RESEARCH PAPER

Differences between water permeability of astomatous and stomatous cuticular membranes: effects of air humidity in two species of contrasting drought-resistance strategy

Jana Karbulková¹,*, Lukas Schreiber², Petr Macek¹,³ and Jiří Šantrůček¹,⁴

¹ Faculty of Science, University of South Bohemia, Braníšovská 31, 37005, České Budějovice, Czech Republic
² Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany
³ Institute of Botany, Academy of Sciences, Dukelská 135, 37982, Třeboň, Czech Republic
⁴ Institute of Plant Molecular Biology, Biology Centre ASCR, Braníšovská 31, 37005, České Budějovice, Czech Republic

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Abstract

Cuticular water permeabilities of adaxial and abaxial leaf surfaces and their dependence on relative air humidity (RH) applied in long-term and short-term regimes have been analysed for Hedera helix, native in a temperate climate, and Zamioculcas zamiifolia, native in subtropical regions. The water permeability of cuticular membranes (CM) isolated from the adaxial (astomatous) and abaxial (stomatous) leaf sides was measured using a method which allowed the separation of water diffusion through the remnants of the original stomatal pores from water diffusion through the solid cuticle. The long-term effects of low (20–40%) or high (60–80%) RH applied during plant growth and leaf ontogeny ('growth RH') and the short-term effects of applying 2% or 100% RH while measuring permeability ('measurement RH') were investigated. With both species, water permeability of the solid stomatous CM was significantly higher than the permeability of the astomatous CM. Adaxial cuticles of plants grown in humid air were more permeable to water than those from dry air. The adaxial CM of the drought-tolerant H. helix was more permeable and more sensitive to growth RH than the adaxial CM of Z. zamiifolia, a species avoiding water stress. However, permeability of the solid abaxial CM was similar in both species and independent of growth RH. The lack of a humidity response in the abaxial CM is attributed to a higher degree of cuticular hydration resulting from stomatal transpiration. The ecophysiological significance of higher permeability of the solid stomatous CM compared to the astomatous CM is discussed.

Key words: Cuticular permeability, Hedera helix, leaf cuticle, pores, relative humidity, stomatal conductance, Zamioculcas zamiifolia.

Introduction

The plant cuticle is the uppermost layer on the outer surface of leaves forming the interface between epidermal cells and the atmosphere. More than 400 million years ago it contributed to the colonization of the mainland by plants. The cuticle forms an effective barrier protecting plants from the uncontrolled loss of water, ions, and nutrients and it reduces infection by pathogens (Kerstiens, 1996a; Bird and Gray, 2003). Because of these barrier properties the availability of CO₂ for leaves decreased. Therefore, the leaf cuticle is perforated with stomatal pores (Woodward, 1998). During water stress, when stomata are closed, plant survival depends on the amount of water lost through the cuticle. From a whole-plant point of view, the interplay between stomatal regulation and cuticular water permeability is therefore essential (Kerstiens, 1996a).

Since the cuticle has properties resembling those of a solution-diffusion membrane, diffusing molecules pass through it as individual molecules following a random path in a mostly lipophilic chemical environment. This
means that hydrophobic and uncharged molecules penetrate more easily than water and other polar species. Schreiber et al. (2001) suggested the existence of two parallel pathways for water diffusion across the cuticle. One pathway is formed by the lipid fraction of the cuticle (i.e., the solution-diffusion type) whereas the second type of pathway is of a more localized nature and exists along hydrated polar groups (-OH and -COOH; polysaccharide microfibrils), which form pores that enable the transit of polar organic compounds and inorganic ions.

There is no experimental evidence to date that phytophysic foliage allows plants to adapt their cuticular permeance to changes in evaporative demand (Riederer and Schreiber, 2001). In an experiment where only growth RH was varied, Geyer and Schönherr (1990) did not detect any significant effect on water permeability of Citrus aurantium cuticles. However, water permeability of cuticles exposed to different short-term vapour pressure deficits (VPD) or RH levels sensitively responded to VPD or RH changes (Hoad et al., 1997; Schreiber et al., 2001).

Analysing interspecific variation holds more promise for explaining ecophysiological adaptations. Plants of different origin were surveyed by Schreiber and Riederer (1996); the lowest permeabilities were recorded for evergreen leaves (mostly from tropical plants), while the highest permeabilities were recorded for mesomorphic leaves of deciduous temperate plants. Since they investigated plants grown under identical climatic conditions, differences in water permeability were explained by genetic differences.

Cuticular permeability differs not only between plants, their organs and their developmental stages, but also between both sides of the same leaf. Present knowledge of cuticular permeability is based mainly on measurements with astomatous leaf surfaces or astomatous cuticular membranes (reviewed in Kerstiens, 2006). In situ measurements with intact stomatous cuticles are difficult, because stomata can contribute to the apparent cuticular transpiration due to imperfect closure (Burghardt and Riederer, 2003; Beyer et al., 2005). This most probably led to an overestimation of cuticular permeability in a number of studies in the past (Kerstiens, 1996a). Several direct (using radiolabelled weak organic acids; e.g., Schreiber, 1990) and indirect techniques (extrapolation of cuticular conductance from linear regression of stomatal density and total surface conductance; e.g., Knoche et al., 2000) have been used to estimate cuticular permeability of intact stomatous leaf or fruit surfaces. Recently, Šantruček et al. (2004) presented a technique that used isolated cuticles and allowed the water flow across the remnants of stomatal pores (‘stomatal transpiration’) to be distinguished from the flow across the solid phase of stomatous cuticles (‘cuticular transpiration’). This method enables a comparison of water permeabilities in isolated CM from both sides of the leaf while avoiding errors caused by stomatal transpiration.

It was the objective of this study to investigate the relationship between cuticular water permeability of adaxial and abaxial leaf sides, leaf life-history, RH, and drought survival. In order to estimate drought strategy effect on cuticular permeability, two species with different drought tolerant and avoidace (temperate and tropical) were both grown in two different environmental regimes characterized by low and high humidity. In addition, different ambient humidities applied during the measurement of cuticular water permeability simulated natural short-term changes in VPD at the leaf surface. The following hypotheses were to be investigated. (i) Cuticular water permeability of the adaxial CM differs from that of the solid abaxial CM. (ii) This difference is constant, irrespective of plant drought resistance strategy. (iii) Cuticular water permeability is not affected by growth conditions. (iv) Cuticular water permeability increases with increasing RH applied during the measurement.

Materials and methods

Experimental design

Two hypostomatous evergreen species were selected for this study: the temperate liana Hedera helix L. (ivy) and the tropical plant Zamioculcas zamifolia (Lodd.) Engl., which occurs naturally in Eastern Africa and Madagascar (Grivet and Petit, 2002; Holtum et al., 2007). Plants of both species were grown for one year in two separate glasshouses with contrasting humidities, ‘wet’ (60–80% RH) or ‘dry’ (20–40% RH), under natural irradiance and semi-controlled temperature (fluctuating between 18°C and 32°C during the year). Plants in the dry glasshouse were watered once a day with a limited volume of tap water just sufficient to avoid wilting. Plants in the wet glasshouse were watered two to three times per day. Full-grown leaves were collected from both species in autumn 2004. Cuticular membranes were isolated from both leaf sides. Water permeances were measured under two different levels of humidity (<2% and 100% RH).

The following leaf characteristics related to drought resistance strategy were estimated:

Degree of sclerophylly (Sc; g m⁻²)

\[ Sc = \frac{DW}{A} \quad (1) \]

Degree of succulence (Su; g m⁻²)

\[ Su = \frac{FWs - DW}{A} \quad (2) \]

where FWs (g) is a fresh weight of water-saturated leaves cut under water and stored for 12 h in the dark in glass covered with a plastic bag, DW (g) dry weight, and A (m²) projected leaf area (according to Burghardt and Riederer, 2003). Stomatal density (mm⁻²) was determined with a light microscope.

Isolation of cuticular membrane (CM)

CM was isolated by immersing leaf discs (diameter 2 cm) in an aqueous solution of 2% (v/v) cellulase (Celluclast, Novo Nordisk, Bagsværd, Denmark) and 2% (v/v) pectinase (Trelonol Super DF, Erbßlöf, Geisenheim, Germany) in 0.01 M citric buffer (Merck, Germany; pH 3.0, adjusted with KOH). The detailed procedure is described in Schönherr and Riederer (1986). Sodium azide (NaN₃,
were flushed with the new gas for 10 min. The amount of 3H$_2$O in the receiver was 35 mm, which corresponded to a boundary resistance thickness of the unstirred air layer between the cuticle and the equilibrium with laboratory temperature (25°C). Compartments of a stainless-steel transport chamber kept in thermal accumulation in the filter paper in the receiver compartment was solid membrane (Šantrúček et al. 2004). Verification of this method and further details are given in Šantrúček et al. (2004).

In equations (3) and (4) it is assumed that water flux across the solid membrane (F$_c$) is not affected by the type of gas in which the isolated CM is immersed. The ratio of water fluxes across the pores filled with helium and nitrogen is given by the ratio of water diffusivities:

$$ F_{3He} \div F_{2N} = D_{3He} \div D_{2N} \cong 3.6 $$

where $D_{3He}$ and $D_{2N}$ are binary diffusion coefficients of water in helium and nitrogen. The diffusion of water vapour in helium is 3.6 times faster than in nitrogen (Cussler, 1987). With the ratio $D_{3He} \div D_{2N}$ called $R$, the parameter $k = R(R-1)$ can be defined. Substituting $F_{3He}$ in equation (4) by equation (5) and combining equations (3) and (4), equation (6) is obtained. It describes the water flux $F_c$ across the solid phase of the stomatous or astomatous cuticle:

$$ F_c = k \times F_{2N} - (k-1) 	imes F_{3He} $$

Verification of this method and further details are given in Šantrúček et al. (2004).

(b) Measurements: Cuticular transpiration was measured using $^3$H-labelled water (specific activity 925 MBq g$^{-1}$, Hartmann Analytik, Braunschweig, Germany). The CM was mounted between the two compartments of a stainless-steel transport chamber kept in thermal equilibrium with laboratory temperature (25°C, Fig. 1). The thickness of the unstirred air layer between the cuticle and the receiver was 35 mm, which corresponded to a boundary resistance of 1483 s m$^{-1}$ in nitrogen and 719 s m$^{-1}$ in helium. However, the boundary resistance represents only 2.5% of the total resistance of the stomatal membrane. There was no pressure difference between donor and receiver compartments. The donor compartment contained liquid $^3$H-labelled water. The receiver compartment contained nitrogen or helium gas and either filter paper soaked with 35 μl of deionized water, leading to RH=100%, or glycerol-saturated filter paper resulting in RH <25%. High-vacuum grease (WackerChemie, Burghausen, Germany) was used to mount and seal the CM between the two compartments of the transport chamber. The morphologically inner side of the CM was oriented towards the donor compartment and the morphologically outer side was in contact with the gas-filled receiver compartment at low (2%) or high (100%) humidity. Before each measurement, donor and receiver compartments were flushed (100 ml min$^{-1}$) simultaneously for 3 min with helium or nitrogen (purity >99.99%). Before replacing helium by nitrogen or vice versa, both compartments were flushed with the new gas for 10 min. The amount of $^3$H$_2$O accumulating in the filter paper in the receiver compartment was measured by sampling and replacing the absorbing paper every 10 min for 30 min. The filter paper containing $^3$H$_2$O was quickly transferred to a scintillation vial with 4 ml of scintillation cocktail (UltimaGold XR, Canberra Packard). The amount of radioactivity was quantified in a scintillation counter (TriCarb 1600, Packard). All individual CM samples were measured in nitrogen and in helium at both measurement RH levels. The effect of measurement RH on cuticular permeability is given as the ratio of cuticular permeability at 100% measurement RH and cuticular permeability at 2% measurement RH.

Since the filter paper in the receiver contained only traces of the radioactivity provided in the donor and since the filter paper was frequently replaced, the driving force $\Delta c$ for $^3$H$_2$O diffusion across the cuticle is given by the concentration of $^3$H$_2$O (cpm m$^{-3}$) in the donor compartment. Criccular permeability is characterized by the permeance $P$ (m s$^{-1}$) calculated according to equation 7:

$$ P = \frac{F}{A \times \Delta c} $$

where $F$ (flux) is given by the slope of the regression line fitted to the linear plot of $^3$H$_2$O penetrated versus time (cpm s$^{-1}$ and $A$ (m$^2$)) is the exposed area of the CM. Permeances $P$ are calculated as ‘vapour-based’ values with the driving force given by the water concentration in the gas phase. $P$ can be converted to equivalent ‘liquid-based’ values, where the driving force is given by the concentration of water in liquid water (=density of water), by dividing $P$ by the liquid/air partition coefficient of water, which amounts to 43 384 at 25 °C. Total permeance $P_t$ of the cuticle describes the transport of water across the solid phase of the cuticle and the gas phase formed by stomata if present. It is calculated from the total flux $F_t$. The permeance $P_t$ exclusively describes the transport of water across the solid cuticle. It is calculated from $F_c$, which is obtained from equation 6.

Sample size and statistics

Water flux was measured with 4–7 CM samples for each species, each leaf side, and each glasshouse. To validate that fluxes across a solid membrane in helium and nitrogen atmospheres were equal, fluxes across paraffilm discs were measured. There was no difference...
between water fluxes in the two different gas atmospheres \((n=5, t=1.54, P=0.20)\). Results with CM were initially analysed using a multifactorial hierarchical ANOVA with four factors: species, glasshouse type, leaf side, and (measurement) RH. As the effect of RH was measured with identical CM samples, this treatment was nested within the others (i.e. species, glasshouse, leaf side). In all analyses, most of the interactions of leaf side \(x\) factor were significant. This indicated that astomatous CM from the adaxial leaf side showed a different response to the other experimental factors compared with stomatous CM from the abaxial leaf side. Therefore, in the following statistical analyses only three factors (species, glasshouse and RH – nested in the interaction of the former two factors) were used and data for each leaf side were evaluated separately. For the characterization of the anatomical properties of the leaves, 10 replicates from each plant and each growth condition were used. Stomatal density was calculated from observation of 10 CM samples (10 graticule fields per sample) in each combination of plant and glasshouse. Data were analysed using ANOVA with 2 factors (species and glasshouse type). To satisfy the ANOVA of plant and glasshouse. Data were analysed using ANOVA with 2 factors (species and glasshouse type). To satisfy the ANOVA model assumptions, some variables (total permeance, solid phase permeances the two-way repeated measures ANOVA was used (to ensure performance of the paired comparison of solid and glasshouse interaction on total water permeance \((P<0.001)\). If the adaxial cuticle is considered to be a porous membrane and the solid phase permeance \(P_c\) is calculated from the flux \(F_c\) according to equation 6, then \(P_c\) should correspond to \(P_t\). This holds when comparing total and solid permeances across species identity and glasshouses \((F=0.40, P=0.54); \text{ Fig. 3}\).

### Results

#### Water diffusion through the adaxial astomatous CM

The permeance \(P_t\) of astomatous cuticles changed significantly with all factors (species, growth RH, and measurement RH; Table 1; Fig. 2). Comparing the \(P_t\) values of both species averaged across all growth RH and measurement RH treatments, the value for \(Z. zamiifolia\) \((2.55 \times 10^{-7} \pm 1.91 \times 10^{-7} \text{ m s}^{-1})\) was 13.5 times smaller than for \(H. helix\) \((3.44 \times 10^{-6} \pm 2.18 \times 10^{-6} \text{ m s}^{-1})\). The interaction of species and glasshouse was not significant (Table 1). The Tukey test indicated that \(H. helix\) plants from the wet glasshouse had a higher \(P_t\) than plants from the dry glasshouse \((P=0.035)\), while in \(Z. zamiifolia\) the difference between glasshouses was not significant \((P=0.960)\). \(P_t\) of \(H. helix\) growing in either the wet or dry glasshouse was significantly different from \(Z. zamiifolia\) in both cases \((P<0.001)\). If the adaxial cuticle is considered to be a porous membrane and the solid phase permeance \(P_c\) is calculated from the flux \(F_c\) according to equation 6, then \(P_c\) should correspond to \(P_t\). This holds when comparing total and solid permeances across species identity and glasshouses \((F=0.40, P=0.54); \text{ Fig. 3}\).

#### Water diffusion through the abaxial stomatous CM

Mean total permeance \(P_t\) (average value across all growth RH and measurement RH treatments) of \(Z. zamiifolia\) and \(H. helix\) was 1.64 \(\times 10^{-5} \pm 7.65 \times 10^{-6} \text{ m s}^{-1}\) and 1.67 \(\times 10^{-5} \pm 7.67 \times 10^{-6} \text{ m s}^{-1}\), respectively. Permeance \(P_c\) of the solid cuticle was not significantly different between the two species. It was 4.20 \(\times 10^{-6} \pm 2.61 \times 10^{-6} \text{ m s}^{-1}\) for \(Z. zamiifolia\) and 6.19 \(\times 10^{-6} \pm 4.93 \times 10^{-6} \text{ m s}^{-1}\) for \(H. helix\). \(P_c\) was also independent of growth RH and measurement RH.

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**Table 1.** Results of ANOVA analysing effects of species, growth conditions (glasshouse) and measurement RH (RH) and species \(\times\) glasshouse interaction on total water permeance \((P_t)\) of adaxial CM, and total and solid phase permeance \((P_c)\) of abaxial CM

| Treatment                  | \(P_t\) (adaxial CM) | \(P_t\) (abaxial CM) | \(P_c\) (abaxial CM) |
|----------------------------|----------------------|----------------------|----------------------|
|                            | \(F\)                | \(P\)                | \(F\)                | \(P\)                |
| Species                    | 459.650              | <0.001               | 0.003                | 0.957               |
| Glasshouse                 | 5.184                | 0.030                | 5.410                | 0.026               |
| RH                         | 11.324               | <0.001               | 1.261                | 0.305               |
| Species \(\times\) glasshouse | 2.359               | 0.135                | 5.761                | 0.022               |
When comparing stomatous and astomatous CM, \( P_t \) of the astomatous CM was significantly lower than \( P_c \) of the stomatous CM (\( t = 4.32, P < 0.001 \)). Depending on humidity in the glasshouse, \( P_c \) of the stomatous CM was 2.7 to 3.0 times (\( H. helix \)) and 15.8 to 22.7 times (\( Z. zamiifolia \)) higher than \( P_t \) of the astomatous CM (Fig. 4).

**Short-term humidity effect**

Effect of measurement RH did not differ between species (\( F = 1.72, P = 0.20 \)); the interaction of species and leaf side was also insignificant (\( F = 0.03, P = 0.87 \)). However, both leaf sides responded to humidity changes differently (\( F = 8.47, P = 0.006 \)), i.e. the increase in permeance of the adaxial CM was higher than that of the abaxial CM. \( P_t \) of astomatous CM increased with increasing measurement RH (Fig. 2). The magnitude of the effect of measurement RH depended on growth RH (\( F = 6.51, P = 0.016 \)). The effect was strongest with material from the wet glasshouse (Fig. 5). \( P_t \) or \( P_c \) of the abaxial CM did not respond to measurement RH (under any growth RH).

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**Other leaf characteristics**

Leaves detached from \( Z. zamiifolia \) had significantly higher \( Su \) than \( H. helix \) leaves (\( F = 198.8, P < 0.001 \)), while \( Sc \) was higher in \( H. helix \) leaves (\( F = 58.3, P < 0.001 \); Table 2). Stomatal density differed between both species in a manner dependent on growth conditions (interaction glasshouse \( \times \) species; \( F = 76.7, P < 0.001 \)). In \( Z. zamiifolia \), stomatal density was about 5–10 times lower than in \( H. helix \) (\( F = 1884, P < 0.001 \)). Growth at high RH increased stomatal density in \( H. helix \) (\( F = 57.9, \)
Table 2. Degree of sclerophyll (Sc, dry weight per area), degree of succulence (Su, saturated water content per area) and stomatal density (number of stomatal pores mm$^{-2}$) in H. helix and Z. zamiifolia CM grown in dry and wet glasshouses

Mean values ±SD are shown.

| Leaf characteristics | Dry glasshouse | Wet glasshouse |
|----------------------|---------------|---------------|
|                      | Z. zamiifolia | H. helix | Z. zamiifolia | H. helix |
| Sc (g m$^{-2}$)      | 66.6±7.7      | 176.9±68.6   | 70.6±14.4     | 127.5±21.0 |
| Su (g m$^{-2}$)      | 533.0±64.4    | 328.6±67.2   | 621.6±77.6    | 199.1±29.7  |
| Stomatal density (mm$^{-2}$) | 31±6         | 143±47      | 25±5          | 234±49      |

$P<0.001$) and decreased it in Z. zamiifolia ($F=21.7$, $P<0.001$; Table 2).

Discussion

Differences between adaxial and abaxial cuticular permeability

At identical vapour pressure gradients, water flux across adaxial CM was higher than that across adaxial CM, obviously due to the presence of stomatal pores on the abaxial leaf side. Total permeance $P_t$ of the stomatous CM is composed of $P_e$, the permeance of the remnants of the stomatal pores, and $P_c$, the permeance of the solid cuticle. Under favourable conditions, the water flux through stomatal pores in an intact sun-exposed leaf makes up roughly 95% of total water loss (Goodwin and Jenks, 2005). A somewhat different situation might appear with closed stomata. Using conventional gas exchange measurements (LI-6400, Li-Cor, USA), it was determined that $P_t$ of intact Z. zamiifolia leaves increased from $1.5\times10^{-5}$ to $1.0\times10^{-2}$ m s$^{-1}$ with stomatal opening (J Kubášek, unpublished data) and $P_t$ of intact H. helix leaves increased from $5.0\times10^{-5}$ to $4.16\times10^{-3}$ m s$^{-1}$ with stomatal opening (Šantrůček et al., 2004). $P_c$ of the abaxial CM represented about 25% to 33% of $P_t$. The abaxial solid cuticle was more permeable than the adaxial CM in both species (Fig. 4). The ratio of abaxial to adaxial permeances under low growth RH was 2.7 and 15.8 for H. helix and Z. zamiifolia, respectively.

Due to the interference of stomatal pores, direct comparisons of water permeability of astomatous adaxial and stomatous abaxial cuticles are rare. A higher permeability of the abaxial leaf side for non-volatile chemicals has been described in the past (Price, 1982; Schreiber, 1990; Šantrůček et al., 2000; Schlegel et al., 2006). Water diffuses across cuticles using two parallel paths (sensu Schreiber et al., 2001), a random lipophilic one and hydrophilic one along hydrated polar pores. Thus, a higher permeability of the abaxial CM should be due to (i) a different density of polar pores and/or their transport properties or (ii) a faster diffusion of water through the lipophilic fraction of the cuticle. A much higher density of polar aqueous pores was in fact observed over guard cells and trichomes (Schlegel et al., 2005). The presence of aqueous pores was also reported for astomatous cuticles of Pyrus communis and Populus canescens (Schönherr, 2000; 2002; Schönherr and Schreiber, 2004). However, rate constants of the same penetrating compounds were significantly higher for stomatous Vicia faba leaves than for astomatous Pyrus and Populus CM (Schönherr, 2006). But very little is known about the structure and transport properties of polar pores (Schreiber, 2005). The adaxial side of the hypostomatous leaves is covered with a continuous cuticle, while on the abaxial leaf side the cuticle also covers the sloping ventral walls of the guard cells and partially lines the substomatal cavity (Pesacreta and Hasenstein, 1999). The functional property of the guard cell cuticle and leaf internal cuticle is largely unknown. Osborn and Taylor (1990) found different types of cuticular ultrastructural organization between the adaxial and the abaxial CM. The possible existence of guard cell-specificity of cuticular transport properties may play an important physiological role in the short-term control of leaf water loss and stomatal sensitivity to CO$_2$ via humidity-sensing stomata (Kerstiens, 1996a, b; Talbott et al., 2003).

Surprisingly, there was large variability between $P_t$ and $P_c$ in the adaxial astomatous CMs (Fig. 3). While one can only speculate about the precise reason for this observation, micro-damage, broken trichomes or aphid incision might be possible explanations. However, no damage was noticed with a light microscope prior to the permeance measurements. Šantrůček et al. (2004) observed pores allowing gas-phase diffusion in the wax-free cuticular matrix membrane, but not in the intact CM, where the pores present are probably covered or filled by waxes. The current state of knowledge does not allow us to arrive at safe conclusions about the existence of gas-filled pores in astomatous CM. Hence, further experiments on the water permeability of astomatous CM exposed to gases of
Drought adaptation strategy and CM permeability

During a survey study of plants from different origins, Schreiber and Riederer (1996) found the highest cuticular resistance to water loss in tropical epiphytes and CAM species while temperate crops are known for much lower cuticular resistance. Therefore, the adaxial cuticle of *Z. zamiifolia*, adapted to arid subtropical areas, has a lower mean permeance than that of *H. helix*, native in regions with a temperate climate. Succulent leaf blades of *Z. zamiifolia* with high relative water content (RWC ~99%) indicate a drought avoidance strategy (Larcher, 1995). In addition, Holtum et al. (2007) observed a facultative switch to CAM during drought in *Z. zamiifolia*. Finally, a very low stomatal density (Table 2) that is a common feature of succulent leaves, means fewer leaky evaporative sites. These adaptations improve *Z. zamiifolia* survival during the dry season in dry lowland forests and savannas. Conversely, *H. helix* uses the drought-tolerance strategy, including osmotic adjustment (indicated by low osmotic potential at turgor loss) and a high degree of sclerophyll (Burghardt and Riederer, 2003). As a temperate perennial evergreen plant, *H. helix* has to overcome winter droughts in north-east European countries. At the opposite geographical distribution, summer drought probably limits its distribution in the Mediterranean area (Metcalfe, 2005).

Interestingly, both species investigated here had very different permeabilities of adaxial CM but similar permeabilities of the solid phase of abaxial CM. Due to the presence of stomata and overlapping diffusion shells of high humidity close to the abaxial leaf surface, the evaporation demand should be substantially lower at the stomatous cuticle–air interface than at the upper leaf surface (under calm or moderately windy conditions). Water diffusing through stomatous CM travels mainly through the stomatal pores, producing a humid boundary layer and, presumably, also increases the permeability of the adjacent cuticle (Burkhardt et al., 1999). Such a mechanism is consistent with the lack of response in abaxial CM to short-term humidity changes. It could indicate a common demand for minimal transpiration flux essential for nutrient and water acquisition from roots. Snyder et al. (2003) pointed out significant night-time transpiration at species with a range of life forms and origin which was associated with higher day-time transpiration values.

Effect of RH during leaf ontogeny

Elevated air humidity during leaf development increased cuticular permeability, especially at the adaxial (astomatous) leaf side. Although Geyer and Schönherr (1990) did not find a growth humidity effect on the permeability of adaxial *Citrus aurantium* CM, there is an important difference between the two experiments: while the *Citrus* plants were generously watered, our plants growing in the dry glasshouse experienced water shortage.

Elevated air humidity may affect stomatal density either positively or negatively (Bakker, 1991; Hirai et al., 2002; Torre et al., 2003; Klooster and Palmer-Young, 2004). Such an inconsistency of effects of growth humidity on stomatal density was also observed in the present experiment. Decreased stomatal density but increased size of guard cells (and presumably also maximum aperture size) in *Z. zamiifolia* from the wet glasshouse could explain higher total water permeability of abaxial CM due to the less efficient obstructions caused by remnants of the internally-extending cuticle. Consequently, there was no significant difference in $P_e$ between stomatous CM from the wet and dry glasshouses (Fig. 3C, D). On the other side, increased stomatal density in *H. helix* in the wet glasshouse did not affect $P_t$ or $P_c$. Similar mean values of $P_c$ under all experimental regimes suggest a high surface humidity (humid boundary layer) in contact with the cuticle between stomata, even at low stomatal density and low ambient air humidity.

Effect of short-term (measurement) RH

Saturation of the air space in the receiver compartment with water vapour increased the permeability of adaxial CM by a factor of up to four (CM from the wet glasshouse, Fig. 5). Similarly, Schreiber et al. (2001) showed a two to three fold increase in permeability of isolated astomatous CM of several species when humidity rose from 2% to 100%. How can this effect be explained? At higher RH, water content in the cuticle increases due to binding of water molecules to polar functional groups, non-esterified carboxyl and/or hydroxyl groups of cutin monomers (Schreiber, 2006). Hence, it forms additional polar pathways and opens further the ones that are initially present (Burghardt and Riederer, 2006). The positive correlation between water permeability and air humidity is based mainly on physical processes, for example, swelling. Different humidity effects between species are affected by the varied content of polar functional groups and polysaccharide microfibrils (Van Hove and Adema, 1996; Schönherr, 2006). If the carboxy groups in CM were methylated, the water permeance was reduced to about 50% (Schreiber et al., 2001).

Several studies on the influence of cuticular permeability on the stomatal response to VPD have been made, although the role of cuticular permeability remains controversial. Recently Eamus et al. (2008) manipulated cuticular transpiration by three independent methods and compared them with mathematical models. Cuticular transpiration influenced stomatal conductance by a feedback mechanism, i.e. VPD affects cuticular transpiration and hence the ability of the epidermal cells to supply...
water to guard cells. This was previously reported by indirect measurements showing different permeabilities of guard cell cuticles depending on pore aperture and cell turgor (Schlegel et al., 2005). The spatial resolution of cuticular permeability on stomatous leaf surfaces and the cell type-specific response of the cuticle to ambient humidity require further investigation.

Conclusions
Data presented here reveal consistently higher water permeability of the solid phase of abaxial stomatous CM than of adaxial astomatous CM isolated from the same leaves of H. helix or Z. zamiifolia, irrespective of plant growth conditions and measurement air humidity. The differences in cuticular permeability between adaxial and abaxial cuticles may be linked to the humid boundary layer created by vapour escaping from stomatal apertures. The proportional effect of measurement RH on the permeability of the astomatous cuticle in both species could indicate a mechanism of cuticular control of leaf water status. The lack of a difference between permeabilities of the solid part (i.e. the membrane located between the stomatal pores) of abaxial stomatous cuticles in the two species and under the different measurement RH levels indicates that the stomatous cuticles posses different structural and functional properties than the astomatous cuticles from the upper leaf surface. The long-term (growth) elevation of ambient RH increased cuticular water permeability and the strength of its response to the alteration of short-term RH in adaxial astomatous but not in abaxial stomatous CM. The difference of an order of magnitude between permeabilities of the adaxial cuticle in Z. zamiifolia and H. helix corresponds to their adaptation to arid and temperate regions, respectively, and water stress avoiding (Z. zamiifolia) and tolerating (H. helix) strategies. The lateral heterogeneity of stomatous cuticles needs to be addressed for a proper evaluation of the roles which guard and subsidiary cells play in sensing the environment.

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