have the potential to enhance the hydrophilic character of the cutin matrix, resulting in higher elasticity and plasticity (Bargel and others 2004; Bargel and Neinhuis 2005). This interpretation was also proposed by Marga and others (2001) for the cuticle of the thistle *Cirsium horridulum* Michx. For a conclusive judgment, however, the number of free hydroxyl groups in the corresponding cuticle and the actual amount of water in the membrane has to be determined, because the ability to absorb and bind water as well as the extent of hydration seems to be crucial to cuticular material properties.

Hydration of the cuticle also seemed to increase the apparent plastic residue, which may be caused by swelling of the cuticle. This effect is particularly obvious in ivy cuticles (Figure 4b). Swelling of the CM could be caused by water molecules sorbed to certain polar domains of the cuticle (Chamel and others 1991), presumably at the sites of free hydroxyl groups of the cutin matrix, and may also be embedded between carboxyl groups of the polyester and hydroxyl groups of polysaccharides via hydrogen bonds (Marechal and Chamel 1996). Importantly, the polysaccharides incorporated into the CM may contribute to a large extent to the water status of the plant cuticle, depending on the polysaccharide content (Chamel and others 1991; Dominguez and Heredia 1999), as also discussed by Bargel and Neinhuis (2004) for tomato fruit cuticle. With respect to the rheological properties, the cuticle as the boundary layer between plant and environment therefore seems to be able to adapt quickly to changes of hydration as mediated either by internal (that is, water supply) and external factors such as light intensity, temperature, and wind. The natural state of cuticular hydration is crucial to fully interpret the mechanical behavior of the plant cuticle, but these data are not currently available, and further studies on the details are necessary.

Influence of Temperature on the Extensibility of Isolated Cuticles

The influence of temperature on the rheological properties of the cuticle was analyzed for the tomato fruit CM at different temperature regimes by regulating the water temperature in the reservoir. These experiments were of a comparative and semi-quantitative nature to show a general trend, and no percentage values for temperature dependence of De_1 and De_2 are provided. Generally, an increase in temperature resulted in increased maximum extensibility of the tension-stressed cuticles (Figure 5), and the response of the CM was gradual, depending on the temperature regimes. Larger increases in temperature, that is, from 7°C to 25°C and from 25°C to 30°C, resulted both in instantaneous larger extension steps as well as in increased creep rates. The latter were pronounced toward the higher temperatures. A second setup was conducted to reveal responses to smaller increases in temperature in the range between 20°C and 23°C in 1°C steps. These temperature increases induced a step-

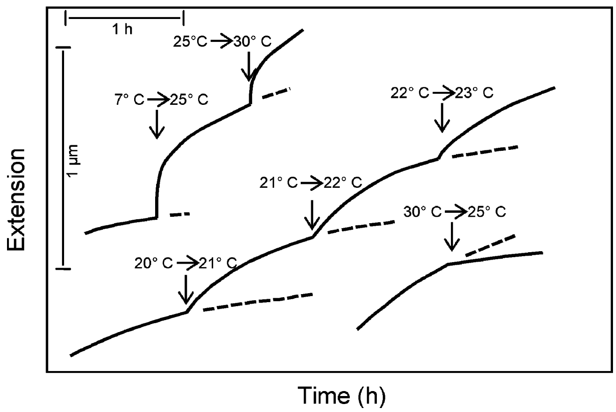

Figure 5. Representative displacement versus time diagrams of submerged, hydrated isolated CM from fruits of *L. esculentum* in dependence of temperature changes as indicated by arrows. Dotted lines illustrate extension if the temperature remained unchanged. Different temperature regimes were tested, and temperature changes as small as 1°C could be detected in the load-creep behavior.

by-step increased extensibility, which was dominated mainly by a creep component. A third setup aimed at revealing changes in the creep behavior of tomato fruit CM if the temperature was exemplary decreased from 30°C to 25°C. This immediately resulted in reduced maximum extensibility. Thus, temperature changes in either direction were reflected spontaneously by an increase or a decrease of the CM extensibility. Similar results could be obtained for the leaf CMs (results not shown).

Temperature-dependent changes in the extensible properties of technical polymers are generally known (Drozdov 2001); however, they have not been directly shown for the plant cuticle. The rheology of the plant cuticle is similar to semicrystalline technical polymers above the glassy state (Nielsen and Landel 1994; Petracek and Bukovac 1995). In particular, the glass transition temperature of mature tomato fruit cuticle has been determined to be –30°C, and that of isolated cutin at –47°C (Luque and Heredia 1997). Additional phase transitions in the tomato fruit cuticle have been reported to occur in the range between 30° and 45°C, and in the isolated biopolyester cutin around 23°C (Luque and Heredia 1997). Phase transitions of cuticles have also been reported for other species, including *Hedera* and *Nerium*, and transitions between 16.3° and 38°C and between 40° and 50°C have been measured (Eckl and Gruler 1980; Schreiber and Schönherr 1990). These phase transitions should provide conclusions on the rigidity of the biopolymer and hence of its material behavior. Below the transition temperature, the mobility of moieties within and rotation along the aliphatic chains of the polyester is restricted, and above the transition temperature, the segmental mobility, rotation, and translational motions between the aliphatic chains present in the cuticle increase. At low temperatures the CM is in a rigid state, whereas at higher temperatures part of the membrane is fluid (Eckl and Gruler 1980). Therefore, the cuticle would exhibit an increased extensibility due to higher viscous flow near or above the specific phase-transition temperatures and a reduced extensibility below the specific phase-transition temperatures. For the experimental conditions in this study, this might be the case for ivy cuticles, reflecting a thermodynamic explanation of the reduced applicable force in the load-creep test as compared to the other species tested. The effect of temperature on the mechanical properties might also hold true for mature tomato fruit CM, because in the study of Matas and others (2004b) the thermograph showed a steady increase in the specific heat capacity with temperature increases ranging from 0° to 40°C. Water changes the rheological properties of the CM, because phase-transition temperatures of hydrated cuticles are shifted toward lower temperatures (Eckl and Gruler 1980; Matas and others 2004b). Thus, both hydration and temperature have a significant influence on the rheological properties of the plant cuticle.

The question remains whether the effect of temperature on the rheological properties of tomato fruit cuticles is of importance for the whole fruit, as well as for leaves. The cuticle as the outermost layer of plants is directly exposed to various environmental influences. Apart from hydration, plant surfaces are most immediately exposed to changes in temperature—in the short term as well as the long term. Changes in temperature should have a great influence on organ growth. It is conceivable that plants can profit from the thermoelastic rheological properties to the extent that at higher temperatures, good hydration conditions provided, growth may be facilitated by higher extensibility of the cuticle in connection with a generally higher metabolic activity. A correlation between temperature and tomato fruit growth has been previously analyzed (Adams and others 2001). In contrast, low temperatures, which affect growth negatively, should be expected to result in mechanically more resistant cuticles, and should therefore lead to a more rigid organ appearance. Eckl and Gruler (1980) pointed out that the large thermal expansion coefficient of the CM could create microfractures. These could enable the membrane to remain mechanically stable instead of experiencing abrupt failure while the membrane enlarges during growth above the phase-transition temperatures without loosing its physiological integrity. On the other hand, both water and temperature are two of the main abiotic stress factors associated with fruit cracking that occurs in tomatoes. This has been repeatedly demonstrated, for example in studies on the influence of rain and direct sunlight on cuticle cracking (Emmons and Scott 1997), water vapor pressure deficit (Bertin and others 2000), or watering techniques and temperature (Pascual and others 2000). One can speculate that the increased total extensibility, creep component, and higher plastic deformation at higher temperature regimes and hydration reflect weakening of the biopolyester, which could result in mechanical failure and cracking under severe circumstances. Recently, Bargel and Neinhuis (2004) showed that hydration not only caused lowered moduli of elasticity, but also reduced the strength of the tomato fruit cuticle throughout fruit growth and ripening. Despite the availability of many articles in the literature, cracking of fruits still remains an important field for further research, although the mechanical properties of the cuticle and the interplay between both temperature and hydration provide hints for the basis of this widespread and economically important problem.

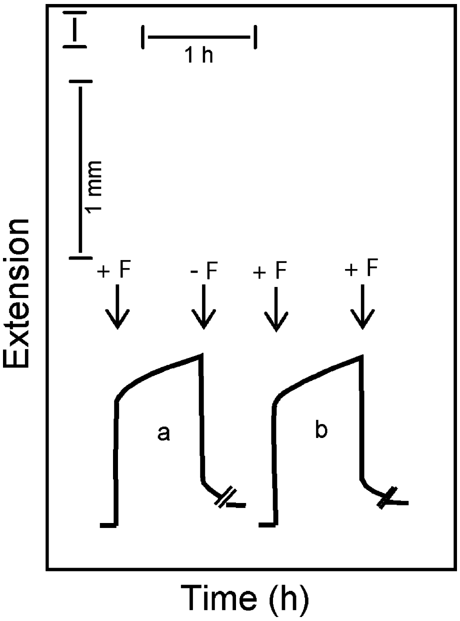

Figure 6. Representative displacement versus time diagrams of hydrated isolated cuticles **(a)** compared to hydrated fruit skins of *L. esculentum* **(b)**. The samples were subjected to a constant force of 0.78 N, applied (+F) and removed (–F), as indicated by arrows.

Rheological Properties of the CM versus Epidermis Cell Walls

To estimate the contribution of the cuticle to the mechanical behavior of the epidermis, the creep behavior of tomato fruit skin samples comprising the cuticle, epidermal layer, and a few additional subdermal cell layers was compared with isolated cuticles from the same batch of samples. No significant differences could be detected between the extension behavior of the fruit skin and the isolated CM (Figure 6), implying that at least at the ripe fruit stage, to a large extent the cuticle mirrors the rheological properties of the fruit skin. It has been argued that the epidermis is of importance in determining the rate of expansion during growth (Thompson 2001; Andrews and others 2002). Moreover, Matas and others (2004a) demonstrated that the tomato fruit cuticle is an important structure of the mature fruit, and Bargel and Neinhuis (2005) showed that the tomato fruit cuticle is of increasing importance for the structural integrity of the fruit skin and, hence, of the whole fruit. This is mainly due to ethylene-dependent modifications of texture, cell wall ultrastructure and composition during ripening of a large variety of climacteric fruits, among them tomato, caused by the activity of several enzymes (see for example, Fischer and Bennett 1991). Thus, the structural components necessary to reinforce the cell wall and the adhesion of cells in the fruit pericarp are degraded during the ripening process and, consequently, the fruit softens. Pectic polysaccharides play a crucial role in cell wall adhesion (Seymour and others 2002). Pectin content on the one hand, and cellulose and hemicellulose content and composition on the other hand affect the mechanical properties of tissues (Whitney and others 1999; Marga and others 2003). In contrast, the cuticle seems to remain largely unaffected by the ripening-related cell wall–modifying enzymatic activity, but it is subject to cuticle-specific alterations that tend to stiffen the membrane (Baker and others 1982; Bargel and Neinhuis 2004, 2005). Thus, the results presented here provide further evidence that the cuticle contributes significantly to the mechanical behavior of the epidermis and is thus important for the structural integrity of the tomato fruit. Possibly this relevance may apply to the cuticles of fruits of other species.

CONCLUSIONS

It has been shown that isolated leaf and fruit CM from three species and from ripe tomato fruit can be characterized by visco-elastic behavior, whereas their rheological properties differ in a species-specific way. Moreover, the cuticle is a flexible biopolymer whose mechanical behavior can be dynamically modified by the abiotic factors water and temperature, and it seems to be determined by its chemical composition and molecular architecture. Hydration of CM samples increased the apparent plastic component; as a consequence, water acts as a plasticizer of the cuticle. From the results presented here, it can be inferred that the plant cuticle plays an important role for the structural integrity of organs, particularly in berry-type fruits, without prominent internal reinforcing elements as found in tomato fruits. Depending on the developmental, as well as environmental conditions, the cuticle inevitably has to accommodate growth of organs, shrinkage/expansion cycles, or external factors such as wind loads and high temperatures without the loss of physiological integrity. The cuticle may therefore constitute part of a sophisticated ''blueprint'' of plants.

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