



## Research Article

# Effectiveness of cuticular transpiration barriers in a desert plant at controlling water loss at high temperatures

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**Abstract.** Maintaining the integrity of the cuticular transpiration barrier even at elevated temperatures is of vital importance especially for hot-desert plants. Currently, the temperature dependence of the leaf cuticular water permeability and its relationship with the chemistry of the cuticles are not known for a single desert plant. This study investigates whether (i) the cuticular permeability of a desert plant is lower than that of species from non-desert habitats, (ii) the temperature-dependent increase of permeability is less pronounced than in those species and (iii) whether the susceptibility of the cuticular permeability barrier to high temperatures is related to the amounts or properties of the cutin or the cuticular waxes. We test these questions with *Rhazya stricta* using the minimum leaf water vapour conductance ( $g_{\min}$ ) as a proxy for cuticular water permeability.  $g_{\min}$  of *R. stricta* ( $5.41 \times 10^{-5} \text{ m s}^{-1}$  at 25 °C) is in the upper range of all existing data for woody species from various non-desert habitats. At the same time, in *R. stricta*, the effect of temperature (15–50 °C) on  $g_{\min}$  (2.4-fold) is lower than in all other species (up to 12-fold). *Rhazya stricta* is also special since the temperature dependence of  $g_{\min}$  does not become steeper above a certain transition temperature. For identifying the chemical and physical foundation of this phenomenon, the amounts and the compositions of cuticular waxes and cutin were determined. The leaf cuticular wax ( $251.4 \mu\text{g cm}^{-2}$ ) is mainly composed of pentacyclic triterpenoids (85.2% of total wax) while long-chain aliphatics contribute only 3.4%. In comparison with many other species, the triterpenoid-to-cutin ratio of *R. stricta* (0.63) is high. We propose that the triterpenoids deposited within the cutin matrix restrict the thermal expansion of the polymer and, thus, prevent thermal damage to the highly ordered aliphatic wax barrier even at high temperatures.

**Keywords:** Aliphatic compounds; cuticular transpiration; cuticular wax; cutin; desert; minimum conductance; plant cuticle; temperature; transition temperature; triterpenoids.

## Introduction

The efficient control of water loss to the atmosphere is one of the foremost prerequisites for plant survival and

competitiveness in any environment but particularly in deserts. Stomata and cuticle together enable plants to maintain a favourable water status. All above-ground

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primary parts of terrestrial higher plants are covered by a cuticle. This structure with thicknesses ranging from  $<1\ \mu\text{m}$  to  $>20\ \mu\text{m}$  (Jeffree 2006) consists of a matrix of polymeric cutin (cross-linked  $\text{C}_{16}$  and  $\text{C}_{18}$  hydroxy fatty and hydroxyepoxy fatty acids) with cuticular waxes embedded within or deposited onto its surface. The typical compounds of plant cuticular waxes are long-chain aliphatics and cyclic molecules, especially pentacyclic triterpenoids (Jetter *et al.* 2006).

The water permeability of the cuticle determines the minimum and inevitable water loss when the stomata are maximally closed at drought stress. Therefore, a low cuticular water permeability is one of the main factors supporting the survival and viability of plants under scarce water supply. Leaf cuticular water permeabilities [see Supporting Information File 1] differ widely among species and range from  $10^{-7}$  to  $10^{-4}\ \text{m s}^{-1}$  (Riederer and Schreiber 2001).

The extraction of the cuticular waxes with organic solvents leads to increases in the cuticular water permeability by up to several orders of magnitude (Schönherr 1976; Schönherr and Lenzian 1981), which demonstrates that the waxes constitute the main transport-limiting barrier of cuticles. The thickness of the cuticle or the amount of total cuticular waxes associated with it are not related to cuticular water permeability (Schreiber and Riederer 1996; Riederer and Schreiber 2001). So, the frequent statements in textbooks and in the scientific literature that thicker cuticles or cuticles containing more wax are better barriers against transpiration than thin ones with only small amounts of wax (e.g. Purves *et al.* 2004; Poorter and Garnier 2007; Lüttge 2007; De Micco and Aronne 2012; Smith *et al.* 2012; Jones 2013) are not supported by experimental evidence. The chemical composition of cuticular waxes varies considerably among species. Despite their physiological and ecological importance, the relationship between the composition of cuticular waxes and the water permeability is not yet sufficiently understood (Schönherr 1982; Riederer and Schneider 1990; Kerstiens 2006).

Cuticular permeability is not a fixed entity but is subject to environmental influence. Temperature has a pronounced effect on cuticular water permeability. Particularly in species experiencing high and fluctuating temperatures, the temperature dependence of permeability is a major parameter influencing the physiological and ecological relevance of the cuticle. The effect of temperature on cuticular permeability is significant. In the species studied so far, cuticular permeability increased slightly at temperatures from 15 to  $\sim 35\ ^\circ\text{C}$  while in the higher temperature range (above  $\sim 35\ ^\circ\text{C}$ ) a drastic increase in the cuticular water permeability was observed (Riederer 2006; Schreiber 2001).

In deserts, high air temperatures, strong irradiation and substantially reduced transpirational cooling due to stomatal closure can cause leaves to reach temperatures up to  $50\ ^\circ\text{C}$  (Smith 1978). As stated by Goodwin and Jenks (2005), it is hard to reconcile the steep increase of cuticular water permeability above the transition temperature observed with many non-desert plant species with the successful adaptation of xerophytes to high-temperature climates. From the knowledge currently available it seems conceivable that at high temperatures the cuticular transport barrier progressively fails and that the survival of the plant is imperilled by excessive cuticular transpiration. This scenario is aggravated by the fact that very often the relative humidity of the air considerably decreases when temperature raises in the course of a day. So, not only a higher permeability of the cuticle but also a higher driving force at elevated temperature lead to a heightened transpirational water loss that may become critical for the plant. However, the impact of temperature on the cuticular water permeability of hot-desert plants has so far not been investigated. Also, there is no study dealing with the cuticular water permeability of a hot-desert plant and its relationship to cuticle chemistry. Consequently, Goodwin and Jenks (2005) argued that studies be needed to elucidate the composition and structure of the cuticles from xerophytic plants and their association with temperature-dependent water permeability.

As a first step to close this gap in knowledge, we investigated the minimum leaf water conductance within the range of ecologically relevant temperatures of the hot-desert plant *Rhazya stricta*. The minimum leaf water conductance at maximally closed stomata is a good proxy for cuticular permeability (Burghardt and Riederer 2003). We also analysed the qualitative and quantitative composition of the cutin and the cuticular waxes of *R. stricta*. This species is an excellent model for this kind of investigations because it is a non-succulent, perennial shrub widespread in the deserts of the Arabian Peninsula and ranging into Iran, Southern Afghanistan and Pakistan (Abd El-Ghani 1997; Deil and Al-Gifri 1998; Emad El-Deen 2005; Al Khamis *et al.* 2012). *Rhazya stricta* has recently been identified as a species with a photosynthetic physiology functional without significant limitations under typical desert conditions of daily extremes of heat, light intensity and low humidity (Lawson *et al.* 2013; Yates *et al.* 2014). The field studies of these authors in the native habitat in Saudi Arabia showed a remarkable high-temperature tolerance of *R. stricta* with no decrease in photosynthetic capacity observed up to leaf temperatures of  $45\ ^\circ\text{C}$ . Since equivalent adaptations on the level of the cuticular transpiration barrier may have evolved, this species seems to be a first choice for testing

hypotheses on the potential adaptations of cuticular properties to hot-desert growing conditions.

In this work, we look for an answer to the fundamental question whether *R. stricta* as a typical woody hot-desert plant maintains a cuticular transpiration barrier able to control effectively water loss even at very high temperatures. We hypothesize that in *R. stricta* when compared with woody non-desert plants: (i) the cuticle is more efficient as a barrier to water loss, (ii) the cuticle is more resistant to thermal stress and (iii) the amounts and/or composition of cuticular waxes and cutin are peculiar. From these hypotheses, we predict that in comparison with woody non-desert species, in *R. stricta* (i) the minimum leaf conductance at a given temperature is lower, (ii) the temperature-dependent increase of minimum conductance is less pronounced and (iii) the composition of the cuticle is qualitatively and/or quantitatively different. We test Hypothesis (i) by measuring the minimum leaf water conductance at 25 °C and compare it with literature data, Hypothesis (ii) by analysing the effect of temperature on minimum conductance and also compare it with literature data and Hypothesis (iii) by qualitatively and quantitatively determining the composition of the cutin and the cuticular waxes for comparison with that of other species. We also consider the role certain major cuticular wax components of *R. stricta* may have for the physical stability of the cuticle.

## Methods

### Field site and plant material

Leaves of *R. stricta* (Apocynaceae) were obtained from original growing sites located at ~80 km north-east of Riyadh, Saudi Arabia (25°24'35.51"N, 47°14'32.61"E and 25°22'53.80"N, 47°14'20.52"E). The two sampling sites are 3 km apart and at the same altitude. All measured properties did not differ significantly between the two populations. The area lies in the tropical arid climate zone. Macroclimatic data are available from a standard meteorological weather station at Riyadh. The mean annual rainfall (1979–2009) is 88.9 mm (Almazroui et al. 2012). The mean annual temperature (1984–2013) is 26.8 °C. The monthly mean of the maximum temperature is 43.7 °C for the warmest month during August (Krishna 2014). The mean annual relative humidity (1980–2000) is 30.4% (Al-Ghobari 2000). Terminal shoots bearing 10–15 fully developed, undamaged leaves from three to five individual plants per site were harvested from November to February (2012–14). The shoots were immediately put into plastic bags and transported to the laboratory within 24 h at room temperature. Afterwards, the samples were stored at 4 °C for up to 3 weeks. *Rhazya stricta* leaves are

very robust, and neither their visual appearance nor minimum conductance or chemical composition of cuticle components changed with storage time.

Basic leaf morphological traits (mean saturated and dry leaf weights, leaf area shrinkage during leaf dehydration and stomatal densities for the adaxial and abaxial leaf surfaces) of the plant material used in the experiments were determined [see Supporting Information File 2]. The leaves were further characterized by the analysis of pressure–volume curves. Values for the relative water deficit (RWD) at the turgor loss point, the osmotic potential at the turgor loss point, the osmotic potential at full saturation, the apoplastic water fraction and the modulus of elasticity were obtained [see Supporting Information File 3].

### Minimum leaf water conductances

The leaf thermal and hydric tolerances were established to delimit the ecophysiological allowable ranges of temperature and dehydration to be used in experimentation. The maximum quantum yield of photosystem II in the dark-adapted state was determined as a vitality parameter at different temperatures and dehydration levels. The maximum thermal and hydric tolerances were 50.8 °C and a RWD of 0.78, respectively [see Supporting Information File 4].

The minimum water conductance of whole leaves ( $g_{\min}$ ) of *R. stricta* was determined. It is defined as the 'lowest conductance a leaf can reach when stomata are completely closed as a result of desiccation stress' (Körner 1995). For mechanistic studies looking for relationships between the barrier properties and the chemical and physical characteristics of the cuticle, measuring the water permeability of isolated, astomatous cuticles would be preferable. However, the leaves of *R. stricta* are amphistomatous, and no cuticles without stomatal pores can be obtained. Burghardt and Riederer (2003) compared  $g_{\min}$  measured with whole leaves according to the protocol employed in the present work with the permeabilities of the isolated astomatous leaf cuticles of five woody plant species. In four species (*Acer campestre*, *Fagus sylvatica*, *Quercus petraea* and *Ilex aquifolium*)  $g_{\min}$  and the water permeability of the isolated cuticles were equal. This suggests that the residual stomatal contribution to  $g_{\min}$  was negligible and that the assumption that  $g_{\min}$  can be used as a reliable proxy for cuticular permeability in *R. stricta* is reasonable.

Leaf minimum water conductances were obtained from the consecutive mass loss of desiccating leaves in the dark at low humidity (Burghardt and Riederer 2003). The wounds of cut petioles of saturated leaves were sealed with a high-melting paraffin wax (melting point 68 °C, Fluka, Neu-Ulm, Germany). The sealed leaves were

placed in an incubator (IPP 110, Memmert) with controlled temperature. Leaf minimum conductances were determined at 15, 20, 25, 30, 35, 40, 45 and 50 °C for characterizing the effect of temperature on cuticular permeability. The air temperature in the incubator was checked with a digital thermometer (Testoterm 6010, Testo, Lenzkirch, Germany). Silica gel (Applichem, Darmstadt, Germany) was used to control the humidity of the atmosphere in the incubator. The actual fresh weights of the desiccating leaves were determined as a function of time of desiccation using a balance (Sartorius MC-1 AC210S, Sartorius, Göttingen, Germany, precision 0.1 mg). The transpiration rate (or flux density of water vapour,  $J$  in  $\text{g m}^{-2} \text{s}^{-1}$ ) was obtained from the change in fresh weight ( $\Delta\text{FW}$  in g) with time ( $t$  in s) across the two-sided leaf area ( $A$  in  $\text{m}^2$ ):

$$J = \frac{\Delta\text{FW}}{\Delta t \times A} \quad (1)$$

The transpiration rate at each dehydration level was corrected for leaf area shrinkage [see Supporting Information File 2]. The water vapour conductance ( $g$  in  $\text{m s}^{-1}$ ) was calculated from the transpiration rate ( $J$ ) divided by the driving force of transpiration. The driving force is the difference between the concentration of water vapour (in  $\text{g m}^{-3}$ ) in the leaf ( $c_{\text{wv leaf}}$ ) and in the surrounding atmosphere ( $c_{\text{wv air}}$ ):

$$g = \frac{J}{c_{\text{wv leaf}} - c_{\text{wv air}}} = \frac{J}{a_{\text{leaf}} \times c_{\text{wv sat leaf}} - a_{\text{air}} \times c_{\text{wv sat air}}} \quad (2)$$

The water activity of the air ( $a_{\text{air}}$ , dimensionless) and hence the air water vapour concentration ( $c_{\text{wv air}}$ ) over silica gel are close to zero (Slavík 1974). The water vapour concentration in the leaf is given by the product of the water activity in the leaf ( $a_{\text{leaf}}$ ) and the water vapour saturation concentration at leaf temperature ( $c_{\text{wv sat leaf}}$ ). The water activity in the leaf for each dehydration level was deduced from the respective measured water potential ( $\Psi_{\text{leaf}}$ , [see Supporting Information File 3]) which can be converted to the corresponding leaf water activity according to:

$$\Psi_{\text{leaf}} = \frac{R \times T}{V_{\text{w}}} \times \ln a_{\text{leaf}} \quad (3)$$

where  $R$  is the gas constant,  $T$  is the absolute temperature and  $V_{\text{w}}$  is the molar volume of water (Nobel 2009). Leaf temperature was measured by a leaf temperature sensor with custom-made leaf clip (Walz, Effeltrich, Germany), and the corresponding water vapour

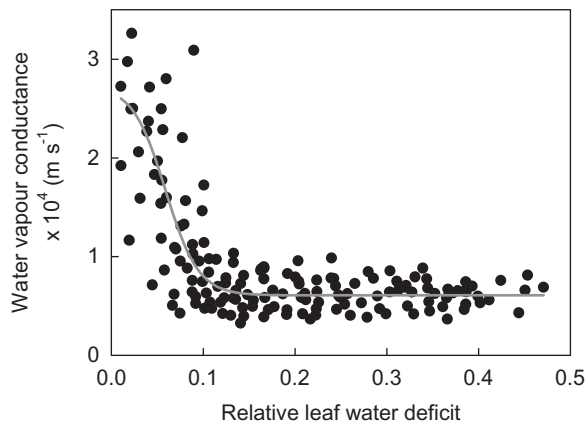
saturation concentrations at leaf temperature were derived (Nobel 2009). The boundary layer conductance was determined using a wet filter paper with the same morphology as *R. stricta* leaves. The wet filter paper was assumed to behave like a water surface and, thus, water conductance was solely controlled by the boundary layer resistance. The evaporation rate was determined gravimetrically in analogy to the method used for the leaves. From the evaporation rate, the conductance was derived assuming a maximum driving force between the wet paper and the dry atmosphere (Grace 1989). The boundary layer conductance was  $6 \times 10^{-3} \text{ m s}^{-1}$  and did not significantly change with temperature. The boundary layer acts as resistance in series, and leaf conductances were corrected accordingly (Slavík 1974).

The conductance of a leaf at a given dehydration state was plotted versus the equivalent RWD [see Supporting Information File 2]. At low RWDs, conductances are high and subsequently decline with progressive leaf dehydration until a plateau is reached where they are no longer sensitive to the further decline of RWD. The constant conductance is commonly interpreted as being the result of maximum stomatal closure. It is called the minimum conductance ( $g_{\text{min}}$ ). The RWD at the stomatal closure point ( $\text{RWD}_{\text{SC}}$ ) was determined from the leaf drying curves as mean values of the conductances at the breaking point between the declining branch and the plateau.

### Chemical composition of cuticular waxes and cutin

Waxes and cutin were obtained from isolated cuticles (Schönherr and Riederer 1986) and analysed by gas chromatography/mass spectrometry (see Smirnova et al. 2013 for details). Leaf discs (area  $1 \text{ cm}^2$ ) were incubated and vacuum-infiltrated in a solution of pectinase ( $10 \text{ g L}^{-1}$ , Trenolin Super DF, Erbslöh, Geisenheim, Germany) and cellulase ( $10 \text{ g L}^{-1}$ , Celluclast, Novozymes, NCBE, University of Reading, UK) in citric acid buffer ( $0.01 \text{ mol L}^{-1}$ , Applichem, Darmstadt, Germany) adjusted to pH 3. Isolated cuticular membranes were obtained within 2 weeks. After washing in borate buffer ( $0.01 \text{ mol L}^{-1}$ , Applichem) adjusted to pH 9 and subsequently in deionized water, the cuticular membranes were air-dried and stored for further use.

Cuticular waxes were extracted by immersing the cuticular membranes two times into 5 mL chloroform ( $\geq 99.8\%$ , Roth) for 5 min at room temperature. Additional extraction steps did not increase the total wax yield. Tetracosane ( $\geq 99.5\%$ , Sigma-Aldrich, Steinheim, Germany) as an internal standard was added to the combined extracts that subsequently were reduced to dryness under a gentle stream of  $\text{N}_2$ .



**Figure 1.** Leaf conductance to water vapour as a function of the relative water deficit (RWD). Each point represents an individual measurement obtained from leaf drying curves at 30 °C with a total of 12 leaves. An exponential four-parameter sigmoid curve is fitted merely to guide the eye. The RWD at maximum stomatal closure is marked by the transition between the declining phase and the plateau phase of leaf conductances. After maximum stomatal closure, leaf conductances remained at a minimum and were constant.

For cutin analysis, the wax-free polymer matrix membranes were depolymerized with a Boron trifluoride-methanol solution (10%  $\text{BF}_3$  in MeOH, Fluka) at 70 °C for 16 h. Dotriacontane ( $\geq 98.0\%$ , Sigma-Aldrich) as an internal standard was added before depolymerization. Subsequently, a saturated aqueous solution of NaCl (Applichem) was added, and the resulting mixture was extracted three times with  $\text{CHCl}_3$ . The combined  $\text{CHCl}_3$  extracts were dried over anhydrous  $\text{Na}_2\text{SO}_4$  (Applichem), and  $\text{CHCl}_3$  was completely removed under a gentle flow of  $\text{N}_2$ .

Wax and cutin samples were derivatized for gas chromatography with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Marchery-Nagel, Düren, Germany) in dry pyridine ( $\geq 99.5\%$ , Roth) for 30 min at 70 °C. Quantitative analysis was carried out with a gas chromatograph equipped with a flame ionization detector and an on-column injector (7890A, Agilent Technologies, Waldbronn, Germany). Separation of compounds was achieved on a fused-silica capillary column (DB1-ms, 30 m length  $\times$  0.32 mm ID, 0.1  $\mu\text{m}$  film, Agilent Technologies) with hydrogen as a carrier gas. The temperature programme for wax analysis was: injection at 50 °C, after 2 min with 40  $\text{K min}^{-1}$  to 200 °C, after 2 min with 3  $\text{K min}^{-1}$  to 320 °C and 30 min at 320 °C. The temperature programme for cutin analysis was: injection at 50 °C, after 1 min with 10  $\text{K min}^{-1}$  to 150 °C, after 2 min with 3  $\text{K min}^{-1}$  to 320 °C and 30 min at 320 °C. Qualitative analysis was carried out with a gas chromatograph (6890N, Agilent Technologies) equipped with a mass spectrometric detector (5975 iMSD, Agilent

Technologies) under the same gas chromatographic conditions except that helium was used as carrier gas.

### Statistical analyses

Statistical analyses were performed using SigmaPlot 13 (Systat Software, San José, CA, USA). The minimum conductances were tested for normal distribution with the Shapiro-Wilk normality test. Data were normally distributed. The statistical parameters (analysis of variance statistics for the regression, regression degree of freedom is 1 for simple linear regressions) for the regression equations were calculated. Pearson product-moment correlation coefficients were determined. Linear polynomial equations  $\pm$ SE of regression (leaf shrinkage, pressure-volume analysis) were used to correct the minimum conductance.

## Results

### Leaf drying curves and minimum conductances

Detached leaves of *R. stricta* were exposed to dry air, and the water loss was measured as a function of dehydration time. For each pair of consecutive data points along the leaf drying curve, transpiration (water loss) rates were calculated according to Equation 1. The respective minimum water vapour conductance was derived from the individual transpiration rates (Equation 2). High conductances characterized the initial phase of the leaf drying curves. Subsequently, with progressing leaf dehydration, conductances declined until a plateau was reached where they were no longer sensitive to a further decline of RWD (Fig. 1). This constant and low conductance is termed the minimum conductance ( $g_{\text{min}}$ ) at maximum stomatal closure.

High temperatures are a predominant stress factor in the hot and dry natural habitat of *R. stricta*. For this purpose, leaf drying experiments were performed in the range of air temperatures from 15 to 50 °C. For calculating conductances, the actual leaf temperature at a given air temperature was used because it is this value that determines the driving force for transpiration during the phase of maximum stomatal closure. Leaf temperatures were only slightly lower than the corresponding air temperatures at 15° and 20 °C. Increasing air temperatures raised the leaf-air temperature differences ( $\Delta T_{\text{leaf-air}}$  in K) that finally reached a value of  $-1.36$  K at an air temperature of 50 °C (Table 1).

The RWD at which stomata were maximally closed ( $\text{RWD}_{\text{SC}}$ ) was derived from the transition between the declining phase and the plateau phase of the curve (Fig. 1). In the temperature range from 15° to 40 °C, stomata closed maximally at RWDs between 0.1 and 0.2. At higher

**Table 1.** Leaf to air temperature difference during the phase of maximum stomatal closure as a result of desiccation stress, minimum conductances ( $g_{\min}$ ) and RWDs at maximum stomatal closure ( $\text{RWD}_{\text{SC}}$ ) obtained from leaf drying curves depend on air temperature ( $T_{\text{air}}$ ). Each value represents the mean  $\pm$  SD ( $n \geq 17$ ). Minimum conductances are expressed in velocity and molar units, a conversion table can be found in [Percy et al. \(1989\)](#).

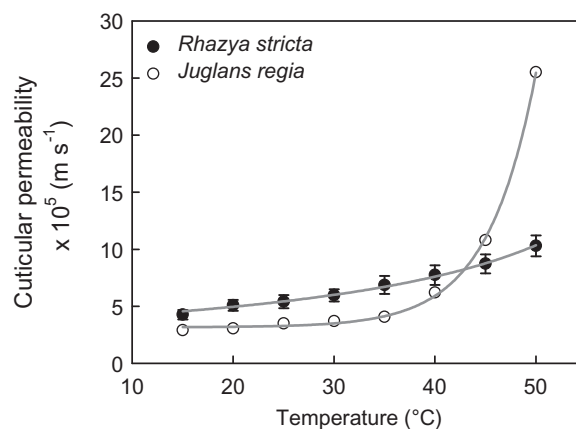
$T_{\text{air}}$ (°C)	$\Delta T$ (K)	$g_{\min} \times 10^5$ ( $\text{m s}^{-1}$ )	$g_{\min} \times 10^3$ ( $\text{mol m}^{-2} \text{s}^{-1}$ )	$\text{RWD}_{\text{SC}}$
15	-0.13 ( $\pm 0.20$ )	4.27 ( $\pm 1.03$ )	1.81	0.12 ( $\pm 0.03$ )
20	-0.11 ( $\pm 0.19$ )	5.08 ( $\pm 1.15$ )	2.12	0.12 ( $\pm 0.03$ )
25	-0.29 ( $\pm 0.23$ )	5.41 ( $\pm 1.36$ )	2.22	0.15 ( $\pm 0.04$ )
30	-0.37 ( $\pm 0.24$ )	5.96 ( $\pm 1.30$ )	2.40	0.14 ( $\pm 0.04$ )
35	-0.42 ( $\pm 0.24$ )	6.87 ( $\pm 1.86$ )	2.73	0.18 ( $\pm 0.04$ )
40	-0.64 ( $\pm 0.34$ )	7.74 ( $\pm 2.21$ )	3.02	0.19 ( $\pm 0.04$ )
45	-0.94 ( $\pm 0.37$ )	8.72 ( $\pm 2.09$ )	3.35	0.25 ( $\pm 0.06$ )
50	-1.36 ( $\pm 0.42$ )	10.30 ( $\pm 2.48$ )	3.88	0.45 ( $\pm 0.09$ )

temperatures, maximum stomatal closure shifted to higher RWDs up to 0.45 at 50 °C (Table 1). The minimum conductance at 25 °C was  $5.41 (\pm 1.36) \times 10^{-5} \text{ m s}^{-1}$  ( $n = 17$ ). Minimum conductances increased with air temperatures by a factor of 2.4 between 15 and 50 °C from  $4.27 \times 10^{-5} \text{ m s}^{-1}$  to  $1.03 \times 10^{-4} \text{ m s}^{-1}$  (Table 1). For comparison, also, the corresponding minimum conductances in molar units are given in Table 1. The cuticular permeability of *R. stricta* continuously increased with temperature without an abrupt change in slope (Fig. 2).

### Chemical composition of cuticular waxes and cutin

Plant cuticles consist of a polymer matrix (cutin) and associated solvent-soluble lipids (cuticular waxes). The waxes and the cutin of the *R. stricta* leaf cuticles were analysed qualitatively and quantitatively to detect potential relationships between cuticular transpiration and the composition of the cuticle. The total leaf cuticular wax coverage (Table 2) was  $251.41 (\pm 38.97) \mu\text{g cm}^{-2}$  (mean  $\pm$  SD,  $n = 5$ ). The cuticular wax was mainly composed of pentacyclic triterpenoids (85.2% of the total wax) with ursolic acid (40.8% of total wax, 48.0% of triterpenoids), echinocystic acid (20.9 and 24.5%) and oleonic acid (19.1 and 22.4%) as the primary constituents. Only a minor fraction of the wax contained long-chain aliphatic compounds with chain lengths ranging from  $\text{C}_{20}$  to  $\text{C}_{33}$  (3.4% of the total wax). Within this fraction, alkanes were the main compound class (2.5% of the total wax, 73.6% of the aliphatics), and hentriacontane was the major constituent (0.9 and 27.8%) of the aliphatic fraction (Table 2). The weighted average chain-length of the very-long-chain aliphatic wax components was 28.5.

Total coverage of cutin monomers was  $340.60 (\pm 33.14) \mu\text{g cm}^{-2}$  ( $n = 5$ ). Various  $\omega$ -hydroxy fatty acids



**Figure 2.** Minimum conductances ( $g_{\min}$ ) from *Rhazya stricta* leaves obtained from leaf drying curves as a function of air temperature showed a continuous change of cuticular permeability over the whole temperature range from 15 to 50 °C. The cuticular permeance obtained from isolated cuticular membranes from *Juglans regia* leaves (Burghardt and Riederer 2006), a characteristic representative of deciduous trees from a temperate climate, increases steeply above 35 °C.

with a mid-chain hydroxyl group were predominant (57.1% of the total cutin) followed by  $\omega$ -hydroxy fatty acids with a mid-chain epoxy group (23.0%). 9/10,16-Dihydroxyhexadecanoic acid (49.7% of the total cutin) and 9,10-Epoxy-18-hydroxyoctadecanoic acid (22.3%) were the major constituents.  $\text{C}_{16}$  monomers were predominant over  $\text{C}_{18}$  monomers at a ratio of 1.7 (Table 3).

### Discussion

In the ecological literature and textbooks, it is often claimed that desert plants are specifically adapted to reduce transpiration by a combination of a particularly

**Table 2.** The leaf cuticular waxes of *Rhazya stricta* are composed of a relatively small aliphatic (3.4% of total wax) and a very large triterpenoid (85.2%) fraction. Each value represents the mean  $\pm$  SD ( $n = 5$  biological replicates).

Compound class	Chain length	Coverage ( $\mu\text{g cm}^{-2}$ )
Alkanes		
	23	$0.36 \pm 0.11$
	25	$0.30 \pm 0.20$
	26	$0.29 \pm 0.17$
	27	$0.20 \pm 0.12$
	28	$0.13 \pm 0.07$
	29	$0.28 \pm 0.11$
	30	$0.22 \pm 0.04$
	31	$2.38 \pm 0.75$
	32	$0.37 \pm 0.10$
	33	$1.76 \pm 0.72$
Total		$6.30 \pm 2.15$
Primary alcohols		
	20	$0.64 \pm 0.43$
	22	$0.42 \pm 0.31$
	24	$0.07 \pm 0.02$
	28	$0.14 \pm 0.03$
	29	$0.10 \pm 0.01$
	30	$0.40 \pm 0.10$
Total		$1.78 \pm 0.78$
Fatty acids		
	20	$0.23 \pm 0.04$
	22	$0.14 \pm 0.05$
	24	$0.11 \pm 0.05$
Total		$0.48 \pm 0.12$
Total aliphatics		$8.56 \pm 2.93$
Triterpenoids		
Taraxerol		$0.95 \pm 0.19$
$\alpha$ -Amyrin		$0.27 \pm 0.07$
Erythrodiol		$2.60 \pm 1.26$
Uvaol		$2.27 \pm 0.32$
Oleanolic acid		$48.05 \pm 6.18$
Betulinic acid		$2.47 \pm 1.09$
Ursolic acid		$102.69 \pm 12.73$
Echinocystic acid		$52.56 \pm 12.57$
Hederagenin		$2.26 \pm 0.60$
Total triterpenoids		$214.12 \pm 31.20$
Not identified		$28.72 \pm 6.34$
Total wax		$251.41 \pm 38.97$

efficient stomatal control and the presence of a cuticular transpiration barrier with extra-low permeability. Intuitively, this seems to be plausible, but it has never been tested whether the cuticular transpiration barrier of desert plants is indeed more efficient than that of species growing in more humid habitats. The results obtained in the present study with *R. stricta* growing in very hot and arid regions allow testing this assumption.

### Is the cuticle of *R. stricta* specifically efficient as a barrier to water loss?

To answer this question, we measured the cuticular transpiration of *R. stricta* leaves under strictly controlled conditions. At 25 °C, the minimum leaf conductance to water vapour is  $5.41 (\pm 1.36) \times 10^{-5} \text{ m s}^{-1}$  ( $n = 17$ ). This means that it is not specifically efficient as a barrier to water loss because it lies within the upper range of cuticular permeabilities and  $g_{\text{min}}$  measured so far for a large set of woody species from various habitats (Fig. 3, [see Supporting Information File 5]). The median of cuticular permeabilities of 12 deciduous and 13 evergreen woody species is  $2.10 \times 10^{-5}$  and  $1.05 \times 10^{-5} \text{ m s}^{-1}$ , respectively, which is lower than the mean  $g_{\text{min}}$  for *R. stricta*. The upper quartiles of the data used for comparison and the variability of the *R. stricta* data partially overlap. It is difficult to assess this finding because only scarce information on  $g_{\text{min}}$  of desert plants is available. Körner (1994, 1995) reported an average value of  $6.4 \times 10^{-5} \text{ m s}^{-1}$  for seven unspecified evergreen desert shrub species. Minimum conductances were also published for the desert grasses *Digitaria californica* ( $6.09 \times 10^{-5}$  and  $7.72 \times 10^{-5} \text{ m s}^{-1}$ ) and *Eragrostis lehmanniana* ( $6.51 \times 10^{-5} \text{ m s}^{-1}$ , Smith et al. 2006). The  $g_{\text{min}}$  of *R. stricta* agrees with this very limited pool of data for desert species. From the distribution of the data [see Supporting Information File 5] the probability can be estimated that for not yet studied woody plants a cuticular permeability lower than that of *R. stricta* will be found in the future. This probability is 0.93. So, the prediction that the minimum conductance of *R. stricta* leaves is lower than that of non-desert plants and the common notion that desert species should have an exceptionally effective cuticular transpiration barrier are not corroborated by our data.

### Is the cuticle of *R. stricta* more resistant to thermal stress than that of other species?

Very high air temperatures are characteristic of the climate of hot deserts and represent a pronounced stressor for all organisms living there. Organisms whose barrier against transpirational water loss consists of waxes embedded within a polymer matrix (plants and

**Table 3.** The leaves of *Rhazya stricta* are covered by 340.60  $\mu\text{g cm}^{-2}$  of total cutin polymer that belongs to the widespread  $\text{C}_{16}/\text{C}_{18}$  type. Each value represents the mean  $\pm$  SD ( $n=5$  biological replicates).

Cutin monomers	Coverage ( $\mu\text{g cm}^{-2}$ )
Fatty acids	
Hexadecanoic acid ( $\text{C}_{16}$ )	1.13 $\pm$ 0.30
Octadecanoic acid ( $\text{C}_{18}$ )	0.79 $\pm$ 1.09
Octadec-9-enoic acid ( $\text{C}_{18:1}$ )	0.71 $\pm$ 0.32
Nonadec-10-enoic acid ( $\text{C}_{19:1}$ )	0.63 $\pm$ 0.24
Eicosanoic acid ( $\text{C}_{20}$ )	Traces
Docosanoic acid ( $\text{C}_{22}$ )	0.33 $\pm$ 0.31
$\omega$ -Hydroxy fatty acids	
16-Hydroxyhexadecanoic acid ( $\text{C}_{16}$ )	1.71 $\pm$ 0.22
16-Hydroxyhexadec-9-enoic acid ( $\text{C}_{16:1}$ )	0.61 $\pm$ 0.27
18-Hydroxyoctadeca-9,12-dienoic acid ( $\text{C}_{16:2}$ )	2.77 $\pm$ 0.18
$\omega$ -Hydroxy fatty acids with midchain hydroxyl group	
9 or 10,15-Dihydroxypentadecanoic acid ( $\text{C}_{15}$ )	2.34 $\pm$ 0.15
9 or 10,16-Dihydroxyhexadecanoic acid ( $\text{C}_{16}$ )	169.11 $\pm$ 22.95
9 or 10,17-Dihydroxyheptadecanoic acid ( $\text{C}_{17}$ )	1.03 $\pm$ 0.24
9 or 10,18-Dihydroxyoctadecanoic acid ( $\text{C}_{18}$ )	7.19 $\pm$ 0.89
9,10,18-Trihydroxyoctadecanoic acid ( $\text{C}_{18}$ )	14.77 $\pm$ 7.52
9,10,18-Trihydroxyoctadec-12-enoic acid ( $\text{C}_{18:1}$ )	0.12 $\pm$ 0.20
9 or 10,19-Dihydroxynonadecanoic acid ( $\text{C}_{19}$ )	traces
$\omega$ -Hydroxy fatty acids with midchain epoxy group	
9,10-Epoxy-18-hydroxyoctadecanoic acid ( $\text{C}_{18}$ )	75.88 $\pm$ 10.02
9,10-Epoxy-18-hydroxyoctadec-12-enoic acid ( $\text{C}_{18:1}$ )	2.46 $\pm$ 0.56
$\omega$ -Hydroxy fatty acids with midchain oxo group	
16-Hydroxy-9/10-oxo-hexadecanoic acid ( $\text{C}_{18}$ )	6.19 $\pm$ 2.15
$\alpha,\omega$ -Dicarboxylic acids	
Hexadecane-1,16-dioic acid ( $\text{C}_{16}$ )	0.76 $\pm$ 0.22
Octadecane-1,18-dioic acid ( $\text{C}_{18}$ )	Traces
$\alpha,\omega$ -Dicarboxylic acids with midchain hydroxyl group	
7(8)-Hydroxyhexadecane-1,16-dioic acid ( $\text{C}_{16}$ )	4.37 $\pm$ 2.21
7(8)-Hydroxyoctadecane-1,18-dioic acid ( $\text{C}_{18}$ )	2.76 $\pm$ 0.42
7(8),9(10)-Dihydroxyoctadec-12-ene-1,18-dioic acid ( $\text{C}_{18:1}$ )	0.51 $\pm$ 0.07
Phenolics	
Coumaric acid	15.61 $\pm$ 1.35

(continued)

**Table 3.** Continued

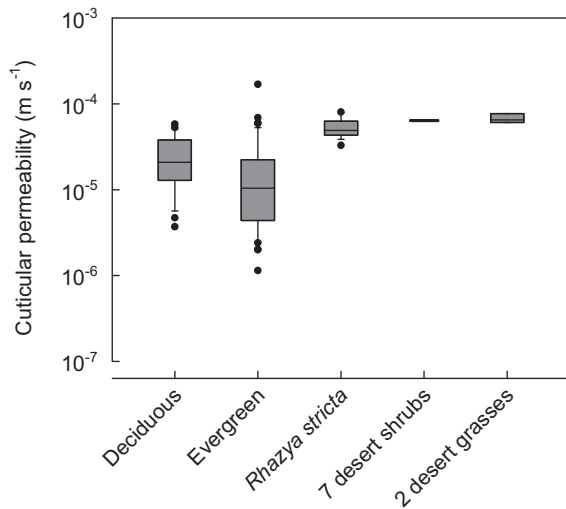
Cutin monomers	Coverage ( $\mu\text{g cm}^{-2}$ )
Coumaric acid derivative	14.09 $\pm$ 3.35
Glycerol	0.23 $\pm$ 0.03
Unidentified compounds	14.49 $\pm$ 2.97

arthropods; Hadley 1972, 1981, 1989) are prone to adverse temperature effects. The waxes undergo a solid to liquid transition in the range from 70 to 85 °C in plants (Merk *et al.* 1998) and, for instance, from 35 to 40 °C in the desert *Drosophila mojavensis* (Gibbs *et al.* 1998). These transitions may compromise the functioning of the waxy transpiration barrier. In the past, some work has been devoted to answering the question to what degree the cuticular transpiration barrier of plants is critically affected by high temperatures. However, in contrast to its ecological and physiological relevance, no work on this topic has so far been done with desert plants.

The present work shows that the temperature dependence of the leaf  $g_{\text{min}}$  of the hot-desert plant *R. stricta* is strikingly different from that of other woody plants from temperate habitats studied so far. In comparison with *Juglans regia* (Burghardt and Riederer 2006), a characteristic representative of deciduous trees from a temperate climate, the change in cuticular permeability is continuous over the whole temperature range from 15 to 50 °C while the permeability of the latter species increases steeply above 35 °C (Fig. 2). This difference becomes even more apparent when the factors are compared by which the cuticular permeabilities of *R. stricta* and non-desert woody plants increase from 15 to 50 °C (Fig. 4). While for *R. stricta* this factor is 2.4, the cuticular permeabilities of deciduous and evergreen non-desert plants (data from Riederer 2006) increase by factors of 12 and 9 (medians), respectively. From the distribution of the values from non-desert plants, the probability can be estimated that for not yet studied woody plants factors higher than that of *R. stricta* will be found in the future. This probability is 0.86. The results from the temperature-dependent measurements of cuticular permeability corroborate the hypothesis that the *R. stricta* leaf cuticle is more resistant to thermal stress than that of woody species from non-desert habitats.

Our data cannot only be used to describe temperature effects on permeability, but they also allow a deeper analysis of the nature of the thermal effects on the properties of the transpiration barrier. Like all processes involving the diffusion of molecules in any medium





**Figure 3.** Comparison of literature data of cuticular permeabilities and/or minimum conductances with the minimum conductance obtained from *Rhazya stricta* leaves. The mean values of 12 deciduous woody plant species (21 data points) and the mean values of 13 evergreen woody plant species (44 data points) are compared with 17 single measurements conducted with *R. stricta* leaves. For desert plants, the mean minimum conductances of two desert grass species (three data points) and an average value for seven unspecified evergreen desert shrubs, are available for comparison. For individual values [see Supporting Information File 5].

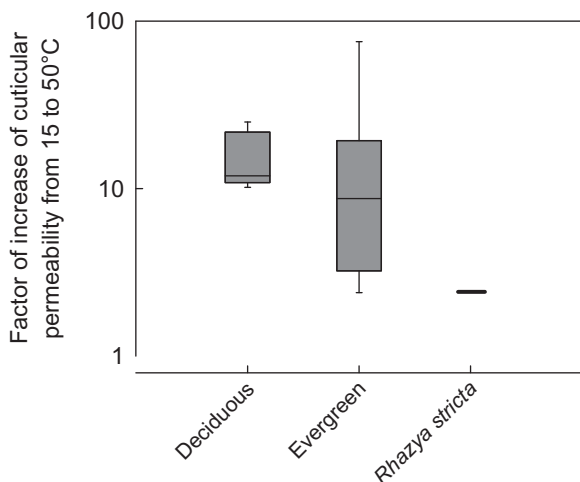
(solid, liquid or gaseous), cuticular water permeability inevitably increases with temperature. For instance, the diffusion coefficient of water molecules in water increase from  $1.77 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$  at  $15 \text{ }^\circ\text{C}$  to  $3.96 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$  at  $50 \text{ }^\circ\text{C}$  (Holz et al. 2000). The velocity of diffusion in a liquid is mainly determined by the viscosity of the medium and, therefore, increases when the viscosity decreases at higher temperatures. For plant cuticles, this relationship does not apply. Here, the diffusion of water takes place in an organic solid (cutin, waxes). The mechanisms governing diffusion process in this case are fundamentally different from that in a liquid. In organic solids, the concept of non-Stokesian diffusion applies. This theory states that the mobility of water molecules in a solid medium, e.g. a polymer, the lipid parts of biomembranes or waxes depends on the availability of free volumes, i.e. ‘holes’ in between the hydrocarbon chains (Stein 1986). During their thermal motion water molecules jump from one of these holes to another and, thus, proceed in the medium. Diffusion will be high if there are many holes if they appear and disappear at high rates and if the probability that a hole large enough to accommodate the diffusing water molecule appears is also high (Stein 1986). The appearance and disappearance of free volumes within an aliphatic solid is due to the thermal motion of its hydrocarbon chains. At higher temperature, the hydrocarbon chains move faster (especially laterally),

open up more free volumes at a higher frequency and the holes appearing become larger. This is the mechanism by which temperature accelerates the diffusion of water across solid organic materials including the plant cuticle. Consequently, temperature effects on cuticular permeability can be analysed based on this concept.

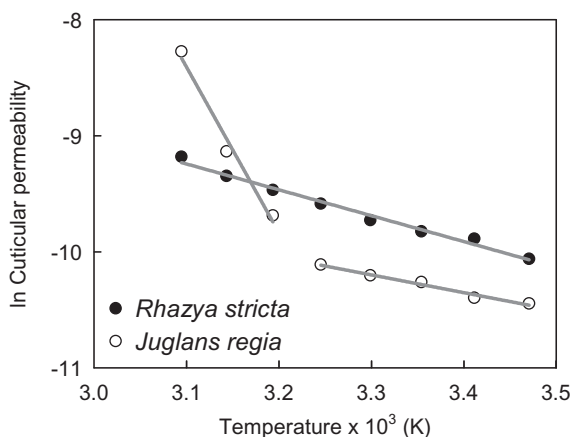
The velocity of a water molecule crossing the cuticle and, as a result, cuticular permeability depends on the rate by which the molecule jumps from one free volume to another. This means that the temperature-dependence of cuticular permeability can be analysed by the Arrhenius formalism which relates rate constants to temperature. A plot of the natural logarithm of the rate constant (in this case cuticular permeability) vs. the reciprocal of the absolute temperature generally yields a straight line. Arrhenius plots are linear as long as the diffusion mechanism remains the same irrespective of changing temperature. A sharp deviation from linearity with a marked shift in the slope of the Arrhenius plot indicates a change in the diffusion mechanism mostly due to a change in the properties of the surroundings of the diffusing water molecule.

The present work’s results show that the minimum conductance of *R. stricta* steadily increases with temperature from  $15$  to  $50 \text{ }^\circ\text{C}$  (Fig. 2). In contrast to this, the cuticular permeability of *J. regia* leaves raises only slightly between  $15$  and  $35 \text{ }^\circ\text{C}$  and at higher temperatures quite steeply (Fig. 2). When the data from both species are plotted according to the Arrhenius formalism, the difference between the two types of temperature dependence becomes even more evident (Fig. 5). While the plot for *R. stricta* is linear over the whole temperature range, the plot for *J. regia* is divided into two branches one ranging from  $15$  to  $35 \text{ }^\circ\text{C}$  and the second from  $40$  to  $50 \text{ }^\circ\text{C}$ . Such biphasic Arrhenius plots have also been obtained for all the woody species used for comparison (Fig. 4). So, it can be concluded that in contrast to non-desert woody species, the diffusion of water across the *R. stricta* cuticle is controlled over the whole range of ecophysiological relevant temperatures by a barrier which does not abruptly change its physical properties at a given temperature. This further supports the hypothesis that the cuticle of *R. stricta* is more resistant to thermal stress than that of other comparable species.

Is it possible to deduce from this finding insights into what makes *R. stricta* cuticles different from others regarding thermal stability? Several hypotheses have been put forward to explain the steep increase of cuticular permeability at temperatures above  $35 \text{ }^\circ\text{C}$  and the concomitant sharp upward bend of the Arrhenius plot observed in all species investigated so far. Eckl and Gruler (1980) attributed it to phase changes of the cuticular waxes. It was also argued that the steep increase



**Figure 4.** Comparison of literature data of the factor of increase of the cuticular permeabilities from 15 to 50°C with the increase factor obtained for *Rhazya stricta* leaves. The factors of five deciduous woody plant species and nine evergreen woody plant species are compared with the factor obtained with *R. stricta* leaves.



**Figure 5.** Arrhenius graphs created by plotting the natural logarithm of cuticular permeabilities versus the inverse absolute temperature. Each point represents for a given temperature the mean minimum conductance of *Rhazya stricta* leaves ( $\ln g_{\min} = -2.332 (\pm 0.338) - (2229.163 (\pm 103.223) \times 1/T)$ ,  $\pm$ SE of regression,  $F = 466.370$ ,  $P < 0.001$ ,  $r^2 = 0.987$ ,  $n = 8$ ) and the mean cuticular permeance of *Juglans regia* leaves (Burghardt and Riederer 2006).

of water permeabilities at higher temperatures be due to the swelling of the polysaccharide fraction of the cuticular matrix opening up new pathways for the diffusion of water from the outer epidermal cell wall to the surrounding atmosphere (Riederer 2006). Finally, another hypothesis proposes that the transition is caused by divergent extents of the thermal volume expansion of the cuticular matrix and the associated waxes stressing the wax deposited in the cutin and thus leading to imperfections of the waxy transpiration barrier (Schreiber and Schönherr 1990). The latter hypothesis postulates that mechanical

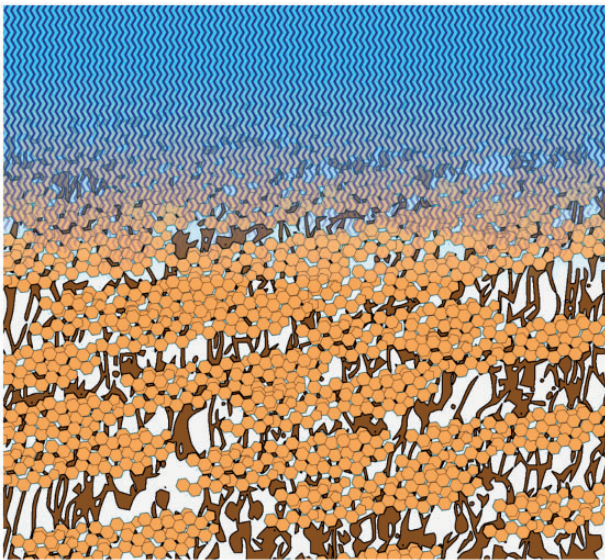
forces act within the cuticle which, at a given transition temperature, open up new pathways for the diffusion of water from the leaf interior to the atmosphere. So in *R. stricta*, the lack of a sudden much steeper increase of water permeability at higher temperatures may be due to a higher mechanical stability of its cuticle. This proposal can be discussed in the context of the chemical composition of the cuticle and its effect on its internal structure and mechanical properties.

### Is the composition of the cuticle of *R. stricta* qualitatively and/or quantitatively different from that of other species?

One of the starting hypotheses of this study was that the amounts and/or composition of cuticular waxes and cutin of *R. stricta* are peculiar in adaptation to its hot and arid habitat. The chemical analysis of the cutin polymer of *R. stricta* leaves does not support that hypothesis as it reveals a typical profile of cutin monomers dominated by C<sub>16</sub> and C<sub>18</sub>  $\omega$ -hydroxy fatty acids substituted with mid-chain hydroxyl and epoxy, and end-chain hydroxyl groups. This mixed C<sub>16</sub>- and C<sub>18</sub>-type cutin with 9/10,16-Dihydroxyhexadecanoic acid and 9,10-Epoxy-18-hydroxyoctadecanoic acid as primary monomers (Table 3) is widespread in the leaf cuticles of many plant species irrespective of their habitats (Holloway 1982, 1984; Pollard et al. 2008). So, the cutin composition of *R. stricta* cannot be interpreted as being specific for this hot-desert plant.

The cuticular wax composition of *R. stricta* is qualitatively also quite common. The aliphatic wax fraction consists of long-chain *n*-alkanes, primary alkanols and alcanoic acids that occur in the majority of plant species (Baker 1982; Jetter et al. 2006). Also, the chain-lengths and chain-length distributions of the aliphatic compound classes are not unusual (Jetter et al. 2006). The notion that desert plants tend to have longer average chain-lengths than plants from cooler climates (Bush and McInerney 2015) is not supported by the data from *R. stricta*. The qualitative composition of the cyclic fraction of the wax is not peculiar. The cyclic constituents of the *R. stricta* leaf cuticular wax are taraxerol,  $\alpha$ -amyrin, erythrodiol, uvaol, oleanolic acid, betulinic acid, ursolic acid, echinocystic acid and hederagenin, which are quite common in many other plant species (Buschhaus and Jetter 2011; Szakiel et al. 2012).

In contrast to the qualitative the quantitative composition of the cuticular wax of *R. stricta* is uncommon although not singular. The wax belongs to a less common type consisting to an extremely high proportion of pentacyclic triterpenoids. In *R. stricta*, triterpenoids make up 85.2% of the total wax. In contrast, the aliphatic fraction



**Figure 6.** Schematic model depicting the localization and functioning of triterpenoid fillers in the cuticular matrix. Triterpenoids (orange) form extended deposits within the cutin polymer (brown) which fixate the polymer strands. This mechanical enhancement reduces the thermal stress on the layer of aliphatic cuticular waxes (blue) thus maintaining its barrier properties even at elevated temperature.

is minor and contributes a low fraction of only 3.4% to the total wax (Table 2). This quantitative composition sets *R. stricta* apart from many woody species investigated so far where the leaf cuticular wax is mainly composed of aliphatic components (Merk et al. 1998; Wissemann et al. 2007; Dragota and Riederer 2008; Jetter and Riederer 2016).

The knowledge of potential functions of triterpenoids has deepened over the last years. Experiments with the fruits of tomato wax mutants (Vogg et al. 2004; Leide et al. 2007, 2011), *Arabidopsis thaliana* mutant leaves (Buschhaus and Jetter 2012) and eight evergreen woody species (Jetter and Riederer 2016) suggest that the contribution of triterpenoids to the efficacy of the transpiration barrier is small or absent. These findings agree with a model for the molecular structure of cuticular waxes proposed earlier (Riederer and Schneider 1990; Riederer and Schreiber 1995). According to this model, cuticular waxes are multiphase systems with highly ordered crystalline zones and amorphous zones in between. The crystalline zones of the wax are thought to consist of long-chain aliphatic molecules while chain ends, short-chain aliphatics and cyclic compounds should accumulate in the amorphous zone located in between the crystallites. Casado and Heredia (1999) and Tsubaki et al. (2013) argued that triterpenoids have a low-molecular order and consequently can be expected to pertain to the amorphous zone. This model also predicts that it is

mainly the aliphatic fraction of the cuticular waxes, which represents the cuticular transpiration barrier that was confirmed by Jetter and Riederer (2016). When this model was established no species were known with cuticular waxes dominated to a very high degree by triterpenoids. So, the structural model for the cuticular wax has to be extended for taking into account very high relative contents of triterpenoids. In *R. stricta*, triterpenoids are 25 times more abundant than the aliphatic compounds and, thus, this material must be predominantly present in largely pure deposits and not mixed with the aliphatic portion of the wax.

So, the question is what functions these extensive triterpenoid deposits may have in the cuticle. Triterpenoids are nearly exclusively located within the cutin polymer matrix (Jetter et al. 2000; Buschhaus et al. 2007; Jetter and Riederer 2016). Exceptions are species whose leaves or stems are covered by extensive deposits of epicuticular triterpenoid crystals (Markstädter et al. 2000). Recently, Tsubaki et al. (2013) studied the fruit of *Diospyros kaki* where the triterpenoids are also mainly deposited in the interior of the cuticle. They proposed that triterpenoids and cutin be highly compatible with each other. So, triterpenoids and cutin intimately intermix, and the triterpenoids fill voids between the cutin polymer strands. This increases the density and mechanical strength of the cuticular matrix as a whole. Similar suggestions have been made for the functioning of the triterpenoid oleanolic acid in the grape berry cuticle (Casado and Heredia 1999) and of flavonoids in tomato fruit cuticles (España et al. 2014).

Frequently, dense materials of organic or inorganic origin, called fillers, are added to amorphous technical polymers for enhancing their strength under mechanical or thermal stress (Xanthos 2010; McCrum et al. 1997). These fillers occupy partially voids between the polymer strands and thereby fixate the latter's location within the polymer network during stress. Triterpenoids can be considered as perfect fillers for a biological amorphous polymer like cutin. Their melting points are high (e.g. 285–288 °C for ursolic acid, Windholz et al. 1983) and their structural arrangement remains unaffected by temperatures up to 100 °C (Casado and Heredia 1999). Consequently, triterpenoid deposits are solid over the whole range of temperatures even a desert plant like *R. stricta* may experience. The mass-based triterpenoid-to-cutin ratio of *R. stricta* is 0.63 which means that 63% of the cutin polymer is associated with agglomerations of triterpenoids that fix the position of the cutin chains and, thus, reinforce the cuticular matrix over the whole range of physiological temperatures (Fig. 6).

So, we formulate the hypothesis that the intracuticular triterpenoids counteract as fillers the thermal expansion

of the cutin polymer which, in the absence of reinforcement, might mechanically stress the waxes embedded within the cutin. This stress may introduce imperfections into the waxy transpiration barrier and, thereby, open up new pathways for the diffusion of water molecules to the atmosphere. This assumption is supported by the work of Schreiber and Schönherr (1990) who studied the thermal expansion of the isolated cuticles of 12 plant species. They found that at higher temperatures the ‘volume expansion of the polymer matrix exceeded that of the waxes because the moderately increasing expansion coefficients of the waxes could not compensate for the sudden increase of the expansion coefficient of the polymer matrix’ and concluded that the difference between the two volume expansion coefficients may lead to defects (Schreiber and Schönherr 1990).

In this context, it is intriguing that in contrast to the other species studied so far the minimum leaf conductance of *R. stricta* does not abruptly increase above a certain transition temperature (Fig. 2). The functionality of the cuticular transpiration barrier of *R. stricta* is preserved even at elevated temperatures. Furthermore, the linearity of the Arrhenius plot (Fig. 5) indicates that in contrast to all other species the diffusion mechanism and/or the environment are identical from 15 to 50 °C. This implies that elevated temperature does not induce the formation of imperfections in the wax barrier of *R. stricta* and, therefore, does not negatively affect the efficacy of the cuticular transpiration barrier. We argue that the reason for this unusual behaviour of *R. stricta* be the reinforcement of the cutin matrix by extensive deposits of triterpenoids acting as fillers and thus protecting the cutin polymer against a thermal expansion exceeding that of the waxes forming the diffusion barrier. Obviously, this is very advantageous for this plant growing in a very hot and arid habitat.

## Conclusions

In conclusion, based on the results and interpretations of this work with the representative hot-desert shrub *R. stricta*, the cuticle, its composition and water permeability in the future may be considered in a broader ecological context. Unfortunately, this is currently only rarely the case. Cuticular features may be recognized as relevant contributions to plant traits ensuring the competitiveness and even survival of a plant under adverse environmental conditions, especially under heat and drought stress. The extent and time to which sufficient amounts of water are available in the photosynthesizing parts largely depend on the efficacy of the control of water loss at maximum stomatal closure. Moreover, this

efficacy essentially depends on cuticular water permeability. However, it is not only cuticular permeability *per se* which is important. In all species exposed to pronounced levels and rapid changes in temperature, the thermal stability of the cuticular transpiration barrier is decisive, maybe even to a higher degree than baseline cuticular permeability.

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## Contributions by Authors

M.R., M.B. and R.H. conceived the research. A.-C.S., M.R., M.B., J.T., A.B. and J.L. designed and performed the experiments and analysed the results. A.-C.S. wrote the first draft and M.R., M.B. and J.L. contributed to revisions. All authors read and approved the manuscript.

## Conflicts of Interest Statement

None declared.

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## Supporting Information

The following additional information is available in the online version of this article —

**File 1.** Use and physical meaning of the terms cuticular permeability, minimum conductance and permeance.

**File 2.** Morphological leaf traits.

**File 3.** Leaf water potential and pressure-volume analysis.

**File 4.** Leaf thermal and hydric tolerances.

**File 5.** Literature data used to test whether the leaf cuticular water permeability of *R. stricta* is lower than that

of woody species from non-desert habitats. The median plus upper and lower confidence limits ( $P=0.05$ ) of cuticular permeances or minimum leaf conductances ( $g_{\min}$ ) of 12 deciduous woody plant species (21 data points), the median plus upper and lower confidence limits ( $P=0.05$ ) of 13 evergreen woody plant species (44 data points), an average minimum conductance for seven unspecified evergreen desert shrubs and the mean minimum conductances of 2 desert grasses (3 data points) are documented.

## Literature Cited

- Abd El-Ghani MM. 1997. Phenology of ten common plant species in Western Saudi Arabia. *Journal of Arid Environments* **35**:673–683.
- Al-Ghobari HM. 2000. Estimation of reference evapotranspiration for Southern region of Saudi Arabia. *Irrigation Science* **19**:81–86.
- Al-Khamis HH, Al-Hemaid FM, Ibrahim ASS. 2012. Diversity of perennial plants at Ibex Reserve in Saudi Arabia. *The Journal of Animal and Plant Sciences* **22**:484–492.
- Almazroui M, Nazrul Islam M, Jones PD, Athar H, Ashfaqur Rahman M. 2012. Recent climate change in the Arabian Peninsula: seasonal rainfall and temperature climatology of Saudi Arabia for 1979–2009. *Atmospheric Research* **111**:29–45.
- Baker EA. 1982. Chemistry and morphology of plant epicuticular waxes. In: Cutler DF, Alvin KL, Price CE, eds. *The plant cuticle*. London: Academic Press, 139–165.
- Burghardt M, Riederer M. 2003. Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants in relation to stomatal closure and leaf water potential. *Journal of Experimental Botany* **54**:1941–1949.
- Burghardt M, Riederer M. 2006. Cuticular transpiration. In: Riederer M, Müller C, eds. *Biology of the plant cuticle*, Vol. **23**. Oxford: Blackwell Publishing, 292–311.
- Buschhaus C, Herz H, Jetter R. 2007. Chemical composition of the epicuticular and intracuticular wax layers on the adaxial side of *Ligustrum vulgare* leaves. *New Phytologist* **176**:311–316.
- Buschhaus C, Jetter R. 2011. Composition differences between epicuticular and intracuticular wax substructures: how do plants seal their epidermal surfaces? *Journal of Experimental Botany* **62**:841–853.
- Buschhaus C, Jetter R. 2012. Composition and physiological function of the wax layers coating *Arabidopsis* leaves:  $\beta$ -amyirin negatively affects the intracuticular water barrier. *Plant Physiology* **160**:1120–1129.
- Bush RT, McInerney FA. 2015. Influence of temperature and  $C_4$  abundance on  $n$ -alkane chain length distributions across the central USA. *Organic Geochemistry* **79**:65–73.
- Casado CG, Heredia A. 1999. Structure and dynamics of reconstituted cuticular waxes of grape berry cuticle (*Vitis vinifera* L.). *Journal of Experimental Botany* **50**:175–182.
- Deil U, Al-Gifri A-N. 1998. Montane and Wadi vegetation. In: Ghazanfar SA, Fisher M, eds. *Vegetation of the Arabian Peninsula*. Dordrecht: Kluwer Academic Publishers, 125–174.
- De Micco V, Aronne G. 2012. Morpho-anatomical traits for plant adaptation to drought. In: Aroca R, ed. *Plant responses to drought stress*. Berlin: Springer, 37–61.
- Dragota S, Riederer M. 2008. Comparative study on epicuticular leaf waxes of *Araucaria araucana*, *Agathis robusta* and *Wollemia nobilis* (Araucariaceae). *Australian Journal of Botany* **56**:644–650.
- Eckl K, Gruler H. 1980. Phase transitions in plant cuticles. *Planta* **150**:102–113.
- Emad El-Deen HM. 2005. Population ecology of *Rhazya stricta* Decne. in Western Saudi Arabia. *International Journal of Agriculture and Biology* **7**:932–938.
- España L, Heredia-Guerrero JA, Segado P, Benítez JJ, Heredia A, Domínguez E. 2014. Biomechanical properties of the tomato (*Solanum lycopersicum*) fruit cuticle during development are modulated by changes in the relative amounts of its components. *New Phytologist* **202**:790–802.
- Gibbs AG, Louie AK, Ayala JA. 1998. Effects of temperature on cuticular lipids and water balance in a desert *Drosophila*: is thermal acclimation beneficial? *Journal of Experimental Biology* **201**:71–80.
- Grace J. 1989. Measurement of wind speed near vegetation. In: Pearcy RW, Ehleringer JR, Mooney HA, Rundel PW, eds. *Plant physiological ecology, field methods and instrumentation*. New York: Chapman and Hall, 57–73.
- Goodwin SM, Jenks MA. 2005. Plant cuticle function as a barrier to water loss. In: Jenks MA, Hasegawa PM, eds. *Plant abiotic stress*. Oxford: Blackwell Publishing, 14–36.
- Hadley NF. 1972. Desert species and adaptation. *American Scientist* **60**:338–347.
- Hadley NF. 1981. Cuticular lipids of terrestrial plants and arthropods: a comparison of their structure, composition, and waterproofing function. *Biological Reviews* **56**:23–47.
- Hadley NF. 1989. Lipid water barriers in biological systems. *Progress in Lipid Research* **28**:1–33.
- Holloway PJ. 1982. The chemical constitution of plant cutins. In: Cutler DF, Alvin KL, Price CE, eds. *The plant cuticle*. London: Academic Press, 45–85.
- Holloway PJ. 1984. Cutins and suberins, the polymeric plant lipids. In: Mangold HK, Zweig G, Sherma J, eds. *CRC handbook of chromatography, lipids*, Vol. **1**. Boca Raton: CRC Press, 321–345.
- Holz M, Heil SR, Sacco A. 2000. Temperature-dependent self-diffusion coefficients of water and six selected molecular liquids for calibration in accurate  $^1\text{H}$  NMR PFG measurements. *Physical Chemistry Chemical Physics* **2**:4740–4742.
- Jeffree CE. (2006) The fine structure of the plant cuticle. In: Riederer M, Müller C, eds. *Biology of the plant cuticle*. Oxford, UK: Blackwell Publishing, 11–125.
- Jetter R, Schäffer S, Riederer M. 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. *Plant, Cell and Environment* **23**:619–628.
- Jetter R, Kunst L, Samuels AL. 2006. Composition of plant cuticular waxes. In: Riederer M, Müller C, eds. *Biology of the plant cuticle, annual plant reviews*, Vol. **23**. Oxford: Blackwell Publishing, 145–181.
- Jetter R, Riederer M. 2016. Localization of the transpiration barrier in the epi- and intracuticular waxes of eight plant species: water transport resistances are associated with fatty acyl rather than alicyclic components. *Plant Physiology* **170**:921–934.
- Jones HG. 2013. *Plants and microclimate: a quantitative approach to environmental plant physiology*. Cambridge: Cambridge University Press. pp 147, 261, 262.
- Kerstiens G. 2006. Water transport in plant cuticles: an update. *Journal of Experimental Botany* **57**:2493–2499.

- Körner C. 1994. Scaling from species to vegetation: the usefulness of functional groups. In: Schulze ED, Mooney HA, eds. *Biodiversity and ecosystem function*. Berlin: Springer, 117–140.
- Körner C. 1995. Leaf diffusive conductances in the major vegetation types of the globe. In: Schulze ED, Caldwell MM, eds. *Ecophysiology of photosynthesis*. Berlin: Springer, 463–490.
- Krishna LV. 2014. Long term temperature trends in four different climatic zones of Saudi Arabia. *International Journal of Applied Science and Technology* 4:233–242.
- Lawson T, Davey PA, Yates SA, Bechtold U, Baeshen M, Baeshen N, Mutwakil MZ, Sabir J, Baker NR, Mullineaux PM. 2013. C<sub>3</sub> photosynthesis in the desert plant *Rhazya stricta* is fully functional at high temperatures and light intensities. *New Phytologist* 201:862–873.
- Leide J, Hildebrandt U, Reussing K, Riederer M, Vogg G. 2007. The developmental pattern of tomato fruit wax accumulation and its impact on cuticular transpiration barrier properties: effects of a deficiency in a beta-ketoacyl-coenzyme A synthase (LeCER6). *Plant Physiology* 144:1667–1679.
- Leide J, Hildebrandt U, Vogg G, Riederer M. 2011. The positional sterile (ps) mutation affects cuticular transpiration and wax biosynthesis of tomato fruits. *Journal of Plant Physiology* 168:871–877.
- Lüttge U. 2007. *Physiological ecology of tropical plants*. Berlin: Springer Science & Business Media, 328.
- Markstädter C, Federle W, Jetter R, Riederer M, Hölldobler B. 2000. Chemical composition of the slippery epicuticular wax blooms on *Macaranga* (Euphorbiaceae) ant-plants. *Chemoecology* 10:33–40.
- McCrum NG, Buckley CP, Bucknall CB. 1997. *Principles of polymer engineering*. Oxford: Oxford University Press.
- Merk S, Blume A, Riederer M. 1998. Phase behaviour and crystallinity of plant cuticular waxes studied by Fourier transform infrared spectroscopy. *Planta* 204:44–53.
- Nobel PS. 2009. *Physicochemical and environmental plant physiology*, 4th edn. Oxford: Academic Press.
- Pearcy RW, Ehleringer JR, Mooney HA, Rundel PW. 1989. *Plant physiological ecology, field methods and instrumentation*. London, UK: Chapman and Hall.
- Pollard M, Beisson F, Li Y, Ohlrogge JB. 2008. Building lipid barriers: biosynthesis of cutin and suberin. *Trends in Plant Science* 13: 236–246.
- Poorter H, Garnier E. 2007. Ecological significance of inherent variation in relative growth rate and its components. In: Pugnaire FI, Valladares F, eds. *Functional plant ecology*. Boca Raton: CRC Press, 67–100.
- Purves WK, Sadava D, Orians GH, Heller HC. 2004. *Life: the science of biology*. 7th ed. Sunderland: Sinauer Associates, 772.
- Riederer M, Schneider G. 1990. The effect of the environment on the permeability and composition of *Citrus* leaf cuticles: 2. Composition of soluble cuticular lipids and correlation with transport properties. *Planta* 180:154–165.
- Riederer M, Schreiber L. 1995. Waxes—the transport barriers of plant cuticles. In: Hamilton RJ, ed. *Waxes: chemistry, molecular biology and functions*. Dundee: The Oily Press, 131–156.
- Riederer M, Schreiber L. 2001. Protecting against water loss: analysis of the barrier properties of plant cuticles. *Journal of Experimental Botany* 52:2023–2032.
- Riederer M. 2006. Thermodynamics of the water permeability of plant cuticles: characterization of the polar pathway. *Journal of Experimental Botany* 57:2937–2942.
- Schönherr J. 1976. Water permeability of isolated cuticular membranes: the effect of cuticular waxes on diffusion of water. *Planta* 131:159–164.
- Schönherr J, Lenzian K. 1981. A simple and inexpensive method of measuring water permeability of isolated plant cuticular membranes. *Zeitschrift für Pflanzenphysiologie* 102:321–327.
- Schönherr J. 1982. Resistance of plant surfaces to water loss: transport properties of cutin, suberin and associated lipids. In: Lange OL, Nobel PS, Osmond CB, Ziegler H, eds. *Encyclopedia of plant physiology. Physiological plant ecology II, Water relations and carbon assimilation*, Vol. 12B. Berlin: Springer, 153–179.
- Schönherr J, Riederer M. 1986. Plant cuticles sorb lipophilic compounds during enzymatic isolation. *Plant, Cell and Environment* 9:459–466.
- Schreiber L, Schönherr J. 1990. Phase transitions and thermal expansion coefficients of plant cuticles. *Planta* 182:186–193.
- Schreiber L, Riederer M. 1996. Ecophysiology of cuticular transpiration: comparative investigation of cuticular water permeability of plant species from different habitats. *Oecologia* 107:426–432.
- Schreiber L. 2001. Effect of temperature on cuticular transpiration of isolated cuticular membranes and leaf discs. *Journal of Experimental Botany* 52:1893–1900.
- Slavík B. 1974. *Methods of studying plant water relations*. Berlin: Springer.
- Smirnova A, Leide J, Riederer M. 2013. Analysis of flower cuticular waxes and cutin monomers. *Bio-Protocol* 3:e899.
- Smith SD, Monson RK, Anderson JE. 2012. *Physiological ecology of North American desert plants*. Berlin: Springer Science & Business Media. pp 229, 231.
- Smith SE, Fendenheim DM, Halbrogge K. 2006. Epidermal conductance as a component of dehydration avoidance in *Digitaria californica* and *Eragrostis lehmanniana*, two perennial desert grasses. *Journal of Arid Environments* 64:238–250.
- Smith WK. 1978. Temperatures of desert plants: another perspective on the adaptability of leaf size. *Science* 201:614–616.
- Stein WD. 1986. *Transport and diffusion across cell membranes*. New York, Academic Press.
- Szakiel A, Pączkowski C, Pensec F, Bertsch C. 2012. Fruit cuticular waxes as a source of biologically active triterpenoids. *Phytochemistry Reviews* 11:1–22.
- Tsubaki S, Sugimura K, Teramoto Y, Yonemori K, Azuma J. 2013. Cuticular membrane of *Fuyu* persimmon fruit is strengthened by triterpenoid nano-fillers. *PLoS One* 8:e75275.
- Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M. 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid  $\beta$ -ketoacyl-CoA synthase. *Journal of Experimental Botany* 55:1401–1410.
- Windholz M, Budavari S, Blumetti RF, Otterbein ES. 1983. *The Merck Index*. New York: Merck & Co.
- Wisemann V, Riedel M, Riederer M. 2007. Matroclinal inheritance of cuticular waxes in reciprocal hybrids of *Rosa* species, sect. *Caninae* (Rosaceae). *Plant Systematics and Evolution* 263:181–190.
- Yates SA, Chernukhin I, Álvarez-Fernández R, Bechtold U, Baeshen M, Baeshen N, Mutwakil MZ, Sabir J, Lawson T, Mullineaux PM. 2014. The temporal foliar transcriptome of the perennial C<sub>3</sub> desert plant *Rhazya stricta* in its natural environment. *BMC Plant Biology* 14:2.
- Xanthos M. 2010. Modification of polymer properties with functional fillers. In: Xanthos M, ed. *Functional fillers for plastics*. New Jersey: Wiley & Sons, 19–41.