Faculty of Science
University of South Bohemia

# The role of plant cuticle in water loss protection

Ph.D. Thesis

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(shortened electronic version)

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# Annotation

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A central aim of this thesis was studying plant regulation of transpiration water loss. I focused first on changes in cuticular water permeability and second, on changes in cuticular chemical composition under simulated drought stress. Finally, the obtained findings were capitalized on in a case study of altitudinal distribution of congeneric treeline species.

# Declaration

I hereby declare that this Ph.D. Thesis has been fully worked out by myself and the named co-authors, and with the use of the cited references.

I declare that in accordance with the Czech legal code § 47b low No. 111/1998 in its valid version, I consent to the publication of my Ph.D. Thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in the public access to the STAG database run by the University of South Bohemia in České Budějovice on its web pages.

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Jana Macková

České Budějovice, 3rd January 2010

# Author contribution statement

This is to confirm, that Jana Macková (formerly Karbulková) significantly contributed to developing new method, obtaining results and that she participated on data explanation in the following research paper:

Šantrůček. J., Šimáňová E., Karbulková J., Šimková M. and Schreiber L., 2004. A new technique for measurement water permeability of stomatous cuticular membranes isolated from *Hedera helix* leaves. Journal of Experimental Botany 55, 1411–1422.

Further, I confirm, that Jana Macková (Karbulková) has main portion on preparing this publication and the submitted manuscript:

Karbulková J., Schreiber L., Macek P. and Šantrůček J., 2008. Differences between water permeability of astomatous and stomatous cuticular membranes: Effects of air humidity in two species of contrasting drought resistance strategy. Journal of experimental Botany 59, 3987–3995.

Macková J., Vašková M., Macek P., Hronková M., Schreiber L. and Šantrůček J. Plant response to drought stress simulated by ABA application: changes in chemical composition of cuticular waxes. Submitted to Environmetnal and Experimental Botany.

Doc. Ing. Jiří Šantrůček, CSc. (supervisor)

Hereby I confirm, that Jana Macková significantly contributed to measuring and collecting data in the field, laboratory analyses and manuscript preparation in the following research paper:

Macek P., Macková J. and de Bello F., 2009. Morphological and ecophysiological traits shaping altitudinal distribution of three *Polylepis* treeline species in the dry tropical Andes. Acta Oecologica 35, 778–785.

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# Contents

# Introduction

Plant cuticle is a unique polymer layer which allowed colonization of dry land by plants. It creates an effective barrier protecting plants from excessive water, ion and nutrient loss and infection by pathogens (Kerstiens, 1996a; Riederer and Schreiber, 2001). In simplified words, cuticle forms 'plant skin' and in fact, the Latin name 'cuticula' means really 'thin skin'.

Cuticular ecophysiological traits, e.g. water permeability, are affected both by cuticular chemical composition and its physical properties. Furthermore, these traits can also be influenced by surrounding environmental conditions. Conversely, environmental conditions are imprinted through $CO_2$ fixation mechanisms into isotopic composition of the cuticle as in the chronicle of plant-environment relationship.

## *1. Cuticle composition*

Cuticular membrane is an extracellular product of epidermis formed during growth period of different aerial organs of the plant. It is a thin (0.1–10 μm) continuous membrane consisting of a polymer matrix (cutin) entwined with polysaccharides and cuticular waxes (solvent soluble lipids; Schönherr and Bukovac, 1973; Nawrath, 2002; Riederer and Schreiber, 2001; Neinhuis et al., 2001). Insoluble cutin polymer, the major structural cuticle component, is formed by cross-linking hydroxylated fatty acids via intermolecular ester bonds leading to a three-dimensional structure. Cutin monomers of the $C_{16}$ and $C_{18}$ classes are synthesized from the acetyl coenzyme A (CoA) esters of palmitic acid and oleic acid, respectively, by multiple hydroxylation and epoxidation reactions (Hauke and Schreiber, 1998; Nawrath, 2002).

Cuticular waxes represent multiphase system, consisting at least of two important structural fractions. A crystalline fraction is composed of long aliphatic chains (alkanes, alcohols, aldehydes, fatty acids and esters) assembled in a reticular lattice, while an amorphous zone forms an interspace between chain ends, functional groups, short-chain aliphatic compounds and non-aliphatic compounds (Kerstiens, 1996b; Kolattukudy, 1996). Wax precursors, fatty acids up to chain length $C_{18}$, are synthesised in plastids from CoA. Elongation processes are catalysed by fatty acid elongases, a multienzyme complex located in endoplasmatic reticulum (Kunst and Samuels, 2003). Final differentiated aliphatic chains (alkans, aldehydes, alcohols, acids and esters) are transported to plasmatic membrane and extracted across cell wall. Due to their lipophilic character, the transfer across plasmatic membrane and cell wall is mediated by ABC transporters and LTP proteins (Treviño and O'Connell, 1998; Pighin et al., 2004; Samuels et al., 2008).

### *2. Cuticle properties: water permeability*

The cuticle forms a contact zone between plant cells and environment and its main function is protection against water loss. Cuticle physical properties depend on genetic predisposition and environmental conditions (e.g. temperature, humidity, irradiance). Under the ambient conditions, the air humidity is below 100% and water flows from inner part to outer surface where it evaporates. However, when air humidity is close to 100%, the opposite situation can also be observed. Cuticular permeability to water is usually characterized by the variable permeance ($P$), which represents the ratio of water flow rate density to driving force, i.e. concentration difference (Kerstiens, 1996b; Riederer and Schreiber, 2001). Water and other solutes get across cuticles by diffusion, which is based on random molecular motions over small molecular distances (Schreiber and Schönherr, 2009). Interestingly, cuticular water permeability is not correlated to cuticle thickness or to wax coverage (Kerstiens, 1996a; Schreiber and Riederer, 1996; Riederer and Schreiber, 2001). This is likely because the effectiveness of cuticular barrier is determined not only by the amount of waxes, but also by their chemical composition, physical arrangement and molecular organization (Schreiber et al., 1996). Membrane permeability in general, and specifically cuticle permeability, are affected by partition coefficients (i.e. how many penetrating molecules enter cuticle at a given external concentration) and component's mobility (i.e. rate of penetrating molecules across the cuticle, Kerstiens, 2006). The increase of partition coefficients eventually leads to a relatively high cuticular permeability for extremely lipophilic compounds. On the other hand, polar electrolytes and ionic species have small partition coefficients leading to extremely low solubility in cutin and cuticular waxes and thus cuticle represents an effective barrier for these compounds (Schreiber et al., 1996; Buchholz et al., 1998). Diffusant's mobility depends on tortuosity of the diffusion pathway, i.e. on spatial ordering of aliphatic domains and cyclic compounds. The investigation of cuticle of *Cirsium horridulum* showed that hydroxyl groups enhance cuticular hydrophilic character and elasticity (Marga et al., 2001). Further, Hauke and Schreiber (1998) documented that waxes with chain length larger that $C_{27}$ have been related to lower cuticular transpiration.

Plant cuticle controls the movement of water between outer cell wall of the epidermis and the atmosphere. The mechanism of water transport across cuticle is a simple diffusion process along a gradient of the chemical potential of water (Niederl et al., 1998). This model explains cuticular permeability rather for lipophilic organic non-electrolytes, but reaches its limits when polar compounds are included (Riederer and Schreiber, 2001). Schreiber at al. (2001) published a new model of two parallel pathways of water diffusion across the cuticle. One pathway proceeds through the lipid fractions of the cuticle and it is easily accessible to lipophilic solutes. The second one is made up along hydrated polar groups (-OH and -COOH, polysaccharide microfibrils) which form pores and allow transit of water-soluble organic compounds and inorganic ions. Nevertheless, until now, it is not clear how these polar pathways develop and where they are localized (Schreiber, 2005). Earlier data suggested that the high density of polar pathways may be localized above anticlinal cell walls of epidermal cells, above guard cells and in the vicinity of cuticular ledges of stomata (Beyer et al., 2005; Schlegel et al., 2005). On the other hand, aqueous pores were also observed in astomatous cuticles of *Pyrus communis* and *Populus canescens* (Schönherr, 2000; 2002). However, rate constants of the same penetrating compounds were higher for stomatous cuticles than for astomatous ones (cf. Schönherr, 2006).

### *2.1 Effect of temperature*

Temperature is a predominant physical factor influencing permeance of the barrier. Leaf surface temperature primarily depends on air temperature and wind velocity, irradiation and transpiration rate. With increasing temperature, diffusion coefficients increase (size selectivity decreased), while partition coefficients between membrane and adjacent phases decrease (Baur and Schönherr, 1995; Baur et al., 1997; Buchholz et al., 1998; Schönherr et al., 2001). Since temperature has stronger effect on diffusion than on partitioning, the water permeance increases with temperature (Riederer and Schreiber, 2001). Nevertheless, permeance of water across cuticle increases nonlinearly with rising temperature; the slopes are becoming steeper at temperatures higher than 35°C (Schreiber, 2001). It seems that at temperature above 30–40°C increased volume expansion of cutin polymer causes defects in transport limiting barrier for water (Schreiber and Schönherr, 1990), while no change was observed in cuticular permeability for lipophilic substances.

### *2.2 Effect of humidity*

Cuticular water permeability significantly increases with increasing air humidity (Slavík, 1973; Hoad et al., 1997; Pospíšilová et al., 1999; Schreiber et al., 2001; Karbulková et al., 2008 - study II). This is caused by non-esterified, free carboxyl groups present in cutin polymer matrix and other water sorbing polar groups, which in turn increase the water permeability of polar domains of the cuticle (Schönherr, 2000). The total water diffusion is arranged in two parallel pathways: the humidity-sensitive polar path and more frequently used humidity-independent, non-polar path formed by the lipophilic wax components. Therefore, the humidity effect on permeability is small. Whereas increasing humidity causes only 2–3 times higher permeability across polar pores, after increasing of temperature (above 30°C) or plasticizer application cuticular permeability can increase by factors between 10 and 1000 (Schreiber et al., 2001). The humidity effect on cuticular water permeability is small also in comparison to that on polar polymer film. It also indicates that polar pathway across plant cuticle forms only minor part of overall permeability (Riederer and Schreiber, 2001; Schönherr, 2000).

### *2.3 Methods of cuticular permeability measurement*

Present knowledge of the cuticular permeability is based especially on measurements with astomatous leaf surface or astomatous cuticular membranes. Water and other solutes pass through cuticle by diffusion. The flow of molecules across cuticle (*F*) is expressed as amount of penetrating compound per time. If the flow is divided by area of the transport, the flux (*J*) is obtained (Schreiber and Schönherr, 2009). Then, cuticular permeability can be characterized by the permeance (*P*) calculated as a ratio of *J* and difference of penetrating compound concentration in donor and receiver, i.e. concentration gradient.

Classic gravimetric methods on intact leaves are based on measurements of transpiration curves, i.e. sequent weighing of excised, originally fully saturated, leaves (Slavík, 1958a; 1958b; 1965). Gasometric methods are based on measurements of water vapor concentration in the air flowing around measured leaves (Slavík, 1965). Disadvantage of theses methods is usually overestimation of the results due to imperfect stomatal closure (Hoad et al., 1996; Burghardt and Riederer, 2003; Beyer et al., 2005).

Thirty years ago, laboratory of prof. Schönherr started with measurements of enzymaticaly isolated cuticular membranes. The high accuracy of such measurements, as compared to measurements of intact leaves, is the main advantage of using isolated membranes. It is possible to use several detection methods for measuring compound flow across membrane: gravimetry (Schreiber and Riederer, 1996), spectrophotometry (Schreiber et al., 1995) or labeling (Schreiber et al., 2001). But still, these methods allow measuring of astomatous cuticular membranes only.

Recently, we published a newly developed approach which allows to distinguish the flow of water across stomatal pores ('stomatal transpiration') from the flow across cuticular solid phase ('cuticular transpiration') in the stomatous cuticle (Šantrůček et al., 2004 - study I). The principle of this method is based on a fact that water vapor diffusivity in gas phase can be manipulated by using inert gasses with various molecular mass (e.g. helium, nitrogen, xenon) while diffusivity of water vapour in the solid phase is not affected.

By applying this method, it is possible to measure and compare cuticular water permeabilities from stomatous and astomatous leaf sides. Nonetheless, this method is applicable only to isolated cuticular membranes. An alternative techniques, suitable for intact leaves, used chlorophyll fluorescence imaging

(Šantrůček et al., 2000) or absorption of radio-labeled compounds ($^{14}C$ 2,4-dichlorophenoxyacetic acid, pentachlorphenol) through stomatous leaf side (Schreiber and Schönherr, 1992; Schreiber, 1994).

### *3. Plant cuticle as a leaf chronicle*

Onthogeny of cuticular membrane starts at very early stage of leaf life. Cuticular waxes, especially in mature leaves, are formed mainly from newly assimilated carbon. Similarly to leaf tissue, wax synthesis and its $^{13}C$ signal is affected by environmental conditions (Conte et al., 2003). The waxes are formed exclusively in epidermal cells (Kunst and Samuels, 2003; Shepherd and Griffiths, 2006). Their precursors are synthesized from photosynthates assimilated in chloroplasts of cells located presumably in the close vicinity of epidermal cells. Hence, the isotopic composition of abaxial and adaxial waxes reflects the $^{13}C/^{12}C$ ratio of $CO_2$ in the nearest chloroplasts (provided the constant fractionation during carboxylation and wax biosynthesis).

Water shortage causes stomatal closure and consequently decreases the $CO_2$ concentration in the leaf ($c_i$). Since the ratio between internal and ambient $CO_2$ concentration ($c_i/c_a$) reflects the balance between net photosynthesis and stomatal conductance, it affects discrimination ($\Delta$) against carbon isotope $^{13}C$ in plants. Therefore, waxes might be used for more accurate estimates of seasonal changes of stomatal aperture, especially in locations were the seasonality is driven by rainfall pattern.

## Aims of the thesis

A central aim of this thesis was studying plant regulation of cuticular transpiration water loss. I focused first on changes in cuticular water permeability and second, on changes in cuticular chemical composition under simulated drought stress. Finally, the obtained findings were capitalized on in a case study of altitudinal distribution of congeneric treeline species.

This thesis consists of four original papers:

**I.** A new technique for measurement of water permeability of stomatous cuticular membranes isolated from *Hedera helix* leaves.

**II.** Differences between water permeability of astomatous and stomatous cuticular membranes: Effects of air humidity in two species of contrasting drought resistance strategy.

**III.** Plant response to drought stress simulated by ABA application: changes in chemical composition of cuticular waxes.

**IV.** Morphological and ecophysiological traits shaping altitudinal distribution of three *Polylepis* treeline species in the dry tropical Andes.

The first, mainly methodological, study presents newly developed method which allows to separate water vapor flux across the cuticle between stomata and cuticular solid phase. It is for the first time when a comparison of water permeability of astomatous cuticle with permeability of solid cuticle from stomatous leaf side was performed.

The second study evolves measurements of water permeability of stomatous and astomatous cuticles. The main focus insists on effect of short-term and long-term (growth) relative humidity on cuticular water permeability of stomatous and astomatous cuticular membranes in two different plant species. More specifically, plants with different drought resistance strategy were compared to elucidate their differences also in water permeability under different humidity conditions.

The third study includes research of abscisic acid (ABA) effect on chemical composition of cuticular waxes. The main question was, whether a plant can regulate abundance of particular components or carbon chain length to improve water loss protection under simulated drought conditions.

The last study presents results of ecophysiological measurements on treeline *Polylepis* species. Here, the measured leaf traits have been related to water and/or temperature limitation and species altitudinal distribution. To assess long-term (seasonal) stomatal functioning, the isotopic composition of abaxial cuticular waxes was used.

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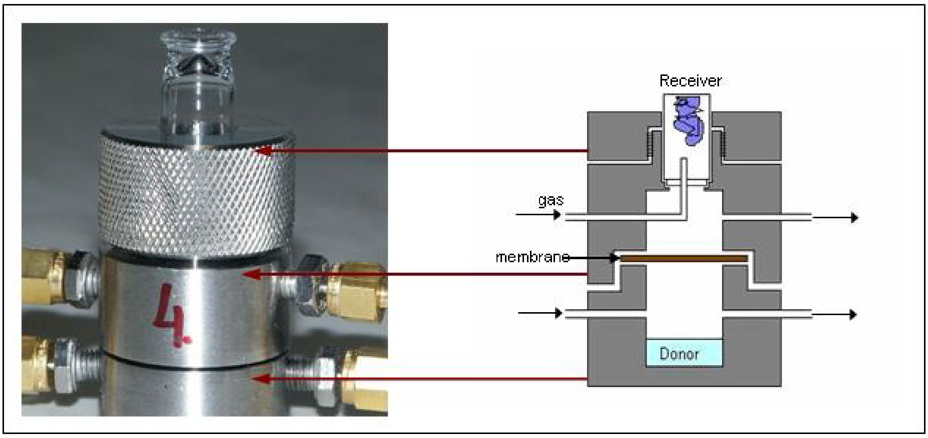

An updated version of chamber for measurements of water vapor diffusion across cuticular membranes in different gases. The donor compartment is filled with $^3H_2O$ water and receiver is a vial with rolled filter paper saturated with non-radiolabeled water.

# A new technique for measurement of water permeability of stomatous cuticular membranes isolated from *Hedera helix* leaves

Jiří Šantrůček, Eva Šimáňová, Jana Karbulková, Marie Šimková and Lukas Schreiber

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## Abstract

Transpiration of cuticular membranes isolated from the lower stomatous surface of *Hedera helix* (ivy) leaves was measured using a novel approach which allowed a distinction to be made between gas phase diffusion (through stomatal pores) and solid phase diffusion (transport through the polymer matrix membrane and cuticular waxes) of water molecules. This approach is based on the principle that the diffusivity of water vapour in the gas-phase can be manipulated by using different gases (helium, nitrogen, or carbon dioxide) while diffusivity of water in the solid phase is not affected. This approach allowed the flow of water across stomatal pores ('stomatal transpiration') to be calculated separately from the flow across the cuticle ('cuticular transpiration') on the stomatous leaf surface. As expected, water flux across the cuticle isolated from the astomatous leaf surface was not affected by the gas composition since there are no gas-filled pores. Resistance to flux of water through the solid cuticle on the stomatous leaf surface was about 11 times lower than cuticular resistance on the astomatous leaf surface, indicating pronounced differences in barrier properties between cuticles isolated from both leaf surfaces. In order to check whether this difference in resistance was due to different barrier properties of cuticular waxes on both leaf sides, mobility of $^{14}$C-labelled 2,4-dichlorophenoxy-

butyric acid ($^{14}$C-2,4-DB) in reconstituted cuticular wax isolated from both leaf surfaces was measured separately. However, mobility of $^{14}$C-2,4-DB in reconstituted wax isolated from the lower leaf surface was 2.6 times lower compared with the upper leaf side. The significantly higher permeability of the ivy cuticle on the lower stomatous leaf surface compared with the astomatous surface might result from lateral heterogeneity in permeability of the cuticle covering normal epidermal cells compared with the cuticle covering the stomatal cell surface.

# Nová technika měření propustnosti pro vodu u průduchových kutikulárních membrán izolovaných z listů břečťanu *Hedera helix*

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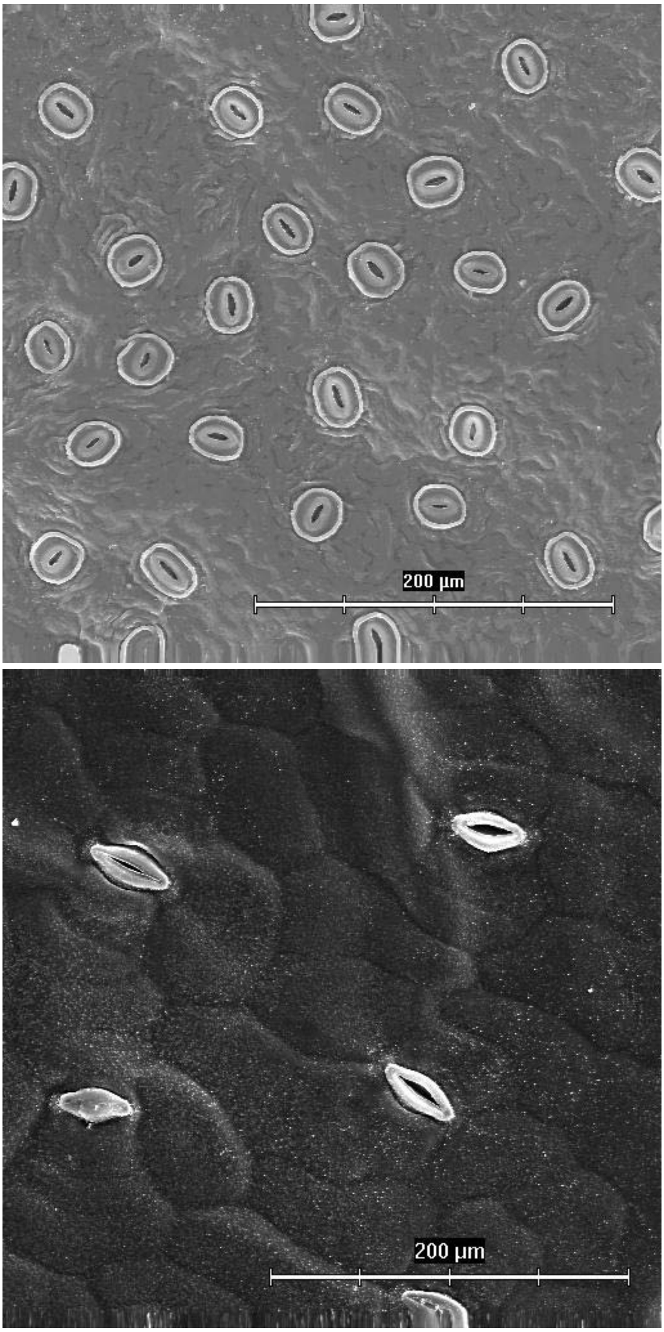
## Shrnutí

Pro měření toku vodní páry přes kutikulární membránu izolovanou ze spodní (průduchové) strany listu břečťanu (*Hedera helix*) byla použita nově vyvinutá metoda umožňující rozdělení celkového toku vodní páry přes kutikulu na průduchovou a kutikulární transpiraci. Metoda je založena na rozdílné difusi vody v plynné fázi a přes membránu. Zatímco rychlost difuse v plynné fázi závisí na použitém plynném mediu (helium, dusík, $CO_2$), difuse přes pevnou fázi (membránu) není změnou media ovlivněna. Z našich výsledků je patrné, že pevná fáze spodní průduchové kutikuly je 11x propustnější pro vodu než neprůduchová kutikula. Ovšem testovaná mobilita $^{14}C$-značené 2,4-dichlorophenoxy-máselné kyseliny v rekrystalizovaných voscích izolovaných z obou stran listu ukazuje nižší propustnost u vosků ze spodní stany listu. Tento rozdíl je pravděpodobně dán rozdílnými vlastnostmi kutikuly pokrývající normální epidermální buňky a kutikuly pokrývající svěrací buňky.



SEM micrographs of stomatal pores in cuticular membrane isolated from the abaxial surface of (a) *Hedera helix* and (b) *Zamioculcas zamiifolia* leaf. The pore viewed from the physiologically outer side of the cuticle.

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

Jana Karbulková, Lukas Schreiber, Petr Macek and Jiří Šantrůček

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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 analyzed for *Hedera helix*, native in a temperate climate, and *Zamioculcas zamiifolia*, native in subtropical regions. Water permeability of cuticular membranes (CM) isolated from the adaxial (astomatous) and abaxial (stomatous) leaf sides was measured using a method which allowed separating 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 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.

# Rozdílná propustnost abaxiální a adaxiální listové kutikuly: Vliv vlhkosti na propustnost u dvou druhů s kontrastní strategií v hospodaření s vodou

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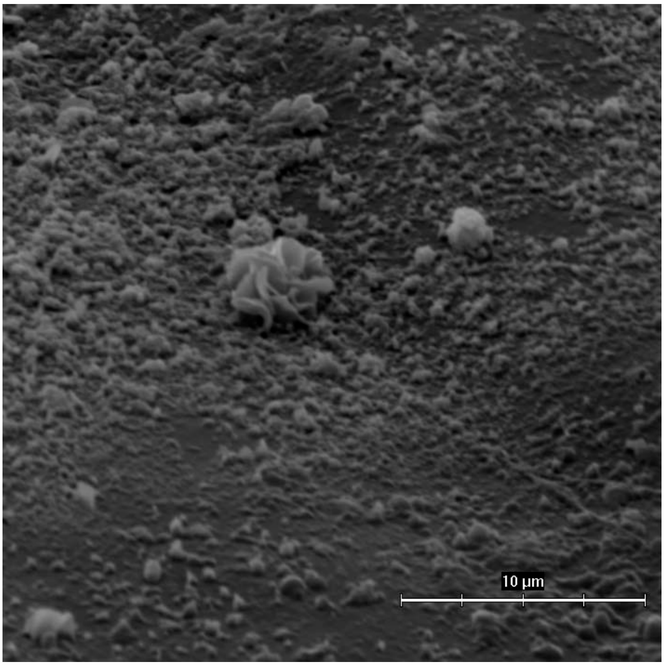
## Shrnutí

Rozdíly v propustnosti rostlinné kutikuly pro vodu byly zjištěny mezi neprůduchovou částí spodní průduchové kutikuly a svrchní neprůduchovou kutikulou u břečťanu (*Hedera helix*) a zamioculcas (*Zamioculcas zamiifolia*) za všech měřených podmínek (krátkodobé a dlouhodobé působení vysoké a nízké relativní vlhkosti). Rozdílný vliv relativní vlhkosti na propustnost neprůduchové kutikuly obou druhů indikuje jejich rozdílné mechanismy v hospodaření vodou. Dlouhodobé působení vysoké relativní vlhkosti se projevilo vyšší propustností neprůduchové kutikuly, zejména u břačťanu. Naopak propustnost průduchové kutikuly se v různých vlhkostních podmínkách měnila minimálně. To může být spojeno s vyšší vlhkostí hraniční vrstvy způsobenou průduchovou transpirací. Řádové rozdíly mezi propustností neprůduchové kutikuly mezi druhy koresponduje s jejich rozdílnou životní historií a adaptací na subtropické a temperátní klima; zároveň indikuje rozdílné mechanismy vyrovnávání se s vodním stresem - toleranci u *Hedera helix* a avoidanci u *Zamioculcas zamiifolia*.



SEM micrograph of epicuticular waxes on the cuticular membrane surface isolated from *Zamioculcas zamiifolia* leaf.

# Plant response to drought stress simulated by ABA application: changes in chemical composition of cuticular waxes

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## Abstract

Plant cuticles form the interface between epidermal plant cells and the atmosphere. The cuticle creates an effective barrier against water loss, bacterial and fungal infection and also protects plant tissue from UV radiation. It is composed of the cutin matrix and embedded soluble lipids also called waxes. Chemical composition of cuticular waxes and physiological properties of cuticles are affected by internal regulatory mechanisms and environmental conditions (e.g. drought, light, humidity). Here, we tested the effect of drought stress simulation by the exogenous application of abscisic acid (ABA) on cuticular wax amount and composition. ABA-treated plants and control plants differed in total aboveground biomass, leaf area, stomatal density and aperture, and carbon isotope composition. They did not differ in total wax amount per area but there were peculiar differences in the abundance of particular components. ABA-treated plants contained significantly higher proportions of aliphatic components characterized by chain length larger than $C_{26}$, compared to control plants. This trend was consistent both between and within different functional groups of wax components. This can lead to a higher hydrophobicity of the cuticular transpiration barrier and thus decrease cuticular water loss in ABA-treated plants. At both ABA-treated and control plants alcohols with chain length $C_{24}$ and $C_{26}$ were predominant. Such a shift towards wax compounds having a higher

average chain length under drought conditions can be interpreted as an adaptive response of plants towards drought stress.

# Změny v chemickém složení kutikulárních vosků v reakci na stres suchem simulovaný aplikací ABA

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(Rukopis podaný do Environmental and Experimental Botany)

## Shrnutí

Rostlinná kutikula tvoří rozhraní mezi rostlinou a atmosférou. Její hlavní funkcí je ochrana proti ztrátě vody. Její chemické složení a vlastnosti závisí často na podmínkách prostředí (světlo, sucho apod.). Zde jsme testovali, zda sucho simulované aplikací kyseliny abscisové (ABA) ovlivní množství a složení kutikulárních vosků. Z výsledků vyplývá, že rostliny ošetřené ABA se lišily od kontrolních v růstových parametrech (množství nadzemní biomasy, listová plocha, izotopové složení listů, velikost a četnost průduchů), ale nelišily se v množství kutikulárních vosků na jednotku plochy. Avšak, rostliny s ABA obsahovaly více alifatických složek s uhlíkovým řetezcem delším než $C_{26}$. Kumulace dlouhořetězcových alifatických látek může vést k vyšší hydrofobicitě kutikuly a tím snižovat kutikulární propustnost pro vodu.

Flowering *Polylepis rugulosa* from the west slope of Cordillera Occidental, 3720 m asl, January 2007

# Morphological and ecophysiological traits shaping altitudinal distribution of three *Polylepis* treeline species in the dry tropical Andes

Petr Macek, Jana Macková and Francesco de Bello

Acta Oecologica 35, 778-785, 2009

## Abstract

Numerous species of the genus *Polylepis* form the highest treeline in the world, with striking dissimilarities in their upper altitudinal limits. The commonly accepted hypothesis is that growth at a treeline is limited by temperature. Here, using *in situ* records of various morphological and ecophysiological traits, we aimed to identify other factors influencing altitudinal distribution of three congeneric species from the dry tropical Andes: *Polylepis rugulosa, Polylepis tarapacana* and *Polylepis tomentella*. While *P. tarapacana* and *P. tomentella* reach their altitudinal limit at around 5000 m asl, *P. rugulosa* does not thrive above 4300 m, but precipitation is markedly lower in its distribution area. The three species responded to altitude by a change of morphological (e.g. decreased tree height and leaf size) and ecophysiological (e.g. decrease of transpiration rate, nutrient concentration or enrichment in the $^{13}$C isotope) traits, and this response was generally more pronounced in *P. rugulosa*. In comparison with *P. tarapacana* and *P. tomentella*, *P. rugulosa* displayed higher transpiration rates. Waxes from the abaxial (stomatous) leaf side of *P. rugulosa* were most strongly enriched in $^{13}$C. Furthermore, leaves of all species studied here had exceptionally low N and P concentrations. Trade-offs linked to changes in leaf area (e.g. bigger leaves, higher photosynthetic capacity but

elevated transpiration) seem to drive differentiation and adaptations to altitude among these three congeneric species. We hypothesize that, while the upper distribution limit of *P. tarapacana* and *P. tomentella* is largely driven by low temperature, water is an important additional factor controlling the altitudinal distribution of *P. rugulosa*. Our results suggest that water stress needs to be taken into account among the factors shaping the altitudinal distribution of tropical treeline species.

# Vliv morfologických a ekofyziologických vlastností tří blízce příbuzných druhů rodu *Polylepis* na výškovou distribuci hranice lesa v oblasti suchých tropických And

Petr Macek, Jana Macková and Francesco de Bello

Acta Oecologica 35, 778-785, 2009

## Shrnutí

Srovnáním ekofyziologických a morfologických vlastností tří příbuzných druhů rodu *Polylepis* tvořících hranici lesa jsme se pokusili vysvětlit jejich různé výškové rozšíření (horní limit od 4300 do >5000 m.n.m.). Hranice lesa je obecně ovlivněna teplotou. Pomocí gazometrických charakteristik a izotopového složení kutikulárních vosků jsme se pokusili identifikovat alternativní faktory. Zatímco výškový limit u *P. tarapacana* a *P. tomentella* je ovlivněn především teplotou, dostupnost vody hraje klíčovou roli ve výškové distribuci *P. rugulosa*. Stress nedostatkem vody by měl být brán v potaz jako faktor ovlivňující hranici lesa v tropických horách.

## Summary and perspectives

Plant cuticle represents the exciting world of first several micrometers separating plant from the atmosphere. If we start from the beginning, the oldest cuticular remnants bear date to late Silurian period. Analyses of cuticular remnants offers useful tool to study ecophysiological adaptations of first vascular plants. From stable isotope studies it is possible to make paleoclimate reconstructions or estimate water availability during plant life (Xie et al., 2004; Polissar et al., 2009; cf. study IV). Interestingly, the stomatal pores evolved simultaneously with cuticle; this supports hypothesis, that genes involved in wax biosynthesis share high homological structure with stomatal distribution genes (cf. Bird and Gray, 2003). The major role of cuticle is the same since its origin in prehistoric plants: water loss protection. Since transpiration fluxes are controlled by both stomata and cuticle, only the optimized coordination of both parts can lead to an effective protection.

Cuticular water permeability was in interest of many researchers in the last sixty years. Numerous studies investigated mechanisms of water diffusion across the cuticle (e.g. Becker et al., 1986; Riederer and Schreiber, 1995; Schreiber et al., 2001). Different permeability as an ecophysiological adaptation to various origins was surveyed by Schreiber and Riederer (1996). Nevertheless, a question remained, whether upper and lower side of hypostomatous leaves contribute to water permeability equally. Available methods did not allow to separate stomatal residual water loss from the cuticular one, so far. A novel method presented in study I brought a useful tool for separation of water flux diffusing across stomata from cuticular-solid-phase flux. For the first time, it is now possible to directly compare permeability of astomatous cuticular membrane with permeability of solid stomatous cuticle in the same leaf. On the example of *Hedera helix*, we documented that astomatous (adaxial) cuticle was less permeable than stomatous (abaxial) one (study I). On the other hand, we found discrepancy in diffusion of $^{14}$C labeled 2,4-DB in reconstructed waxes isolated from both leaf sides. We suggested that lateral heterogeneity of cuticular conductance, e.g. differences in transport properties of epidermal, subsidiary and guard cell cuticle played significant role in this case (study I).

One of the main environmental factors affecting water permeability is relative humidity. Short-term humidity effect (minutes, hours) enhances water permeability 2–3 times (Schreiber et al., 2001; study II). It seems that this response is based on physical processes and its proportion is affected by varied content of polar functional groups and polysaccharide microfibrils (Van Hove and Adema, 1996; Schönherr, 2006). On the contrary, long-term humidity effect (months, years) on cuticular water permeability is rather species-specific. According to our study of two evergreen species with contrasting origin, *Hedera helix* and *Zamioculcas zamiifolia*, it seems that permeability response is connected to drought resistance strategy and/or species practice to cope with long-term (periodical) humidity changes (study II). It is also interesting that humidity effect was more pronounced at adaxial (astomatous) cuticles than at abaxial (stomatous) cuticles (study II). It is probably due to higher degree of cuticular hydratation resulting from stomatal transpiration.

Environmental factors related to air humidity, e.g. drought stress, affect also cuticular thickness, total wax amount and chemical composition (Jenks et al., 2001; Shepherd and Griffiths, 2006, Bringe et al., 2006; Koch et al, 2006). Nevertheless, at *in vitro* cultivations, usually characterized by high air humidity and lower light intensity, the situation is strikingly different. Less developed cuticle was commonly documented (e.g. Pospíšilová et al., 1999). This leads to an insufficient cuticular water loss protection contributing to wilting after fast plant transfer to the field. However, when acclimatization with gradual lowering air humidity is included, the cuticle thickness and wax content can change during several days after transplantation from *in vitro* to the field conditions. Acclimatization can be accelerated by hardening of plants by decreasing the transpiration rate by antitranspirants, e.g. abscisic acid (ABA; Pospíšilová, 1996; Pospíšilová et al., 1998). Similarly, we documented, that drought stress simulated by ABA application can affect percent abundance of wax component substance classes and the abundance of the components with different carbon chain lengths (study III). Since increasing amount of wax chain length larger that $C_{27}$ correlates to decreasing cuticular transpiration (Hauke and Schreiber, 1998), the quantitative changes in wax composition indicate that simulated water stress may reduce cuticular permeability to water. On molecular level, CER6 gene seems to be plausible candidate regulating wax production under ABA treatment (Hooker et al., 2002). In our study III, ABA affected not only total aboveground biomass, leaf carbon isotope composition and wax chemical composition but also stomatal density and size. Negatively correlated stomatal density and stomatal size seems to improve water use efficiency, because the smaller stomata are, the faster they respond (Franks and Beerling, 2009). Effective stomatal regulation contributes to better leaf water economy.

Drought stress results not only in different abundance of chemical compounds but also in their carbon isotope composition. On leaf level, drought causes stomatal closure and consequently reduction of leaf internal $CO_2$ concentration. Plant discrimination to $^{13}C$ is proportional to ratio of $CO_2$ concentration inside and outside the leaf ($c_i/c_a$; Farquhar and Richards, 1984). It means that drought affected plants are more enriched in $^{13}C$ than well watered plants (cf. study III, study IV). Furthermore, the same principle is valid on chemical compound level. Wax precursors are largely made *de novo* from newly and localy assimilated carbon. Therefore, their isotopic composition corresponds to (i) composition of assimilated $CO_2$ and, (ii) to local $c_i/c_a$, reflecting stomatal aperture. Since wax turnover is shorter than leaf lifetime, but still long enough to cover variability between seasons, waxes might be used for more accurate estimate of seasonal changes of stomatal aperture in some species (study IV).

Presented results show cuticle as a heterogeneous system reflecting plant requests for water loss protection from several points of view. Although these studies certainly contributed to understanding of cuticular role in plant water management, they also opened a range of new questions for future research.

Studies of environmental impacts on chemical composition and regulation of wax biosynthesis on molecular level seems one promising field. The use of different wax mutants allows following the particular steps in wax biosynthesis under different treatments. In our case, the combination of control, ABA sensitive and ABA non-responsive plants allows us to separate ABA effect on gene expression.

Since water diffusion across cuticular membrane uses both lipophilic and hydrophilic pathways, it is necessary to know aqueous (polar) pore structure and its transport properties which have not been studied directly yet. Aqueous pores are formed by permanent dipoles such as hydroxyl, amino, and carboxyl groups. These aqueous are formed only in the presence of water and have been localized using ionic fluorescent dyes, silver nitrate, and mercuric chloride (Weichert et al., 2004; Schlegel et al., 2005; Schreiber et al., 2006; Schönherr, 2006). The presence of aqueous pores is crucial for ecophysiological role of plant cuticle in water loss protection. The pores can shrink or disappear under very low air humidity, which results in decrease of water permeability. Further, the aqueous pores facilitate non destructive penetration of large ionic molecules to leaves, stems, and flowers (Schönherr, 2006). Therefore, it is important to know whether and to what extent are the hydrophilic paths characteristic for the cuticles of all plant (Schreiber, 2005).

Among the other interesting field of research are functional properties of guard cell cuticle. It seems that possible existence of guard cell-specificity of cuticular transport properties may play an important physiological role in short-term control of leaf water loss. This might be important for stomatal sensitivity to $CO_2$ via humidity-sensing stomata (Kerstiens, 1996a; 1996b; Talbott et al., 2003).

Stable isotope techniques are widely used methods allowing research in many scientific fields. In combination with other techniques, e.g. nuclear magnetic resonance, they offer more sensitive tool in biochemistry, geochemistry and also in plant physiology. Furhtermore, the use of compound-specific analysis allows following a specific molecule or component in plant material analysis (cf. Chikaraishi and Naraoka, 2003; Smith and Freeman, 2006). In wax studies it provides comparison of wax components associated with different biosynthetic pathways, i.e. acetogenic, mevalonic-acid and non-mevalonic-acid pathways (Chikaraishi et al., 2004).

Cuticular research, as was documented here, represents still a very exciting and moving scientific field with numerous potential practical and theoretical outcomes.

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