Effects of inorganic salts on water permeability of isolated cuticular membranes

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Effects of inorganic salts on water permeability of isolated cuticular membranes

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Tag der Promotion:

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ABBREVIATIONS

A	Area
CEC	Cation exchange capacity
c.i	Confidence intervals
CM	Cuticular membrane
dpm	Disintegration per minute
Δc	The differences between the donor and receiver concentration
F	Flow rate
^3H_2O	Tritiated water
K	Selectivity coefficient
IEP	Isoelectric point
MBq	Megabequerel
MX	Polymer matrix
P	Permeance
POD	Point of deliquescence
R	Cuticular resistance
r^2	Correlation coefficient
RH	Relative humidity
S.D	Standard diviation

1 INTRODUCTION

1.1 The plant cuticle

Water is essential for life and it plays a major role in all physiological processes of the plant cell. Thus, both shortage and excess of water can cause physiological problems for plants. To control or avoid negative environmental conditions, plants, like all other living organisms, have developed a suite of physiological, anatomical and morphological adaptations. Most plant species possess specific adaptations to their habitats. One basic adaptation of plants for their survival on the mainland is the plant cuticle. Studies of Silurian and Devonian plant fossils showed that cuticles are very resistant and the oldest known cuticles are over 400 million years old (Woodward 1998, Edwards et al. 1996*)*. Early studies on the nature of cuticles were started in the 20th century (Kolattukudy 1981). The cuticle is defined as a heterogeneous, extracellular biopolymer (Schönherr and Huber 1977, Kirsch et al. 1997), which is synthesized by epidermal cells (Marga et al. 2001).

The cuticle covers all primary above-ground parts of the plants, such as leaves and fruits (Schönherr 1976a*,* Marga et al. 2001, Round et al. 2000, Jetter and Schäffer 2001, Neinhuis et al. 2001, Niederl et al. 1998*)* but not woody stems and wounds (Kerstiens 1996). It forms the interface between the plant cell and the atmosphere (Niederl et al. 1998, Luque et al. 1995, Jetter and Schäffer 2001). The cuticle 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 1976a). The cuticle also acts as the first protective barrier against UV radiation (Mariani and Wolters-Arts 2000) and it reduces leaching, e.g. it protects leaves from an excessive loss of ions and nutrients (Niederl et al. 1998).

1. 2 Structure of the plant cuticle

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 cutin polymer, which makes up the bulk of the cuticular membrane (Schönherr 1976b), forms the mechanically stable polymer matrix (Round et al. 2000), which is attached

to the epidermal cell wall with a pectinaceous layer (Kolattukudy 1981) and presumably other cell wall carbohydrates. It is a lipophilic, amorphous polymer membrane (Holloway 1982). Cutin is composed of mainly C_{16}- and C_{18}-hydroxy fatty acids cross-linked by ester bonds (Kolattukudy 1981, Riederer and Schreiber 2001). Polysaccharides, such as pectin, crystalline cellulose and hemicelluloses are also embedded in the polymer matrix (Jeffree 1996, Schönherr and Baur 1996). In addition, polyuronic acids, proteins and phenolic compounds can be found in cutin (Schönherr 1976b). Cutin amounts range from 20 % to 84 % by weight of the isolated cuticles (Schönherr 1976b).

The second important fraction of the cuticle is composed of soluble lipids. These represents a complex mixture of aliphatic and cyclic compounds and they are often called cuticular waxes (Schönherr and Riederer 1989). These lipids consist of intracuticular waxes, which are embedded within the cutin polymer matrix and of epicuticular waxes, which are deposited as thin films and aggregates on the leaf and fruit surfaces. The structure is summarized in Figure 1.

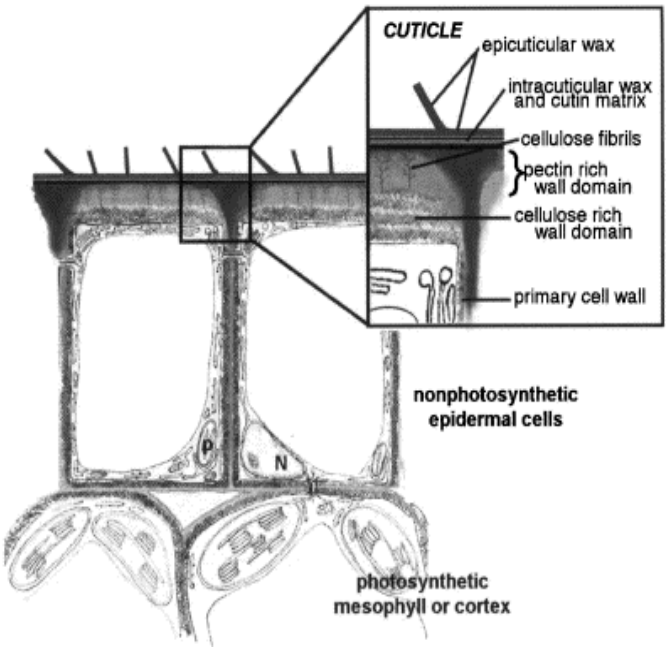

Figure 1. Schematic drawing of the structure of the cuticular membrane showing the components of the cuticle: the cuticle proper (cutin) forms an electron dense layer over the epidermal cells; both, intracuticular waxes and epicuticular waxes form the surface lipids (from Kunst and Samuels 2003).

Cuticular wax is a general term for a complex heterogeneous mixture of very long-chain (C_{20} - C_{34}) fatty acids and their derivatives (Rhee et al. 1998). They are synthesized from C_{16}- and C_{18}-precursors that are produced in the plastids (Bird and Gray 2003). In addition varying proportions of cyclic compounds such as pentacyclic triterpenoids and hydroxycinnamic acid derivatives (Riederer and Markstädter 1996) are part of the wax. 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 upper leaf side has usually more epicuticular wax crystals compared to the lower side. The formation of cuticular waxes has always been discussed with the problems of their movement through the cuticle (Neinhuis et al. 2001). Neinhuis et al. (2001) suggested that the molecules, which finally form the cuticular waxes diffuse through the cuticle as molecules dissolved in water.

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).

1.3 Function of the plant cuticle

The plant cuticle forms the interface between the aerial environment and the living cells of the plant. Therefore, the cuticle has to manage multiple physiological and ecological functions. It is an effective barrier to the transport of solutes and gases in and out of the leaf (White et al. 2002) and it plays an important role during the foliar uptake of agrochemicals (Burghardt et al. 1998). It reduces leaching and thus prevents leaves from an excessive loss of ions and nutrients (Tyree et al. 1992, Niederl et al. 1998). It also presents the major barrier to penetration of leaf tissues by a variety of environmental chemicals such as sulfuric and nitric

acid, when the plants are exposed to these acids (Hauser et al. 1993). Furthermore, it forms the primary barrier against bacterial and fungal attacks and reduces the infection of plants by pests and pathogens. The cuticle can also protect the photosynthetic tissues from excess light by reflecting and scattering and subsequently attenuating the light to such an extent that it causes no damage to the tissues.

1.4 Transport of molecules across cuticles

When analysing the permeation of solutes and water molecules across the plant cuticle, it can be treated as a homogeneous solubility/mobility membrane (Riederer and Schreiber 1995). In this case, the transport across the plant cuticle is simply occurring along the chemical potential that is caused by the difference of the concentrations of the permeating molecules between the inside leaf and the outside of the leaf.

The mechanism of foliar penetration consists of two phases; surface adsorption (an initial phase), and cuticular penetration. It is initiated when a droplet of water containing some solute comes in contact with the cuticle (Schönherr and Riederer 1989). The permeating molecules are sorbed by the membrane on one side, penetrate it, dissolved as single molecules within the membrane phase, and they leave the membrane on the other side. However, this model can be only used with lipophilic solutes and it reaches its limits when polar compounds are considered (Riederer and Schreiber 2001).

Alternatively, a model suggesting two parallel paths of diffusion across the plant cuticle was suggested (Schönherr 2000, Riederer and Schreiber 2001). The first pathway, similar to that described above, is formed by the amorphous phases of cutin and wax, which can be used only by lipophilic solutes. The second path is formed by polar pores of molecular dimensions filled with water, which can be penetrated by water, and polar charged organic as well as inorganic compounds (Riederer and Schreiber 2001). The diameter of polar pores in isolated cuticular membranes devoid of cuticular waxes was determined using organic molecules of known diameter. The pore radius was estimated to be around 0.45 nm for *Citrus* and *Allium* (Schönherr 1976c). Schönherr (1976a) argued that these pores are dynamic structures and they arise only on hydration of polar functional groups in the polymer matrix. Due to very small radii of the pores, the molecule size is one of the important properties that determine mobility of polar solutes in the cuticle. Thus, only small molecules can diffuse in these pores (Schönherr and Riederer 1989).

The barrier properties of the cuticle depend to a large extent on cuticular waxes. Therefore, the transport across the plant cuticle mainly depends on the wax layer, which consists of crystals that are embedded within a cutin matrix of amorphous material. The crystals (or impermeable flakes; Riederer and Schreiber 1995) reduce the volume of the barrier available for diffusion and lead to a highly tortuous paths across it (Fig. 2).

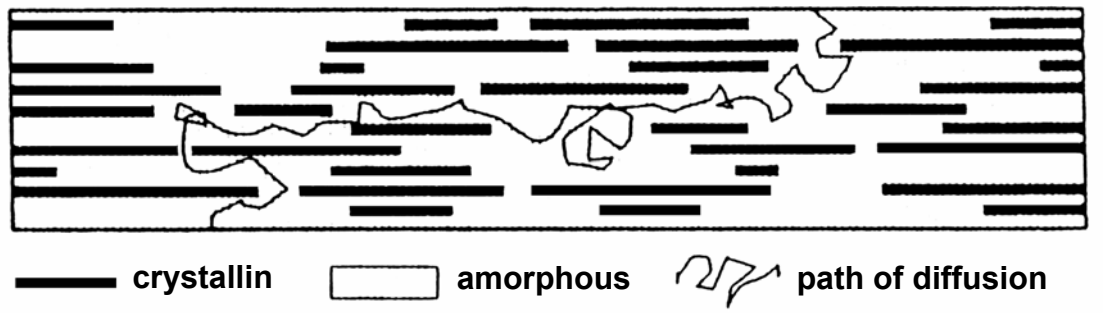

Figure 2. Tortuosity of the pathway through the cuticular membrane; The solute molecule move through the amorphous wax and jump from vacancy to vacancy. Dependent on crystalline wax formation and their distribution, crystalline waxes reduce the volume of the amorphous phase available for diffusion (from Riederer and Schreiber 1995).

1.5 Water permeability

The permeance is a parameter that is characteristic for a given type of cuticle, a given solute (or solvent) and at a given temperature (Schönherr and Riederer 1989). The permeance is a useful parameter for describing permeability of cuticular membranes and it is defined as follows:

$$P = F / (A \cdot \Delta c) \qquad (1)$$

F ($g \cdot s^{-1}$) represents the flow rate, A (m^2) the exposed area of the cuticle and Δc ($g \cdot m^{-3}$) the concentration difference between donor and receiver compartments also called the driving force for diffusion.

Water permeability of isolated cuticular membranes has been studied extensively in the last years, especially from an ecophysiological point of view. Water permeabilities of plant cuticles from different species are highly variable. They differ not only among different species, but also deffer within the same species. They can even vary within the isolated

cuticles obtained from the same organ (leaf or fruit). Interspecific variability varies over 2.5 orders of magnitude (Riederer and Schreiber 2001). Cuticular water permeability is not correlated to the thickness or to wax coverage of the cuticle (Riederer and Schreiber 2001). The differences of water permeabilities are caused by ecophysiological adaptations that are genetically fixed. In adaptation to their habitats, ever green epiphytic or climbing plants growing naturally in tropical climates and species adapted to dry climates exhibited the lowest water permeabilities. In contrast the highest water permeances were observed with the deciduous plants growing in temperate climates (Schreiber and Riederer 1996). Studies of fruit cuticles indicated that their water permeances were about 10 times higher than those of leaf cuticles with highest water permeabilities (Riederer and Schreiber 2001). Cuticular permeability is influenced by physical (temperature, humidity, pH) and chemical (adjuvants, pollutants) factors. Many studies and investigations of cuticular permeability showed that water permeability was increased by increasing temperature (Schönherr and Baur 1996), relative humidity (Schreiber et al. 2001) and by increasing pH (Schönherr 1976a).

1.6 Cuticular resistance

Cuticular resistance (**R**) is defined as driving force per unit flux, and its dimension is $s \cdot m^{-1}$ (Schönherr 1982). In other words, the resistance of membrane is the reciprocal value of the permeance (Schönherr and Riederer 1989). It is defined as:

$$R = 1/P \tag{2}$$

Permeances of cuticles observed so far range from about $10 \cdot 10^{-6}$ to $10 \cdot 10^{-10}$. The range of resistances is therefore $10 \cdot 10^{6}$ to $10 \cdot 10^{10}$ (Schönherr and Riederer 1989).

1.7 Effect of wax extraction on cuticular water permeability

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). 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 above, 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).

1.8 Effect of cations on water permeability

The polymer matrix contains polar pores due to the presence of polar functional groups in the cutin monomers. Hydration of these groups causes a swelling of the CM and affects size or number of these pores. Since the hydrated diameter of many ions is smaller than the pore size (Tyree et al. 1990), it is highly probable that ions penetrate these pores. Both components of the salt (cations and anions), were found to penetrate the plant cuticle in equivalent amounts. A number of investigations showed that cations can affect cuticular water permeability rather than anions (Schönherr 1976a, Beyer 2002).

The concentration of fixed charges, e.g. free carboxylic groups, in a polymer is an important property affecting the sorption and transport of water and ions. The salt ions affect the cation exchange capacity (CEC) of the polymer matrix of the cuticle. The charge of the ion influences the CEC. Generally, ions with higher valance will exchange for those of lower valance. For ions with the same valance, the effective hydrated cation radius is of importance. Small cations (with small cation radii) are more strongly hydrated, in other words, their hydration is higher than those of the large cations. When small cations sorb to the polymer matrix, they attract more water molecules, cause increased swelling of the polymer matrix, and as a consequence water permeability of the membrane is increased. Swelling and pore volume of polymer matrix have been shown to depend on the kind of cations. Schönherr and Bukovac (1973) reported that at a constant pH value and salt concentration, the exchange capacity of tomato cuticles was dependent upon the counter ions. The ion exchanger prefers divalent cation over monovalent if ion selectivity coefficient (K) > 1 and monovalent over divalent if K < 1 (Schönherr and Bukovac 1973*)*. Since activity coefficients in the polymer

are not known, molalities (m) are used to determine the selectivity coefficient of cations. For example, the selectivity coefficient of Ca^{++} and Na^{+} is defined by:

$$K^{Ca} = \{m_{Ca} / (m_{Na})^2\} \cdot \{(m_{Na})^2 / m_{Ca}\} \quad (3)$$

1.9 Effect of humidity on water permeability

Water permeability of cuticles increases also with increasing air humidity. This was demonstrated by using isolated cuticular membranes by a number of investigators *(*Schönherr and Schmidt 1979, Schönherr and Merida 1981, Schreiber et al. 2001*)*. The effect of humidity is caused by water molecules sorbing to the polar sites of the cuticle, which leads to the formation of polar pores, and eventually, increasing water permeability. As stated above in paragraph (1.8), permeation of some kinds of cations to cuticular membranes increases also water permeability. With increasing humidity, rates of salt penetration increase, due to dissolution of salt residues on the surface of the cuticle *(*Schönherr 2000, 2001*)*. This process is controlled by the point of deliquescence (POD) of the salt (Schönherr and Luber 2001), which is defined as the conversion of a solid substance into a liquid as a result of absorption of water vapour from the air. The salt residue could sorb the moisture from the air depending on humidity and hygroscopicity of the salt. When the humidity is above the POD, the salt residues on the cuticle dissolve and penetration occurs, while below a solid crystalline residues are formed and the uptake process stops (Schönherr and Luber 2001).

1.10 Effect of pH on water permeability

The membrane permeability may be affected by solution pH in three ways (Schönherr and Riederer 1989): direct effect of pH, effect on the driving force via electrical potentials, and change of the properties of the solutes by dissociation. The cuticles are polyelectrolytes and their isoelectric point (IEP) is around pH 3 (Schönherr and Huber 1977). Above this point, when pH increases, the cuticles carry fixed negative charges. These charges are an important characteristic affecting the water content of the polymer matrix via swelling (Şahin et al. 2002). Unionized carboxyl groups are little hydrated (Schönherr and Riederer 1989), and when the pH increase, the ionization degree of these functional groups will increase, they become able to attract more water molecules to the polymer matrix (swelling) and subsequently water permeability will be increased.

The radius of the water filled pores is not pH dependent. With increasing pH level, the number of pores increased but not their radii. Schönherr (1976a) reported that the number of pores per cm² was increased from $5 \cdot 10^{10}$ to around $16 \cdot 10^{10}$ when the pH level was increased from 3 to 9. Beyer et al. (2002) reported that pH gradients between donor and receiver solutions are also very important to sorption of cations to plant cuticles, which reduced water uptake of the cuticles.

1.11 Aim of the present study

Clearly, cuticular transpiration is one of the important biological processes affecting plant viability especially under water stress conditions. This physiological process is influenced by a number of environmental factors that can cause an increase or a decrease of transpiration rates. There is more information available about permeation of water across plant cuticles and the effect of adjuvants, temperature, humidity and wax extraction. Less information is available on the effect of ionic compounds on cuticular permeability. Therefore, the objective of our study was to investigate the effects of different salts on water permeability of different isolated plant cuticles. We focused on cations because the earlier studies found them to be more effective than anions in increasing and decreasing water permeability of isolated cuticles.

2 MATERIALS AND METHODS

2.1 Plant material

Fully expanded healthy leaves of *Hedera helix*. L and *Prunus laurocerasus* L. were sampled from mature plants from Bonn city, Germany, in June 2002 and November 2002, respectively. The leaves were visually investigated to exclude any damages or infections by microorganisms.

Mature fruits of tomato *Lycopersicon esculentum* Mill. were purchased on the market in January 2003. Fruits were selected for uniformity of development, size (47 -57 mm) and absence of defects by visual inspection.

Prunus laurocerasus cuticular membranes were used in experiments to determine the effect of K_2CO_3 on water permeability at different relative humidities (radioactive experiments). They were isolated previously from plants grown in the Botanical Garden of Würzburg University.

Further, previously isolated cuticular membranes of 12 species (Tab. 1) were used in experiments investigating polar pores in cuticles.

Table 1. Scientific names of plant species used in the experiments investigating polar pores in cuticles.

	species
1	*Nerium oleander* L.
2	*Stephanotis floribunda* Brongn
3	*Ligustrum* cf. *vulgare* L.
4	*Juglans regia* L.
5	*Forsythia intermedia* L.
6	*Vinca major* L.
7	*Malus* cf. *domestica* Borkh. var. *gloster*
8	*Syringa vulgaris* L.
9	*Pyrus communis* L. cv. *conference*
10	*Citrus aurantium* L.
11	*Populus canescens* (Aiton) Sm.
12	*Prunus domestica* L.

2.2 Isolation of cuticles

The isolation of cuticles has been carried out according to the method described by Schönherr and Riederer (1986). The leaves were washed with water, dried with soft tissue and left at room temperature for a few hours until they were completely dry. The lower stomatous sides of the leaves were marked with a water-insoluble pen to separate adaxial and abaxial cuticles after isolation. Disks of 20 mm diameter were punched out from the leaves and tomato 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 liter of the enzyme solution.

Depending on the species, cuticles could be isolated after several days to several weeks. 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. Isolated cuticles will be called cuticular membranes (CMs).

2.3 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 were again dried under a stream of air. In the following, dewaxed cuticles will be called polymer matrix membranes (MX).

Wax coverage of the three species (*Hedera helix* L., *Prunus laurocerasus* L., and *Lycopersicon esculentum* L.) was determined gravimetrically. 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).

2.4 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 these experiments (Fig. 3). Each chamber consists of two parts: (1) a metal ring serving as a cover to fix the membrane on the chamber and (2) a chamber functioning as a reservoir for the donor solution. The edges of the transpiration chambers in contact with CM or MX were sealed with high vacuum silicone grease (Wacker Chemie, Burghausen, Germany). The chambers were filled with 900-1000 µl of deionized water or buffer that served as a donor solution. CMs or MXs were mounted on the transpiration chambers with their morphological outer or inner surface (depending on the experiment) facing the atmosphere as shown in Figure 3.

These chambers were placed upside down in closed polyethylene boxes above silica gel. In order to prevent damage of the membranes; a flat metal net was placed between the chambers and silica gel granules. The chambers prepared in this way were incubated in an incubator (Binder, Tuttlingen, Germany) 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).

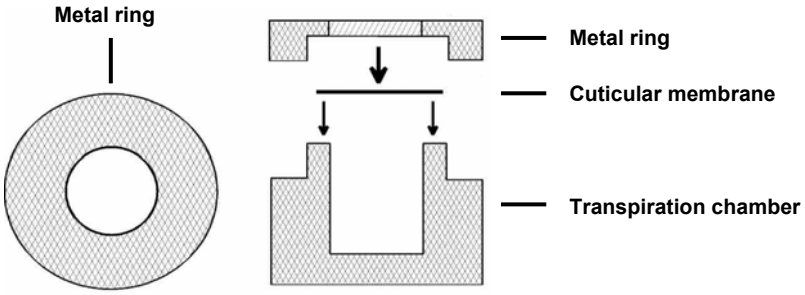

Figure 3. Schematic drawing of cross section of transpiration chamber used in measuring water permeability and salt effects.

Amounts of water diffused across the membranes were summed up and plotted as a function of time (Fig. 4). Rates of water loss were calculated from linear regression lines fitted to the plotted data. The water concentration over silica gel is negligible, therefore, water density in the chambers (1000 kg / m³) was used as driving force for transpiration, and the exposed area of the cuticle to the atmosphere was 1.13 cm². Permeance (P*)* was calculated using the equation (1).

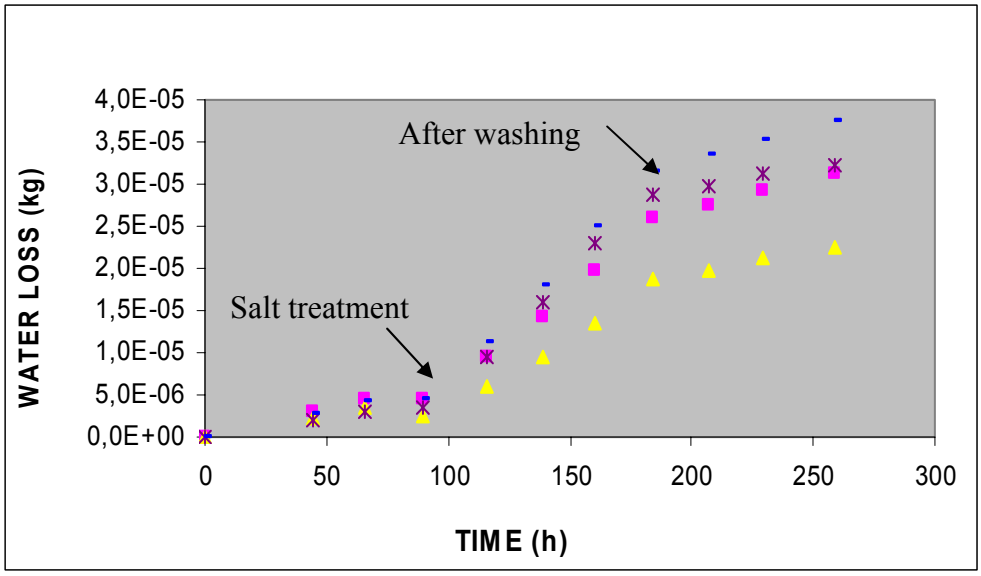

Figure 4. The transpiration rates of four selected CMs of *Prunus laurocerasus* before treatment and after treated with 0.2 M Cs_2CO_3 and after washing. Rates of water flow were determined by a linear regression lines for each treatment separately.

2.5 Experiments analysing the interaction of salts with isolated cuticular membranes

2.5.1 Effects of different salts on cuticular water permeability

After water permeability of each single membrane (CM or MX) had been measured, 200 µl of 0.2 M aqueous salt solutions were applied on the outer surface of the membranes (Tab. 2). In parallel, five chambers of each species were treated with 200 µl deionized water as a control. The chambers were left between 6 to 24 hours at room temperature until the water of the treating solution had evaporated. They were incubated again at 25 ± 0.5 C° as described above and transpiration was measured for the following 4 to 5 days as described above. Permeance was calculated by using the same method and equation (1) that was used before. Between 10 to 15 chambers were used for each treatment.

Table 2. The salts used in salt effect experiments and their pH values.

salt	pH	mw ($g \cdot mol^{-1}$)
$AlCl_3$ [a]	2.9	133.34
NH_4Cl [b]	5.6	53.49
NH_4NO_3 [a]	5.7	80.4
$(NH_4)_2SO_4$ [b]	6.0	132.14
KNO_3 [a]	6.6	101.11
$CaCl_2.2H_2O$ [a]	6.6	147.02
KCl [a]	6.7	74.55
$NaNO_3$ [a]	7.0	84.99
NaCl [a]	7.0	58.44
K_2CO_3 [b]	11.0	138.21
Cs_2CO_3 [a]	11.2	325.85

[a] Fluka, Neu-Ulm, Germany

[b] Merck, Darmstadt, Germany

The effect of the salt on water permeability was calculated for each single membrane by dividing the permeance after treatment by that measured before the treatment. To test if the salt effect is reversible or whether there was irreversible damage of the cuticles, the salt residues were washed off from the cuticles again and the experiment was continued in order to determine the permeance after washing off the salt.

2.5.2 Study of the effect of K_2CO_3 on water permeability of *Prunus laurocerasus* L. CM at different air humidities

Cuticular transpiration of *Prunus laurocerasus* CMs was measured at different relative humidities as described by Schreiber et al. (2001) using tritiated water (Hartmann Analytika, Braunschweig, Germany). In this experiments, the system consisted of three parts: (1) the same transpiration chambers which had been used before with the gravimetric method, (2) slightly differently designed stainless steel lids and (3) polyethylene scintillation vials (Canberra Packard, Dreieich, Germany) (Fig. 5). After adding 900 µl of donor solution mixed with traces of ^3H_2O (specific activity: 925 MBq g^{-1}), the CMs were mounted on the transpiration chambers with their morphological outer surface facing towards the atmosphere. Subsequently, covered

lids were carefully fixed to the chambers using vaccum grease. Finally, chambers were turned upside down and the grease-covered outer surfaces of the lids were placed on the top of scintillation vials containing dry silica gel.

The chambers prepared in this way were incubated at 25 C° for equilibration. Further polyethylene scintillation vials were prepared containing either 100 µl of glycerol, glycerol/water mixtures or pure water. Thus, different air humidities (RH) were adjusted: pure glycerol = 2% RH, 60 µl glycerol and 40 µl water = 60% RH, 30 µl glycerol and 70 µl water = 90% RH, pure water = 100% RH. At the same time these reservoirs at the bottom of the scintillation vials served as the receiver for the radioactive water. Before the experiment was started the atmosphere in the vials was equilibrated overnight at 25 ± 0.5 C°. During the experiment, the transpiration chambers were removed carefully from the scintillation vials containing silica gel and put on top of the scintillation vials containing glycerol (2 % relative humidity) and they were incubated again. After defined time intervals (30, 60 and 90 min) scintillations vials with the same RH were replaced by new vials 3 times. Then transpirations chambers were put on scintillation vials having a higher humidity.

This was repeated with all 4 humidities between 2% and 100% RH. The amount of ^3H_2O, which had diffused across the cuticle into the vials was counted using a scintillation counter (model 1600 CA, Canberra Packard, Dreieich, Germany) after adding scintillation cocktail (Permafluor, Canberra Packard). Different amounts of cocktail were added depending on the amount of glycerol: 7 ml with pure glycerol, 5 ml with 60 and 30 µl glycerol and 2 ml with pure water. Plotting the amounts of radioactive water which had diffused across the cuticles at each air humidity versus time gave good linear transpiration kinetics ($\mathbf{r^2}$ was better than 0.99 in all cases). The permeance was determined using equation 1 with the flow rate F given as $dpm \cdot s^{-1}$, the donor activity Δc given as $dpm \cdot m^{-3}$ and the area of the cuticle given as 1.13 cm^{-2}.

After measuring the transpiration of *Prunus laurocerasus* L. at all 4 different air humidities, the CMs were treated with 0.2 M K_2CO_3. Chambers were left at room temperature in the fume hood until the water was evaporated. Then they were stored again on scintillation polyethylene vials containing silica gel at 25 C° for equilibration. Finally, a new set of scintillation vials with the same 4 different relative air humidities was used to determine water permeability after K_2CO_3 treatment.

Finally, K_2CO_3 was washed off from the CMs, they were stored again in the incubator and transpiration was measured again as described above. The effect of K_2CO_3 before and after treatment and after washing was calculated in the same way as described in paragraph 2.4.

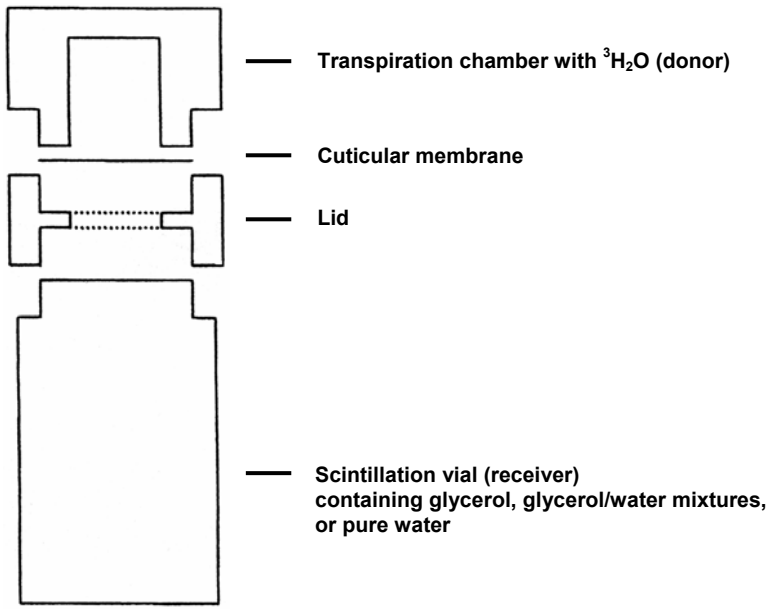

Figure 5. Schematic drawing of the experimental set-up used for the experiment of the effect of K_2CO_3 at different relative air humidities.

2.5.3 Investigation of the effect of AgCl precipitations on cuticular water permeability

Deionized water as control and 0.01 M NaCl solutions were used in a different set of experiments measuring water permeabilities of isolated cuticles. After measuring water permeability, outer surfaces of the cuticles were treated with 0.01 M $AgNO_3$ for 24 hours. This allowed counter diffusion of Cl ions from the inside and Ag ions from the outside, leading to insoluble AgCl precipitations within the cuticle. After this treatment transpiration was measured again and the effect of AgCl crystallites on cuticular flow of water was calculated as described in paragraph 2.5.1.

Subsequently, water permeability of CMs and MXs, which had been treated with $AgNO_3$, was measured again after treatment with 0.2 M K_2CO_3 and after washing off the K_2CO_3 crystallites.

2.5.4 Microscopic investigation of AgCl precipitations in isolated cuticular membranes

For each species 2 to 3 CMs free of any visible defects were selected and incubated with their morphological inner side in Petri dishes containing a 0.01 M NaCl solution. A droplet of $AgNO_3$ was added on the outer surface of the cuticle and the Petri dishes were closed and left at room temperature overnight. The $AgNO_3$ solutions were washed off the next day and the AgCl precipitations within the CMs were investigated using a light microscope (Axioplan, Zeiss, Germany). Untreated areas of the membranes served as controls. Pictures of treated and untreated areas of the membranes were recorded using a digital camera (Nikon digital camera DXM1200). Correlation between crystal size or number of crystals and effects of this treatment on cuticular water permeability was determined.

2.6 Effects of different pH values on cuticular water permeability

Three different pH values were used in this experiment to test the effect of the pH of the donor solution on water permeability of the CMs of *Prunus laurocerasus* L.*, Hedera helix* L. and *Lycopersicon esculentum* Mill. (citric buffer, Fluka, Neu-Ulm, Germany, adjusted to pH 2.9 and 6.9 with NaOH; Disodiumhydrogenphosphat buffer, Merck, Darmstadt, Germany, adjusted to pH 10.9 with NaOH). In this experiment transpiration chambers were used having a sample port in the side of the chamber (Fig. 6). This allowed to exchange the donor solutions and to add donor solutions with varying pH values. Using these chambers, cuticular water permeability of the same cuticle was measured varying the pH values of the donor. Measurements were started with pH 2.9, continued with pH 6.9 and finished with pH 10.9. In these experiments morphological inner sides as well as morphological outer sides facing the donor solutions were tested.

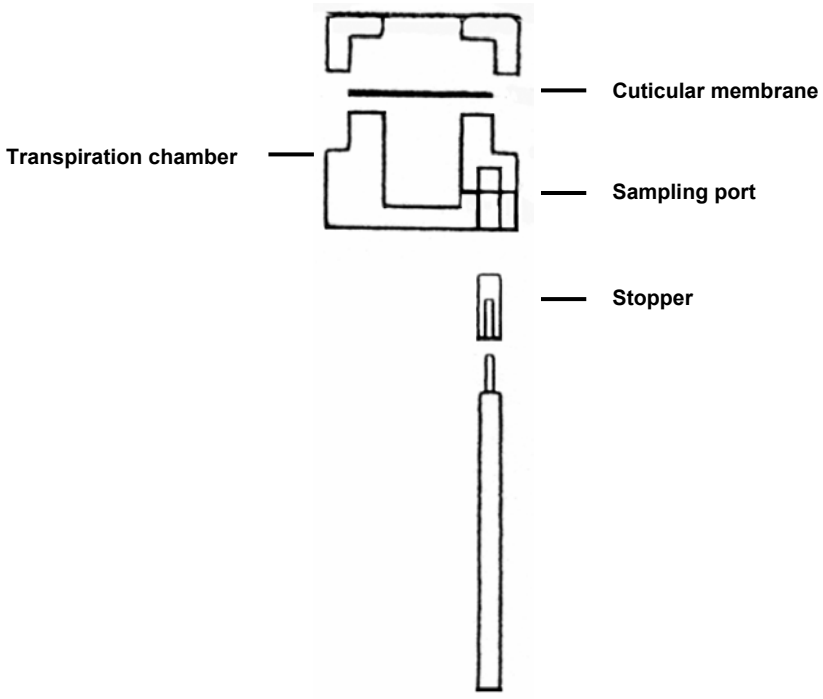

Figure 6. Schematic drawing of transpiration chambers used in experiments testing the effect of pH on water permeability of cuticles. The chambers used had a sample port allowing changes of the donor solution.

In order to test the effect of 0.2 M K_2CO_3 on water permeability of cuticles at three different pH values of the donor, between 12 to 15 chambers were prepared as described above. The permeance and the effect of K_2CO_3 was determined by the method described in paragraph 2.5.1.

2.7 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. Statistical calculations were done using the Microsoft Excel software.

3 RESULTS

3.1 Water permeances of *Hedera helix, Prunus laurocerasus*, and *Lycopersicon esculentum*

Water permeances ($m \cdot s^{-1}$) are presented in Table 3. Permeances of MXs were higher than those of CMs (Tab. 3). 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*.

Table 3. 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	MX
	P ($m \cdot s^{-1}$) ± ci	P ($m \cdot s^{-1}$) ± ci
Hedera helix	$5.7 \cdot 10^{-11} \pm 7.2 \cdot 10^{-12}$	$2.4 \cdot 10^{-8} \pm 1.1 \cdot 10^{-9}$
Prunus laurocerasus	$1.3 \cdot 10^{-10} \pm 9.1 \cdot 10^{-12}$	$8.6 \cdot 10^{-9} \pm 2.4 \cdot 10^{-10}$
Lycopersicon esculentum	$3.9 \cdot 10^{-9} \pm 9.7 \cdot 10^{-10}$	$1.7 \cdot 10^{-8} \pm 7.5 \cdot 10^{-10}$

3.2 Salt effects on water permeability of *H. helix, P. laurocerasus*, and *L. esculentum*

3.2.1 Effect of different salts on cuticular permeability of CM

The effects of salts on water permeability of isolated CM of the three species varied depending on the salts and the species (Fig. 7). While most of the salts (KCl, KNO_3, $(NH_4)_2SO_4$, $NaNO_3$, NaCl, NH_4Cl, $AlCl_3$, and NH_4NO_3) had no pronounced effect, $CaCl_2$, K_2CO_3 and Cs_2CO_3 were very efficient in increasing cuticular water permeabilities of *H. helix, P. laurocerasus*, and *L. esculentum* (Fig. 7). The effects of $CaCl_2$ were 2.41±0.26, 1.29±0.11, and 1.55±0.31 for the three CMs. The effects of K_2CO_3 were 1.43±33, 1.68±0.13 and 2.63±0.28 and the effects of Cs_2CO_3 were 1.52±0.18, 2.60±0.29 and 2.50±0.48. The effects were significantly decreased again by washing the CM with water, although initial low permeances were not fully established again (Fig. 8). Treatment of the CM with deionized water as a control did not influence cuticular water permeability of all three species tested (Fig. 7).

3.2.2 Effect of selected salts on water permeance of MX

MX membranes of the three species *Hedera helix*, *Prunus laurocerasus* and *Lycopersicon esculentum* were treated with the three effective salts $CaCl_2$, K_2CO_3 and Cs_2CO_3. There was no effect on water permeability when MXs of all three species were treated with $CaCl_2$ and there was also no effect when *H. helix* and *P. laurocerasus* MXs were treated with K_2CO_3 and Cs_2CO_3 (Fig. 9). MX of *L. esculentum*, however, was affected by the treatment with K_2CO_3 and Cs_2CO_3 (Fig. 9). Cuticular water permeability was increased by factors of 1.63±0.12 and 1.45±0.9, respectively.

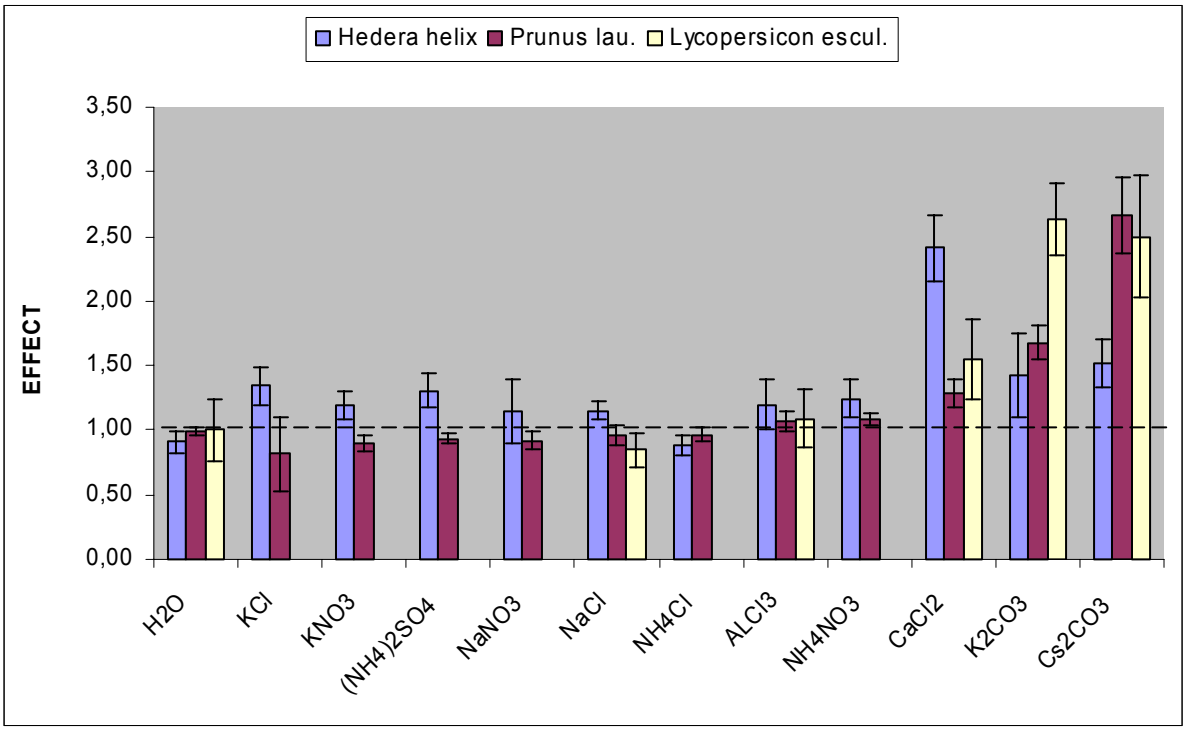

Figure 7. Effect (P_2/P_1) of different salt solutions on water permeability of *H. helix, P. laurocerasus*, and *L. esculentum* CMs. The outer surface of the CM was treated either with water as control or with the different salt solutions (0.2 M). Highest effects with *H. helix* CMs were observed after $CaCl_2$ treatment. Highest effects with *P. laurocerasus* CMs were observed after Cs_2CO_3 treatment and highest effects with *L. esculentum* CMs were observed after K_2CO_3 and Cs_2CO_3 treatment. Between 10 to 15 CMs were investigated. Results represent means with 95% confidence intervals.

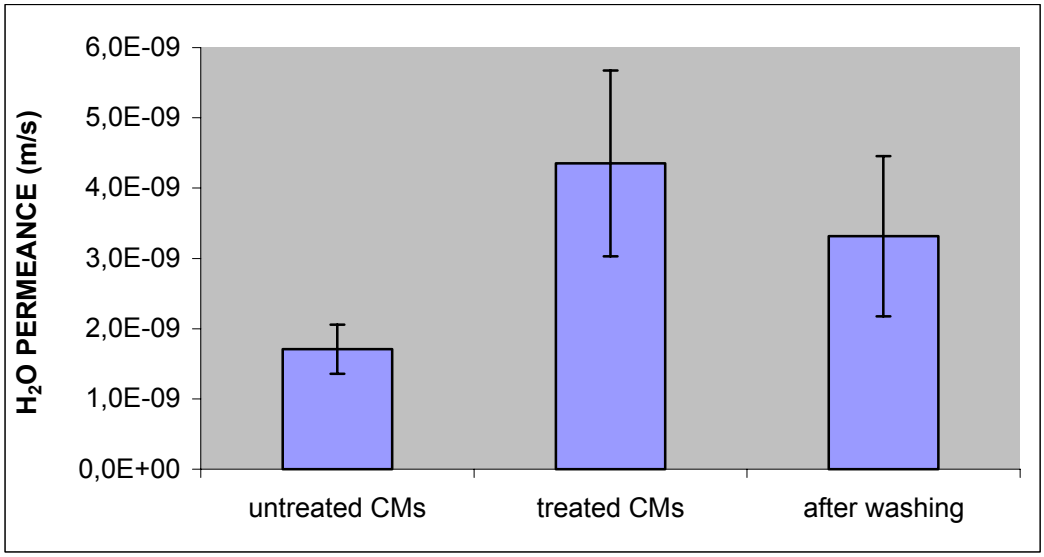

Figure 8. Effect of treatment of *Lycopersicon esculentum* CM with Cs_2CO_3 and subsequent treatment with deionized water. Removal of the salt with water decreased cuticular permeability again although initial low water permeability was not restored again. Results are means of 12 CM with 95% confidence intervals.

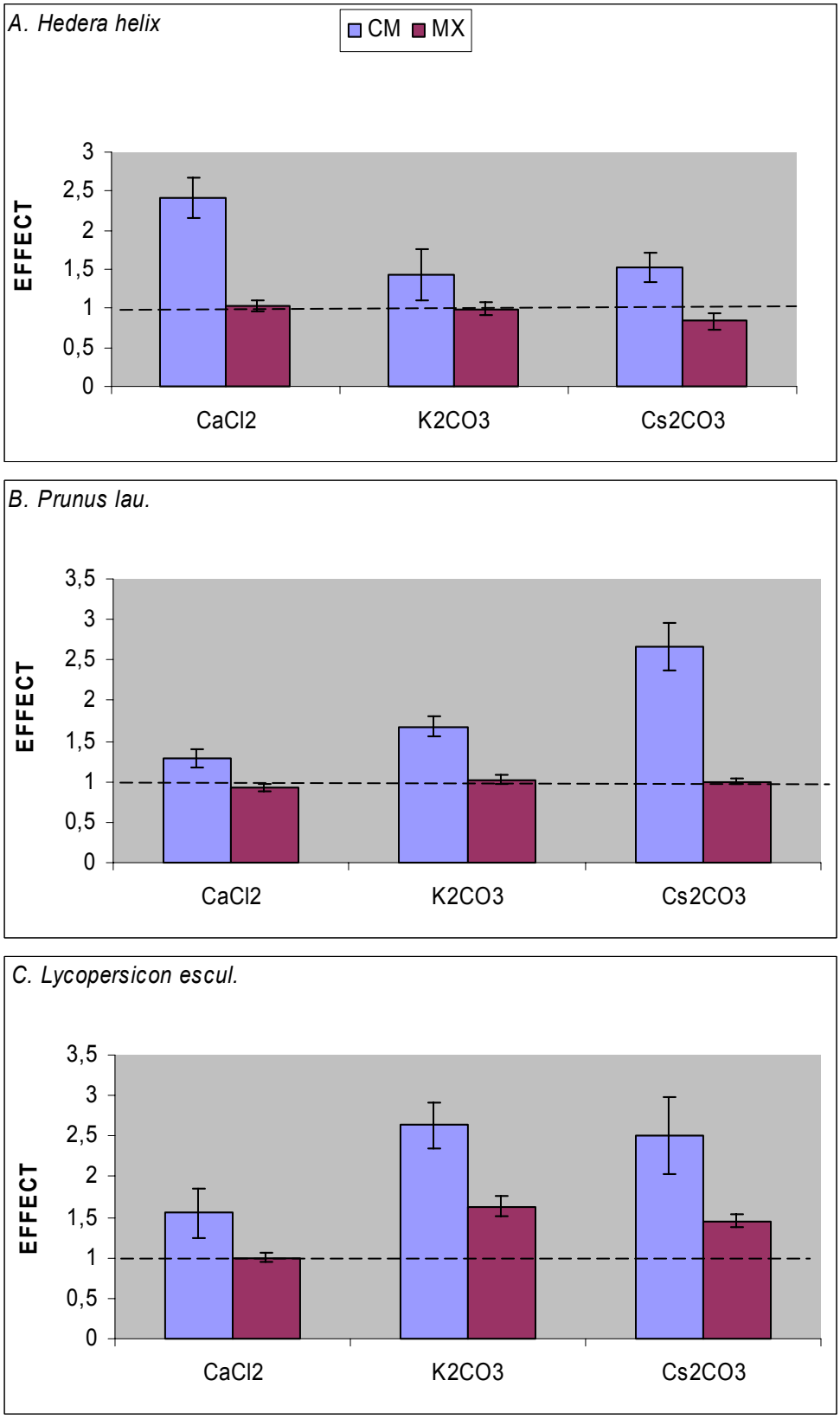

Figure 9. The effect (P_2/P_1) of 0.2 M of three selected salts $CaCl_2$, K_2CO_3 and Cs_2CO_3 on water permeability of *Hedera helix, Prunus laurocerasus* and *Lycopersicon esculentum* CM and MX. Only water permeability of *L. esculentum* MX was affected after treatment with K_2CO_3 and Cs_2CO_3. Error bars present 95% confidence intervals.

3.2.3 Effect of different salt concentrations on water permeability of outer and inner CM surfaces

Treating outer surfaces of *P. laurocerasus* CMs with different amounts of Cs_2CO_3 showed that water permeability was increased by increasing the amounts of salt applied ($mol \cdot m^{-2}$). A linear correlation ($r^2 = 0.99$) between increase of water permeability and amounts of salt applied were observed (Fig. 10). The effects of increasing cuticular water permeability were 2.66±0.29, 4.96±0.71 and 6.19±1.30 when CMs were treated with 0.35, 0.7 and 1.06 $mol \cdot m^{-2}$, respectively.

Treatment of the outer and the inner morphological surfaces of the CM of the three species *H. helix*, *P. laurocerasus.* and *L. esculentum* with different concentrations of K_2CO_3 (0.02, 0.2 and 2 M) showed that inner surfaces of *H. helix* (Fig. 11a) and *P. laurocerasus* (Fig. 11b) were more sensitive than the outer surfaces, whereas there was no pronounced difference with *L. esculentum* (Fig. 11c).

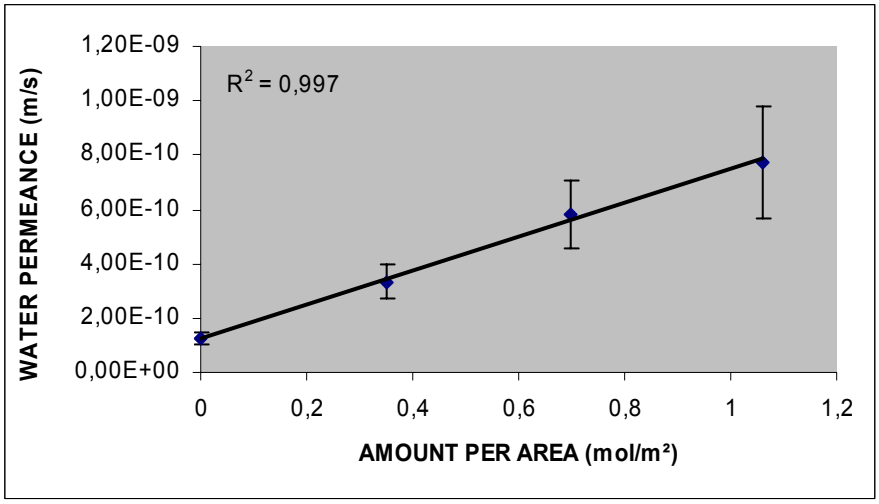

Figure 10. The effect of increasing amounts of Cs_2CO_3 on water permeability of *Prunus laurocerasus*. 200, 400, and 600 µl of Cs_2CO_3 were applied to the outer surface of the CM. Permeance increased from $1.25 \cdot 10^{-10}$ $m \cdot s^{-1}$ before treatment to $7.74 \cdot 10^{-10}$ $m \cdot s^{-1}$ after treatment with highest amounts of Cs_2CO_3. Results are means of 15 CMs with 95% confidence intervals.

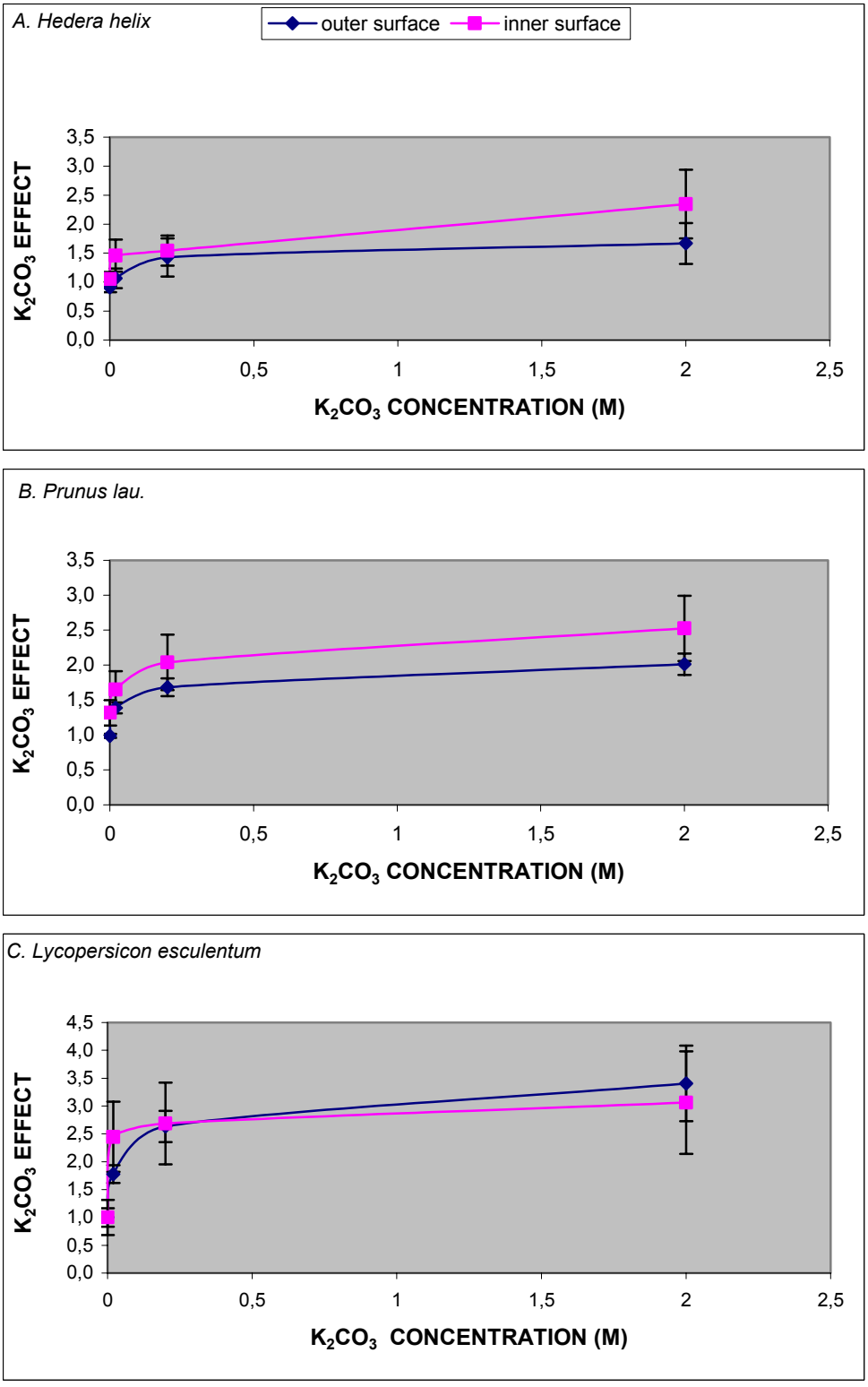

Figure 11. The effect of increasing K_2CO_3 amounts applied either on the morphological outer side or on the morphological inner side on water permeability of *Hedera helix, Prunus laurocerasus* or *Lycopersicon esculentum* CM. Applied amounts were 0.02, 0.2 and 2 M, respectively. Error bars present 95% confidence intervals.

3.2.4 The effect of K_2CO_3 on water permeability of *Prunus laurocerasus* L. at different relative air humidities

Varying air humidity between 2 and 100%, cuticular water permeability of *P. laurocerasus* slightly increased by a factor of 1.2 (Tab. 4 and 5). When CM were treated with 0.2 M K_2CO_3, water permeability of *P. laurocerasus* increased and decreased again after treatment with deionized water (Tab. 4 and 5). The effect of K_2CO_3 was highest, when relative humidity was increased from 2% to 60% (Fig. 12). Increasing relative humidity up to 90% and 100% did not lead to a further increase in water permeability (Tab. 4 and Fig. 12). Washing the CMs decreased the effect on cuticular water permeability again (Fig. 13).

Table 4. The water permeance ($m \cdot s^{-1}$) of *Prunus laurocerasus* CMs treated with 0.2 M K_2CO_3 at different relative air humidity (RH). Permeances are means of 13 CMs with 95% confidence intervals (ci).

RH (%)	untreated CMs P ($m \cdot s^{-1}$) ± ci	treated CMs P ($m \cdot s^{-1}$) ± ci	after washing P ($m \cdot s^{-1}$) ± ci
2	$3.34 \cdot 10^{-10} \pm 1.0 \cdot 10^{-10}$	$6.83 \cdot 10^{-10} \pm 1.80 \cdot 10^{-10}$	$4.43 \cdot 10^{-10} \pm 1.39 \cdot 10^{-10}$
60	$3.38 \cdot 10^{-10} \pm 1.08 \cdot 10^{-10}$	$1.31 \cdot 10^{-09} \pm 3.30 \cdot 10^{-10}$	$5.41 \cdot 10^{-10} \pm 1.63 \cdot 10^{-10}$
90	$3.52 \cdot 10^{-10} \pm 1.29 \cdot 10^{-10}$	$1.16 \cdot 10^{-09} \pm 2.83 \cdot 10^{-10}$	$6.65 \cdot 10^{-10} \pm 1.93 \cdot 10^{-10}$
100	$4.01 \cdot 10^{-10} \pm 1.45 \cdot 10^{-10}$	$1.26 \cdot 10^{-09} \pm 3.17 \cdot 10^{-10}$	$7.66 \cdot 10^{-10} \pm 2.45 \cdot 10^{-10}$

Table 5. Effect of 0.2 M K_2CO_3 on water permeability of *Prunus laurocerasus* CMs at different relative air humidity (RH). The mean values of 13 CMs are given with 95% confidence intervals (ci).

RH (%)	untreated CMs effect ± ci	treated CMs effect ± ci	after washing effect ± ci
2	1.00 ± 0.30	2.24 ± 0.45	1.39 ± 0.30
60	1.02 ± 0.06	4.57 ± 1.40	1.69 ± 0.31
90	1.04 ± 0.09	3.87 ± 1.02	2.05 ± 0.42
100	1.20 ± 0.13	3.67 ± 1.01	2.03 ± 0.43

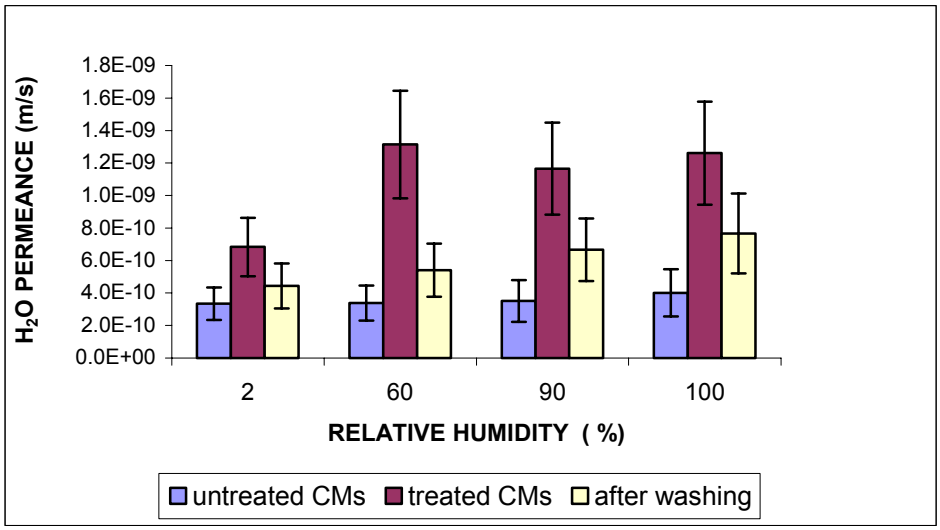

Figure 12. Water permeability of *Prunus laurocerasus* CMs at different air humidities before and after treatment with 0.2 M K_2CO_3 and after washing. Error bars are 95% confidence intervals.

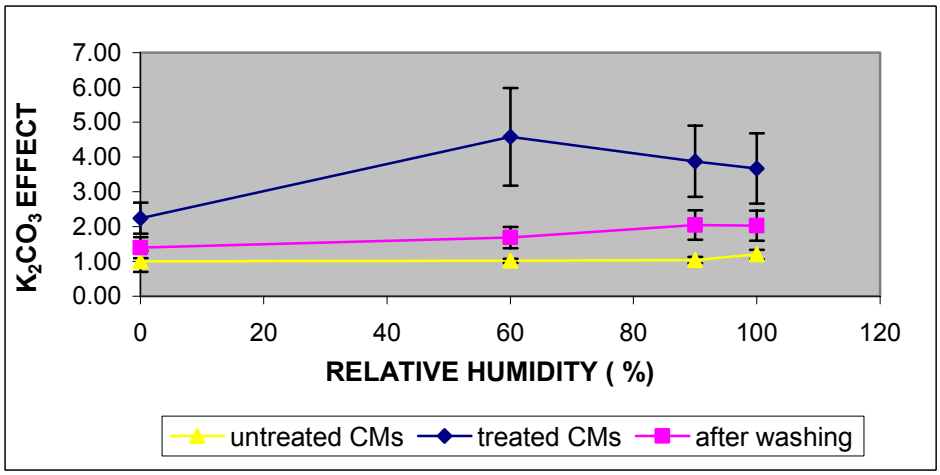

Figure 13. The relative effect on water permeability of *Prunus laurocerasus* before and after treatment with 0.2 M K_2CO_3 at different air humidities. Results are means of 13 CMs with 95% confidence intervals.

3.2.5 The effect of AgCl precipitations on cuticular water permeability

In order to check whether AgCl precipitations in isolated cuticles could effect cuticular water permeability, cuticular transpiration was first measured using 0.01 M NaCl solutions as

donor. Cuticular transpiration of *Populus canescens* using 0.01 M NaCl or deionized water as donor solution gave the same results, since permeances were $3.33 \cdot 10^{-9}$ m·s^{-1} for NaCl and $3.32 \cdot 10^{-9}$ m·s^{-1} for deionized water.

After treating cuticles having 0.01 M NaCl at the inner side of the CM with 0.01 M $AgNO_3$ from the outer side of the CM for 24 hours, cuticular transpiration was measured again. From 15 species investigated, cuticular transpiration of 13 species significantly decreased by factors between 1.13 to 0.38 (Fig. 14). The largest decrease of cuticular water permeability was observed with CM isolated from *Prunus domestica* fruits and *Populus canescens* leaves. In contrast, *Nerium oleander* and *Hedera helix* CMs were not affected by the treatment (Fig. 14 and Tab. 6).

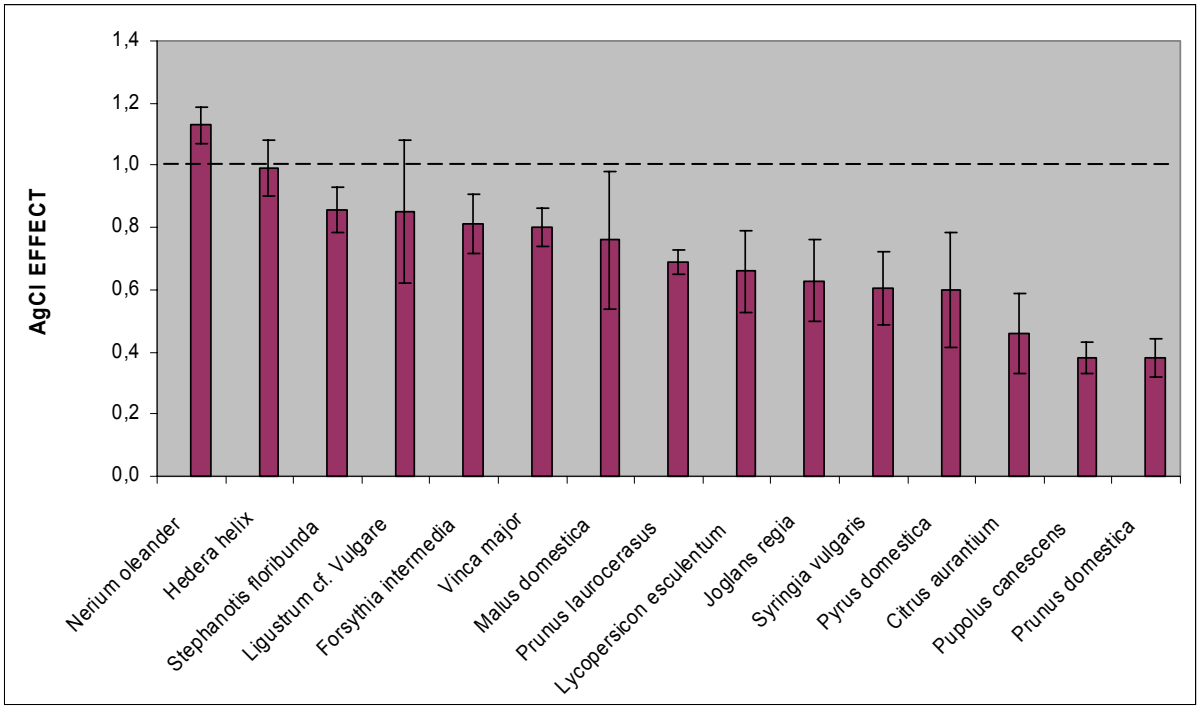

Figure 14. The effect (P_2/P_1) of AgCl precipitations on water permeability of isolated cuticles from 15 different plant species. The values represent means with 95% confidence intervals.

There was no correlation between initial permeance of isolated cuticles before treatment and the effect of AgCl precipitations on water permeability. The initial permeances ranged from $6.56 \cdot 10^{-11}$ m·s^{-1} for *Nerium oleander* leaf CM to $3.45 \cdot 10^{-9}$ m·s^{-1} for *Prunus domestica* fruit CM (Fig. 15).

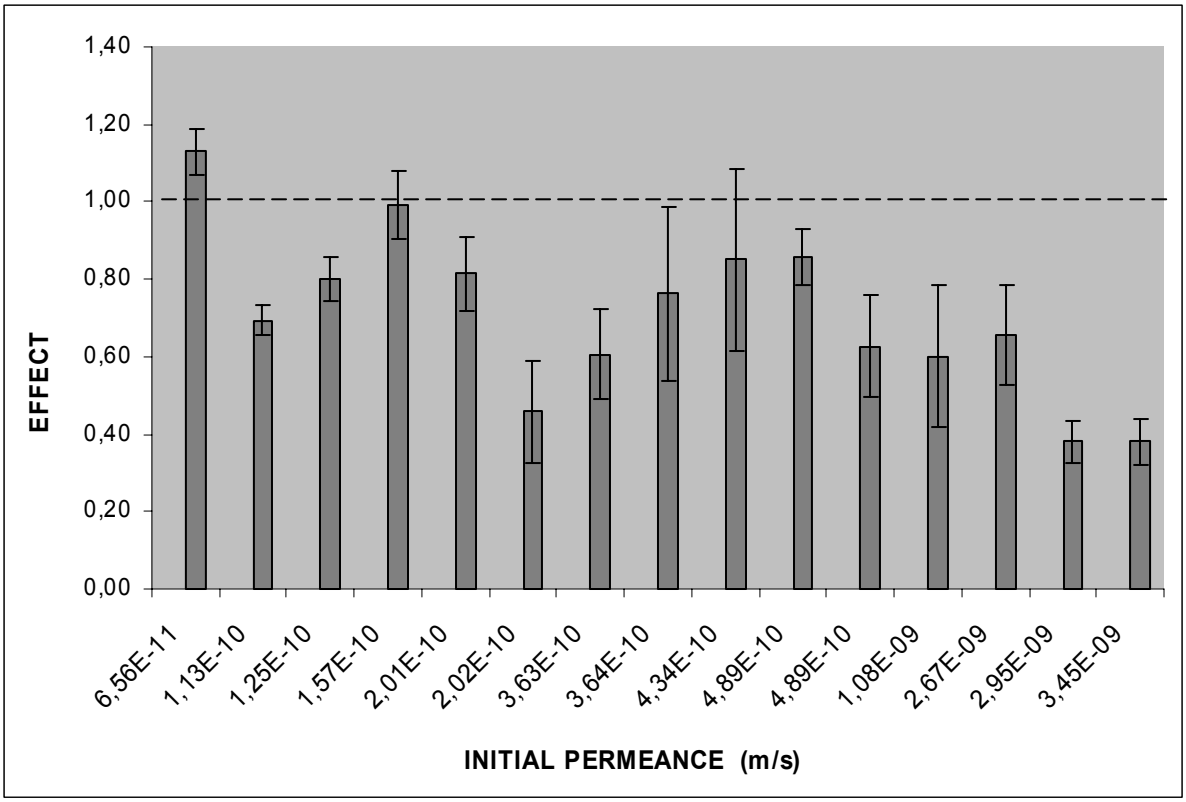

Figure 15. The correlation between the effects (P_2/P_1) of AgCl precipitations and the initial permeances on water permeability of 15 species. Error bars represent 95% confidence intervals. The initial permeance for each species corresponds to the order of the plant names in Table 6.

Interestingly, neither the MX membranes of *Prunus laurocerasus* nor *Hedera helix* were affected by AgCl precipitations in the membrane. The effects of the treatment for both MXs were 1.02 ± 0.04 and 0.95 ± 0.05 respectively. Treatment of the cuticles having AgCl precipitations with 0.2 M K_2CO_3, resulted in significant increases of cuticular transpiration with 14 of the 15 investigated species (Tab. 6).

Table 6. The effect (P_2/P_1) of AgCl precipitations and 0.2 M K_2CO_3 on cuticular water permeability of 15 different species. After determination of the effect of AgCl precipitations, 200µl of 0.2 M K_2CO_3 were added on the outer surface of the CM and the effect of K2CO3 was established again. Results are means of 10 to 20 CMs with 95% confidence intervals.

species	effect of AgCl precipitations Effect (P_2/P_1) ± ci	effect of K_2CO_3 Effect (P_3/P_1) ± ci
Nerium oleander	1.13 ± 0.06	3.22 ± 0.39
Hedera helix	0.99 ± 0.09	1.24 ± 0.12
Stephanotis floribunda	0.86 ± 0.07	1.50 ± 0.19
Ligustrum cf. *vulgare*	0.85 ± 0.23	3.27 ±0.95
Juglans regia	0.63 ± 0.13	6.27 ± 1.5
Forsythia intermedia	0.81 ± 0.09	1.34 ± 0.17
Vinca major	0.80 ± 0.06	2.40 ± 0.27
Malus domestica	0.76 ± 0.22	1.84 ± 0.42
Prunus laurocerasus	0.69 ± 0.04	1.14 ± 0.05
Lycopersicon esculentum	0.66 ± 0.13	1.52 ± 0.38
Syringa vulgaris	0.61 ± 0.12	4.08 ± 1.36
Pyrus communis	0.60 ± 0.18	1.10 ± 0.10
Citrus aurantium	0.46 ± 0.13	0.72 ± 0.20
Populus canescens	0.38 ± 0.05	16.25 ± 5.20
Prunus domestica	0.38 ± 0.06	13.09 ± 3.50

Transpiration rates of *Populus canescens* CMs with AgCl precipitations, strongly increased after treatment with K_2CO_3 (Fig. 16). The effect decreased again after washing, although original low permeabilities were not completely restored (Fig. 16).

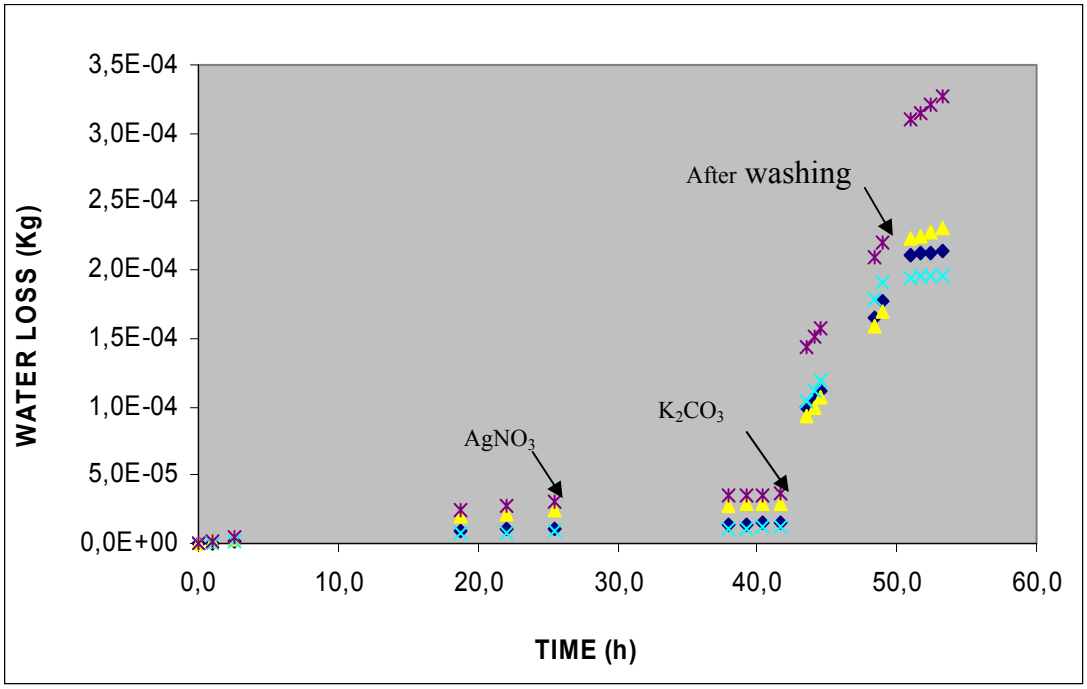

Figure 16. Transpiration rates of four selected *Populus canescens* CMs. 1000 µl of 0.01 M NaCl was used as donor solution. Transpiration was measured again after adding 200 µl (0.01 M) $AgNO_3$ to the outer surface of the CMs for 24 hours. Subsequently transpiration was measured after treatment with 0.2 M K_2CO_3 and after washing.

Plotting the effects of AgCl precipitations on transpiration of each single cuticle versus its initial resistance, weak positive correlation were obtained with *Populus canescens*, *Citrus aurantium* and *Prunus domestica* (Fig. 17). Plotting the mean values of the effects of all species versus their initial resistances again resulted in a weak positive correlation, indicating that species with higher cuticular water permeability showed larger effects of AgCl precipitations (Fig. 18).

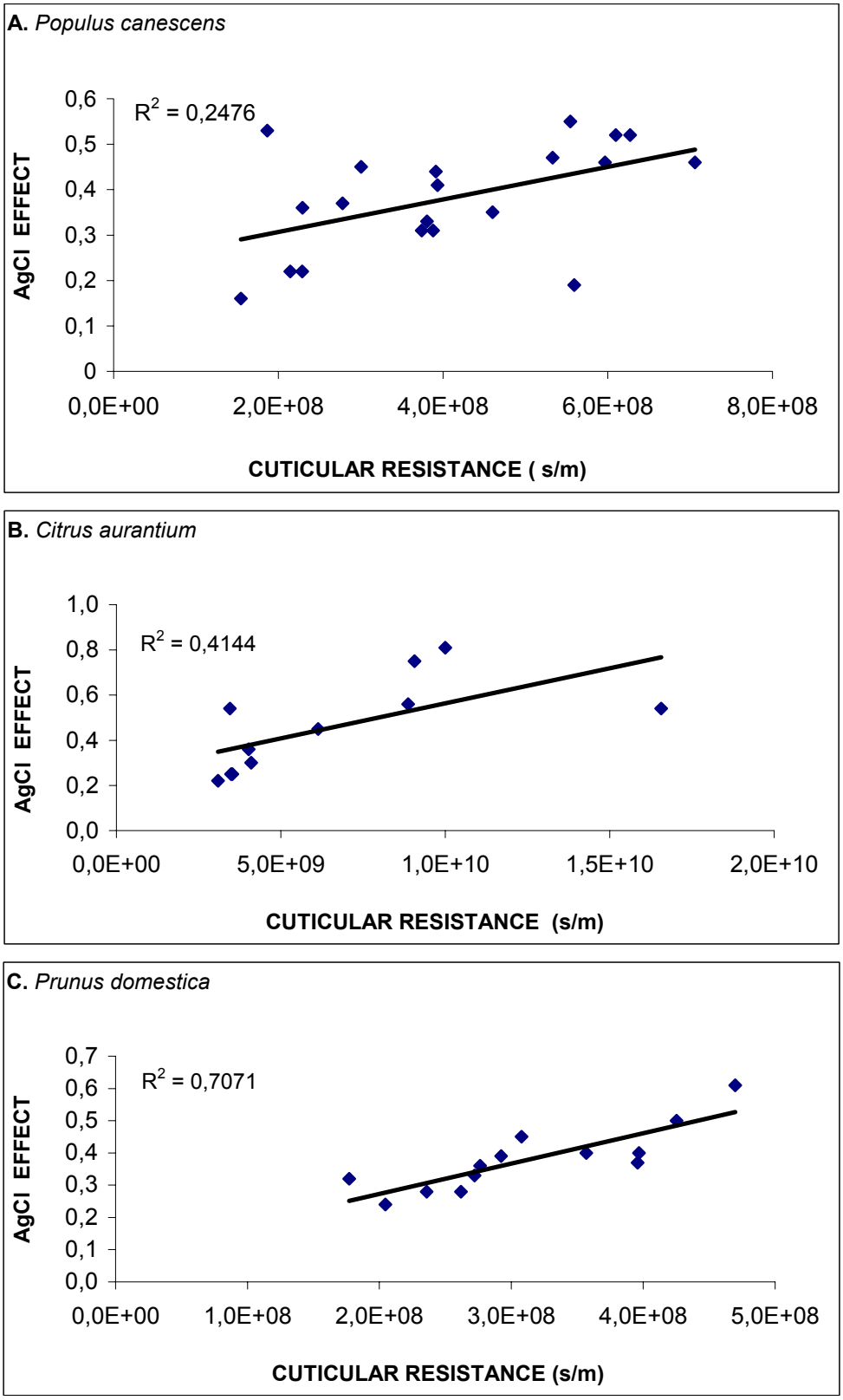

Figure 17. Correlations between effects of AgCl precipitations on cuticular water permeability and initial cuticular resistances of the three species *Populus canescens*, *Citrus aurantium* and *Prunus domestica*.

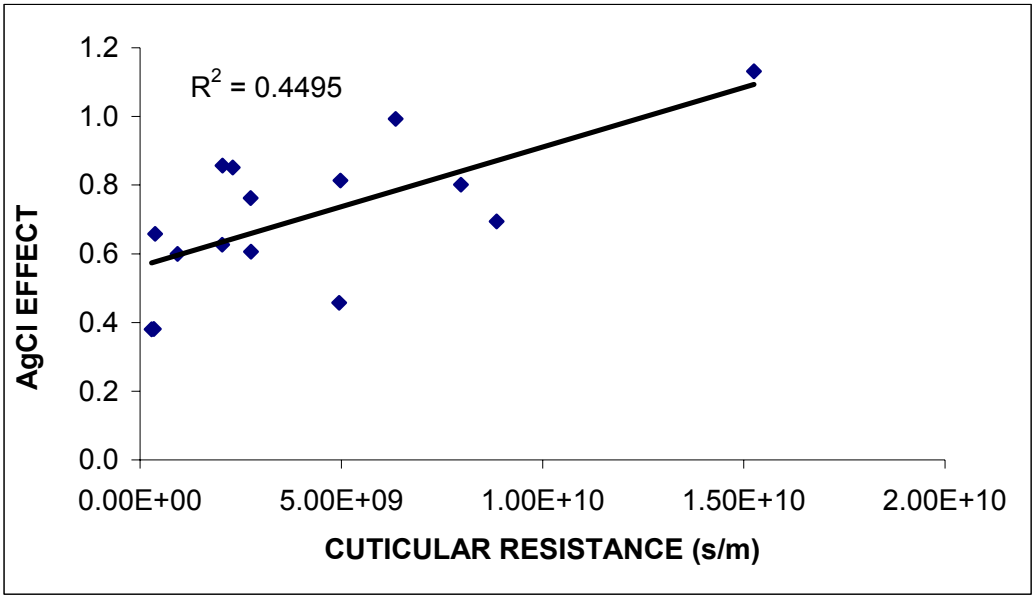

Figure 18. Correlations between effects of AgCl precipitations on cuticular water permeability and initial cuticular resistances of all 15 species investigated.

Light microscopic investigation of CMs allowed to visualize AgCl precipitations as black crystallites. With the exception of *Nerium oleander*, all species investigated showed these AgCl precipitations after counter diffusion of NaCl versus $AgNO_3$. Untreated areas of the cuticles served as control (Fig. 19). The largest crystals were observed in CMs of *Prunus domestica* fruits. Smallest crystals were observed with *Vinca major* CMs (Tab. 7). MX of *Prunus* and *Hedera* was also characterized by the formation of AgCl crystals (Tab. 7).

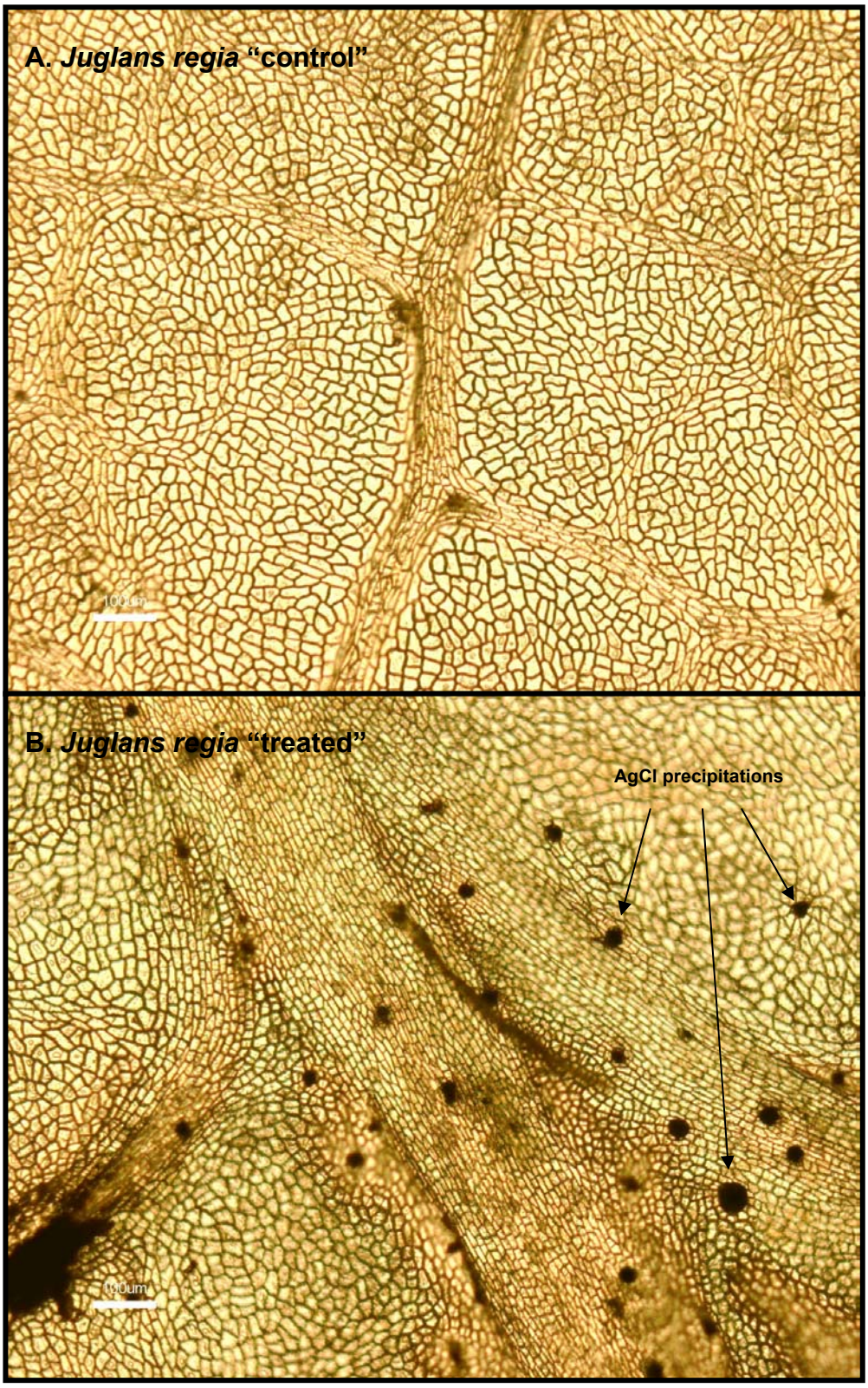

Figure 19. Formation of AgCl crystals in the pores of isolated cuticles of *Juglans regia*. (A) untreated CMs showed no crystals. In contrast, they appeared in treated CMs (B). 0.01 M NaCl was used as donor solution and 0.01 M $AgNO_3$ was added on the outer surface of the CMs.

Table 7. The crystal size of AgCl (µm) and their number per unit area (mm).

Species	Effect of AgCl precipitations	AgCl crystals			
	Effect(P2/P1)± c.i	No./ mm	S.D.	diameter (µm)	S.D.
Nerium oleander	1,13 ± 0.06	0,00	0,00	0,00	0,00
Hedera helix	0,99 ± 0.09	1556,90	80,00	48,45	13,71
Stephanotis floribunda	0,86 ± 0.07	1063.30	146.40	28.07	7.20
Ligustrum cf. Vulgare	0,85 ± 0.23	4670,71	635,60	36,03	9,95
Forsythia intermedia	0,81 ± 0.09	2792,73	254,44	27,16	5,52
Vinca major	0,80 ± 0.06	14401,35	389,23	18,18	5,86
Malus cf. domestica	0,76 ± 0.22	2574,55	423,07	31,75	8,28
Prunus laurocerasus	0,69 ± 0.04	4370,71	177,00	21,76	6,00
Lycopersicon esculentum	0,66 ± 0.13	1890,91	272,12	62,50	20,74
Juglans regia	0,63 ± 0.13	16606,96	3500,63	34,60	14,39
Syringia vulgaris	0,61 ± 0.12	21537,15	4509,35	20,92	9,24
Pyrus communis	0,60 ± 0.18	2509,09	243,93	28,57	5,50
Citrus aurantium	0,46 ± 0.13	3067,71	118.20	66,67	38,64
Populus canescens	0,38 ± 0.05	19772,66	3058,83	34,19	10,02
Prunus domestica	0,38 ± 0.06	1570,91	87,27	90,48	11,00
Prunus laurocerasus MX	1,02 ± 0.04	4670,71	182.56	11,76	4,00
Hedera helix MX	0,95 ± 0.05	20069,44	7686,01	21,27	9,09

3.3 Effect of different pH levels of the donor on cuticular transpiration

Water permeability was weakly increased when the morphological inner surfaces of the CMs of *Hedera helix*, *Prunus laurocerasus* and *Lycopersicon esculentum* were facing different pH values between 2.9 and 10.9 (Fig. 20). When the morphological outer surfaces faced different pH values, water permeabilities of *Hedera helix* and *Prunus laurocerasus* were weakly increased, whereas *Lycopersicon esculentum* showed a slight decrease going from pH 2.9 to 10.9 (Fig. 20). However, due to the large error bars differences were not statistically significant on the 95% level.

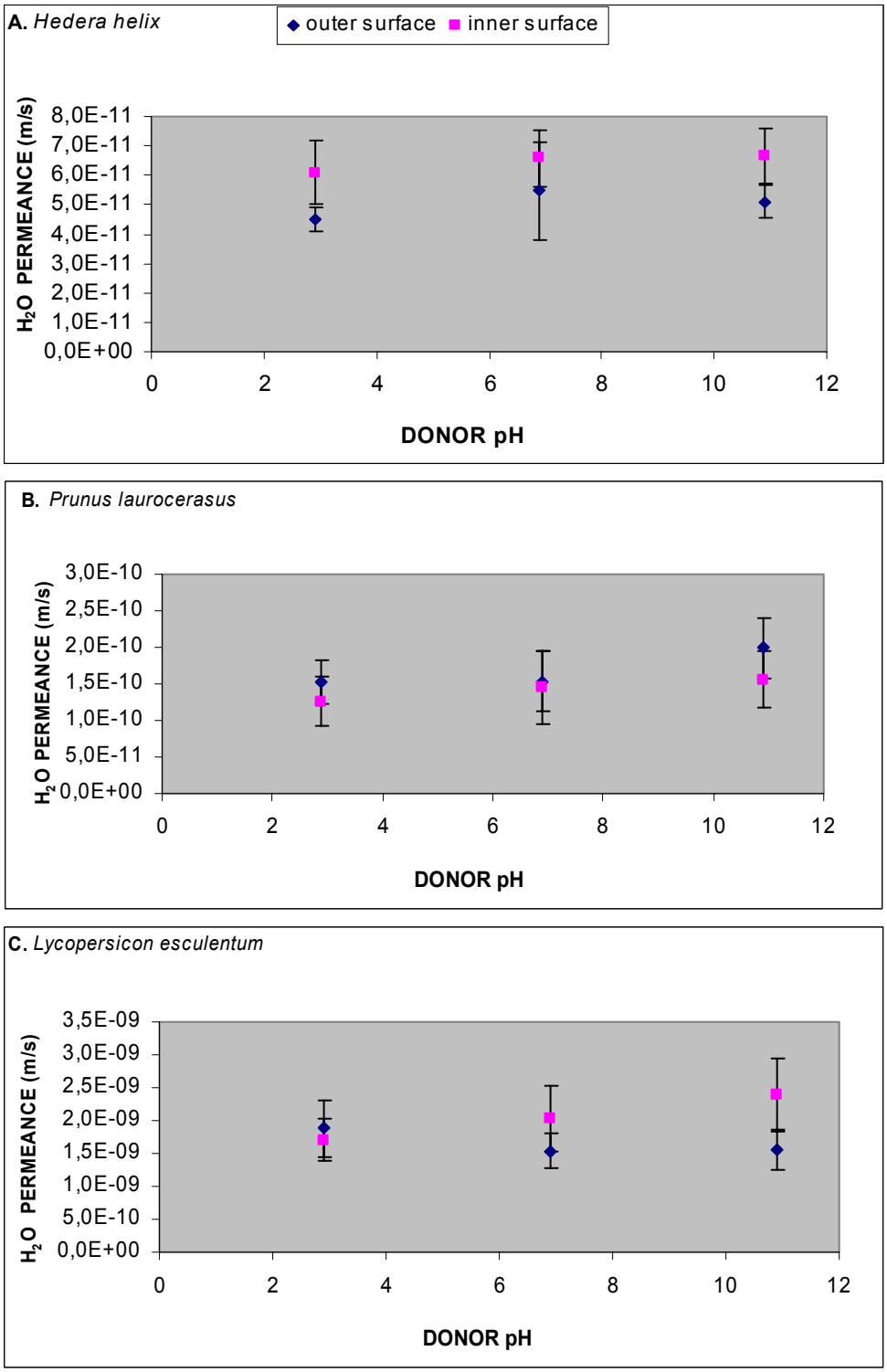

Figure 20. The effect of increasing donor pH on water permeability of *Hedera helix*, *Prunus laurocerasus* and *Lycopersicon esculentum* mounted to the transpiration chambers with the morphological inner side facing the donor and vice versa. No statistically significant differences permeances were obtained with different pH values and different orientations of the isolated CMs. Results are means of 10 to 13 CMs and error bars represent 95% confidence intervals.

4. DISCUSSION

It is well known from the literature that cuticular permeability of different plant species can vary significantly (Riederer and Schreiber 2001). This large variability has been interpreted as an adaptation to the natural habitats of the species and to the type or plant organs analysed (Schreiber and Riederer 1996). Species from tropical habitats had lowest cuticular permeabilities, whereas species from humid climates had significantly higher rates of cuticular water permeability. Highest rates of cuticular transpiration were found with fruit cuticular membranes and this was explained as an adaptation to the short life span of fruits compared to leaves. The three species, *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum*, which were used in most of the studies of this thesis, fit to this classification of cuticular water permeabilities. *Hedera* and *Prunus* as those species with evergreen leaves had by one order of magnitude lower water permeabilities compared to *Lycopersicon* fruit cuticular membranes (Tab. 3). Furthermore, the large increase of cuticular transpiration after wax extraction demonstrated the pivotal role of the cuticular waxes in forming the transpiration barrier of plant cuticles (Schönherr 1982, Tyree et al. 1990).

The simple and inexpensive system used to measure cuticular transpiration in this study, allowed to analyse cuticular water permeability with a high number of parallels and a series of species. Due to its simplicity, relevant boundary conditions of the experimental setup, such as size of the driving forces, cuticle area across which diffusion occurs, and temperature is always known and controlled. The biggest advantage of this system is represented by its sensitivity since combined replicates can be used. One and the same cuticle can be analysed before (as a control) and after a certain treatment (e.g. changes in temperature), and thus possible effects can be determined with high accuracy at statistically high levels. Using this system, effects of inorganic salt solutions, different pH values and different humidities on cuticular transpiration were analysed.

In the past a large amount of information was collected about cuticular permeability of lipophilic non-electrolytes (Schönherr and Riederer 1989). Significantly less is known about permeability of polar ionic compounds across isolated plants cuticles and interaction of ionic compounds with cuticles (Schönherr 2001). Thus, in this thesis the effect of various inorganic salts on cuticular transpiration was analysed. The question was, to what extent inorganic salts could eventually increase rates of cuticular water permeability and whether there are any

possibilities to decrease rates of cuticular water permeability by the deposition of salt crystal into the cutin polymer. These questions were analysed using a series of cuticles isolated from different leaves and fruits. In the following the discussion will focus on various mechanisms of ion interaction with the plant cuticle and to what extent these interactions could lead to increased or decreased cuticular permeabilities of water.

4.1 Effect of cation size on cuticular water permeability

The size of polar pores in *Citrus* and *Allium* cuticles was estimated to be around 0.45 nm (Schönherr 1976c). Since the hydrated diameter of many ions is below 0.8 nm (Tyree et al. 1990), it is highly probable that ions can sorb and move within these pores by diffusion. The data of this study show that the effects of various ions on cuticular water permeability are weakly correlated with the radii of the ions of different charges in all three species (Fig. 21). Much better correlation was obtained, when only monovalent cations, having the largest effects on cuticular water permeability, were selected for the correlations (Fig. 22). Here a clear correlation becomes visible showing that the effect on cuticular water permeability increased with increasing radius of the respective monovalent ion.

From these findings it must be concluded that polar inorganic ions are sorbed to the lipophilic cuticles. As a consequence polarity of the cuticle is increased and increasing amounts of water are sorbed to the cuticular membrane. This leads to a swelling of the membrane and finally to an increased cuticular transpiration (Figs. 7, and 9). Similar conclusions were drawn in earlier publications, where it was shown that water permeance of cuticular membranes increases by increasing the size of the counter ions sorbed to free carboxylic groups of the cutin polymer (Schönherr and Bukovac 1973).

At a first sight it is puzzling that a lipophilic polymer membrane, such as the plant cuticle, offers sorption sites for polar charged ions. These compounds should not be soluble at all in a lipophilic domain. However, this problem can only be solved assuming there is a pronounced lateral heterogeneity in cuticle structure and function (Schönherr and Schreiber 2004). Besides lipophilic domains forming the largest fraction of the cuticular membrane, there are also polar domains in the transport limiting barrier of the cuticle, which offer sites for the sorption of water and ions. With increasing amounts of ions, increasing amounts of water are sorbed and as a consequence cuticular permeability increase.

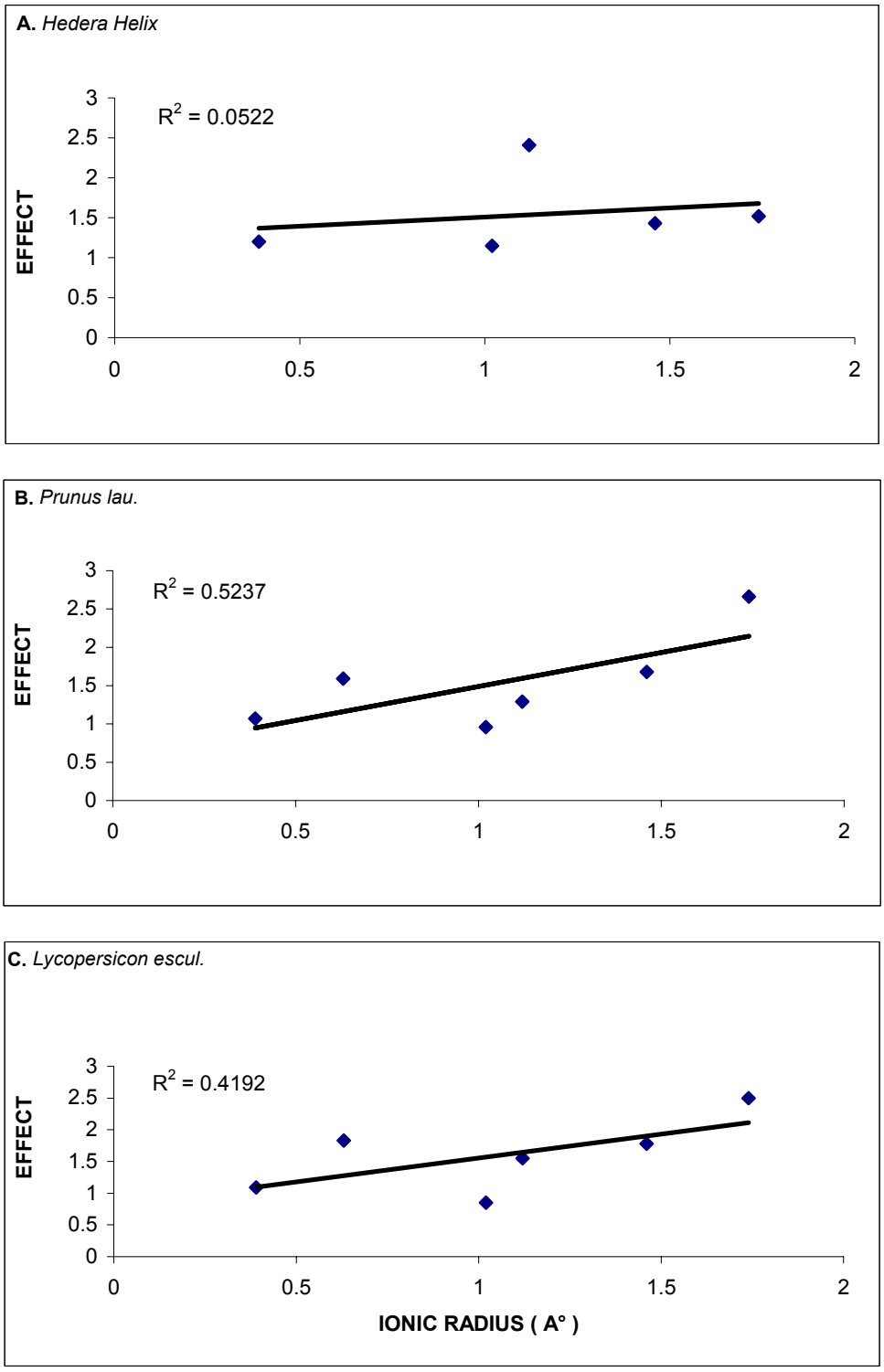

Figure 21. Correlations between the effects of different salts on cuticular water permeability and the ionic radius of the salts. The order of the salts and their radii is; Al^{+++} 0.39, Fe^{+++} 0.63, Na^{+} 1.02, Ca^{++} 1.12, K^{+} 1.46, and Cs^{+} 1.74.

Whereas, the nature of the lipophilic fraction of the cuticle can be described as cutin and wax domains, the structure of the postulated polar domains remains unknown at the moment. It can be speculated that these polar domains within the lipophilic cuticle are formed by polar functional groups of cutin monomers. To a large extent cutin is a polyester of esterified hydroxy fatty acids (Kolattukudy 1981). Polar domains within the lipophilic cutin polymer could be formed by non-esterified free carboxy and hydroxy groups of cutin monomers. This is supported by recent experiments showing that the effect of humidity on cuticular transpiration was reduced by about 50% after methylation of carboxylic groups in plant cuticles (Schreiber et al. 2001). Alternatively, polar sites within the lipophilic plant cuticle could be formed by carbohydrates extending from the outer epidermal cell walls into the cutin polymer and eventually to the outer surface. It is known that plant cuticles contain up to 20 % carbohydrates (Schreiber and Schönherr, 1990).

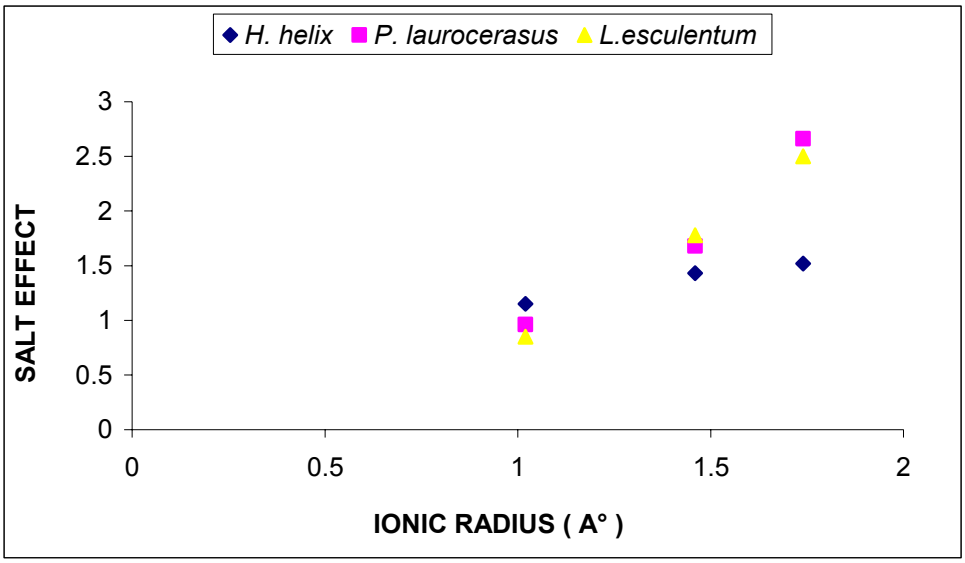

Figure 22. Correlation between the effects of the three salts NaCl, K_2CO_3 and Cs_2CO_3 on cuticular water permeability of three species and the ionic radius of the monovalent cations. Coefficients of determination (r^2) were 0.97, 0.95 and 0.99 for *H. helix*, *P. laurocerasus*, and *L. esculentum*, respectively. The ionic radii 1.02 for Na, 1.46 for K and 1.74 for Cs.

4.2 Effect of wax amounts on cuticular water permeability

The amounts of cuticular waxes covering leaf and fruit surfaces are very different between the different plant species (Tab. 8). 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). Nevertheless, observed differences in the effects of the different cations on water permeability of the different species (Fig. 9) might be due to differences in wax amounts (Smalley et al. 1993). However, as shown in Figure 23 there is no correlation between salt effects and wax amounts of the three species *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum*. Since the wax is even more hydrophobic than cutin, charged molecules such as ions will not sorb to cuticular waxes and thus an effect of wax amounts to the observed effect on cuticular water permeability can not be expected.

Table 8. Amounts of wax (µg · 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 (µg · cm^{-2}) ± ci
Prunus laurocerasus	211.6 ± 65.7
Hedera helix	80.5 ± 9.4
Lycopersicon esculentum	55.4 ± 9.2

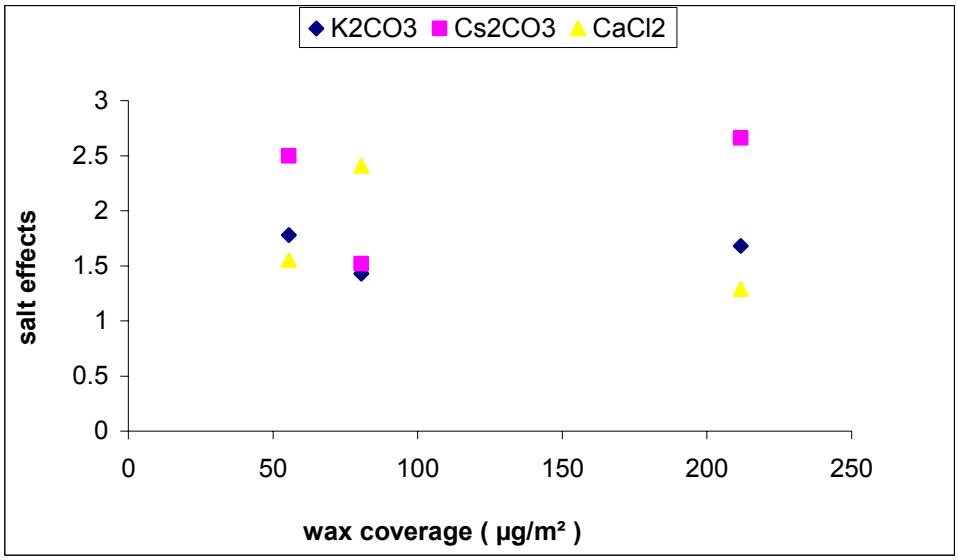

Figure 23. Correlation between the effects of 3 salts on cuticular water permeability of the three species *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum*. and there CMs wax coverage.

It is surprising that effects of the salts on cuticular water permeability of MX membranes are much weaker than on CM (Fig. 9). One would expect that MX membranes offer significantly more polar sites of sorption for ions since the very hydrophobic wax molecules, which are solid and partially crystalline at room temperature and thus do not form sorption sites for ions, have been removed (Schönherr and Bukovac 1973, and Schönherr 1976a). However, overall permeability of the cuticles was significantly increased by more than a factor 100 (Tab. 3). Thus, sensitivity of the cuticles was probably lost and the effects of the salts on cuticular water permeability are not anymore relevant at 100 times higher water permeabilities. Furthermore, it was shown that ion permeability of cuticles was rarely affected by wax extraction (Schönherr 2000, Tyree et al. 1992). This is very good evidence that lipophilic cutin domains and polar charged cutin domains are spatially separated and independent from each other.

4.3 Effect of different salt concentrations on cuticular water permeability

Treating the morphological inner and outer sides of the cuticles of the three species *Prunus laurocerasus*, *Hedera helix* and *Lycopersicon esculentum* with increasing amounts of K_2CO_3 resulted in an increase of the effects on water permeability (Fig. 11). An excellent linearity ($r^2 = 0.99$) was observed when *Prunus laurocerasus* CMs were treated with increasing amounts of Cs_2CO_3 (Fig. 10). In most cases a higher effect was obtained when the morphological inner side of the cuticle was treated with the salt. This can be explained by the fact that the plant cuticle has a pronounced asymmetry (Tyree et al. 1990). The cuticle is more hydrophobic and denser at the morphological outer side and more hydrophilic and polar at the morphological inner side. This is due to the fact that the cutin polymer originally was connected to the carbohydrate cell wall and after isolation there are still carbohydrates attached to the inner surface of the isolated cuticle rendering this side more polar and hydrophilic. Sorption of ions is strongly affected by the amount and density of fixed charges and since they are present in higher amounts at the inner side of the isolated cuticles, effects of salts are more pronounced when they are applied at this side of the cuticle. Applying a mixture of the two salts K_2CO_3 and Cs_2CO_3 (0.2 M) onto *Prunus laurocerasus* CMs showed that the effect was the sum of the effects of the salts when applied separately (Fig. 24). This shows that the salts obviously act independently.

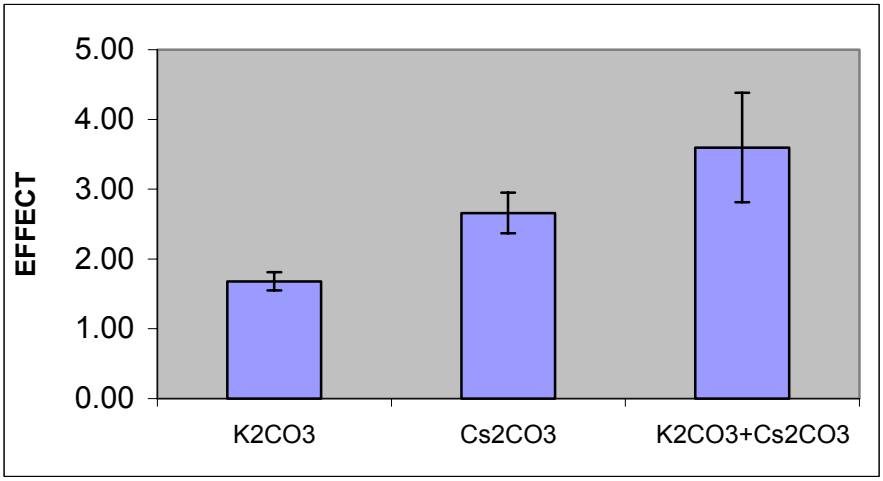

Figure 24. The effect of K_2CO_3 and Cs_2CO_3 applied separately and together on water permeability of *Prunus laurocerasus* CM. Error bars are 95% confidence intervals.

4.4 Effect of different humidities on cuticular water permeability

In order to be able to sorb to the cutin polymer, the salts deposited to the cuticle surface have to be in a liquid state (Schönherr 2001, Schlegel and Schönherr 2002). If they dry out they will crystallize and this renders them completely immobile. Hydration and dissolution of salts is determined by their point of deliquescence "POD" (Schönherr 2001). This point refers to the humidity over a salt solution containing solid salts. When the humidity is above the POD, the salt residue on the cuticle sorbs water from the atmosphere (Schlegel and Schönherr 2002), dissolves and the ions of the salt are mobile and can diffuse into the cutin polymer, while below the POD this process stops.

The effect of K_2CO_3 on cuticular water permeability of *Prunus laurocerasus* was higher than that of KCl and KNO_3 (Fig. 7). These differences can partially be explained by the POD of these salts. The POD of K_2CO_3 is 44%, while KCl has a POD of 86% and that of KNO_3 is 95%. This means that K_2CO_3 is in a liquid state at much lower humidities than KCl and KNO_3 and thus it is more efficient in changing cuticular transport properties for water. This corresponds to reports that rate constants of potassium salt penetration through plant cuticles was increased by increasing humidity (Schönherr and Luber 2001).

In order to analyse to what extent increasing humidities could interact with the salt effects, cuticular water permeability was measured before and after K_2CO_3 deposition to the outer surface of the cuticle of *Prunus laurocerasus* at increasing humidities (Fig. 12). It is evident that much higher water permeabilities were measurable already at much lower

humidities with salt treated cuticles compared to untreated cuticles (Figs. 12 and 13). This observation must be explained by the fact that the presence of water is pivotal for the salts to become mobile and effective. If there is more water available due to high humidities, compared to the small amounts of salts diffusing through the cuticle from the inside, the effects of the salts on cuticular permeability will be more pronounced (Fig. 13).

Thus, in addition to the observation that cuticular permeability is increased by increasing humidity (Schreiber et al. 2001), this effect can significantly be enhanced adding salts to the leaf surface. Here again the question arises, how barrier properties of a lipophilic membrane can be affected by increasing humidities. The answer must be a similar one as already given above. These results are good evidence that there are polar domains in the cuticle where water molecules can sorb and induce swelling, which in turn leads to an increase of cuticular transpiration. This indicates again that there must be a lateral heterogeneity in cuticle structure and that polar domains in the cuticle are sensitive to the sorption of water. This effect is enhanced in the presence of salts.

4.5 Effect of AgCl precipitations on cuticular water permeability

Water permeances of isolated CMs can be blocked by AgCl precipitation within the cuticular membranes (Fig. 14). Decreases in water permeability are most likely caused by blockage of aqueous polar pores traversing the cutin polymer (Schönherr 2000). Due to the counter diffusion of NaCl applied at the inner side of the cuticle and $AgNO_3$ applied at the outer side of the cuticle, Cl and Ag ions meet within the polymer and thus are precipitated. Comparable results reducing cuticular transpiration of cherry fruit cuticles with Al^{3+} and Fe^{3+} precipitates were reported (Beyer et al. 2002). They argued that $FeCl_3$ and $AlCl_3$ ions sorbed to the cutin polymer and were precipitated as insoluble hydroxides in polar domains of the cuticle thus decreasing the free volume of the polymer matrix available for the diffusion of water.

In a similar way as the effect of the salts on cuticular water permeability of MX membranes was rarely measurable (Fig. 9), there was also no effect of AgCl precipitations on MX membranes (Tab. 7). Here again it must be argued that MX membranes have a much higher overall permeability and thus are not anymore sensitive enough (Tab. 7). Among the CM of 15 species investigated, only 2 of them, *Nerium oleander* and *Hedera helix*, were not affected by AgCl precipitations (Tab. 6). Since initial cuticular water permeabilities of these two species were already very low (Fig. 15), the cuticles of these species obviously are very

dense and do not offer enough free volume for AgCl precipitations. However, with the other 13 species pronounced effects of AgCl precipitations on cuticular water permeability were observed (Fig. 14). Reasonable correlations were obtained when effects of AgCl were plotted versus the initial resistances of the investigated cuticles. This shows that cuticles having a very low initial resistance were most sensitive towards AgCl precipitations (Figs. 17 and 18). Those cuticles obviously have a pronounced polar domain offering enough free volume for the deposition of AgCl crystals. Thus, it can be postulated that water permeability should depend on the number and/or the size of polar aqueous pores (Schönherr 1976c), which are probably blocked by AgCl.

A weak correlation was in fact obtained when the size of the AgCl crystals was plotted versus the effects of decreases in cuticular water permeability (Fig. 25 a). This was not the case when the number of AgCl crystals was plotted as a function of the decreasing effects on cuticular transpiration (Fig. 25 b). Obviously, the radius of the pores is more important for transcuticular diffusion of polar and charged molecules than the absolute number of pores. In a following experiment, effects of K_2CO_3 on cuticles with AgCl precipitations were tested (Tab. 6). Since there were still pronounced effects of K_2CO_3 increasing cuticular permeability of water, it must be concluded that not all of the polar pores were fully blocked. Obviously, some of them were still accessible to the applied salt leading to increased rates of cuticular transpiration (Tab. 6).

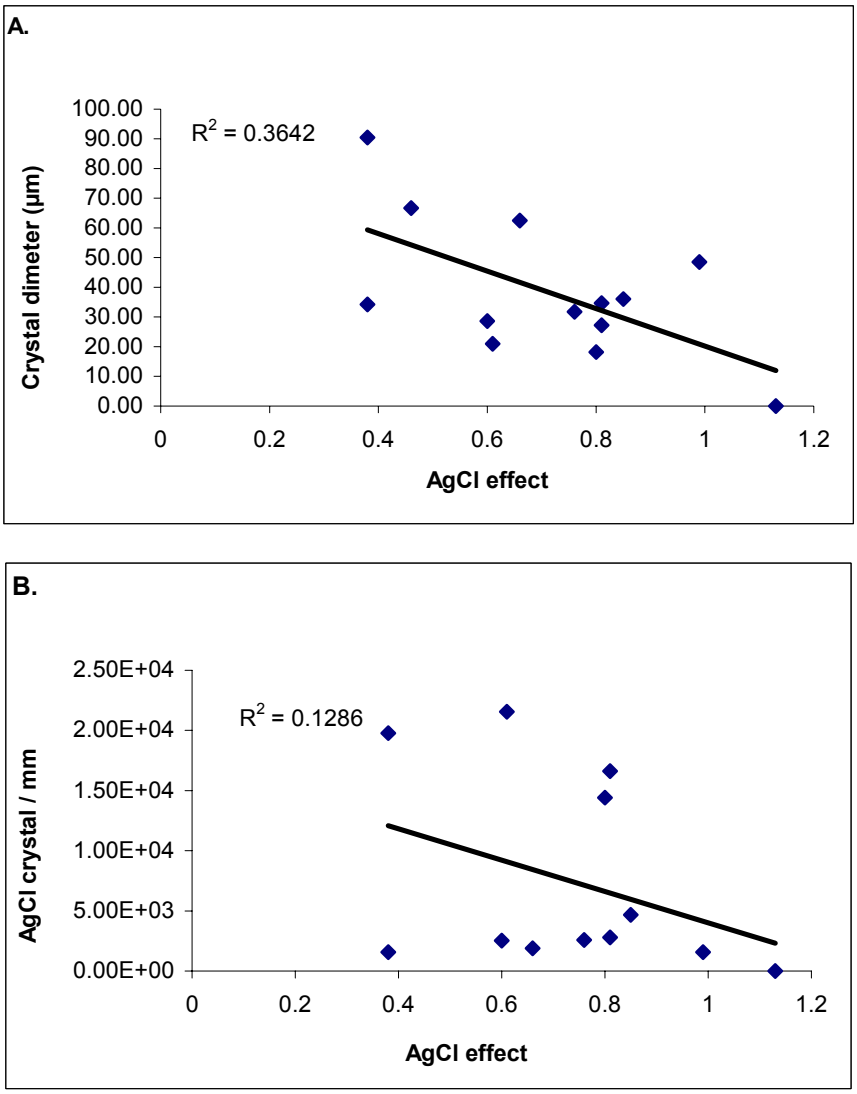

Figure 25. Correlations between size (a) and number (b) of AgCl precipitations in cuticular membranes and their effects on cuticular water permeability.

4.6 Effect of different pH values on cuticular water permeability

Since CMs carry weak acidic charges, the external solution pH could have an effect on the water content of the cuticle and thus increase water permeability. Such an effect was demonstrated for MX and CM membranes (Schönherr 1976a, and Luque et al. 1995). The pH values of the salts used in the experiments presented here are given in Table 2. The salts with the highest effects, K_2CO_3 and Cs_2CO_3, both had the highest pH values (around 11) and for this reason, their effects were probably also due to the pH effect. It was in fact found that water permeability of the cuticles weakly increased, when their inner surfaces were exposed to increasing pH values (Fig. 20).

It was shown previously that increasing water permeability of cuticular membranes was due to a higher water content of the polymer matrix caused by the dissociation of fixed non-esterified carboxylic groups in the cutin polymer (Schönherr 1976a). Since cuticles are polyelectrolytes and since their isoelectric points around pH 3, an increase of the pH leads to the dissociation of the carboxylic groups. This increases the numbers of polar sites in the cuticles significantly since more negative fixed charges are present and more water molecules will be sorbed to the polymer matrix. This in turn leads to an increase in cuticular water permeability.

4.7 Effect of washing off the salt solutions on cuticular water permeability

Finally, in all experiments where cuticles have been treated with salts and as a consequence an increase in cuticular water permeability was observed, effects were again significantly reduced by washing the cuticles (Fig. 9). Although, original low permeabilities were never established again (Fig. 9), the fact that washing reduces cuticular permeability again, indicates that the increase in cuticular water permeability is at least partially reversible. Thus, salt/cutin interactions probably did not lead to irreversible damages of the cutin polymer.

5. SUMMARY

The effect of 0.2 M inorganic salt solutions on cuticular transpiration of isolated cuticular membranes (CM) of the three species *Hedera helix* L., *Prunus laurocerasus* L., and *Lycopersicon esculentum* Mill. was measured. Water permeability was not increased by $AlCl_3$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, KNO_3, KCl, $NaNO_3$ and NaCl. However, when cuticles were treated with $CaCl_2$, K_2CO_3 and Cs_2CO_3, cuticular transpiration was significantly increased. Depending on the species, relative effects of the tested salt solutions varied between 1.5- to 3-fold. Wax-free polymer matrix membranes (MX) of *H. helix* and *P. laurocerasus* were not affected by the 3 salts, whereas, cuticular transpiration of *L. esculentum* MX significantly increased, after treatment with K_2CO_3 and Cs_2CO_3. Effects on cuticular transpiration were positively correlated with the increasing cation radii of the monovalent cations.

Water permeability increased linearly with the amounts of K_2CO_3 applied per cuticle surface area. Treatment of the morphological inner sides of the CM with different concentrations of K_2CO_3 was more effective increasing cuticular water permeability than treatment of the morphological outer sides. There was no correlation between the wax coverage of the cuticles and the effects of the salts. Using three different pH values (2.9, 6.9 and 10.9), an increase of cuticular permeability was observed with *P. laurocerasus* at the high pH value of 10.9. At high external air humidities, effects of K_2CO_3 on water permeability of *Prunus laurocerasus* L. CM were significantly increased by a factor 4.57. Salt effects were partially reversible when cuticles were washed with water. It is argued that inorganic salts were sorbed to polar domains of the cuticles. As a consequence amounts of water sorbed to the cuticles were increased, which in turn increased rates of cuticular transpiration.

Furthermore, it was shown for the first time in this thesis, that polar domains in cuticles, potentially serving as polar paths of diffusion for polar, charged molecules like inorganic salts, could be blocked by the precipitation of AgCl crystallites in the cuticular membranes of 15 species. AgCl precipitations significantly reduced cuticular transpiration between 2 to 3-fold in 13 of the15 species investigated. Strongest effects were observed with *Prunus domestica* fruit CM and *Populus canescens* leaf CM. Investigation of treated CM by light microscopy clearly showed black AgCl precipitations and effects on water permeability of cuticles increased with increasing diameters of the AgCl precipitations.

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