Effect of temperature on cuticular transpiration of isolated cuticular membranes and leaf discs

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Abstract

Cuticular transpiration was measured in the temperature range between 10 °C and 55 °C using tritiated water and five species (Vinca major L., Prunus laurocerasus L., Forsythia intermedia L., Citrus aurantium L., and Hedera helix L.). Cuticular water permeabilities measured with isolated cuticular membranes were not different from cuticular water permeabilities measured with leaf discs. Depending on the species cuticular water permeabilities increased by factors between 12 (V. major) to 264 (H. helix) when temperature was increased from 10 °C to 55 °C. Arrhenius plots (lnP versus 1/T) of all investigated species were characterized by phase transitions occurring in the temperature range of 30–39 °C. Activation energies for water permeability across plant cuticles below and above the midpoint of phase transition were calculated from Arrhenius plots. Depending on the species they varied between 26 (F. intermedia) to 61 kJ mol⁻¹ (H. helix) below the phase transition and from 67 (V. major) to 122 kJ mol⁻¹ (F. intermedia) above the phase transition. Since the occurrence of phase transitions always lead to significantly increased rates of cuticular transpiration it is argued that temperatures higher than 35 °C caused structural defects to the transport-limiting barrier of the plant cuticles of all species investigated.

Key words: Arrhenius plot, cuticular membrane, cuticular transpiration, leaf surface, phase transition, water permeability.

Introduction

Plant cuticles cover leaf surfaces of higher, land-living plants (Kerstiens, 1996a). Cuticles are lipophilic extracellular polymer membranes composed of the cutin polymer (Holloway, 1993) and cuticular waxes (Bianchi, 1995). It is well established that transport properties of plant cuticles are largely determined by cuticular waxes forming solid, partially crystalline, aggregates deposited to the outer parts of the cutin polymer serving as a mechanically stable polymer matrix (Riederer and Schreiber, 1995). Plant cuticles protect leaves of higher, land-living plants from uncontrolled losses of water and ions and they establish a mechanical barrier preventing most micro-organisms from infecting leaves (Kerstiens, 1996b).

Under severe environmental conditions of water shortage stomates will be closed and the residual amount of water lost from the leaf surfaces is determined by rates of cuticular transpiration (Schönherr, 1982). Since water shortage often occurs in parallel with high temperatures, it is important to know how cuticular transpiration is influenced by increasing temperature. In the past it was shown with isolated Citrus aurantium L. cuticles that cuticular transpiration strongly increased with increasing temperatures (Schönherr et al., 1979). Furthermore, Arrhenius plots revealed phase transitions occurring at temperatures around 40 °C, indicating structural changes in the transport-limiting barrier of plant cuticles at higher temperatures (Eckl and Gruler, 1980; Schreiber and Schönherr, 1990). In this study a new experimental technique was used that allowed the measurement of cuticular transpiration of isolated plant cuticles and leaf discs. It was the aim of this study to find out whether the temperature-dependent behaviour of cuticular transpiration can also be detected with further plant species and to what extent intact leaves behave in a similar way to isolated cuticles.
Materials and methods

Plant materials

Fully expanded leaves of the five species *Vinc a major* L., *Prunus laurocerasus* L., *Forsythia intermedia* L., *Citrus aurantium* L., and *Hedera helix* L. were collected in the Botanical Garden of Würzburg. Leaf discs (2 cm diameter) were punched out using a cork borer. They were either directly used in the experiments or adaxial, stomatous cuticles were isolated from the upper leaf sides (according to Schönerr and Riederer, 1986), and the leaf discs immersed in an enzymatic solution of cellulase (Celluclast, Novo Nordisc, Bagsvaerd, Denmark) and pectinase (Trenolin, Erbsloh, Geisenheim, Germany). After a few days cuticles were sampled, washed in deionized water, dried and stored in Petri dishes at room temperature until they were used in the experiments.

Determination of cuticular transpiration

Cuticular water permeability was measured by applying a newly developed method using tritiated water (specific activity: 925 MBq g⁻¹; Hartmann Analytik, Braunschweig, Germany) and either isolated cuticles or leaf discs. 1 ml transpiration chambers (as described previously: Schönerr and Lendzian, 1981; Geyer and Schönerr, 1990; Schreiber and Riederer, 1996) were used with only a minor modification. The metal lids protecting the mounted cuticles were modified in such a way that 24 ml scintillation vials made of polyethylene (Canberra-Packard, Dreieich, Germany) could be firmly fixed to the outer side of the transpiration chambers. This allowed the direct collection of radiolabelled water, which had diffused across the cuticle, in the scintillation vials.

Before starting with the measurements, transpiration chambers and lids were covered with high vacuum silicon grease (Wacker Chemie, Burghausen, Germany) and 800 µl of the donor solution containing radioactive water was pipetted into the chambers. Chambers were covered with either isolated cuticles or leaf discs and lids were carefully put on top of the transpiration chambers. Cuticles were oriented with their physiological inner side facing the donor solution. Leaf discs were mounted on the transpiration chambers with their lower stomatous leaf side facing the donor solution. However, before mounting leaf discs, they were vacuum-infiltrated with donor solution and carefully blotted dry with filter paper. Preliminary experiments had shown that vacuum-infiltration of leaf discs with donor solution strongly facilitated equilibration between donor and leaves resulting in constant driving forces for transpiration while measuring cuticular water permeability. As a control, parafilm discs were measured instead of isolated cuticles or leaf discs. These discs should allow the sealing of the chambers to be checked thus showing that possible effects of temperature on measured rates of transpiration are a function of cuticles and leaf samples and not of the chambers.

Transpiration chambers prepared in this way were fixed upside down to scintillation vials containing silica gel to scintillation vials containing water as the receiver. The tritiated water which had diffused across the cuticle into the gas phase of the vial rapidly equilibrated with the water reservoir at the bottom of the scintillation vial serving as the receiver for the radioactive water. For example, in equilibrium with the gas phase the partition coefficient between water and air is 43 384 at 25 °C (Geyer and Schönerr, 1990). Even if the gas phase has a volume 24 times larger than the water phase (1 ml), in equilibrium 1800 times more water molecules are sorbed to the water phase. Thus, in the temperature range measured here, most of the water will be sorbed to the water reservoir at the bottom of the scintillation vials.

At fixed time intervals (15, 30, 45, and 60 min) transpiration chambers were transferred to new scintillation vials. 5 ml scintillation cocktail (Ultima Gold XR, Canberra-Packard) was added to the used vials and the amount of radioactivity was counted in a scintillation counter (Wallac counter, Model 1409, Turku, Finland). After 60 min transpiration was measured at the next higher temperature. The temperatures investigated were 10, 20, 30, 35, 40, 45, 50, and 55 °C. For each temperature, the total amounts of radioactivity which had diffused through the cuticles to the scintillation vials at a respective sampling time were calculated by summing up the amounts of radioactivity which were obtained at each single sampling time. Plotting the amounts of radioactivity which had penetrated the cuticles at each temperature versus time gave linear transpiration kinetics with coefficients of determination (r²) better than 0.99 as shown several times in recent publications (Schreiber and Riederer, 1996; Kirsch et al., 1997; Niederl et al., 1998). Permeances were calculated from transpiration kinetics according to equation 1:

\[
P = \frac{F}{A \Delta \xi}
\]

(1)

Permeance \( P \) [m s⁻¹] is a measure of the barrier properties of a plant cuticle towards a certain substance such as water, \( F \) [mol s⁻¹] is the linear flow of \(^1\text{H}\)-labelled water across the cuticle and \( A \) [m²] is the area across which transpiration was measured. \( \Delta \xi \) [mol m⁻³] is the driving force which was given by the concentration of \(^1\text{H}\)-labelled water in the donor solution. The advantage of calculating permeances is the fact that they are independent from the area and the driving force. Thus, cuticular transpiration from different species and different experiments can easily be compared on the basis of permeances (Schreiber and Riederer, 1996).

Plotting the logarithms of \( P \) versus the respective temperatures resulted in Arrhenius plots. From the slopes obtained from linear regressions fitted to the Arrhenius plots, activation energies of water transport across the cuticles were calculated according to equation 2:

\[
\ln P = \ln A - \frac{1}{T} \times E_a
\]

(2)

\( P \) [m s⁻¹] is the permeance, \( \ln A \) is the pre-exponential factor of the Arrhenius plot, \( T[K] \) is the temperature, \( E_a[J mol^{-1} K^{-1}] \) is the activation energy of cuticular permeance, and \( R [J mol^{-1} K^{-1}] \) is the gas constant.

Sample size and statistics

Permeances of isolated cuticular membranes and leaf discs were measured at each temperature with at least 15 replicates. Results are given as means with 95% confidence intervals (95% CI). Before carrying out parametric statistics, permeances were subjected to a logarithmic transformation as suggested...
previously (Baur, 1997), who showed that permeances of water measured with isolated cuticles followed a log-normal distribution.

**Results**

Permeances of all five investigated species calculated from the linear slopes of the transpiration kinetics measured at 20 °C varied between $3.93 \times 10^{-11}$ m s$^{-1}$ (H. helix) up to $7.41 \times 10^{-10}$ m s$^{-1}$ (C. aurantium). Permeances of all investigated species strongly increased with temperatures increasing from 10–55 °C as shown for V. major (Fig. 1A–D). There were no major differences in the temperature-dependent behaviour of isolated cuticular membranes or leaf discs (Fig. 1A–D). $P$ of parafilm was 10–100 times lower than the $P$s of the investigated species and there was only a weak increase of water permeability with temperature (Fig. 1C). Maximum effects calculated from the ratios of $P$ measured at 55 °C divided by $P$ measured at 10 °C ranged from a 12-fold increase of

![Fig. 1. Effects of temperature on cuticular water permeability of (A, C, E) isolated cuticular membranes and (B, D, F) leaf discs of Vinca major L. (A, B) Permeances of water [m s$^{-1}$] as a function of increasing temperatures [°C] of (A) five randomly selected cuticular membranes and (B) five randomly selected leaf discs. (C, D) Mean permeances of water [m s$^{-1}$] as a function of increasing temperatures [°C] of (C) cuticular membranes and (D) leaf discs. In (C) water permeance across parafilm serving as control is shown. (E, F) Arrhenius plots (ln$P$ versus $T^{-1}$) of (E) isolated cuticular membranes and (F) leaf discs. Arrhenius plots in (E) and (F) were calculated from (C) and (D), respectively. Each data point in (C) to (F) represents the mean of 20 replicates. Error bars represent 95% confidence intervals.](image)
cuticular transpiration (*V. major*) to a 264-fold increase (*H. helix*) (Table 1). Plotting the logarithms of $P$ versus the reciprocals of $T$ of all investigated species resulted in biphasic Arrhenius plots ($\ln P$ versus $T^{-1}$) of cuticular water permeability of (C) isolated cuticular membranes and (D) leaf discs of *Forsythia*. Each data point in (A) to (D) represents the mean of 18 replicates. Error bars represent 95% confidence intervals.

**Table 1.** Maximum effects of temperature on cuticular permeability of isolated cuticular membranes and leaf discs with 95% confidence intervals

Maximum effects of temperature were calculated dividing the permeance measured at 55 °C by the permeance at 10 °C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Maximum effect of temperature</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cuticular membrane</td>
</tr>
<tr>
<td><em>Vinca major</em></td>
<td>12.3 ± 2.4</td>
</tr>
<tr>
<td><em>Prunus laurocerasus</em></td>
<td>62.2 ± 17.5</td>
</tr>
<tr>
<td><em>Forsythia intermedia</em></td>
<td>32.5 ± 8.5</td>
</tr>
<tr>
<td><em>Citrus aurantium</em></td>
<td>—</td>
</tr>
<tr>
<td><em>Hedera helix</em></td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of temperature on cuticular water permeability of (A, B) *Prunus laurocerasus* L. and (C, D) *Forsythia intermedia* L. (A, B) Arrhenius plots ($\ln P$ versus $T^{-1}$) of cuticular water permeability of (A) isolated cuticular membranes and (B) leaf discs of *Prunus*. (C, D) Arrhenius plots ($\ln P$ versus $T^{-1}$) of cuticular water permeability of (C) isolated cuticular membranes and (D) leaf discs of *Forsythia*. Each data point in (A) to (D) represents the mean of 18 replicates. Error bars represent 95% confidence intervals.

linear parts of the Arrhenius plots below and above the phase transitions (Table 2). They ranged from 26 kJ mol$^{-1}$ (*F. intermedia*) to 61 kJ mol$^{-1}$ (*H. helix*) below the phase transition and from 67 kJ mol$^{-1}$ (*V. major*) to 122 kJ mol$^{-1}$ (*F. intermedia*) above the phase transition.

### Discussion

Permeances of water across cuticles increased nonlinearly with the slopes constantly becoming steeper at temperatures higher than 35 °C (Fig. 1A–D). Parafilm serving as a control in this newly developed experimental systems showed completely different behaviour (Fig. 1C). $P$ was significantly lower by 1–2 orders of magnitude compared to $P$ of cuticles and only a weak, linear increase of $P$ with temperature was observed up to 50 °C (Fig. 1C). From these results obtained with parafilm as a control it can be concluded that the new method presented here is well suited to measuring the temperature dependence of cuticular transpiration.

One of the basic aims of this study was the comparison of isolated cuticular membranes with leaf discs. In general, the results obtained with isolated cuticles are in very good agreement with results obtained with leaf discs and...
with both types of samples a similar reaction of transpiration towards increasing temperature was observed (Figs 1, 2). Furthermore, the maximum effects of temperature (Table 1), as well as the activation energies and temperatures of phase transitions (Table 2) were in good agreement within the range of experimental errors between both types of samples. A good correspondence between the transport properties of isolated plant cuticles and leaf discs has already been reported by measuring the cuticular permeability of lipophilic organic compounds (Kirsch et al., 1997) and it is now confirmed again for cuticular transpiration.

However, having a closer look at the results presented here, it becomes evident that there is a tendency that values obtained with leaf discs are slightly lower compared to isolated cuticles (Figs 1, 2). There may be two reasons for this. Isolating cuticles is a very harsh procedure and thus small non-visible micro-defects could be induced to the cuticular transport barrier of waxes leading to slightly increased rates of transpiration. This interpretation is supported by the observation that freshly isolated cuticles of *C. aurantium* had 1.5–2-fold higher permeabilities than cuticles stored for several weeks (Geyer and Schönhr, 1990). This finding was explained by a healing of small micro-defects in the transport-limiting barrier of the cuticles, which were induced during the isolation procedure.

Alternatively, it cannot be completely ruled out that the assumption about the driving force for transpiration acting across the cuticle of the leaf discs is incorrect. Leaf discs infiltrated with donor solution were mounted in the chambers and it was assumed that the donor solution in the chamber was in equilibrium with the leaf disc. This assumption was based on preceding experiments, which have shown that a stable equilibrium between leaf discs and the donor solution was established by infiltrating leaf discs before starting the experiments. Thus, similar to isolated cuticles the concentration of radioactive water in the donor solution was used as the driving force for calculating of leaf discs. However, it cannot be excluded that there could be some inhomogeneities in the distribution of the radioactive water in the intact leaf leading to locally lower concentrations of radioactivity, which would result in a lower driving force than assumed. This in turn would lead to a systematic deviation of values obtained with leaf discs compared to isolated cuticles.

With all the investigated species, characteristic biphasic Arrhenius plots were obtained (Figs 1E, F, 2, 3)

![Graph](image)

Fig. 3. Effects of temperature on cuticular water permeability of (A) *Citrus aurantium* L. and (B) *Hedera helix* L. (A, B) Arrhenius plots (lnP versus $T^{-1}$) of cuticular water permeability of leaf discs of (A) *Citrus* and (B) *Hedera*. Each data point in (A) and (B) represents the mean of 15 replicates. Error bars represent 95% confidence intervals.

<table>
<thead>
<tr>
<th>Species</th>
<th>Activation energy $E_a$ [kJ mol$^{-1}$] (± ci)</th>
<th>Temperature of phase transition $T_{PT}$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM Below $T_{PT}$</td>
<td>Above $T_{PT}$</td>
</tr>
<tr>
<td><em>Vinca major</em></td>
<td>26.6±2.9</td>
<td>68.7±7.1</td>
</tr>
<tr>
<td><em>Prunus laurocerasus</em></td>
<td>49.6±6.2</td>
<td>89.3±10.6</td>
</tr>
<tr>
<td><em>Forsythia intermedia</em></td>
<td>26.5±7.9</td>
<td>121.6±18.5</td>
</tr>
<tr>
<td><em>Citrus aurantium</em></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Hedera helix</em></td>
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</tr>
</tbody>
</table>

Table 2. Activation energies $E_a$ with 95% confidence intervals and midpoints of phase transitions $T_{PT}$

Activation energies were calculated from the linear parts of the Arrhenius plots (Figs 1E, F, 2A, B, C, D, 3A, B) below and above the midpoints of phase transitions ($T_{PT}$). Midpoints of the phase transitions were calculated from the intercepts of the two linear regression lines fitted to the lower and upper parts of the Arrhenius plots, respectively.
exhibiting phase transitions in the temperature range between 30–39 °C (Table 2). This confirms results obtained with isolated C. aurantiun cuticles and non-isolated cuticles of Allium cepa L. using the different experimental system of mounting the cuticles between aqueous donor and receiver compartments, respectively (Schönherr et al., 1979; Schönherr and Merida, 1981). Thus, these characteristic phase transitions could also be detected with different species and a different experimental set-up. Since barrier properties of plant cuticles are essentially determined by cuticular waxes it was originally argued that the biphasic Arrhenius plots observed were in fact due to temperature-induced phase transitions of cuticular waxes (Eckl and Gruler, 1980). However, by measuring the volume expansion of plant cuticles it was shown later that isolated plant cuticles and wax-free cuticular polymer matrix membranes themselves exhibited characteristic phase transitions in the temperature range between 30–40 °C (Schreiber and Schönherr, 1990).

From these findings it was concluded that, at temperatures above 30–40 °C an increased volume expansion of the cutin polymer caused defects in the transport-limiting barrier leading to additional paths of diffusion for water. It was argued that defects forming at the cutin wax interfaces are responsible for increased rates of cuticular transpiration above the phase transition temperatures (Schreiber and Schönherr, 1990). In contrast to the results obtained here with water, there was no evidence for the occurrence of phase transitions analysing cuticular permeability of lipophilic substances (Baur et al., 1997). Arrhenius plots were linear in the temperature range 20–70 °C or they were even weakly bent towards the x-axis. Comparing this to the results presented here it must be concluded that the temperature-induced defects in the transport-limiting barrier of plant cuticles are most likely additional paths of diffusion at the wax/cutin interfaces of a high polarity, since they are utilized by polar molecules like water but not by lipophilic substances.

Activation energies for cuticular transpiration calculated from the linear parts of the Arrhenius plots below the phase transitions (Table 2) were in a similar order of magnitude, although partially somewhat lower, as the values reported for C. aurantiun cuticles, which were around 50 kJ mol\(^{-1}\) (Schönherr et al., 1979; Schönherr and Merida, 1981). Activation energies for water permeability across a large variety of organic polymer membranes even ranged from –12 to 43 kJ mol\(^{-1}\) (Barrie, 1968). This fairly large variation shows that activation energies, which are obtained by measuring the permeability of a membrane (expressed as permeance [m s\(^{-1}\)] or as permeability coefficient [m\(^2\) s\(^{-1}\)]], are somewhat difficult to interpret. It is even more surprising that activation energies calculated from the linear portions of the Arrhenius plots above the phase transitions (35–55 °C) were all significantly higher compared to activation energies in the lower temperature range (Table 2), because permeances were significantly higher at higher temperatures indicating decreased barrier properties of the cuticles instead of increased barrier properties.

This puzzling observation described above can probably best be explained by the fact that both permeances and permeability coefficients are composed quantities. They both depend on the diffusion coefficient \(D\) [m\(^2\) s\(^{-1}\)] describing the mobility of water in the membrane, and on the dimensionless partition coefficient \(K\) describing the solubility of water in the membrane. Thus, permeances or permeability coefficients are strongly dependent on the individual contributions of \(D\) and \(K\). Furthermore, both parameters will change with temperature. Mobility of water in cuticles will increase with increasing temperatures (Baur, 1997) and \(K\) most probably will decrease with increasing temperatures. Since there is no direct data about the temperature-dependent solubility of water in cuticles, this conclusion is drawn from results reporting the decrease of 4-nitrophenol sorption to cuticles (Riederer and Schönherr, 1986). From this discussion it must be concluded that a more detailed thermodynamic and mechanistic analysis of the effects of temperature on the barrier properties of plant cuticles, especially at higher temperatures above the phase transitions, requires a separate analysis of the effects of temperature on \(D\) and \(K\), which was not the intention of this study.

Nevertheless, activation energies of water diffusion through various lipophilic barriers at 25 °C reported in the literature were 50 kJ mol\(^{-1}\) for phospholipid bilayers (Price and Thompson, 1969) and 31 kJ mol\(^{-1}\) for stearyl alcohol-covered polymer membranes (Kester and Fennema, 1989), which is in a similar range to the values obtained in this study. Activation energies measured for larger lipophilic compounds were much higher in this temperature range ranging from 75–189 kJ mol\(^{-1}\) (Baur et al., 1997). This still allows the conclusion that water diffusion across cuticles in the temperature range between 10–35 °C basically takes place in a lipophilic environment composed of cutin and waxes. This conclusion is strongly supported by co-permeability experiments, where the simultaneous diffusion of \(^3\)H-labelled water together with \(^14\)C-labelled organic compounds of varying lipophilicity was analysed (Niederl et al., 1998). Water permeability was always highly correlated to permeabilities of the organic acids independent from their lipophilicity indicating that transcuticular diffusion is basically in a lipophilic environment.

Trying to analyse the differences of cuticular transpiration observed with the five species, permeances of the different species and samples measured at 20 °C were correlated versus various parameters obtained in this study. Fairly good correlations were obtained when ln \(P\) was plotted versus the phase transition temperature (Fig. 4) and versus the maximum effect of temperature...
Both correlations indicate that cuticles having a very low permeability for water (e.g. *H. helix*) react more sensitively towards an increase in temperature leading to the phase transition, whereas cuticles having a relatively high permeability for water (e.g. *V. major*) are less sensitive. Furthermore, plotting ln *P* versus activation energies calculated from linear portions of the Arrhenius plots in the lower temperature range (10–35 °C) of all five investigated species (*Vinca major* L., *Prunus laurocerasus* L., *Forsythia intermedia* L., *Citrus aurantium* L., and *Hedera helix* L.) and the two different types of samples (isolated cuticular membranes and leaf discs) shows that cuticles having greater activation energies also had lower permeances (Fig. 6), although this correlation is less significant with an *r*² of 0.64.

Coming to a final conclusion it must be mentioned that leaf surface temperatures up to 50 °C and sometimes even higher have been reported for plants growing in hot and dry climates (Lange and Lange, 1963; Kuraishi and Nito, 1980). But even with plants growing in temperate climates leaf surface temperatures between 40–50 °C have been measured (Huber, 1956). This shows that plants sometimes will have to cope with high leaf surface temperatures as used in this study. Nevertheless, it must be mentioned that an estimation of the ecological consequences of increased cuticular transpiration at higher temperatures for plants growing in their natural habitats on the basis of the results obtained in this more mechanistic study is not easily possible. This will need further information such as an analysis of times and duration of exposure to high temperatures in the environment, which is currently under investigation.

Acknowledgements

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