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Epicuticular wax on leaf cuticles does not establish the transpiration barrier, which is essentially formed by intracuticular wax

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ABSTRACT

It is well established that waxes built up the barrier properties of cuticles, since their extraction in organic solvent e.g. chloroform increases diffusion of water and organic compounds by 1–2 orders of magnitude. Leaf surface waxes can be divided in epicuticular (on the surface of the cuticular membrane) and intracuticular (embedded in the cutin polymer) waxes. Until today there are only limited investigations dealing with the question to what extent epi- or intracuticular waxes contribute to the formation of the transpiration barrier. For *Prunus laurocerasus* previous studies have shown that epicuticular waxes do not contribute to the formation of the transpiration barrier. This approach successfully established for *P. laurocerasus* was applied to further species in order to check whether this finding also applies to a broader spectrum of species. Epicuticular wax was mechanically removed using collodion from the surface of either isolated cuticular membranes or intact leaf discs of ten further plant species differing in total wax amounts, wax compositions and transport properties. Scanning electron microscopy, which was performed to independently verify the successful removal of the surface waxes, indicated that two consecutive treatments with collodion were sufficient for a complete removal of epicuticular wax. The treated surfaces appeared smooth after removal. The total wax amounts removed with the two collodion treatments and the residual amount of waxes after collodion treatment were quantified by gas chromatography and mass spectrometry. This showed that epicuticular waxes essentially consisted of long-chain aliphatic molecules (e.g. alkanes, primary alcohols, fatty acids), whereas intracuticular wax was composed of both, triterpenoids and long-chain aliphatic molecules. Cuticular transpiration using combined replicates was measured before and after removal of surface wax. Results clearly indicated that two consecutive collodion treatments, or the corresponding solvent treatments (diethyl ether:ethanol) serving as control, did not increase cuticular transpiration of the ten further leaf species investigated. Our results lead to the conclusion that epicuticular wax does not contribute to the formation of the transpiration barrier of leaves.

1. Introduction

The extracellular plant cuticle covers leaves and fruits and is composed of the insoluble polymer cutin and soluble cuticular lipids called wax. Cutin is a biopolyester mainly consisting of C_{16} and C_{18} hydroxy-fatty acids (Pollard et al., 2008). These fatty acids are cross-linked by ester bounds leading to the mechanically stable cutin polymer network (Espelie et al., 1980; Kolattukudy 1981; Nawrath 2006). Cuticular wax is synthesized in epidermal cells and is composed of a mixture of linear, long-chain aliphatic molecules of different functionalities (e.g. alkanes, aldehydes, fatty acids…), different chain lengths and in some species it also contains significant amounts of pentacyclic triterpenoids (Samuels et al., 2008; Kunst and Samuels, 2009). It is well known, that the cuticular transport barrier for water and solutes is established by cuticular wax. Upon wax extraction rates of cuticular permeability increased by one to two (sometimes three orders) of magnitude (Schönherr and Lendzian, 1981; Schönherr and Riederer, 1989; Schreiber and Schönherr, 2009).

Cuticular wax is embedded in the cutin polymer (intracuticular wax) and deposited on the outer surface (epicuticular wax) of the cuticle (Buschhaus and Jetter, 2011), where it can form characteristic, three-dimensional epicuticular wax crystalloids (Barthlott et al., 1998) leading to the self-cleaning mechanism of plant organs, known as the Lotus effect (Neinhuis and Barthlott, 1997). Epi- and intracuticular wax fractions can vary in their chemical composition. Using FTIR measurements and *Prunus laurocerasus* as a model organism studying wax heterogeneity showed that cyclic triterpenoids are located in deeper regions of the plant cuticle, belonging to the intracuticular wax (Jetter

Abbreviations: CM, isolated cuticular membrane; LD, intact leaf disk; MX, wax-free matrix membrane; P, permeance

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et al., 2000; Weissflog et al., 2010). Long-chain aliphatics like fatty acids, primary alcohols, aldehydes, alkanes and esters are also constituents of the intracuticular wax, but they can also be found in the epicuticular wax fraction (Jetter et al., 2000). This observation is not only true for *P. laurocerasus*, but also for other species (Buschhaus and Jetter, 2011), where most of the triterpenoids are located in the intracuticular wax fraction, whereas long-chain aliphatics can be found in both wax fractions.

Epicuticular waxes do play a crucial role in plant/pathogen recognition (Ringelmann et al., 2009; Hansjakob et al., 2010) and they are obviously also important for protecting plants from abiotic stress factors. In the past miscroscopical investigations were performed, which indicated that different environmental stresses (high UV-light and high temperature) can lead to an increase amount of epicuticular waxes (Grant et al., 1995; Sase et al., 1998; Kim et al., 2007) on the surface of plants described as wax bloom. However, without measuring cuticular transpiration from this observation it is often concluded that enhanced amounts of epicuticular wax should reduce cuticular transpiration.

Thus, these observations, such as (i) differences in wax composition between epi- and intracuticular wax fractions (ii) and increased epicuticular wax amounts upon environmental stress, lead to the question whether the epicuticular wax fraction significantly contributes to the formation of the cuticular transport barrier or not? In the past this question was addressed in a limited number of studies. In some studies it was concluded that epicuticular wax does not contribute to the formation of the transport barrier of leaves (Baur, 1998; Larsson and Svenningsson, 1986), whereas in other studies a two to three fold increase of fruit CM (tomato and cherry) transpiration was described (Knoche et al., 2000; Vogg et al., 2004). In a recent publication (Jetter and Riederer, 2016) it was concluded, that the cuticular transpiration barrier is essentially established by the intracuticular wax fraction, if cuticular wax exclusively consists of long-chain aliphatic wax molecules. In species showing both substance groups, cyclic triterpenoids and long-chain aliphatic molecules in wax, it was concluded that both epi- and intracuticular wax fractions contribute to the formation of the transpiration barrier.

This is in contradiction to another recent publication investigating the localisation of the cuticular transpiration barrier of *Prunus laurocerasus* CMs in detail (Zeisler and Schreiber, 2016). Cuticular wax of *Prunus* is composed of both substance groups, cyclic triterpenoids as well as long-chain aliphatics, and it was found that the cuticular transpiration barrier was exclusively established by the intracuticular wax fraction, even with triterpenoids representing the dominant wax fraction. Therefore, we extended our recent approach investigating the localisation of the cuticular transpiration barrier including a series of 10 further species using a combined and complementary approach consisting of (i) scanning electron microscopy for verifying the successful mechanical removal of epicuticular wax by treating the membranes with collodion, (ii) gas chromatography and mass spectrometry for analyzing wax composition in the different wax fractions obtained by two consecutive collodion treatments as well as residual wax analysis and finally (iii) measuring cuticular transpiration before and after selective removal of waxes. Results indicate that the epicuticular wax fraction does not significantly contribute to the formation of the cuticular transpiration barrier of leaf cuticles, which is established by the intracuticular wax fraction.

2. Materials and methods

2.1. Plant materials

Leaves of *Buxus sempervirens*, *Camellia sinensis*, *Clivia miniata*, *Clusia fluminensis*, *Ficus elastica*, *Hedera helix*, *Monstera deliciosa*, *Philodendron rugosum* and *Schefflera arboricola* were either harvested from plants growing in a rural area outside of Bonn or harvested from plants growing in the Botanical Garden of the University of Bonn. *Solanum lycopersicum* fruits were purchased from a local supplier. Isolation of cuticular membranes was performed by enzymatic digestion as described previously (Schönherr and Riederer, 1986; Zeisler and Schreiber, 2016).

2.2. Mechanical removal of epicuticular wax from CMs using collodion

Removal of epicuticular wax from isolated cuticular membranes (CMs) and intact leaf discs (LDs) from ten different plant species was done using collodion (Fluka) as described recently (Zeisler and Schreiber, 2016). CMs and LDs were mounted to stainless steel transpiration chambers (Zeisler and Schreiber, 2016) leaving an area of 1 square centimetre accessible. Collodion was gently applied (about 10 µL) with a soft brush on the outer cuticle surface of the isolated cuticle and intact leaf samples. After drying (about 30 s.) a thin polymer film with adherent waxes could be removed from the surface of the membranes using fine tweezers. Every membrane used for mechanical removal of the surface waxes (microscopy, analytics and transpiration) was treated twice with collodion. For scanning electron microscopy and transpiration measurements the polymer films were discarded. The polymer films were extracted in chloroform for analytical investigations of the mechanically removed wax amounts.

2.3. Scanning electron microscopy

Samples of CMs and LDs were fixed to aluminium sample holders. In the case of fresh leaf material the leaf discs were first freeze dried (−80° degrees stored over silica gel; before fixation to sample holders). The fixed surfaces of CMs, and LDs were twice half treated with collodion to discriminate between a treated and an untreated area within one and the same sample. All samples were sputtered (Polaron E5100, 15 mA, 30 s, Polaron Equipment LTD.) with a thin layer (18 nm) of gold and investigated at an accelerating voltage of 20 keV by scanning electron microscopy (SEM; Leitz AMR 1000, Leitz).

2.4. Extraction of residual amounts of cuticular wax from CMs and LDs

After two consecutive treatments with collodion, treated areas (1 cm^2) of CMs or LDs were cut out of the transpiration chambers using a scalpel. Intact LDs were incubated in enzymatic solution for cuticle isolation prior to residual wax extraction. Residual wax of CMs was directly extracted. All samples were extracted in chloroform (4 mL) over night at room temperature before further chemical analysis.

2.5. Wax analysis using gas chromatography and mass spectrometry

Wax analysis was conducted as described in detail several times in the past (Kurdyukov et al., 2006; Zeisler and Schreiber, 2016). All collodion samples as well as the residual wax amount extracts were spiked with an adequate amount of tetracosane (50 µL tetracosane of a chloroform solution of 10 mg in 50 mL; Fluka) serving as internal standard for wax quantification. The chloroform volumes of the extracts were reduced to a final volume of 200 µL under a gentle stream of nitrogen gas at 60° C in a heating block. Polar groups like hydroxylic and carboxylic groups of alcohols and acids, being important constituents of waxes, were transformed into the corresponding trimethylsilylethers and −esters by derivatization. Derivatization was done using *N,O*-Bis (trimethylsilyl)trifluoroacetamide (BSTFA; Macherey-Nagel) and Pyridine (Sigma Aldrich) for 45 min at 70 °C.

Wax amount quantification was performed by on-column injection of 1 µL sample in a gas chromatograph equipped with a flame ionization detector (GC-FID; CG-Hewlett Packard 5890 series H, column: 30 m DB-1 i.d. 0.32 mm, film 0.2 µm; J&W Scientific). Identification of wax compounds was achieved by analyzing 1 µL of samples by gas chromatography coupled to mass spectrometry (GC-MS; quadrupole mass