Science & its applications 5:2 (2017) 96-98

Faculty of Science - University of Benghazi

Science & its Applications

journal home page: www.sc.uob.edu.ly/pages/page/77

Wax-extraction effect on water permeances of three isolated plant cuticles.

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ARTICLE INFO

Article history:
Received 11 February 2017
Revised 07 May 2017
Accepted 12 June 2017
Available online 23 June 2017

Keywords:
Plant cuticle; Water permeability; Polymer matrix; Cuticular wax.

ABSTRACT

The cuticle is a heterogeneous, extracellular biopolymer, which is synthesized by epidermal cells. It plays many physiological and ecological roles but its main function is the reduction of water loss from plants when the stomata are closed. Cuticular waxes are essential part of the cuticle structure and their presence is limitation factor in controlling water permeability. Therefore, knowledge about amounts and chemical composition of cuticular waxes is necessary in order to understand their functions. This study was conducted to find out the effect of wax extraction on water permeability of three isolated plant species cuticles. The cuticles were enzymatically isolated and waxes were extracted from the cuticles using chloroform. The results showed that the water permeance was affected by removing waxes from cuticle structure and it was increased by high factor for all treated plants. This study showed no correlation between wax coverage of each single species and its water permeability.

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1. Introduction

One of basic adaptation of plants for their survival on the mainland is the plant cuticle. The cuticle covers all primary above-ground parts of the plants and it forms an effective barrier against desiccation (Marga *et al*., 2001) and thus the main function of the cuticle is the reduction of water loss from plants when the stomata are closed (Schönherr, 1976). The plant cuticle is a hydrophobic, continuous and flexible thin (from 0.1 to 10 µm; Vogg *et al*., 2004) membrane consisting of two lipid fractions; the polymer matrix (cutin polymer or cutin-containing layer) and cuticular waxes which are deposited on the outer surface and embedded in the matrix (Luque *et al*., 1995).

The proportion of these compounds differs among plant species and even among the different tissues of an individual plant (Mariani and Wolters-Arts, 2000). Although these waxes represent a low amount of the total mass of the cuticle, from 1 to 10 % (Walton, 1990), they are responsible for 90 to 99, 9 % of the total resistance of the cuticular membrane to water loss (Riederer and Schreiber, 1995). Removing them from the cuticle using organic solvent such as chloroform has demonstrated their efficiency in forming a barrier. The correlation between the chemical composition of cuticular waxes and their function as a transpiration barrier is still unsolved (Vogg *et al*., 2004).

The Knowledge on amounts and chemical composition of cuticular waxes is necessary in order to understand their functions. These features (amounts and composition) depend on endogenous and exogenous factors (Riederer and Markstädter, 1996). A number of studies have shown that environmental factors such as light, humidity and temperature may influence the amount and composition of cuticular waxes (Riederer and Markstädter, 1996). Dynamic changes of epicuticular waxes during leaf development (aging factor) were also reported (Jetter and Schäffer, 2001).

Riederer and Schreiber (2001) reported that cuticular water permeability is not correlated to the thickness or to wax coverage of the cuticle. From this point of view, we tested in this work the wax coverage amount of different three plant cuticles and their correlation to water permeances for both complete and de-waxed cuticles.

2. Materials and method

2.1 Isolation of cuticles

The isolation of cuticles has been carried out according to the method described by Schönherr and Riederer (1986). Disks of 20 mm diameter were punched out from *Hedera helix* L. and *Prunus laurocerasus* L. leaves and tomato *Lycopersicon esculentum* L. fruits and incubated in an aqueous solution containing 2% (v/v) cellulase (Celluclast, Novo Nordisk, Bagsvared, Denmark) and 2% pectinase (Trenolin, Erbslöh, Geisenheim, Germany) in 0.01 M citric buffer (Merk, Germany; pH 3.0 adjusted with KOH). In order to prevent microbial growth, 1 ml of 1 M Sodium azide (NaN3, Fluka, Neu-Ulm, Germany) was added to 1 litre of the enzyme solution. Cuticles from the adaxial leaf sides were separated from the cellular debris and incubated in 0.01 M borax buffer (Fluka, Germany) adjusted to pH 9 for about one week. Subsequently, the cuticles were incubated again for about 10 days in deionized water. The cuticles were removed from the solution and dried under a stream of pressurised air that helped to flatten the cuticles. They were stored in Petri dishes at room temperature until they were used.

2.2 Wax extraction and polymer matrix preparation

After the cuticles were successfully isolated, a number of CMs (about 100 CMs) were selected by investigating them visually to ensure that they were free from holes or any other defects. 10 to 12 cuticles were immersed in chloroform at room temperature for 16 h to extract cuticular waxes. Subsequently, extracted membranes were transferred via hexane and ethanol (95 %) to deionized water, respectively. Extracted membranes (MX) were again dried under a stream of air. Wax coverage of the three species (*Hedera helix* L., *Prunus laurocerasus* L., and *Lycopersicon esculentum* L.) was determined gravimet-rically. 10 CMs of each species were selected. The difference in the weight before and after wax extraction was used to determine wax coverage using an electronic microbalance (±1µg; Sartorius, MC 21S Göttingen, Germany).

2.3 Measurement of water permeability

Water permeability (cuticular transpiration) of CM and MX membranes was determined using a gravimetric method described previously by Schönherr and Lendzian (1981), and in a slightly modified form by Schreiber and Riederer (1996). Stainless steel transpiration chambers were used in this experiment. The chambers were filled with 900-1000 µl of deionized water that served as a donor solution. CMs or MXs were mounted on the transpiration chambers with their morphological outer surface facing the atmosphere. These chambers were placed upside down in closed polyethylene boxes above silica gel and were incubated at 25±0.5 C°. The incubation period was different depending on the membrane (CM or MX) and the species. The incubation period was overnight in all CMs and between 2 to 3 hours with tomato CMs and all MXs. Water loss was monitored by weighing the chambers every 24 hours for 4 to 5 days when CMs were used and every 2 to 12 hours when MXs were used. Water loss was determined with a microbalance (Sartorius Analytic BP 221S, Göttingen, Germany) connected to a personal computer (SartoConnect version 3, 1).

2.4 Sample size and statistical analysis

Regression equations were fit to transpiration kinetics and means of permeances of 10 to 20 cuticular membranes were calculated. Results are given as means with 95% confidence intervals (ci). Wax coverage was determined from 10 CMs and the results are given as mean values with 95% confidence intervals.

3. Results and discussion

Water permeances ($m \cdot s^{-1}$) are presented in Table 1. The results showed that permeances of MXs were higher than those of CMs. Wax extraction from the CMs led to an increase of **P.** by a factor of 280 for *Hedera helix*, 160 for *Prunus laurocerasus* and 120 for *Lycopersicon esculentum* (Fig. 1).

Table 1. Water permeances ($m \cdot s^{-1}$) of CMs and MXs of three different species. The values are means of 156, 352, and 208 CMs and between 33 to 70 MXs ±95% confidence intervals.

Species	CM P ($m \cdot s^{-1}$) ± ci	MX P ($m \cdot s^{-1}$) ± ci
Hedera helix	$5.7\times10^{-11} \pm 7.2\times10^{-12}$	$2.4\cdot10^{-8} \pm 1.1\times10^{-9}$
Prunus laurocerasus	$1.3\times10^{-10} \pm 9.1\times10^{-12}$	$8.6\cdot10^{-9} \pm 2.4\times10^{-10}$
Lycopersicon esculentum	$3.9\times10^{-09} \pm 9.7\times10^{-10}$	$1.7\cdot10^{-8} \pm 7.5\times10^{-10}$

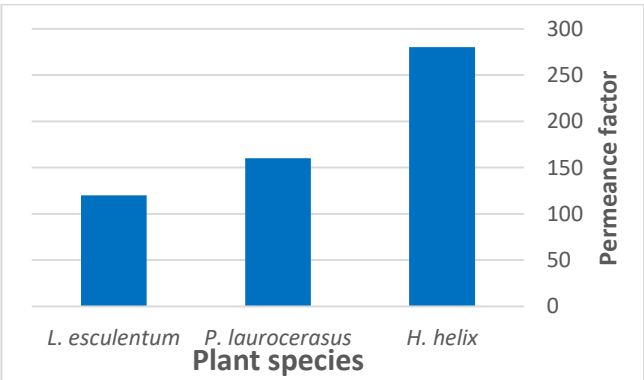

Fig. 1. The effect of wax extraction on water permeability of three different species. The permeances were increased rapidly by the factor 120, 160 and 280 respectively.

The amounts of cuticular waxes covering leaf and fruit surfaces are very different between the different plant species (Table 2). The wax coverage of tomato fruit cuticles was the lowest and it was 55.4 ($\mu g \cdot cm^{-2}$), while the *prunus* cuticles revealed the highest amount. Even though *Hedera helix* cuticles have the highest permeance with factor 280 when waxes were extracted, but its wax coverage was lower than *prunus laurocerasus* and it was 80.5 ($\mu g \cdot cm^{-2}$), while it was 211.6 ($\mu g \cdot cm^{-2}$) for *Prunus laurocerasus* which reflected water permeances of MXs with factor 160 (Table 2).

Table 2. Amounts of wax ($\mu g \cdot cm^{-2}$) of the three species *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum*. Results are means of 10 CMs with 95% confidence intervals.

Species	wax coverage ($\mu g \cdot cm^{-2}$) ± ci
Prunus laurocerasus	211.6 ± 65.7
Hedera helix	80.5 ± 9.4
Lycopersicon esculentum	55.4 ± 9.2

It is obvious that cuticular waxes play an important and a decisive role in determining permeabilities of cuticles. They form the transport barrier even though they make up only a small percentage of the total mass of the cuticle. Extracting the waxes from the cuticle reveals their efficiency as a barrier. The correlation between wax chemical composition and their function as transpiration barrier is poorly understood (Vogg *et al.*, 2004). The effect of epicuticular wax on cuticular permeability is not completely known at this time because of the difficulties in removing epicuticular waxes without affecting intracuticular waxes. Therefore, only the effect of the complete wax extraction has been studied (Schönherr and Riederer, 1989). During last few years, some successful attempts to remove epicuticular waxes from intracuticular wax have been developed, but the lack of information still addressed.

Polymer matrix membranes are membranes where wax has completely been extracted. Their permeances of water and solutes are one to three orders of magnitude higher than those of cuticular membranes (CMs) (Schönherr, 1982). As described previously, two parallel pathways in cuticular membranes for permeating molecules were hypothesized. There are estimations, that the pores occupy about 6 ppm of the surface area of the cuticle (Tyree *et al.*, 1990). Increasing water permeabilities of MXs up to three orders of magnitude, suggest that 100 to 1000 times more pores were exposed by removing cuticular wax (Tyree *et al.*, 1990).

The amounts of cuticular waxes covering leaf and fruit surfaces are very different between the different plant species (Table 2). This can be due to endogenous and/or exogenous factors (Riederer and Markstädter, 1996). Since cuticular waxes form the transport limiting barrier of cuticles, the amount of wax could determine the rates of water permeability of cuticles. However, it was reported that cuticular water permeability was not correlated with the amounts of wax (Riederer and Schreiber, 2001) and our results were agreed with this. Smalley *et al.* (1993) observed differences in the effects of the different cations on water permeability of the different species and this might be due to differences in wax amounts. However, as shown in Fig. 1 there is no correlation between water permeability and wax amounts of the three species *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum*. Zeisler and Schreiber (2016) tested removing waxes from cuticular membranees of *Prunus laurocerasus* leaves and they concluded that epicuticular wax of *Prunus laurocerasus* does not contribute to the formation of the cuticular transpiration barrier, which must be established by intracuticular wax.

Even though plant cuticle structure is already mostly known, but the part which act as barrier is not clear enough. Therefore, future studies must focus on the role of intracuticular waxes and their function they play as barrier for water permeance and other compounds.

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