CUTICULAR TRANSPORT OF HYDROPHILIC MOLECULES WITH SPECIAL FOCUS ON PRIMARY METABOLITES AND ACTIVE INGREDIENTS

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
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KUTIKULÄRER TRANSPORT VON HYDROPHILEN PRIMÄRMETABOLITEN UND AKTIVSUBSTANZEN

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Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Dr. Ulrich Scheer

Gutachter: Prof. Dr. Markus Riederer

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I. CONTENTS

II. FIGURES

III. TABLES

IV. Abbreviations

τ	Tortuosity of the diffusional path length
$\%_{max}$	Maximum cuticular penetration [%]
2,4-D	2,4-dichlorophenoxyacetic acid
95% CI	95% confidence interval
A	Area [m²]
CM	Cuticular membrane
D	Diffusion coefficient [$m^2\ s^{-1}$]
E_A	Activation energy [$kJ\ mol^{-1}$]
F	Flow [$mol\ s^{-1}$]
H(λ)	Diffusional hindrance factor
K	Partition coefficient
k	Rate constant [h^{-1}]
$K_{A/W}$	Air/water partition coefficient
$K_{C/A}$	Cuticle/air partition coefficient
$K_{C/W}$	Cuticle/water partition coefficient
K_M	Michaelis-Menten constant [Substrate concentration]
$K_{MX/W}$	Matrix membrane/water partition coefficient
$K_{O/W}$	1-Octanol/water partition coefficient
l	Limiting skin [m]
m	Mobility (P/K) [$m\ s^{-1}$]
MV	Molar volume [$cm^3\ mol^{-1}$]
MW	Molecular weight [$g\ mol^{-1}$]
MX	Matrix membrane (dewaxed cuticular membrane)
P	Permeance [$m\ s^{-1}$]
pk_a	Dissociation constant
POD	Point of deliquescence
R	Gas constant [$J\ K^{-1}\ mol^{-1}$]
λ	Ratio of the radius of the diffusing molecule and the effective pore radius of the membrane
RH	Relative humidity
SE	Standard error
ß’	Size selectivity [$mol\ cm^{-3}$]
T	Temperature [°C]
$t_{max}/2$	Time needed for half of the maximum cuticular penetration
V_f	Mean free volume [$cm^3\ mol^{-1}$]
v_{max}	Maximum velocity of a catalysed reaction [velocity]
WS	Water solubility [$mol\ kg^{-1}$]
Δ x	Membrane thickness [m]

1. INTRODUCTION

1.1 The plant cuticle – chemistry, fine structure and function

The presence of a superficial membrane on plants has been known for about more than 200 years. Brongniart (1830-34) showed it to be a continuous, non-cellular structure moulded on the epidermal cells and named it 'cuticle'. Brongniart concluded that the membrane was little permeable to liquid or gas, and that its essential function was to protect the plant against excessive evaporation of water.

All primary aerial surfaces of vascular plants and some bryophytes are covered by a thin, continuous film, the cuticle, which is composed of soluble and polymeric lipids (Jeffree, 1996). The cross-linked polymer cutin is in principle of very great molecular weight, constructed of inter-esterified aliphatic hydroxy acids, with chain-length of C_{16} and C_{18} (Jeffree, 1996). The cuticular waxes consist principally of alkanes, alkanols, alkanoic acids, alkylesters and cyclic compounds like triterpenols (Bianchi, 1995). These waxes are embedded within the cutin matrix (intracuticular) and also make up epicuticular films and aggregates (Riederer & Markstädter, 1996). A detailed composition of the cuticle is described elsewhere (Holloway, 1982; Kolattukudy, 1984; Holloway, 1994; Bianchi, 1995; Jeffree, 1996). The thickness of plant cuticles varies from less than 0.1 µm to more than 10 µm (Holloway, 1994), but thickness is not related to permeability (Schönherr, 1982; Schönherr & Riederer, 1989; Kerstiens, 1996).

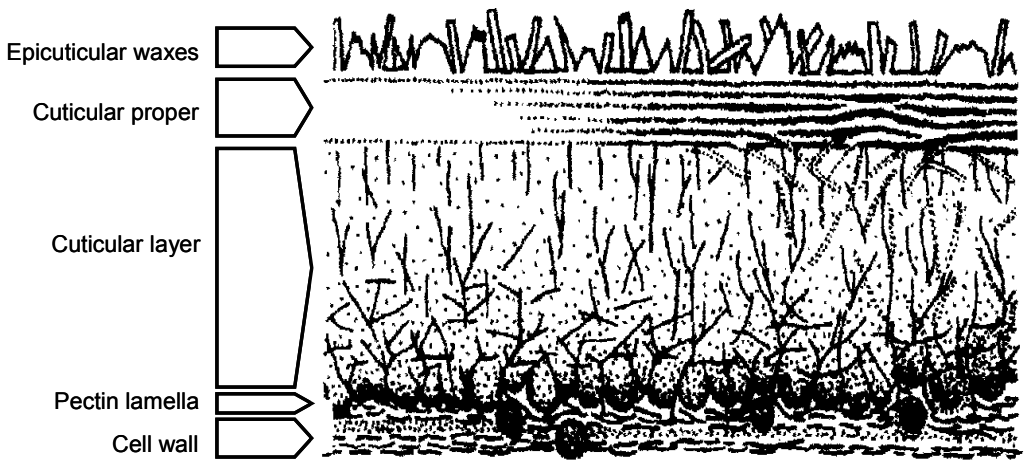


Figure 1.1 Schematic cross-section of the plant cuticle (Taken from Jeffree, 1996).

In most plants the cuticular membrane is not structurally or chemically homogenous but composed of a number of layers (Fig. 1.1) (Holloway, 1982). The first layer in all terrestrial plants is epicuticular wax. Beneath the layer of epicuticular wax is found the cuticle proper which contains no cellulose or cell wall materials. In many species the cuticle proper is of lamellate structure (Jeffree, 1996). Interestingly, there are species which lack a cuticle proper like *Ilex aquifolium, Prunus laurocerasus* as well as *Lycopersicon* fruits (Lendzian & Kerstiens, 1991). Below the cuticle proper one or more cuticular layers may be present and frequently form the bulk of the cuticle when it is fully developed (Holloway, 1982). This reticulate layer contains cutin, embedded waxes and also embedded polysaccharides (Jeffree, 1996). The identity of the polysaccharide microfibrils is poorly defined, but probably includes pectin, crystalline cellulose microfibrils, and hemicelluloses (e. g. xylan and xyloglucan). Such fibrils extending into the cuticular layer were interpreted as extensions from the underlying pectin lamella, which bond the cuticle to the cell wall (Jeffree, 1996). This pectin lamella allows the isolation of many cuticles by pectinolytic enzymes (Orgell, 1955).

> *Zahlreiche Beobachtungen haben gezeigt, dass abgesehen von untergetauchten Wasser- oder Schattenpflanzen, durch die Oberhaut nur wenig Wasser abgegeben wird; dagegen sind die Spaltöffnungen die ‚Tore', durch die der Wasserdampf vorwiegend entweicht.*
>
> *Schmeil, Leitfaden der Pflanzenkunde, 1940*

The cuticle plays an important role as a structural element, holding the cellular tissues compact and firm. Above all, its major role is that of an interface between the body of the plant and its environment. Functions ascribed to the cuticle include the conservation of water in the plant, the prevention of loss of plant components by leaching and the protection of the plant from injuries due to wind and physical abrasion, frost and radiation. The nature of the cuticles also greatly influences the deposition and subsequent behaviour of pesticides, growth regulators, foliar nutritions or other chemicals used on plants. The cuticle provides the first potential barrier to attack by fungi, insects or other pathogens. The various ways in which the cuticle is involved in phenomena at the plant surface can, for convenience of discussion, be classified in three broad categories: physiological functions, interactions with chemicals and interaction with pathogens (Martin & Juniper, 1970).

1.2 Barrier properties of cuticular waxes

The transport limiting barrier in uptake processes are the cuticular waxes (Riederer & Schreiber, 1995), even though they may amount to only a few percent of the mass of cuticles. Therefore, the structure of the waxes will now be discussed shortly. A detailed description of the plant waxes is given by Riederer & Schreiber (1995) and Riederer & Markstädter (1996).

The waxes consist of at least three structurally distinct fractions of different degrees of order and composition. The crystalline fraction of the waxes (zone A) is excluded from any diffusion of molecules. In this zone, the middle portions of the long aliphatic chains of wax constituents are regularly aligned. It was shown that the hydrocarbon chains in the crystalline parts of cuticular wax are assembled in an orthorhombic crystal lattice at lower temperatures and in a hexagonal one at elevated temperatures just below the melting point. These crystalline aggregates form non-accessible flakes which are probably extended parallel to the outer surface of the cuticle. As a consequence of the chain-length distribution of zone A, a volume fraction consisting of chain-ends fills the space between two adjacent flakes (zone B). This zone B is characterised by a much higher degree of motional freedom compared to the rigid arrangement of the crystalline region.

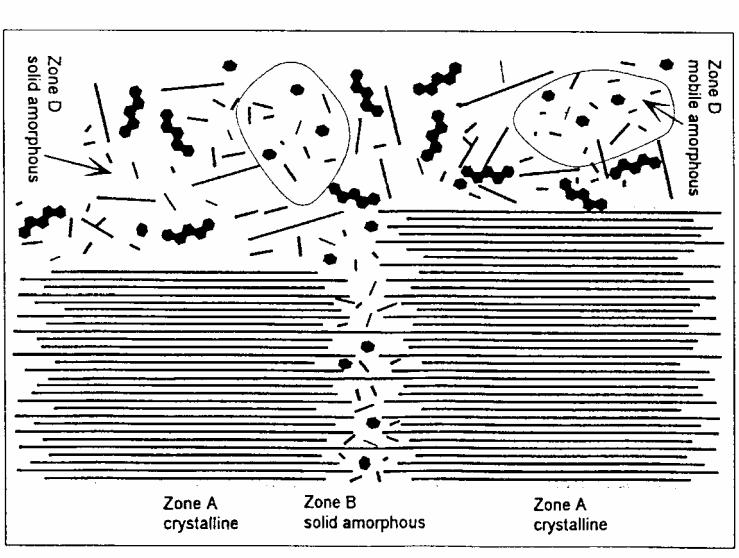


Figure 1.2 Schematic diagram of the molecular structure of plant cuticular wax (Taken from Riederer & Schreiber, 1995).

Zone B has a solid amorphous state. Components of cuticular waxes not incorporated into the orthorhombic crystal lattice of zone A constitute another amorphous zone called zone D. Such components may be excluded from zone A either by their low melting point (e. g. short-chain aliphatics) or for steric reasons (e. g. cyclic compounds). Zone D is a solid amorphous and, with increasing temperature also liquid (mobile) amorphous phase. It fills the voids between chain-ends in zone B and, if present in sufficiently high quantities, may also form clusters outside zone B. Another zone C occurs only in synthetic, not in plant waxes. According to this model, diffusion through the waxes is explainable. For most compounds of biological relevance, zone A is practically an excluded volume because either the penetrant molecules cannot enter the crystalline domains for steric reasons or because their solubility within zone A is extremely low in comparison to the rest of the wax. Therefore, penetrants may diffuse only within the amorphous zones B and D (Riederer & Schreiber, 1995).

1.3 The plant cuticle and its role in plant protection

To guarantee or to improve the performance of pesticides applied *via* leaf surfaces it is a prerequisite to understand the physical and chemical mechanisms which govern uptake by the plant. The cuticle is the interface between the plant and the atmosphere. Therefore, foliarly applied pesticides are encountered by the cuticle at the very beginning of the treatment process. To achieve performance of the herbicides, penetration across the cuticle is indispensable. For that reason, it is absolutely essential to know the composition of the cuticle and the cuticular sites, where penetration takes place. Since the plant cuticle is considered as a solution/diffusion membrane (Vieth, 1991), in the first step dissolution is necessary to ensure diffusion. All the knowledge about the plant cuticle is helpful to understand and to influence the respective penetration processes. A purposeful and responsible application of pesticides is preferable for ecological and economic reasons and also to warrant the health of the nature and the people which deal with them. A huge amount of literature is available which is focused on cuticular transport of pesticides or further compounds. An important tool to improve the performance

of herbicides provides the addition of adjuvants. Modes of action of a broad variety of adjuvants are reviewed by McWorther (1982), Kirkwood (1993), Stock & Holloway (1993), Penner (2000), etc. Continuing cuticular research is essential in order to guarantee sustainable application of pesticides also in the future, while new active ingredients call for favourable application methods.

1.4 Evolution of the pore theory

Foliar transport of hydrophilic compounds as leachates (Tukey *et al.*, 1965) or highly hydrophilic active ingredients (Brian, 1966; 1967) was observed many times. Foy (1964) and Franke (1967) reviewed penetration of herbicides *via* polar and apolar routes through the cuticle. As a polar route Foy suggested cracks, punctures, or areas of leaves not completely covered with wax, then followed by a polar route presumably made up by hydrated cutin and/or the hydrophilic pectic and cellulose portions of the epidermal cell wall. As a result of many investigations, mainly done with electron microscopy, microfibrils traversing the plant cuticle were observed many times (Franke, 1967; Martin & Juniper, 1970; Hoch 1975, 1979; Wattendorf & Holloway, 1980; Merida *et al*., 1981; Wattendorf & Holloway, 1984; Kolattukudy, 1984; Miller, 1986; Jeffree, 1996; Krüger *et al*., 1996; Marga *et al*., 2001) which might serve as possible routes for cuticular penetration of polar molecules. While several cuticular penetration experiments with polar compounds were done, a mechanistic proof with regard to the existence of a polar pathway was outstanding, so far.

In the seventies, some work was done with isolated cuticles and hydrophilic molecules. Schönherr and Bukovac (1970) showed with isolated cuticles from *Allium cepa* bulb scales and leaves areas which are preferentially permeable to mercuric chloride and probably to other polar compounds. In 1976, Schönherr presented two pieces of evidence for the existence of polar pores in the cutin matrix of *Citrus aurantium* leaves. Firstly, the osmotic water permeability was greater than the water diffusion. Secondly, the permeability of the matrix membrane was size-dependent for some polar compounds. While glucose which has a calculated radius of 0.44 nm penetrated the membrane, the latter

was impermeable for solutes with a molecular radius like sucrose (r=0.55 nm) and raffinose (r=0.65 nm). So, Schönherr concluded that the pores in the cutin matrix must have a radius of 0.45 nm. Additionally, he found a pH insensitivity of the pore radius, while the number of pores per area increases with increasing pH. The order of magnitude of the pore number per cm² was given as 10^{10} to 10^{11}.

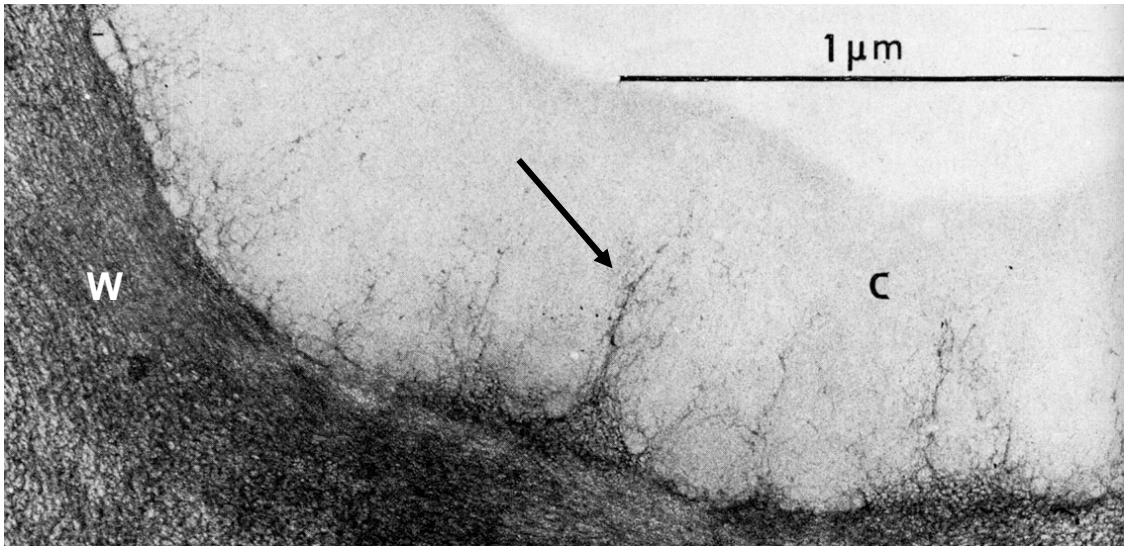


Figure 1.3 Electron micrograph of a section across the cuticle (C) of a celery (*Apium*) petiole. The arrow marks strains originated from the cell wall (W). These strains are stained. (Taken from Martin & Juniper, 1970).

Polar pores also were suggested for *Citrus* cuticular membranes (Schönherr & Schmidt, 1979; Schönherr *et al*., 1979). Several years later, Schönherr (2000) postulated aqueous pores in the cuticles of a few plant species. He detected cuticular penetration of ions, which are restricted to these polar pores. In his experiments he compared the transport properties of calcium chloride and lipophilic compounds. The permeation of the polar ions exhibited no sensitivity to accelerators and temperature. Therefore, the author postulated the presence of an alternative pathway. The existence of polar pores was confirmed for several times (Schönherr 2001, 2002; Schönherr & Luber, 2001; Schlegel & Schönherr, 2002; Schönherr & Schreiber, 2004a & b; Schlegel *et al*., 2005). However, pores of this size may appear and disappear continually as a result of molecular motion of the matrix (Wijmans & Baker, 1995).

1.5. Examples for cuticular transport of hydrophilic molecules

Prediction of the cuticular transport of hydrophilic molecules from their octanol/water partition coefficient results in a noticeable underestimation of the real penetration rate, since this way of prediction implies transport across the lipophilic compartments of the plant cuticle. However, cuticular transport of a huge variety of hydrophilic molecules is well known as it is summarised in the following.

The plant cuticle is a very effective protection against uncontrolled water loss. The cuticle reduces the water evaporation up to 0.1% compared with an uncovered water surface (Pisek & Berger, 1938). It is generally believed that transpiration *via* the cuticle is relatively unimportant compared with loss through the stomata (Martin & Juniper, 1970). Nevertheless water evaporation across the cuticle occurs. The water permeability of astomatous cuticles of an enormous variety of plant species was examined in the past. These values are reviewed by Kerstiens (1996) and Riederer & Schreiber (2001).

Leaching of nutrients and other substances from leaves is well documented. Inorganic minerals, carbohydrates, amino acids and inorganic acids have been leached out from the foliage of more than 140 diverse species by the action of rain, dew and mist (Tukey *et al*., 1965). Stammitti *et al*. (1995) found permeability of fructose, glucose and sucrose across isolated astomatous cuticles of *Prunus laurocerasus*. The occurrence of soluble carbohydrates on leaf surfaces was shown qualitatively and quantitatively (Fiala *et al*., 1990, Derridj, 1996; Mercier & Lindow, 2000). It was found that amounts of sugars on leaves of different plant species are directly correlated with population sizes of bacterial epiphytes (Mercier & Lindow, 2000). The bacterial consumption of sugar in the phyllosphere was proven also (Leveau & Lindow, 2001). Moreover, water soluble metabolites identified from surfaces of apple fruit and leaf stimulates oviposition of *Cydia pomonella* (codling moth) (Lombarkia & Derridj, 2002). Some plants actually invite visits by insects and other organisms by release of highly localised spots of sugars on their leaf surfaces (Leveau, 2004). A correlation between the colonisation of *Pseudomonas* strains on isolated

cuticles as a function of the availability of nitrogen delivering amino acids was shown (Singh *et al*., 2004).

The translocation of minerals is not restricted to the direction from the leaf interior towards the leaf surface. Transport in the opposite direction was shown by foliar nitrogen nutrition as charged nitrate or ammonium (Peuke *et al.*, 1998) or uncharged urea (Knoche *et al*., 1994; Rosecrance *et al*., 1998), additionally. Foliar applied calcium offered as calcium nitrate or calcium chloride prevents damages and diseases, especially on apple fruits (Ferguson & Watkins, 1989). There are some charged, highly hydrophilic active ingredients in plant protection like glyphosate, which is the world's biggest selling and fastest growing agrochemical (Baylis, 2000). Its favourable performance is undisputed which implies cuticular penetration. As a consequence of the Chernobyl disaster Ertel *et al*. (1992) and Malek *et al*. (2002) examined the cuticular permeability of radioactive pollutants ^{137}Cs and ^{90}Sr.

Comparatively little is known about gaseous exchange through cuticles, in contrast to the wealth of information available on exchange through stomata. The extent and mechanisms of cuticular penetration of carbon dioxide is of special interest in relation to photosynthesis (Martin & Juniper, 1970).

1.6 Comparison of the transport properties of the lipophilic and the hydrophilic pathway

This review-chapter compiles mechanistic transport parameters of the lipophilic and the hydrophilic pathway which govern cuticular permeability across the respective pathways. While the lipophilic pathway is relatively well understood, the alternative hydrophilic pathway is the object of current research. The charakterisation of this polar pathway is dominated by the work of Jörg Schönherr, Lukas Schreiber and co-workers. They describe this pathway as a transport across aqueous pores. Therefore, in this chapter, the hydrophilic pathway is mostly called aqueous pores. All following aspects are taken from the literature.

Preconditions for the diffusing molecule for entrance of the polar and the lipophilic pathway. The simplest conceptual model for the penetration of the plant cuticle by non-ionic organic molecules is that of a solution-diffusion membrane. The permeating molecules are sorbed by the membrane on the one side, diffuse across it dissolved as single particles in the membrane and subsequently are desorbed on the other side (Vieth, 1991). This model explains the permeability for lipophilic molecules very well. Decisive parameters describing these transport processes are the lipophilicity and the mobility of the penetrant. The lipophilicity depicts the solubility of the penetrating molecule within the transport-limiting barrier of the plant cuticle and is normally described by the cuticle/water or rather the wax/water partition coefficient (Schönherr & Riederer, 1989). The mobility describes the diffusion of the penetrating compound across the transport-limiting barrier of the cuticle. This parameter is strongly influenced by the molecular size of the compound (Baur *et al.*, 1997). Since solubility is a prerequisite for mobility, it is obvious that the molecule has to be lipophilic to achieve absorption into the lipophilic compartments of the cuticle. Hydrophilic molecules are not absorbed into lipophilic areas (Briggs & Bromilow, 1994). Since charged molecules carry hydration shells (Lieb & Stein, 1986) which cannot be shed, they will not be soluble in the lipophilic cutin and wax domains of the cuticle. Therefore, solutions of charged lipophilic active ingredients like 2,4-dichlorophenoxyacetic acid (2,4-D) have to be buffered (Schönherr, 1993a), because the lipophilic pathway is restricted to uncharged molecules. Ionisable compounds normally achieve their highest octanol/water partition coefficients when they are in a non-ionised state (Chamberlain *et al.*, 1996).

Since cuticular penetration of calcium and glyphosate salts was shown several times (Schönherr, 2000; 2001; 2002, Schönherr & Luber, 2001; Schönherr & Schreiber, 2004a; Schlegel *et al.*, 2005), it has been concluded that ions penetrate *via* an alternative polar pathway. However, it is assumed, that ions penetrate only, when they are accompanied by a counter-ion, since Krüger (1999) observed that in the penetration of $Ca(NO_3)_2$ one Ca^{2+} ion was accompanied by two NO_3^- ions. Diffusion through polar pores probably explains, why electrical potentials arise when salts diffuse across isolated cuticles (Tyree

et al., 1990a). The permeability of alkali metals is rising with decreasing ionic radius and increasing hydrated ionic radius (Tyree *et al*., 1990b). Less work was done with uncharged polar molecules. Schönherr (1976b) and Stammitti *et al*. (1995) could detect permeability of carbohydrates across dewaxed cuticles of *Citrus aurantium* and cuticular membranes of *Prunus laurocerasus*. It was assumed that their penetration has occurred across a polar pathway. Summarising all this findings indicates the exclusion of polar charged and uncharged molecules from the lipophilic pathway, but restriction to the polar pathway. In contrast to that, solution of lipophilic molecules inside the polar pathway is highly improbable which implies restriction to the lipophilic pathway.

Even though there seems to be a clear concept, the penetration pathway of uncharged polar water is a matter of lively debate. Schreiber *et al.* (2001) showed that the penetration of water across the cuticle depends on relative humidity in the adjacent air space. The molecular basis for this effect is the absorption of water molecules by polar functional groups of the transport barrier of the cuticular membrane and the matrix membrane, which finally leads to the formation of polar pores. Schönherr (1976b) found a dynamic nature of these pores. They do not exist in dry cuticles but develop on hydration. The dependence on humidity of cuticular water permeability has been interpreted as evidence that aqueous pores exist in cuticular membranes from at least some plant species (Schönherr & Schmidt, 1979; Schönherr & Merida, 1981; Schönherr, 1982; Kerstiens, 1996) but the effect of humidity on water permeability was very small compared to polymethacrylate membranes (Schönherr, 1982). However, this can be explained by the fact that water diffusion is not limited to aqueous pores. Hence, for water two parallel pathways may exist (Schönherr, 2000). Riederer & Schönherr (1990) showed that cuticular water permeability is very sensitive to plasticisers, which implies water permeation *via* the lipophilic pathway, because polar pores are not influenced by plasticisers (Schönherr, 2000). No indications were found for a significant contribution of the pores in water transpiration (Kerstiens, 1996; Schönherr, 2000; Riederer & Schreiber, 2001). Co-permeability of water and organic compounds of varying lipophilicity was examined, and 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 (Niederl *et al*., 1998). Schreiber (2001) determined the activation energy of water diffusion through cuticles. Compared with measurements taken from the literature made with different lipophilic barriers, he found that the activation energy is in a similar range. So he concluded that water diffusion across plant cuticles in the temperature range from 10 - 35 °C basically takes place in a lipophilic compartment composed of cutin and waxes. Interpreting all these results implies water diffusion across both pathways, or variations due to different plant species.

Influence of temperature. The effect of temperature on the transport properties of the waxes is described by Riederer & Schreiber (1995) in detail. The following part discusses the influence of increasing temperature on the amorphous and the crystalline wax fractions. Cuticular waxes do not have a distinct melting point but gradually liquefy over a wide temperature range (Riederer & Schreiber, 1995). As it is shown in figure 1.4, increasing temperature increases the accessible volume fraction of the waxes. This results in increased mobilities of diffusing organic solutes when temperature increases, too.

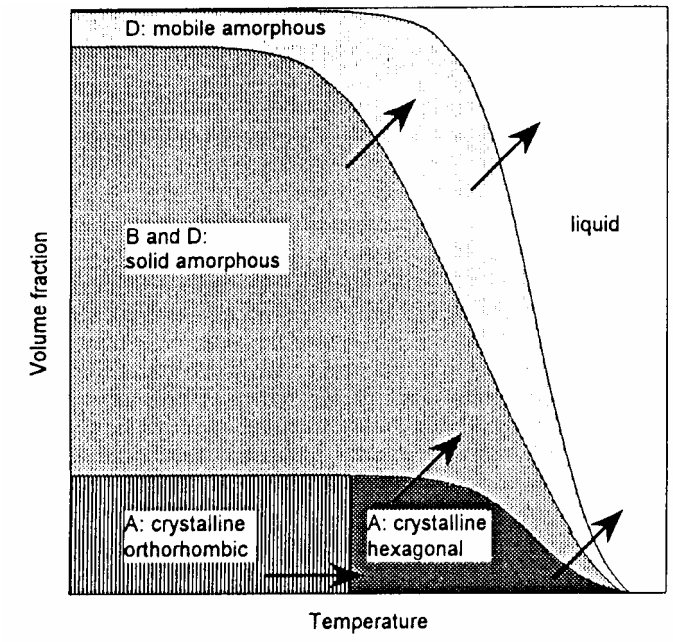


Figure 1.4 Effect of temperature on the volume fractions occupied by the different zones of plant cuticular wax. (Taken from Riederer & Schreiber, 1995). The different wax fractions are discussed in chapter 1.2.

Temperature effects on solute mobilities increases with molar volume of solutes (Baur *et al*., 1997) and is the predominant physical factor influencing the permeance (equation 2.8) of a barrier. The diffusion coefficient of a molecule diffusing in the membrane increases with temperature while its partition coefficient decreases. But temperature has a stronger effect on the diffusion coefficient than on the partition coefficient. That is the reason why permeances generally increase with temperature (Riederer & Schreiber, 2001). These enormous effects of the temperature on the mobility of lipophilic molecules are reflected in high activation energies of diffusion which range between 75 and 189 kJ mol^{-1}, depending on species and solute size (Baur *et al*., 1997). The permeability of poplar cuticles to potassium glyphosate resulted in an activation energy of only 2.92 kJ mol^{-1} (Schönherr, 2002), and the permeability of pear leaf cuticles to calcium chloride (Schönherr, 2002) and potassium carbonate (Schönherr and Luber, 2001) was not affected by temperature at all. This corresponds either with no, or with very low activation energies for diffusion in a hydrophilic compartment.

High temperatures cause defects between the cutin and the wax interfaces. This results in increased transpiration rates (Schreiber & Schönherr, 1990). At temperatures above 30 - 40 °C an increased volume expansion of the cutin polymer causes defects in the transport-limiting barrier leading to additional paths of diffusion for water (Schreiber, 2001). In contrast to these results obtained with water, Baur *et al*. (1997) found no evidences for a phase transition at cuticular membranes for lipophilic substances. Therefore, it must be concluded that 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 utilised by polar molecules like water but not by lipophilic substances (Schreiber, 2001). The influence of high temperatures on the polar pathway is not described in the literature so far.

Influence of accelerators. Accelerators are chemicals absorbed in cuticles which cause an increased fluidity of the waxes and therefore, results in higher mobilities of the diffusing compounds (Schönherr, 1993a). Generally, accelerators and temperature have the same mechanism of action (Riederer &

Schreiber, 1995; Baur *et al*., 1997). The effect of accelerators on the lipophilic pathway is established and described very often (McWorther, 1982; Schönherr, 1993a/b; Gauvrit & Cabanne, 1993; Stock & Holloway, 1993; Stock *et al*., 1993; Tan & Crabtree, 1994; Riederer *et al*., 1995; Schreiber, 1995; Schönherr & Baur, 1996b; Baur & Schönherr, 1997; Mouloungui & Gauvrit, 1998; Burghardt *et al*., 1998; Knoche & Bukovac, 1999; Penner, 2000). No acceleration effects were detected for the permeation of calcium chloride (Schönherr, 2000; 2001) and glyphosate salts (Schönherr, 2002). However, Baur (1999) found an accelerator effect for methylglucose.

Influence of the relative humidity. As mentioned before, hydration of the cuticle depends on the relative humidity (Luque *et al*., 1995; Dominguez & Heredia, 1999). The hydration of the cuticle determines the number and the radius of the pores, as it was found for *Citrus* matrix membranes (Schönherr, 1976b). The uptake of substances through the cuticle is promoted by high relative humidity, since the cuticle then is in its most open and swollen condition while under low relative humidity the cuticle shrinks and the wax deposits are compressed, so that the passage of water soluble substances is impaired (Martin & Juniper, 1970). When matrix membranes from *Prunus* were methylated, a pronounced decrease in the response of cuticular water permeability to air humidity was observed. Humidity-sensitivity of the polar path was also supported by Schreiber *et al*. (2001), but not for the non-polar path. Polysaccharide fibrils are assumed to create the hydrophilic pathway across plant cuticles (see chapter 1.4) and their sensitivity to humidity is well understood.

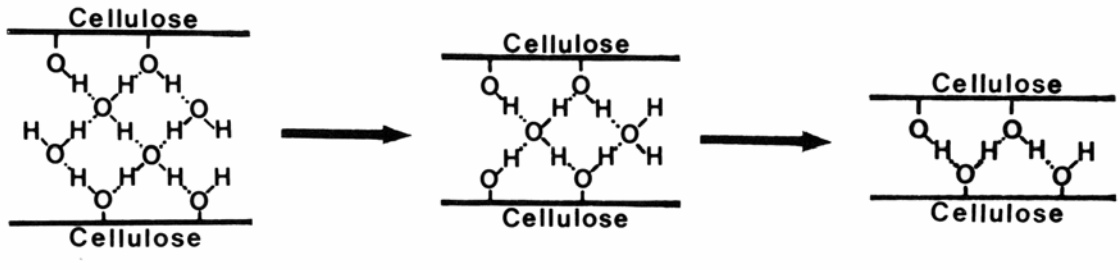


Figure 1.5 Drying of wet cellulose causes shrinking of the fibrils and the formation of H-bonds (···) between adjoining glucose molecules (Taken from Fengel, 1985).

For instance, cellulose takes up water in a typical way for polysaccharides. The uptake of water is conventionally described in terms of the relative weight of water sorbed. Cellulose sorbs up to 25% water at high relative humidity. In a swollen state, interfibrilar vacancies are filled with water. When wet cellulose is drying, water molecules between the strains disappear which causes shrinking of the fibrils and the formation of H-bonds between glucose molecules (Fengel, 1985). This process is illustrated in figure 1.5.

Influence of pH. The effect of the pH should be restricted to the polar pores, because with increasing pH the number of pores, but not the radius increases as it was found with *Citrus* matrix membranes (Schönherr, 1976b). The increase in permeability with increasing pH is a definite evidence for the existence of fixed dissociable groups of the weak acid type in these cuticular membranes. Since sulfur und phosphorus are generally not present in cuticles (Martin & Juniper, 1970) these groups are most likely to be carboxyl (below pH 7) and phenolic hydroxyl groups (above pH 9) (Schönherr, 1976a). Besides that, amino acids occur in the cuticles as components of polypeptides or proteins. The nature or function of these polypeptides or proteins in the cuticles is not known (Schönherr & Huber, 1977). Therefore, the plant cuticle has an isoelectric point around pH 3. Below pH 3 cuticlular membranes carry a net positive charge and are permselective for anions, above pH 3 they carry a net negative charge and are permselective to cations (Schönherr & Huber, 1977). Such non-esterified, free carboxyl groups present in the cutin polymer matrix significantly contribute to the effect of humidity on cuticular water permeability. These and the other polar groups sorb water, which in turn increases the water permeability of polar domains of the cuticle. It is expected that the influence of a variation of pH is restricted mainly to diffusing charged molecules.

Size-limitation of both pathways. The diffusion coefficients of solutes in isolated cuticular membranes (Schönherr & Baur, 1994; Baur *et al*., 1996; Baur & Schönherr, 1997) and reconstituted cuticular waxes (Schreiber, 1995; Schreiber *et al*., 1996; Kirsch *et al*., 1997) exponentially decrease with molecular size. This property is called size-selectivity. No differences in the size-selectivity were found among six species and an average of 0.0095 mol

cm^{-3} was found (Buchholz *et al*., 1998). Size-selectivity can be decreased with plasticisers (Burghardt *et al*., 1998) or with increasing temperature (Buchholz *et al*., 1998). The size-selectivity for the penetration of Ca^{2+} salts across isolated cuticles was significantly less pronounced than size selectivity of lipophilic molecules (Schönherr & Schreiber, 2004). In an earlier work done with dewaxed *Citrus* membranes Schönherr (1976b) postulated an exclusion of hydrophilic molecules with a molecular radius higher than the pore radius (0.45 nm).

Main barrier of permeation. It is well established that the actual transport barrier of the cuticle is formed by cuticular waxes (Schönherr, 1976a) since cuticular permeability increases by factors between 10 to 1000 upon wax extraction (Schönherr & Riederer, 1989). Extracting the waxes from pear leaf cuticles increased the rates of penetration of $CaCl_2$ only about twofold at 90% humidity and about threefold at 50% humidity. This clearly shows that most of the aqueous pores detected in dewaxed membranes were not covered or plugged up by cuticular waxes (Schönherr, 2000). Santier & Chamel (1998) suggested that in the case of a hydrophilic compound like glyphosate the influence of waxes on diffusion is reduced for five species. From these aspects one can conclude that pore-covering waxes and the limited hydration of the cuticle are the main barriers at the polar pathway.

1.7 Objectives of the present work

? Much cuticular research was performed in terms of lipophilic model compounds, less was done with hydrophilic model compounds and very less with hydrophilic primary metabolites. So, which transport mechanisms govern cuticular penetration of primary metabolites, e. g. carbohydrates and amino acids? It is assumed, that the most appropriate assessment of their transport properties calls for a direct comparison with lipophilic compounds, since these mechanisms are pretty well understood.

? The existence of polar pathways is postulated for some species. Has the low permeable cuticle of *Hedera helix* also polar pathways? And how is it with *Vitis vinifera* leaf cuticles?

? The selection of a variety of carbohydrates and amino acids of different molecular mass could provide a very favourable feature, since these molecules have similar chemical and physical properties, but differ in their size. To take this as a basis the question is: Which effect has the molecular size of the hydrophilic molecules on the cuticular transport?

? In addition to that, carbohydrates are uncharged molecules and amino acids are dissociable molecules. Charge has fundamental importance for the cuticular penetration. Are there any differences in the transport properties of both hydrophilic chemical classes?

? The data-set was supplemented by volatile molecules. So how behave volatile polar compounds compared with the other classes?

? Cuticular transport is also of strong interest for the agrochemical industry. Therefore, hydrophilic and lipophilic active ingredients were included. As mentioned above, penetration of lipophilic actives is well understood. But which transport mechanisms govern the uptake of hydrophilic active ingredients? A better understanding could help to improve the uptake rates of agrochemicals.

? To study selected parameters which are involved in cuticular penetration processes of agrochemicals in the field, the suitability of steady state experiments is limited. To make a compromise between application in the laboratory and in the field, a method was developed which enables the examination of selected transport parameters of a highly water soluble herbicide. Therefore, droplets containing herbicides were applied on isolated cuticles from a species which is relevant in crop protection. This middle course allows the examination of single parameters which are involved in cuticular transport processes. This work was focused on some parameters: What is the effect of the relative humidity on the penetration? What happens when the

concentration of the active ingredient or the size of the droplet is changed? Do cuticular waxes also affect the transport of hydrophilic actives? Do additives affect the penetration and what is the mode of action?

? Finally, it is of special interest to obtain information about the molecular dimensions of the putative hydrophilic pathway. Therefore, membrane research from other disciplines might provide useful tools to find answers on some questions: Is it possible to obtain information about the size and the frequency of that pathway? Are there any differences between well permeable cuticles like those from *V. vinifera* and low permeable like those from *H. helix*? Which role play the cuticular waxes?

2. THEORETICAL BACKGROUND

2.1 Stokesian diffusion and non-Stokesian diffusion

Diffusion of molecules within biological membranes depends on the diffusant volumes (Lieb & Stein, 1986). Diffusion is defined as a process that results from the random, chaotic, noncoherent motion of molecules due to their thermal energy (Koch, 1990).

Stokesian diffusion. Diffusion of molecules in a continuous fluid medium is described by the Stokes-Einstein equation. For a sphere of radius (r) moving within such a fluid, the diffusion coefficient (D) is given by:

$$D = \frac{kT}{6\pi\eta r} \qquad \text{(Eq. 2.1)}$$

k is the Boltzmann constant, T is the absolute temperature and η is the coefficient of viscosity. The denominator is simply the factor that Stokes found necessary to describe the frictional drag of a spherical particle diffusing across a viscous medium. The frictional drag between layers of the fluid determines the frictional resistance to the diffusion. This type of diffusion is called Stokesian diffusion and is fulfilled when:

$$D \times \sqrt[3]{MV} = \text{const.} \qquad \text{(Eq. 2.2)}$$

MV is the molar volume of the respective compound. A double logarithmic plot of the diffusion coefficient versus the molar volume results in a slope of $-1/3$. From equation 2.1 it is obvious, that the diffusion coefficient of a molecule in the same medium depends on the molecular size. Since the influence of the temperature is restricted on the viscosity, which also affects the diffusion coefficient, the temperature effect on the diffusion is limited. The activation energies which are originated from variations in the temperature do not depend on the molecular size of the diffusant. This is reflected in activation

energies in the range of 17 to 21 kJ mol^{-1} for molecules with a molecular weight between 20 and 66,000 g mol^{-1} diffusing in water (Lieb & Stein, 1986). According to Stokes-Einstein, the charge of the molecule has no meaning for the respective diffusion coefficient (Atkins, 1987). Another feature of this relation is its dependence upon the diffusant shape. For diffusion of large and small molecules in water, it is always found that departure from a spherical shape results in a reduction in the diffusion coefficient. An exemplary Stokesian diffusion as a function of the molar volume is shown in figure 2.1 (dashed line).

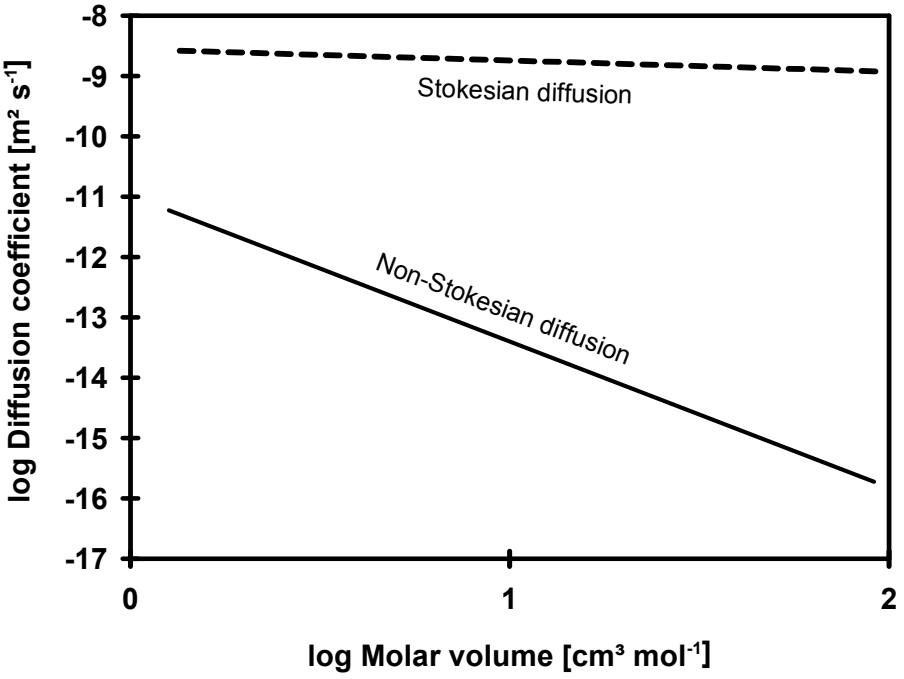


Figure 2.1 Exemplary diffusion in water which is relatively insensitive to size (Stokesian diffusion). The slope of the regression line is $-^1/_3$ and equation 2.2 is fulfilled. Diffusion across a membrane is sensitive to size and equation 2.2 is not fulfilled (non-Stokesian diffusion).

Non-Stokesian diffusion. Diffusion in polymers or biological membranes does not obey the Stokes-Einstein equation, so it is called non-Stokesian diffusion (Fig. 2.1, solid line). The reason is obvious, since the polymer is not a fluid, and its molecules cannot flow around the diffusing particle. However, biological membranes are fluid in the plane of the membrane. In the direction normal to this plane, along which transmembrane diffusion takes place, the lipid hydrocarbon chains are anchored to the membrane/water interface and thus cannot flow past the diffusant. To explain non-Stokesian diffusion, the 'free volume theory' has been found to be suitable. This theory assumes that there is

a large amount of free volume (V_f) in a membrane and this derivation is based on the concept that statistical redistribution of the free volume occasionally opens up voids which are large enough for diffusive displacement (Cohen & Turnbull, 1959). A penetrant can only move, when there is a space available to receive it. These voids are created by the Brownian movement of the molecular segments of the polymer chain. When one of the fluctuations is sufficiently large to contain the diffusing molecule, movement occurs (Vieth, 1991). The total probability (p) of a molecule finding a void of exceeding volume depends on the molar volume (MV) and is given by Cohen & Turnbull (1959):

$$p_{(MV)} = e^{\frac{-MV}{V_f}} \qquad \text{(Eq. 2.3)}$$

The overall diffusion coefficient is proportional to the number of voids, the rate of appearance and disappearance of voids and the probability of a given void having a volume equal to or larger than the diffusant volume (Lieb & Stein, 1986). In recent investigations dealing with molecular diffusion in human skin (Potts & Guy, 1992) and isolated cuticles (Schönherr & Baur, 1994) the following equation was suggested for analysing lipid transport barriers:

$$D = D_0 \times e^{-ß \times MV} \qquad \text{(Eq. 2.4)}$$

D [$m^2\ s^{-1}$] is the diffusion coefficient of the respective compound, D_0 [$m^2\ s^{-1}$] is the diffusion coefficient of a molecule with a molar volume of zero, ß [$mol\ cm^{-3}$] is the size selectivity describing the dependence of D on the size of the molecules, and MV [$cm^3\ mol^{-1}$] is the molar volume of the investigated molecules.

$$V_f = \frac{1}{ß} \qquad \text{(Eq. 2.5)}$$

From the ‘free volume theory’ it is understandable why non-Stokesian diffusion often exhibits selectivities toward diffusant size that are much greater than those possible for Stokesian diffusion. The temperature has a dramatic effect on

non-Stokesian diffusion. Diffusion is increased by temperature due to increased free volume and increasing the rate of the overall formation of holes. Activation energies in polymers and in membranes thus can be larger than diffusion in simple liquids. Another feature is the sensitivity of biological membranes for plasticisers. It is assumed that such compounds make the membrane more flexible by disrupting interactions between the membrane components. The interpretation of the barrier property of the cuticular membrane by the 'free volume theory' is given in chapter 5.2.4.1.

2.2 Mathematical description of cuticular transport

The amount (M) of a compound diffusing across a membrane per unit area (A) and time (t) depends on the barrier properties of the membrane and the driving force. The membrane thickness is Δ x. According to Fick's first law, flow (F) of a compound through a membrane is proportional to the local concentration gradient (Δ c /Δ x).

$$\frac{M}{A \times t} = F = \frac{D}{\Delta x}(c_{Source} - c_{Sink}) = D\frac{\Delta c}{\Delta x} \qquad \text{(Eq. 2.6)}$$

Since the membrane is solid and therefore has another solubility for the penetrating molecules as the adjacent liquid medium, the solubility of the membrane for the respective compound has to be included. The dimensionless membrane/liquid partition coefficient (K) is the ratio of the concentration of the compound in the cuticle to the concentration of the compound in the liquid. If the solute is more soluble in the membrane than in the liquid, the partition coefficient is > 1. When the solute is more soluble in the liquid than in the membrane the partition coefficient is < 1. The importance of the membrane solubility on the flow is shown in figure 2.2. Correcting for differential solubility of solutes results in the following equation, assumed the membrane is homogeneous, that is diffusion and partition coefficients are the same everywhere in the membrane and the same solvent is used on both sides:

$$F = \frac{D \times K}{\Delta x}(c_{Source} - c_{Sink}) = P \times (c_{Source} - c_{Sink}) \qquad \text{(Eq. 2.7)}$$

P is the permeance or the mass transfer coefficient for a given membrane and compound which relates the observed flow to the driving force. The permeance has the dimension of a velocity [m s^{-1}]. As driving force the difference in the concentrations of the adjacent liquids is used. P contains information about solute mobility (D), differential solubility of solutes in the membrane and in the adjacent solutions (K) and the membrane thickness (Δ x), or more precisely the lengths of the diffusion paths in the membrane.

$$P = \frac{D \times K}{\Delta x} \qquad \text{(Eq. 2.8)}$$

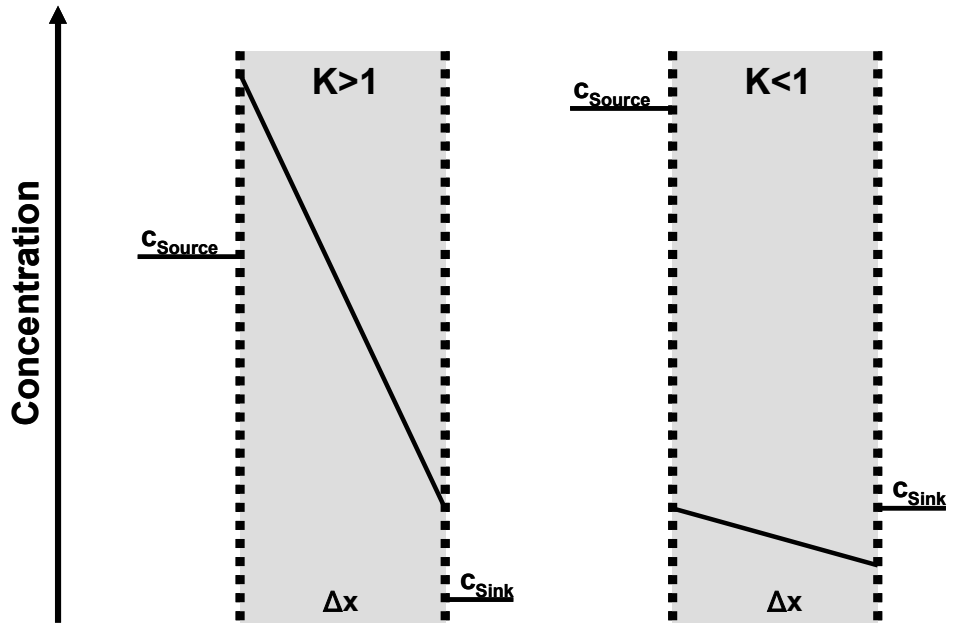


Figure 2.2 Concentration profiles across stirred solutions and homogeneous membranes with a membrane/water partition coefficient K > 1 and K < 1. Δ x is the thickness of the membrane.

For determination of the solute mobility in the wax, a method called unilateral desorption from the outer surface was developed (UDOS) (Bauer & Schönherr,

1992; Schönherr & Bauer, 1992). Applying this method, the rate constant k* is determined, which is directly proportional to D in the limiting skin (ls) of the waxes which are located at the morphological outer surface of cuticles and in the sorption compartment (soco) beneath the waxes which corresponds to the cutin matrix:

$$D = k^* \times l_{ls} \times l_{soco} \qquad \text{(Eq. 2.9)}$$

The cuticular penetration is determined by the limiting skin, because mobility and solubility are much lower than in the sorption compartment. The following equation describes the flow through a synthetic membrane, but can also used for plant cuticles.

$$\frac{M}{A \, x \, t} = F = P(c_{Source} - c_{Sink}) \qquad \text{(Eq. 2.10)}$$

In this equation the flow F is defined as amount (M) which diffuses per area (A) and time (t) and the difference in concentration between donor and receptor is used as the driving force. After substituting equation 2.8 and 2.9 in equation 2.10 it gives:

$$F = \frac{k^* \times l_{ls} \times l_{soco}}{l_{cm}} \times K(c_{Source} - c_{Sink}) \qquad \text{(Eq. 2.11)}$$

Since the thickness of the cuticular membrane is very close to the thickness of the matrix membrane ($l_{cm} \approx l_{soco}$), equation 2.11 can be simplified:

$$F = k^* \times l_{ls} \times K(c_{Source} - c_{Sink}) \qquad \text{(Eq. 2.12)}$$

Defining the driving force gives:

$$F = k^* \times l_{ls} \times (K_{W/W} c_{aq.sol.} - K_{MX/W} c_{apo}) \qquad \text{(Eq. 2.13)}$$

The term in parenthesis represents the driving force, composed of the differential solubilities in the wax and water ($K_{W/W}$), polymer matrix and water ($K_{MX/W}$) and the concentrations in the aqueous solution ($c_{aq.sol.}$) and in the apoplast (c_{apo}). If the solute concentration in the apoplast remains negligible, because uptake into cells and translocation is rapid, the second term in parenthesis vanishes and the driving force depends only on the product $K_{W/W}c_{aq.sol.}$ (Schönherr & Baur, 1996b).

2.3 Established methods for quantifying cuticular transport

The lipophilic pathway across the plant cuticle is relatively well examined. Much work has been done to understand the mechanism governing the cuticular transport *via* this pathway. Several methods were developed to measure and to evaluate the permeability of molecules. An important prerequisite therefore, was the development of an enzymatic method to isolate plant cuticles without modification of their transport properties by Schönherr and Riederer (1986). This allows transport studies across cuticular membranes. Another experimental set-up are measurements using extracted reconstituted plant waxes making up the actual transport barrier of cuticles. This technique was developed by Schreiber & Schönherr (1993). The following table gives a short survey about the established methods and systems.

Table 2.1 Survey of the established methods to describe uptake properties.

System	Experiment	Method described by	Obtained parameter	Comments
Isolated cuticles	Permeation experiment	Schreiber *et al*., 1995	Permeance P [m s^{-1}]	Steady state-conditions
Isolated cuticles	**U**nilateral **D**esorption from the **O**uter **S**urface (UDOS)	Bauer & Schönherr, 1992	Rate constant k* [h^{-1}]	Non-steady state-conditions
Isolated cuticles	**S**imulation **O**f **F**oliar **U**ptake (SOFU)	Baur & Schönherr, 1997	Rate constant k [h^{-1}]	Non-steady state-conditions
Reconstituted waxes	Desorption experiment	Schreiber & Schönherr, 1993	Diffusion coefficient [m^2 s^{-1}]	Maximum effects

Each method has its advantages. From permeation experiments, the velocity of permeation of compounds is obtainable, also in the presence of additives. But this method is restricted to plant species with astomatous cuticles. By desorption experiments done with reconstituted waxes one can determine diffusion coefficients and also wax/water partition coefficients. Addition of additives is feasible. It is also possible to calculate the permeance from parameters available by this method (eq. 2.8). Therefore, it is possible to obtain permeances for species which are not accessible for cuticular studies, because of the occurrence of stomata on the adaxial and on the abaxial side of the leaf. 'UDOS' is limited to relatively lipophilic solutes (Schönherr & Bauer, 1996b), since sufficient sorption of the model compound inside the sorption compartment is a prerequisite for this experiment. If the amount desorbed at the time t is named M_t and the total amount initially sorbed inside the cuticle is M_0, the data can plotted as $-\ln(1-M_t/M_0)$ vs. t, which results in straight lines. The term in parenthesis is equivalent to the fraction of the compound remaining in the cuticular membrane at time t and the slope is the rate constant of desorption k* [h^{-1}] (Schönherr & Baur, 1996b), which is a measure for the mobility of the compound. With 'SOFU' the data are also plotted as $-\ln(1-M_t/M_0)$ vs. time. In this type a first order plot is obtained when the compound is dissolved and transport properties of the cuticles remain constant during the experiment. A linear curve progression is obtainable which allows the determination of rate constants k [h^{-1}]. Both rate constants k* and k should not be confused since the driving forces differ in these two sets of experiments. In 'UDOS' the driving force is the solute concentration in the sorption compartment of the cuticular membrane and the rate constant is marked k*, while in 'SOFU' the driving force is the wax/water or rather the wax/formulation partition coefficient multiplied with the concentration in the adjacent solution and the rate constant is marked k (Schönherr & Baur, 1996b).

3. MATERIAL AND METHODS

3.1 Characterisation of the grapevine cuticle (*Vitis vinifera*)

3.1.1 Isolation of the grapevine cuticles and extraction of the cuticular waxes

All cuticular membranes were isolated from grapevine leaves (*V. vinifera* L. cv. Nelly) ('Rötliche Hausrebe') growing in the Botanical Garden in Würzburg. The grapevine cuticles were isolated according to Schönherr & Riederer (1986). When the isolation of the cuticles was finished, the membranes were stored in borax buffer (10^{-2} M) (Sigma Chemie, Deisenhofen, Germany) which was changed for several times. Storage of dried cuticular membranes was not possible because of their delicate nature. The diameter of the membranes was 25 mm.

The cuticular waxes were extracted from intact leaves by dipping them into chloroform (Roth, Karlsruhe, Germany) for about five seconds. The chloroform/wax extract was filtered three times to remove impurities like dust. Afterwards the extract was evaporated.

3.1.2 Chemical analysis of the cuticular waxes

Prior to gas-chromatographic analysis (GC), chloroform was evaporated from all samples under a gentle stream of N_2 while heating the sample-vials to 50 °C. Then all samples were treated with bis-N,N-(trimethylsilyl)trifluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) in pyridine (30 min at 70 °C) to transform all hydroxyl-containing compounds to the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied by capillary GC (8000Top; Fisons Instruments, Rodano-Milan, Italy) with He carrier gas inlet pressure constant at 30 kPa and a mass- spectrometric detector (70 eV, m/z 50–850, MD1000; Fisons). GC was carried out with temperature-programmed

injection at 50 °C, oven 2 min at 50 °C, raised by 40 °C min^{-1} to 200 °C, held for 2 min at 200 °C, raised by 3 °C min^{-1} to 320 °C, held for 30 min at 320 °C. The quantitative composition of the mixtures was studied by capillary GC (5890 II: Hewlett Packard, Avondale, PA, USA; 30 m DB-1, 0.32 mm i.d., d_f=1 µm: J & W Scientific, Folsom, CA, USA) and flame ionization detection under the same gas-chromatographic conditions as above, but the H_2 carrier gas inlet pressure was programmed for 50 kPa at injection, held for 5 min, raised at 3 kPa min^{-1} to 150 kPa and held for 40 min at 150 kPa. Single compounds were quantified against the internal standard by integrating peak areas.

3.1.3 Preparation of the cuticular membranes for scanning electron microscopy

Dried isolated leaf cuticles of grapevine (*V. vinifera* cv. Nelly) were mounted on aluminium holders, sputter-coated with ~ 3 nm platinum and examined under a Hitachi S-4700 field emission scanning electron microscope (FESEM) at an accelerating voltage of 3.0kV.

3.2 Non-steady state experiments with paraquat

3.2.1 Objectives of the experiment

The objective of these experiments was to examine cuticular penetration processes with a species which is relevant for crop protection and which also reflects transport properties as expected for relevant crops and weeds. As model compound a highly hydrophilic herbicide was used. This compound was applied as aqueous droplets on the top of isolated cuticles. The experimental set-up was a compromise between non-steady state conditions occurring in the field and steady state conditions in the laboratory. From this approach it is possible to obtain information on cuticular transport processes under non-optimised conditions and to understand the meaning of selected parameters examined on cuticular transport processes.

3.2.2 Selection of the model plant

An isolation of the cuticles of the most crops and weeds is not possible, because the membranes are just too thin. Only damaged cuticular membranes can be obtained. Another prerequisite is to have the possibility to handle them without causing cracks. Most plant species are excluded for still another reason, since most of them have stomata on the adaxial and on the abaxial side of the leaves. The cuticle of grapevine was found to be highly suitable, because it is a relevant crop, its cuticles can be isolated and has also no stomata on the adaxial side of the leaf. That is why this species was selected as a model plant. The transport properties of the cuticles isolated are expected to be close to those of relevant crops and weeds.

The isolation of the grapevine cuticles was described in chapter 3.1.1 before. To distinguish between the outer and the inner side of the astomatous cuticle it was tried to find visual differences using a microscope and to find differences in the contact angle of a water droplet which was applied on both sides of the same membrane. Unfortunately, all these attempts failed. Due to the very little amount

of epicuticular wax it was also not possible to see differences in the brightness between both sides, as it is possible for English ivy membranes, for example. In terms of the relative high permeability of grapevine cuticles it is expected, that there are no variations in transport properties caused by the orientation of the membrane.

3.2.3 Development of a method to measure penetration of active ingredients across isolated grapevine cuticles

Due to the thinness of the grapevine membrane it was essential to develop a method which allowed the measurement of cuticular penetration. Most established methods described in the literature (see chapter 2.3) are inapplicable, because they demand more robust membranes. The most important requirement was to find a possibility to handle the membranes and to find a suited desorption medium which does not injure the membrane. A liquid desorption medium is excluded, because it would damage the membranes as a consequence of its weight and movement. To handle the membranes, a carrier was needed. As a very well-suited carrier, fibre rings (12 x 19 mm) (Haas, Nürnberg, Germany) were found to be suitable. The rough but soft microstructure of these rings is the most important reason for its high applicability. Such fibre rings normally are used for sealing tap heads and copper pipes. The cuticles were put in water and trapped under water with a fibre ring (Fig. 3.1). In the next step the cuticle was mounted on the ring. When the cuticles are dried, they are ready for the experiment.

It was also possible to work with dewaxed cuticular membranes (MX) of grapevine. Therefore, the cuticles were mounted on a washer made from zinc coated steel (6.4 x 20 mm) (Würth, Gaisbach, Germany). When the cuticle was completely dried the washer with the membrane was laid in a sufficient amount of chloroform for several hours to extract the soluble cuticular waxes. After that, the washer was taken out of the chloroform and dried again. Afterwards the cuticle was ready for the experiment.

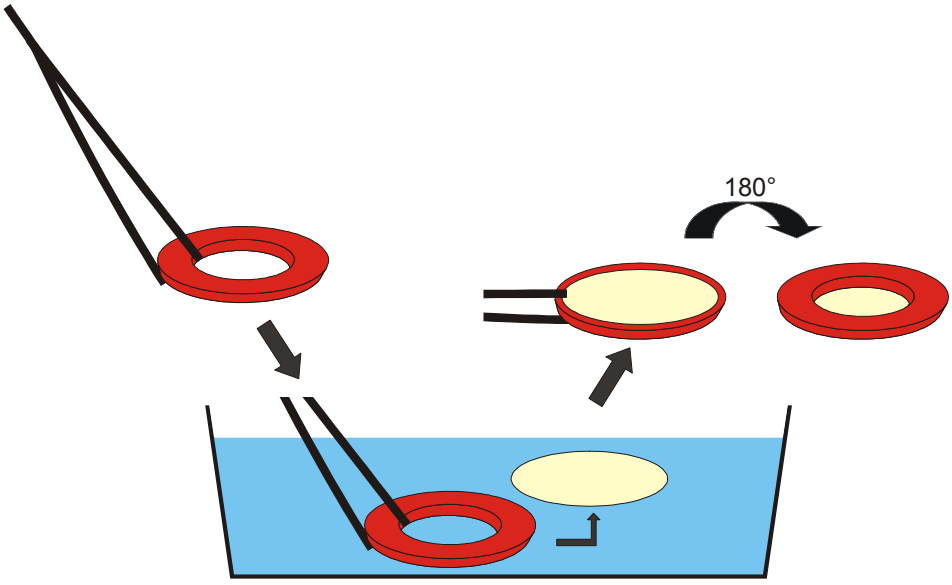


Figure 3.1 Schematic drawing of mounting the grapevine cuticles on a fibre ring.

For the reasons mentioned above it was not possible to use a liquid desorption medium as with most established methods. Instead, a solid desorption medium was searched, where the cuticle is laid on it. Low melting agarose (Roth, Karlsruhe, Germany) was found to be a well suited desorption medium, because it consists for the most part of water and it is solid. The diffusion coefficients of compounds in agarose are only one order of magnitude below liquid water (personal communication Markus Riederer). For that reasons, agarose meets the requirements as a well suited desorption compartment.

All used model compounds were radiolabelled (see chapter 3.2.7). Therefore, quantification of the cuticular penetration was very easy. To detect the total radioactivity absorbed by the agarose, the latter was melted inside a closed scintillation vial at about 75 °C. Low melting agarose has a melting point in the range of 65 °C. Before the melting of the agarose, an adequate amount of scintillation cocktail was added. The complete melting of the agarose was checked, and afterwards the scintillation vial was shaken forcefully. This procedure allows the quantitative detection of the radioactivity of each sample.

3.2.4 Course of a measurement

All experiments made with grapevine cuticles were conducted under non-steady state conditions. When the cuticular membrane was mounted on a fibre ring, it was laid on a cylindrical piece of agarose (diameter 15 mm, height ~15 mm), immediately. The surface of the agarose was wet to ensure intimate contact between the cuticle and the piece of agarose. The membrane was ready for the experiment when it was dried.

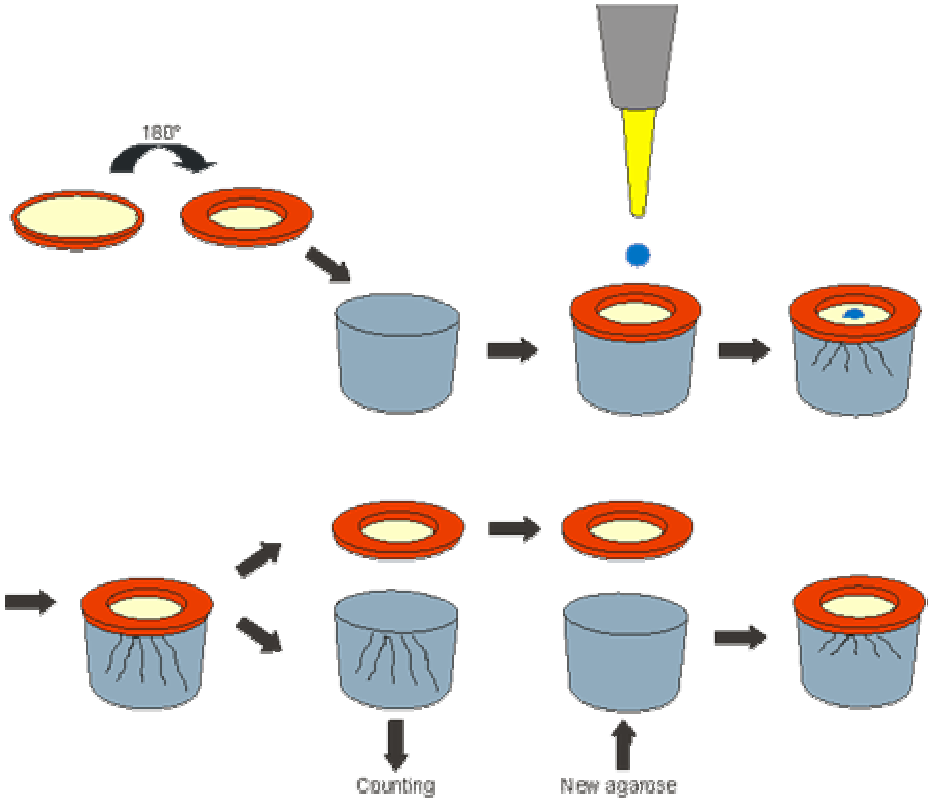


Figure 3.2 Schematic drawing of a droplet experiment. The grapevine cuticle is mounted on a fibre ring. Afterwards it is laid on a cylindrical piece of agarose and an aqueous droplet is applied. The desorption medium is changed for several times.

The measurement was started by application of a 5 µl droplet of the selected compound at time t_0. Figure 3.2 shows a schematic diagram of the course of a measurement. All compounds were applied as an aqueous solution. When water was completely evaporated, the agarose-cylinder was changed for the first time (t_1). The duration of the evaporation depends on the relative humidity and on the addition of adjuvants. The desorption medium was changed for several times (t_2-t_n). When the measurement was terminated the cuticular

membrane was also put in a scintillation vial to measure the residual radioactivity on the surface and inside the membrane to allow the determination of the total amount applied.

3.2.5 Adjustment of the relative humidity

It is highly important to keep the relative humidity constant during the whole duration of the experiment. Therefore, all measurements were conducted in a closable plastic box. The relative humidity inside this box was controlled by a cold trap (KF-18-2) (Walz, Effeltrich, Germany) very accurately. The air with adjusted water content was blown inside the box by a tube. There was also an outlet in the box, to allow a permanent change of the air inside the box. The relative humidity was controllable within minutes. To avoid gradients in temperature and humidity inside the closable room, a slowly rotating ventilator was built in. To check the temperature and the relative humidity at each time, a sensor of a thermo-/hygrometer (Huger Electronics, Germany) was included also.

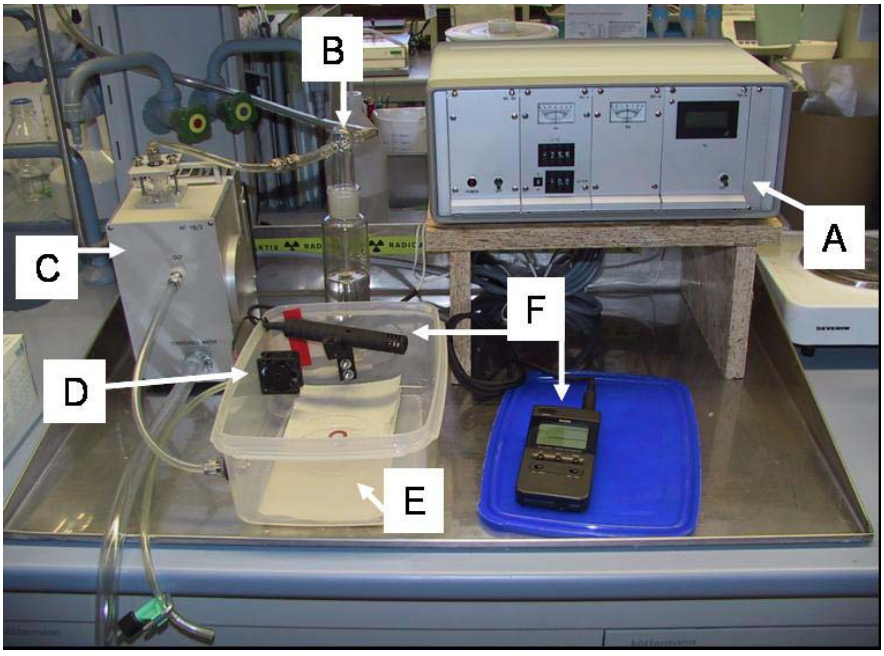


Figure 3.3 Photograph of the experimental set-up in the laboratory. A: Control unit of the cold trap, B: Bottle, filled with water to saturate the air flow, C: Cold trap, D: Ventilator to avoid any gradients inside the box, E: Box, F: Thermo-/hygrometer to check temperature and relative humidity.

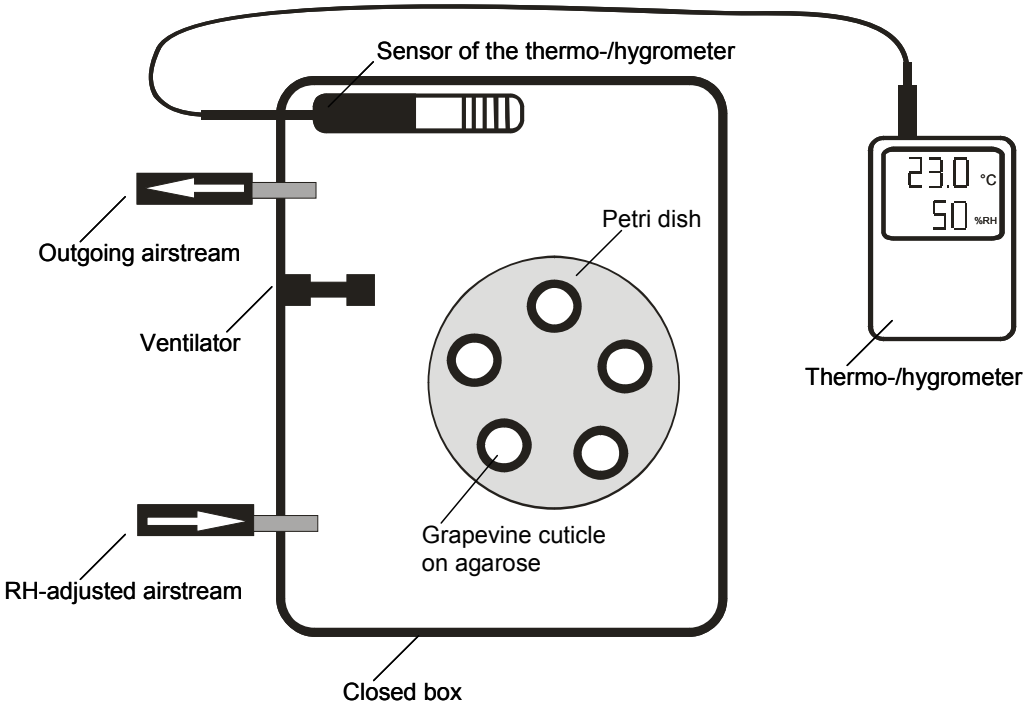


Figure 3.4 Schematic drawing of the closed box, including the ventilator, inlet and outlet apertures and the sensor of the thermo-/hygrometer.

3.2.6 Variation of the experimental conditions

This work was focused on cuticular penetration of paraquat dichloride (Tab. 3.1) across isolated cuticles of grapevine. Some experiments were done with further active ingredients like glyphosate (hydrophilic compound) and clodinafop-propargyl (Tab. 3.1) which is a lipophilic compound as seen from its octanol/water partition coefficient. The experimental set-up described above allows the variation of a huge amount of parameters, e. g. relative humidity, concentration of the active ingredient, droplet size, usage of dewaxed membranes and the use of different additives.

3.2.7 Radiolabelled model compounds

As model compound a charged hydrophilic herbicide was used: paraquat-dichloride. Additional work was done with glyphosate and clodinafop-propargyl.

The formulas are given in table 3.1 and the chemical and physical properties are summarised in table 3.2. All radiochemicals were ^{14}C-labelled and were provided by the Syngenta Isotope Laboratory (Basel, Switzerland).

Table 3.1 Overview about all used radiolabelled active ingredients, formulas and chemical names of the molecules.

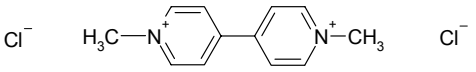
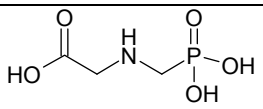
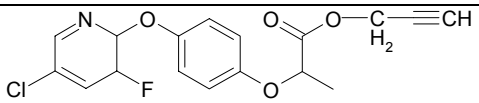
	Formula	Chemical name
Paraquat dichloride		1,1'-dimethyl-4,4'-bipydridinium
Glyphosate		N-(phosphonomethyl)glycine
Clodinafop-propargyl		2-propynyl-(R)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy)-phenoxy]-propionate

Table 3.2 Chemical and physical properties of the active ingredients: Water solubility, octanol/water partition coefficient (log $K_{O/W}$), specific activity and molecular weight.

	Water solubility [g l^{-1}]	log $K_{O/W}$	Specific activity [MBq mg^{-1}]	Molecular weigth [g mol^{-1}]
Paraquat dichloride	700[a]	-2.71[b]	2.03	257.2
Glyphosate	12[a]	-4.0[b]	4.54	169.8
Clodinafop-propargyl	0.0025[a]	3.9[c]	2.08	349.8

[a] The Agrochemical Handbook, 3rd Edition

[b] EPIWIN v3.11 (Calculated)

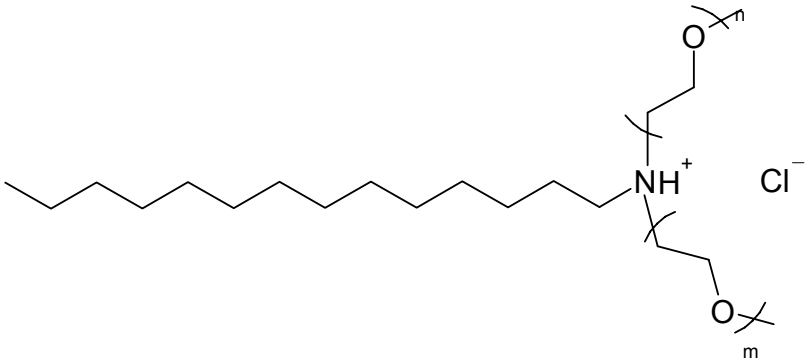
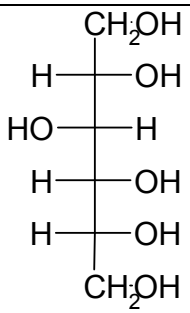
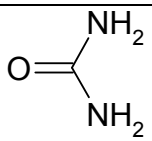
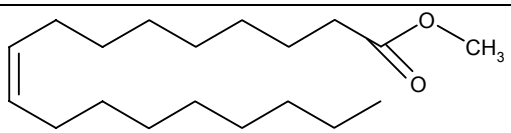
[c] Wirkstoffe in Pflanzenschutz und Schädlingsbekämpfungsmittel (1995)

3.2.8 Usage of additives

Different additives were used in the experiments (Tab. 3.3). Most work was done with ethomeen T/25. Ethomeen T/25 is a tallow amine ethoxylate with an average poly-ethoxy-ethylene-chain lenght of 15. Ethomeen T/25 was acidified with concentrated HCl to neutral pH (7.0), which forms the hydrochloride salt of the amine. This form was used in all experiments. The additive was provided by

Syngenta Crop Protection (Jealott's Hill International Research Centre, UK). Further experiments were done with sorbitol, urea, and methyl oleate. All these additives were purchased from Fluka (Neu-Ulm, Germany). Due to the fact that methyl oleate is not water soluble an emulsifier was necessary. The emulsifier was an anionic surfactant (Gafac RE-610; Rhodia, Paris, France). The concentration of the emulsifier was 10% relative to the amount of methyl oleate used.

Table 3.3 Formulas of the used additives.

Ethomeen T/25 (pH 7)	
Sorbitol	
Urea	
Methyl oleate	

3.2.9 Presentation of the data

All penetration experiments done with isolated grapevine cuticles are plotted as relative cuticular penetration [%] of the respective compound versus time. This

way of presentation allows an easier understanding of the time-course of the penetration process which is differentiated in two distinguishable phases: In the first phase (t_0-t_1) water of the applied droplet evaporates. It is characterised by a liquid or a wet residue. In the second phase droplet water is evaporated. This has enormous consequences for cuticular penetration processes. It was the aim of this work to understand, what was happening in drying droplets in terms of cuticular uptake. That is why recording of all data started with application of the droplets (t_0). Mostly, the first phase is the most important phase for uptake while the second phase is characterised by a continuously decreasing penetration. For that reasons, the curve of cuticular penetration under these conditions follows a hyperbola which approaches a horizontal asymptotic line. This line corresponds to maximum cuticular penetration. To examine the total penetration across the membrane it is necessary to measure a long time interval. Otherwise, the determination of the maximum penetration is imprecise. This behaviour of penetration is comparable to enzyme kinetics where the Michaelis constant and the maximum velocity of the enzyme are of interest. These values are determined by enzyme kinetic studies and include the so-called maximal rate of the catalysed reaction (v_{max}). At this rate the active site is saturated. The Michaelis constant (K_M) is the substrate concentration at which the reaction rate is one-half its maximum value. It is also known as the turnover number. All these values are determined experimentally by recording the progress of an enzyme-catalysed reaction using fixed amounts of an enzyme and a series of different substrate concentrations. v_{max} and K_M also can be determined from linear regression analysis of a plot of reciprocal initial velocity versus reciprocal substrate concentration, a so called Lineweaver-Burk plot. Figure 3.5A displays a schematic diagram of the initial velocity of an enzyme plotted versus substrate concentration. All discussed parameters are drawn in. Figure 3.5B is the reciprocal plot which also shows the parameters mentioned above.

In terms of the similarities of the enzyme kinetics and the line course of the cuticular transport experiments, all measured results were analysed in a double-reciprocal plot of percentage cuticular penetration versus time, additionally. The parameters K_M correspond to that time, when half of the maximum transport is reached ($t_{max}/2$) and v_{max} corresponds to the maximum cuticular penetration of

the examined compound ($\%_{max}$). The following equation describes the penetration kinetics following a hyperbolic curve progression:

$$\text{Penetration}_{(t)} = \frac{\%_{max} \times t}{(\frac{t_{max}}{2}) + t} \qquad \text{(Eq 3.1)}$$

The parameters found with the double reciprocal analysis of transport kinetics allow a more precise evaluation of all conducted experiments. All these data shall give some information in terms of cuticular penetration after droplet application as happening in the field.

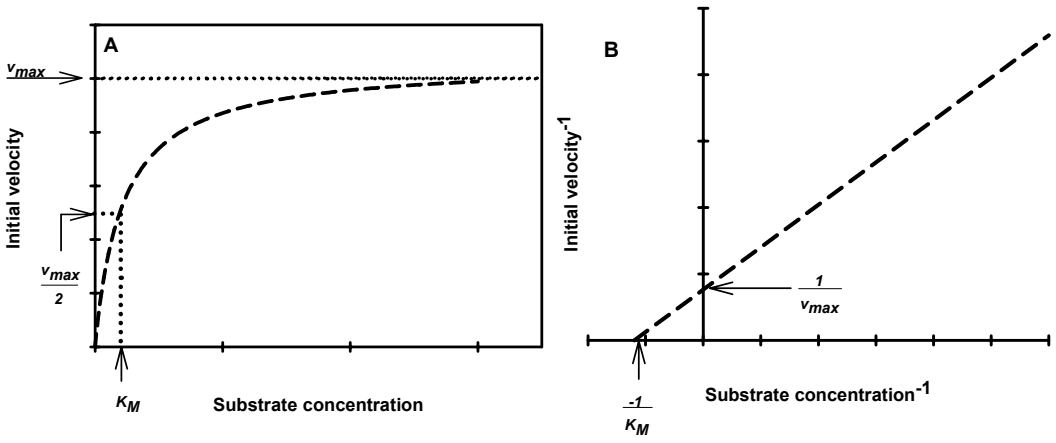


Figure 3.5 **A:** Typical correlation between the substrate concentration and the initial reaction velocity of an enzyme. All parameters marked are explained above. **B:** In a Lineweaver-Burk plot the inverse of the x- and y-intercepts represent the kinetic constants K_M or rather v_{max} .

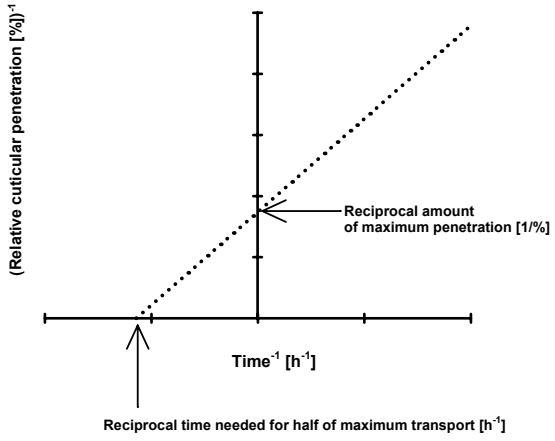


Figure 3.6 Reciprocal plot of percentage cuticular penetration versus time. K_M from Lineweaver-Burk corresponds with time needed for half of maximum cuticular penetration. v_{max} corresponds with maximum penetration.

Additionally, the time course of penetration was analysed by plotting the natural logarithm of the relative amount not penetrated $-\ln(1-M_t/M_0)$ versus time. In this type a first order plot is obtained when the selected compound is dissolved and transport properties of the cuticles remain constant during the experiment. Deviation from linearity signals that one or more conditions for a first order process are not fulfilled. Mostly, a linear curve progression is obtainable which allows the determination of rate constants k [h^{-1}] (see chapter 2.3). In some experiments linearity failed.

3.2.10 Sample size and statistics

At the beginning of the experiment sample sizes were between 12 and 15 replicates. Because of the delicate nature of the membranes each experiment was finished with less cuticles, but at least with eight membranes. Results are given as means, error bars represent standard errors.

3.3 Steady state experiments with focus on primary metabolites

3.3.1 Creation of aqueous leaf imprints

In order to obtain first information on the occurrence of primary metabolites on plant surfaces, imprints of astomatous, adaxial leaf surfaces were made. Leaves were taken from grapevine plants growing in the greenhouse. Advantages of that plant material were the relative high permeability of these cuticles and the high relative humidity in the ambience of the greenhouse as well as clean air. Plants were healthy and only leaves without necrosis etc. were selected. There were also no problems with aphids. About 15 leaves were imprinted on a wet filter paper very carefully to avoid injuries of the plant surface. To stain reducing carbohydrates on the filter paper a glucose-oxidase-peroxidase colour reagent (Siemens & Mitchell-Olds, 1998) was applied on the

whole area of the filter. To avoid any artifacts, one filter paper was dipped in a high concentrated aqueous solution of glucose and another one was wetted with pure water. The presence of reducing carbohydrates on the leaf surfaces was qualitatively detectable when there was a reddish staining.

This experiment was repeated with the only exception that a ninhydrin solution was applied on the filter. Ninhydrin creates a purple dye in the presence of amino acids, urea or ammonia. To obtain a stable colour product a Ninhydrin-Reagent-Set (Fluka, Neu-Ulm, Germany) was used. To ensure that the staining is not an artifact, one filter paper was dipped in a high concentrated aqueous solution of phenylalanine and another one was wetted with pure water. Presence of stable ninhydrin positive compounds on the leaf surfaces were qualitatively detectable when there was a purple staining visible.

3.3.2 Plant material for transport experiments

Adaxial astomatous cuticular membranes (CM) were enzymatically isolated from fully expanded leaves of English ivy *(H. helix* L.*)* and grapevine (*V. vinifera* L. cv. Nelly ['Rötliche Hausrebe']) growing in the Botanical Garden in Würzburg according to the procedure described by (Schönherr & Riederer, 1986). Before they were used for transport measurements the cuticles of English ivy were stored for three months, because it was described by Geyer & Schönherr (1990) that permeances of isolated cuticles decreased by a factor of about two within the first weeks after isolation (Geyer & Schönherr, 1990). Polymer matrix membranes (MX) were obtained by extracting cuticular waxes from the CM of *H. helix* with a sufficient amount of chloroform (Roth, Karlsruhe, Germany) for 12 h at room temperature

3.3.3 Model compounds

A broad variety of model compounds was chosen. Selection was focused on compounds which are of interest in plant biology and plant protection. All

selected molecules are summarised in table 3.1 and figure 3.1. The molar volume was calculated according to McGowan & Sowada (1993), octanol/water partition coefficient and water solubility were estimated using the modelling program EPIWIN v3.11 (freely available from the U.S. Environmental Protection Agency, http://www.epa.gov). Carbohydrates were assumed to be preferentially in the cyclic form (Angyal, 1987). Compounds were classified according to the octanol/water partition coefficient as hydrophilic (log $K_{O/W} \leq 0$) and lipophilic (log $K_{O/W} > 0$).

The set of the hydrophilic compounds contained urea and the carbohydrates erythrose, xylose, glucose, maltose and maltotriose. As volatile compounds water and ethanol were added. Additionally, several amino acids and two hydrophilic herbicides glyphosate and paraquat dichloride were included. In the case of the lipophilic compounds the main focus was on plant protection agents like the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and metribuzin, the fungicide bitertanol and the herbicide safener cloquintocet-mexyl. Furthermore, benzoic acid and salicylic acid were also used.

The weak acids benzoic acid ($pK_a = 4.19$), salicylic acid ($pK_a = 2.97$) and 2,4-D ($pK_a = 2.85$) were measured at pH 2 and the weak base cloquintocet-mexyl ($pK_b = 3.03$) at pH 6 in order to ensure that the compounds are predominately non-ionised (99 % for benzoic acid, 90 % for salicylic acid, 88 % for 2,4-D and 99.9 % for cloquintocet-mexyl). In all calculations only the concentration of the non-dissociated species of these compounds was used. Additionally, benzoic acid was measured at pH 7 when it is ionised for 99.9%. All amino acids were used at their respective isoelectric point.

3.3.4 Quantification of the model compounds

Carbohydrates were quantified using a oxidase-peroxidase colour reagent (sample : colour reagent = 1 : 3 v/v) (Siemens & Mitchell-Olds, 1998). Maltose and maltotriose were digested to their glucose monomers using a α-glucosidase

(Sigma-Aldrich, Taufkirchen, Germany) before the colour reagent was added. The amounts of carbohydrates were assayed with a spectrophotometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) at 490 nm. Calibration curves were added on each microplate (R^2 > 0.98). All amino acids and also urea was quantified using ninhydrin which forms a purple dye. This method was described by Moore & Stein (1954) and Moore (1968). To obtain a stable colour product a Ninhydrin-Reagent-Set (Fluka, Neu-Ulm, Germany) was used (volume ratio sampling probe : colour reagent = 1 : 1 v/v). The amounts of the respective amino acids and urea also were assayed with a spectrophotometer at 570 nm. The volatile compounds ethanol and water were detected gravimetrically using a micro-balance (± 1 μg; Sartorius, Göttingen, Germany). All other compounds were ^{14}C-labelled. The amounts of radioactivity were determined by liquid scintillation counting (Tri Carb 2500, Canberra Packard, Frankfurt, Germany) after addition of scintillation cocktail (Ultima Gold XR, Canberra Packard, Dreieich, Germany) to the samples.

3.3.5 Determination of permeances

Permeability experiments were carried out with transport chambers made of stainless steel (Schreiber *et al*., 1995). The cuticles were mounted between a donor and a receiver compartment with the physiological outer side pointing to the donor compartment. Experiments were initiated by addition of an aqueous solution of the respective compound to the donor chamber. The amounts of molecules permeated were detected in regular time intervals in the receiver chamber, which was filled with an aqueous phospholipid suspension made from soybean lecithin (1 g l^{-1}, Roth, Karlsruhe, Germany) when the experiment was conducted with a lipophilic compound. The lecithin aggregates are large and do not penetrate the CM, but serve as a sorption compartment which keeps their concentration in the surrounding aqueous solution practically zero (Schönherr & Bauer, 1992). For experiments done with hydrophilic compounds deionised water was used as receiver medium. Between the sampling points the transport chambers were put on an orbital shaker at 25 °C to avoid concentration gradients inside both chambers. Linear transport kinetics were obtained in all

cases and permeances (P) were calculated from the flow (F), the exposed area (A) and the concentration difference between the donor and the receiver chamber (Δc):

$$P = \frac{F}{A \times \Delta c} \qquad \text{(Eq. 3.2)}$$

Permeances of the volatile compounds were measured with modified transport chambers lacking a receiver compartment. The flow was determined by measuring the weight loss of the chambers as a function of time due to volatilasation of the compounds by permeation through the cuticular membrane into the surrounding air. The transport chambers were stored in a plastic box over silica gel to adjust a relative humidity of ~ 0%. Permeances of volatiles can be expressed either on the basis of the liquid state or on the basis of the vapor state. The conversion factor is given by the ratio of the corresponding densities amounting to 43,400 for water and 5,500 for ethanol at 25 °C. Permeances referring to the concentration in the liquid state as driving force can be easily converted by multiplication with the corresponding conversion factors.

To measure permeances with thin grapevine cuticles a modification was essential. The cuticles were mounted on a washer made from zinc coated steel (6.4 x 20 mm) (Würth, Gaisbach, Germany). The washer with the cuticle was built in the transport chamber like the robust cuticles of *H. helix*. This alteration reduced the exposed area of the cuticle and the aqueous solution which was essential to avoid diffractions of the sensitive membrane.

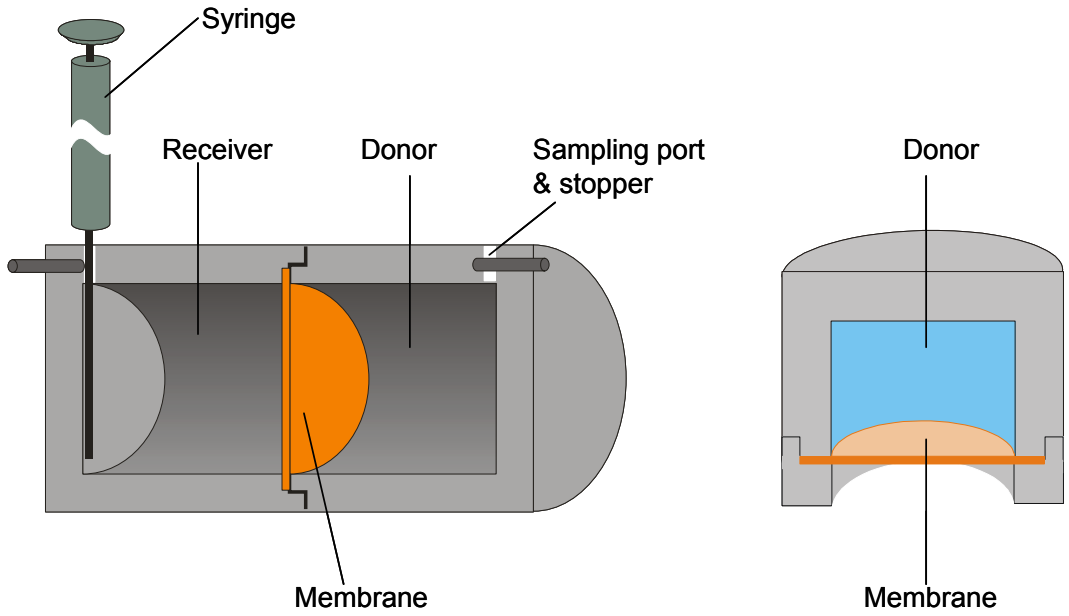


Figure 3.7 Schematic drawing of the experimental set-up for the determination of permeances. Transport chambers are made from stainless steel.

Cuticular permeances of benzoic acid and xylose were measured in the temperature range from 15 °C to 35 °C. Activation energies were calculated from the slopes of the regression lines fitted to Arrhenius plots by plotting the natural logarithm of the permeance versus the reciprocal value of the absolute temperature:

$$\ln P = \ln A - \frac{E_a}{R \times T} \qquad \text{(Eq. 3.3)}$$

Ln A is the pre-exponential factor of the Arrhenius plot, T is the abslolute temperature [K], E_a is the activation energy [kJ mol^{-1}] and R is the gas constant [J mol^{-1} K^{-1}].

Nonionic surfactants act as accelerators of diffusion in the cuticular wax barrier (Schreiber *et al.*, 1996). The effect of the alcohol ethoxylate triethylene glycol monododecylether ($C_{12}E_3$, Fluka, Neu-Ulm, Germany) on the permeance of xylose, benzoic acid and salicylic acid was measured by addition of the

surfactant (0.1 g l^{-1}). In order to obtain maximum effects and to avoid any interactions, $C_{12}E_3$ was applied in the receiver chamber.

3.3.6 Determination of partition coefficients

The cuticle/water partition coefficient ($K_{C/W}$) is defined as the ratio between the equilibrium concentration in the cuticle ($c_{cuticle}$) and the equilibrium concentration in the aqueous phase (c_{water}):

$$K_{C/W} = \frac{c_{cuticle}}{c_{water}} \qquad \text{(Eq. 3.4)}$$

Cuticular membranes were added to aqueous solutions of the respective compound. Equilibrium was achieved by shaking the probes on an orbital shaker for 24 h at 25°C. Afterwards, the cuticular membranes were removed and the concentration in each phase was determined as described in chapter 3.3.4. Sorption of water and ethanol to cuticular membranes was gravimetrically measured from a saturated permanently saturated atmosphere of the volatile compound (c_{air} = 23.1 g m^{-3} for water and c_{air} = 146.8 g m^{-3} for ethanol) yielding the cuticle/air partition coefficient ($K_{C/A}$):

$$K_{C/A} = \frac{c_{cuticle}}{c_{air}} \qquad \text{(Eq. 3.5)}$$

Cuticle/air water partition coefficients can be converted into cuticle/water partition coefficients according to (Merk & Riederer, 1997):

$$K_{C/W} = K_{C/A} \times K_{A/W} \qquad \text{(Eq. 3.6)}$$

The air/water partition coefficient ($K_{A/W}$) is identical to the dimensionless Henry constant ($K_{A/W}$ = 2.3 x 10^{-5} for water and $K_{A/W}$ = 2.0 x 10^{-4} for ethanol). Correspondingly, matrix membrane/water partition coefficients ($K_{MX/W}$) were

measured with the only exception that matrix membranes were used instead of cuticular membranes. Partition coefficients were only determined with English ivy membranes, since usage of grapevine cuticles would require huge amounts of these cuticles due to its thinness.

3.3.7 Sample size and statistics

All permeation experiments and all partition coefficients were based on at least 12 replications. Results are given as means with 95% confidence intervals. Partition coefficients and permeances were tested for normal distribution by the Kolmogorov-Smirnov test which validated the use of parametric statistics in all cases.

Table 3.4 List of all model compounds including molecular weight (MW) [g mol^{-1}], molar volume (MV) [cm^3 mol^{-1}], octanol/water partition coefficient ($K_{O/W}$) and water solubility (WS) [mol kg^{-1}]. MV, $K_{O/W}$ and WS were estimated using the modeling program EPIWIN v3.11.

			MW [g mol^{-1}]	MV [cm^3 mol^{-1}]	log $K_{O/W}$	log WS [mol kg^{-1}]
Small hydrophilics	1	Water	18	17	-1.38	1.74
	2	Ethanol	46	45	-0.14	1.24
	3	Urea	60	47	-1.56	0.85
Carbohydrates	4	D(-) Erythrose	120	80	-1.52	0.92
	5	D(+) Xylose	150	100	-1.98	0.82
	6	D(+) Glucose	181	120	-2.89	0.74
	7	D(+) Maltose	342	223	-5.03	0.47
	8	Maltotriose	504	326	-7.36	0.3
Amino acids	9	Glycine	75	57	-3.21	0.92
	10	L-Alanine	89	71	-2.96	0.61
	11	L-Serine	106	76	-3.07	0.60
	12	L-Valine	117	99	-2.26	-0.30
	13	L-Threonine	119	91	-2.94	-0.09
	14	L-Leucine	131	113	-1.52	-0.94
	15	L-Phenylalanine	165	131	-1.44	-1.77
Hydroph. Al.	16	Glyphosate	169	109	-4.0	-1.15
	17	Paraquat dichloride	257	251	-2.71	0.43
Lipophilics	18	Benzoic acid	122	93	1.87	-1.69
	19	Salicylic acid	138	99	2.24	-1.56
	20	2,4-D	221	138	2.62	-2.82
	21	Metribuzin	214	162	1.49	-2.22
	22	Cloquintocet-mexyl	336	257	5.28	-5.94
	23	Bitertanol	337	267	4.07	-4.70

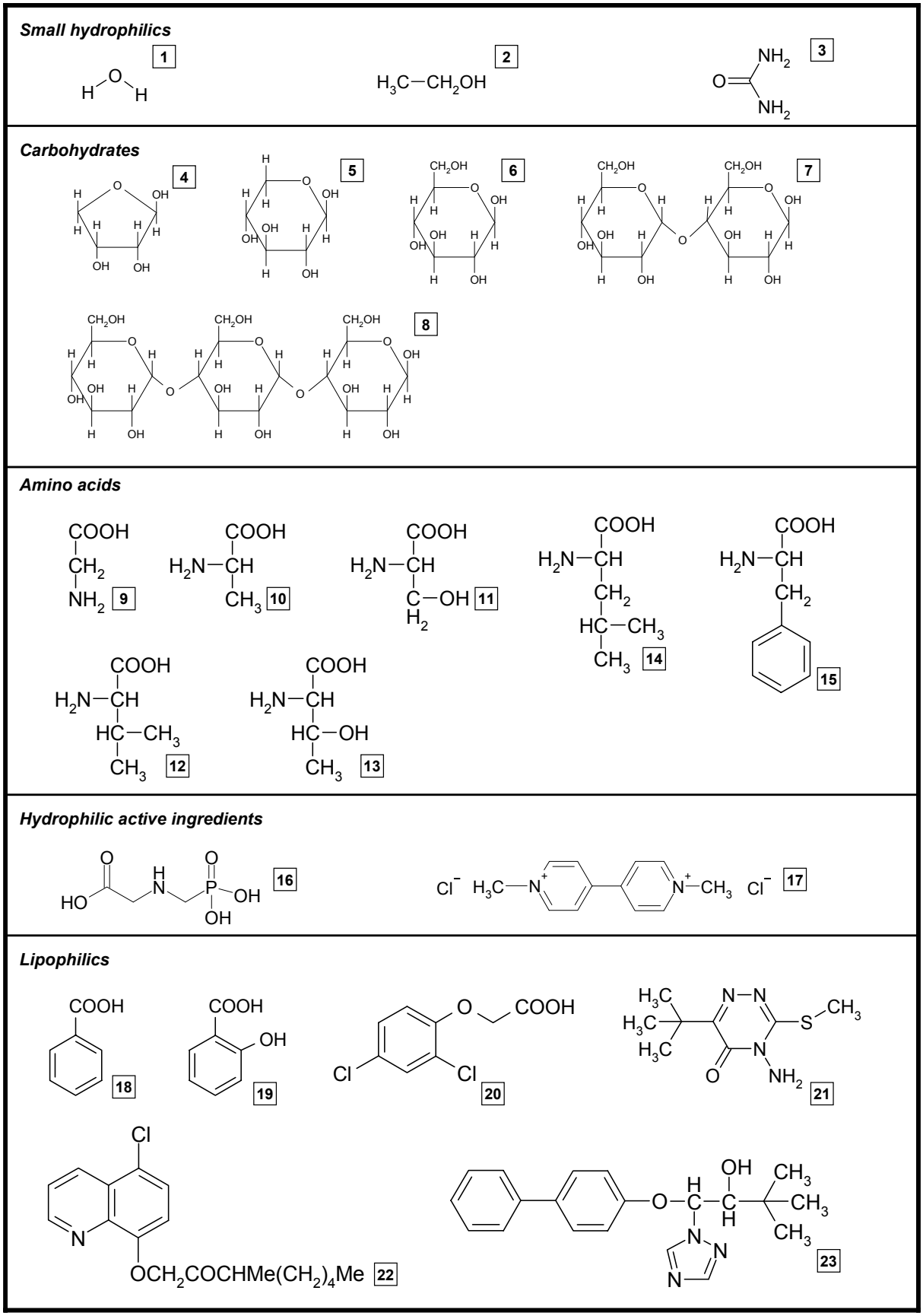


Figure 3.8 Chemical structure of the model compounds. Numbers refer to table 3.4

Sources of supply:

2, 3, 4, 5, 6, 7, 8, 12, 15: Sigma-Aldrich, Taufkirchen, Germany

9, 10, 11, 13, 14: VWR, Darmstadt, Germany

15, 16, 18, 20, 22: Syngenta Crop Protection, Basel, Switzerland

21, 23: Bayer, Leverkusen, Germany

19: Du Pont de Nemours, Dreieich, Germany

Chemical names of the active ingredients:

Glyphosate	N-(phosphonomethyl)glycine
Paraquat dichloride	1,1'-dimethyl-4,4'-bipyridinium
2,4-D	2,4-dichlorophenoxyacetic acid
Metribuzin	4-amio-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one
Cloquintocet-mexyl	5-chloro-8-quinolinoxyaceticacid 1-methylhexyl ester
Bitertanol	1-(biphenyl-4-yloxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol

4. RESULTS

4.1 Characterisation of the grapevine cuticle (*Vitis vinifera*)

4.1.1 Water permeability of isolated grapevine cuticles (*V. vinifera*)

The water permeability is a very useful tool to classify transport properties of plant cuticles. After completed isolation of a new set of cuticles it is very enlightening to determine the water permeability and to compare it with other species. There are some reviews (Kerstiens, 1996; Riederer & Schreiber, 2001) which summarise water permeabilities of a huge variety of plant species.

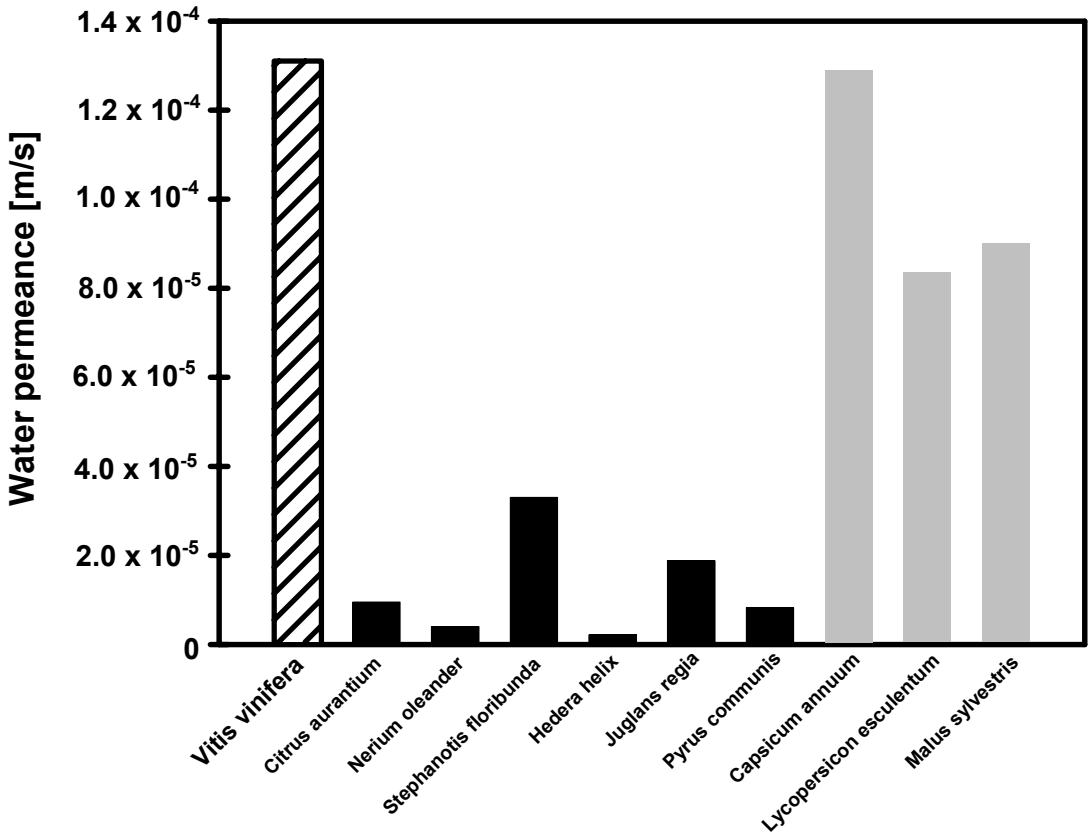


Figure 4.1 Comparison of the water permeance determined for *V. vinifera* leaf cuticles with other species (taken from Riederer & Schreiber, 2001). The selection of the comparative values is based on a comparison with species which are often used in cuticular research. Black bars represent water permeabilities of leaf cuticles, grey bars represent water permeabilities of fruit cuticles.

After succeeded isolation of grapevine leaf cuticles the water permeance was determined. A water permeance of 1.31 x 10^{-4} m s^{-1} ($\pm$ 0.44 x 10^{-4} m s^{-1}) (95% CI) was found. The calculation for this value would be based on a vapour-phase driving force. Based on liquid water the water permeance is 3.02 x 10^{-9} m s^{-1}. This is a relatively high water permeability when compared to data from the literature (Fig. 4.1). The magnitude of 10^{-4} m s^{-1} is mostly reached only by fruit cuticles which generally have higher water permeances. Such high permeabilities of leaf cuticles are not abnormal, but most cuticular research was done with more stable cuticular membranes which are more easy to isolate. Such cuticles very often have low permeabilities. The handling of thicker membranes is much easier and less error-prone. However, it is expected that many species - mainly herb and grass species - have as high water permeabilities as grapevine.

To ensure any alteration of the transport properties of isolated grapevine cuticles, the water permeability of intact leaves was measured, too. Transpiration across the stomatous abaxial leaf cuticle was prevented using adhesive tape. A water permeance of 1.14 x 10^{-4} m s^{-1} ($\pm$ 0.45 x 10^{-4} m s^{-1}) was determined. The permeances found with intact leaves and with isolated cuticles were not significantly different (P = 0.63). This implies that the isolation process does not alter the transport properties of the isolated grapevine cuticles.

4.1.2 Wax coverage of *V. vinifera* cuticles

The mean wax coverage of grapevine leaf cuticles is 17.5 $\pm$ 4.8 µg cm^{-2}. This agrees exactly with Radler (1970). It is a comparably low value, since *H. helix* has a wax coverage of 122 $\pm$ 16 µg cm^{-2}, for example. Converting the wax coverage into thickness of the wax layer results in 0.19 $\pm$ 0.05 µm for grapevine and in 1.35 $\pm$ 0.24 µm for English ivy. Wax density assumed is 0.9 g cm^{-3} (Weast, 1977).

4.1.3 Chemical composition of the cuticular waxes of *V. vinifera*

The cuticular waxes of grapevine leaf cuticles consist of 55% aliphatic compounds and 35% cyclic triterpenoids. 10% of the total wax yield was not identifiable. Main components are primary alcohols and triterpenoids which form all in all 68.5% of the total wax. The residual portion consists of aliphatic compounds like *n*-alkanes, fatty acids, alkylesters and ketons. The four main components are C_{26}-alcohol (10.8%), C_{28}-alcohol (9.2%), β-amyrin (9.7%) and taraxerol (9.6%). Figure 4.2 summarises all compound classes making up the cuticular waxes of *V. vinifera* cv. Nelly. A detailed listing of all identified aliphatic and cyclic wax components is given in tables 4.1 and 4.2.

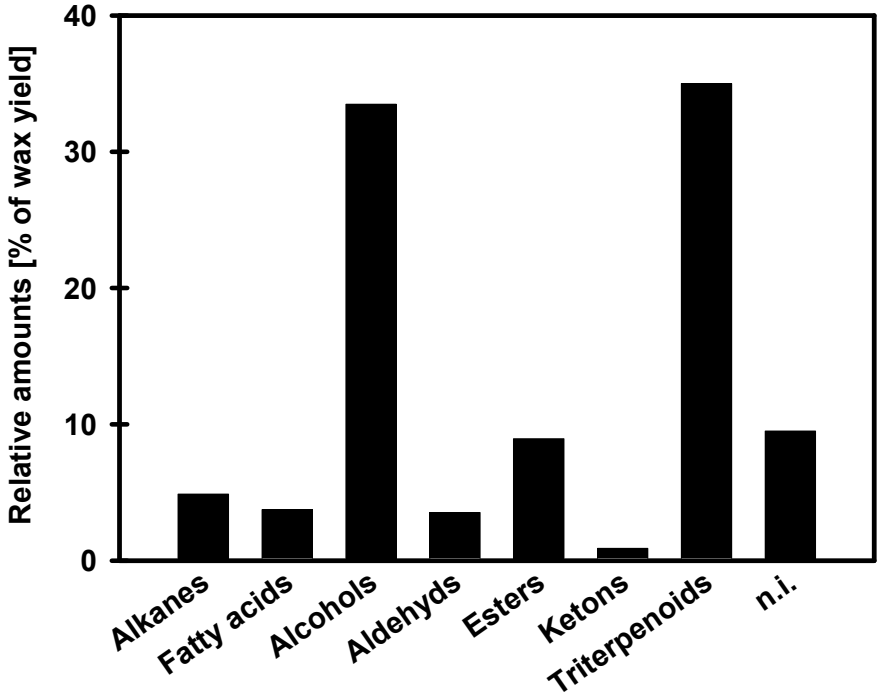


Figure 4.2 Relative constitution of the cuticular waxes of grapevine. The primary alcohols and the cyclic triterpenoids make up the bulk of the waxes.

Table 4.1 Detailed listing of all identified aliphatic wax components of *V. vinifera.* Numbers are relative amounts of the wax yield [%].

Chain-length	Alkanes	Fatty acids	Alcohols	Aldehyds	Ketons	Chain-length	Esters
20		1.2				**38**	0.5
21						**39**	
22		0.2	0.3			**40**	1.9
23		0.1	0.1			**41**	
24		0.5	3.8	0.4		**42**	0.8
25	0.4	0.1	0.3			**43**	
26		0.9	10.8	1.2	0.1	**44**	1.0
27		0.2	0.3	0.2	0.8	**45**	
28	0.1		9.2	1.2		**46**	1.3
29	1.8			0.1		**47**	
30	0.1	0.4	5.1	0.3		**48**	1.2
31	2.5					**49**	
32		0.2	3.8	0.2		**50**	2.3

Table 4.2 Detailed listing of all identified cyclic wax components of *V. vinifera.*

	Relative amounts [% of wax yield]
taraxerol	9.6
α-amyrin	3.8
β-amyrin	9.7
β-sitosterol	0.6
lupeol	1.0
erythrodiol	5.0
uvaol	0.8
oleanolic acid	0.5
terpenes n. i.	3.9

4.1.4 SEM images of isolated *V. vinifera* cuticles

It was the objective of these investigations to get information on the cuticle surface of this model plant. All following pictures are SEM-images from isolated adaxial, astomatous grapevine leaf cuticles. These images display the lower side of the cuticle which was orientated towards the underlying epidermis cells, or the upper side of the cuticle which was the outer surface of the leaf. Very prominent features are the cuticular ledges on the outer side of the adaxial leaf cuticle of grapevine which are no artefacts. Such ledges were also found with cotton leaf cuticles (Oosterhuis, 1998). The thickness of the membrane is 0.7 µm.

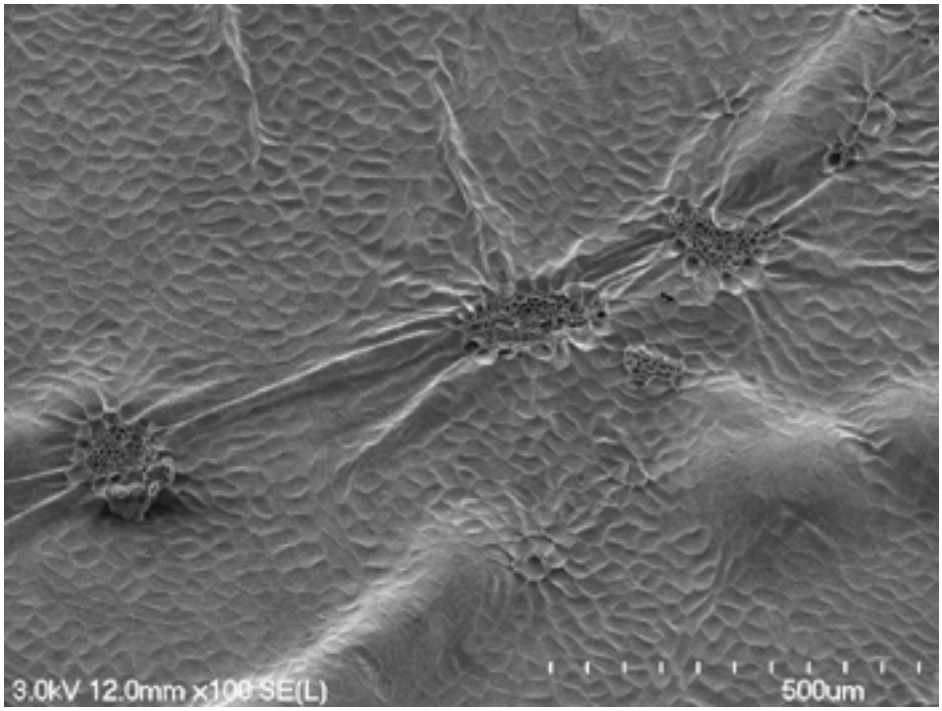


Figure 4.3 Lower side of the isolated grapevine cuticle. Imprints of the epidermis cells and also not fully digested epidermis cells are visible. Magnification ca. 260x.

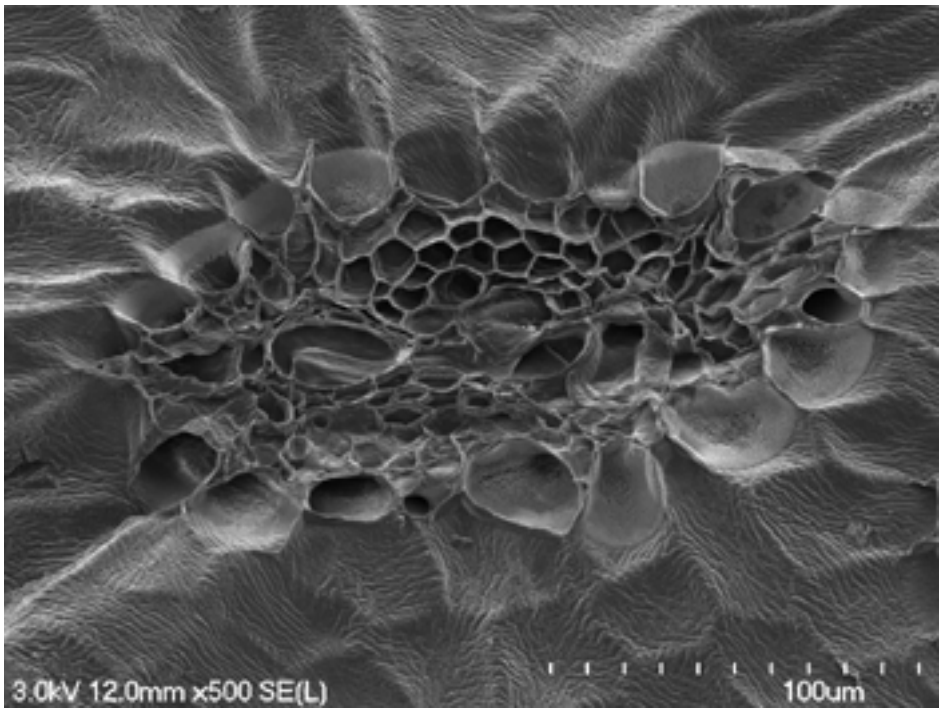


Figure 4.4 Lower side of the isolated grapevine cuticle. Focus on not fully digested epidermis cells. Magnification ca. 1,300x.

Figure 4.5 Lower side of the isolated grapevine cuticle. Focus on a partially digested epidermal cell well. Magnification ca. 7,800x.

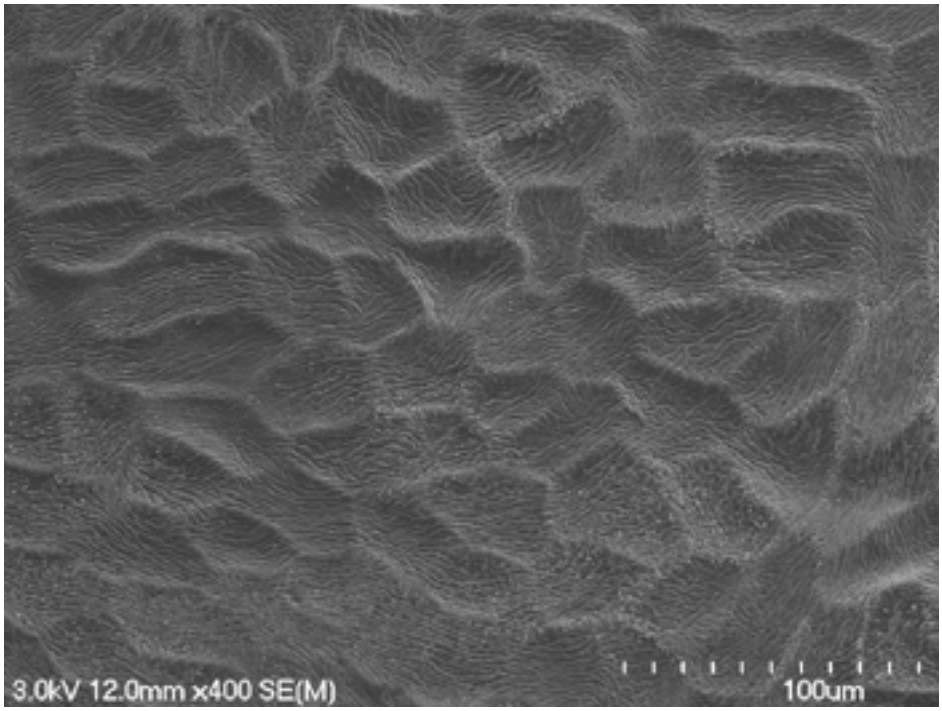


Figure 4.6 Lower side of the isolated grapevine cuticle. Focus on imprints of the epidermis cells. Magnification ca. 1,300x.

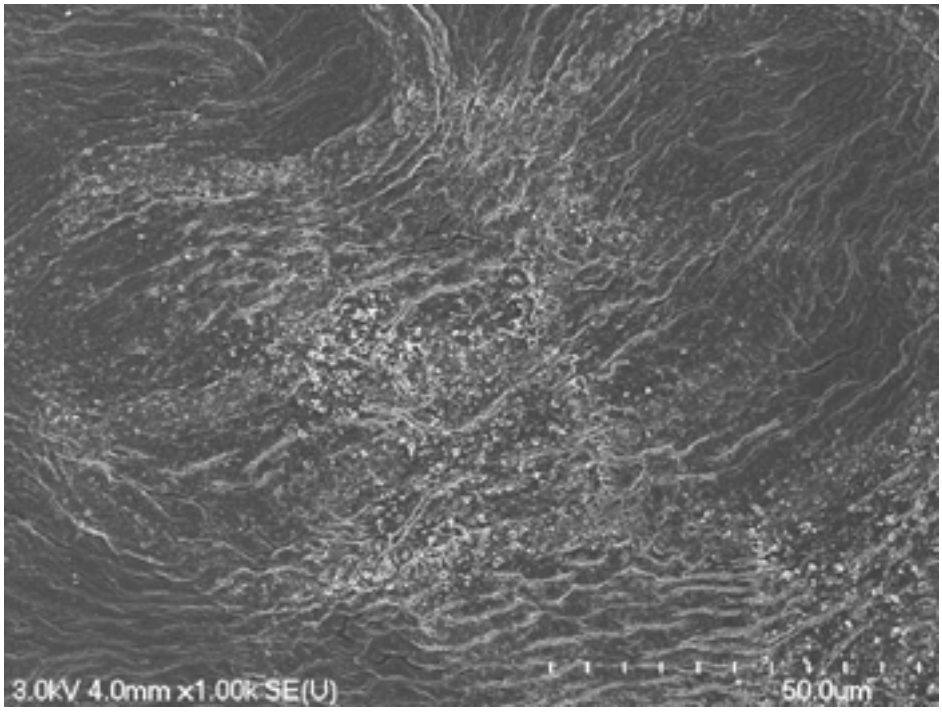


Figure 4.7 Lower side of the isolated grapevine cuticle. Focus on the structure. Magnification ca. 2,600x.

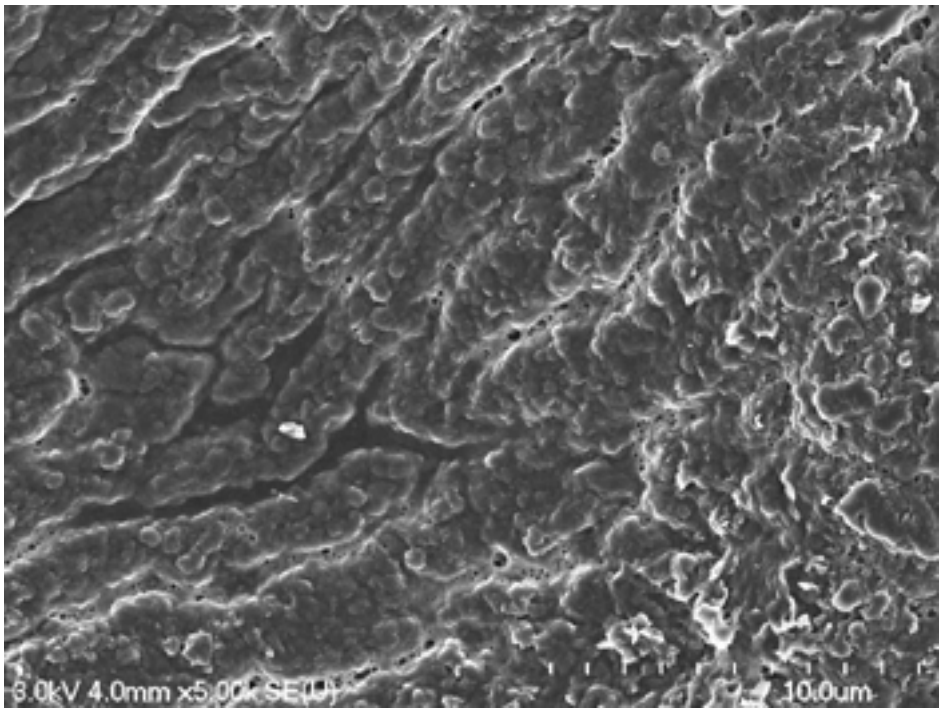


Figure 4.8 Lower side of the isolated grapevine cuticle. Focus on the structure. Magnification ca. 13,000x.

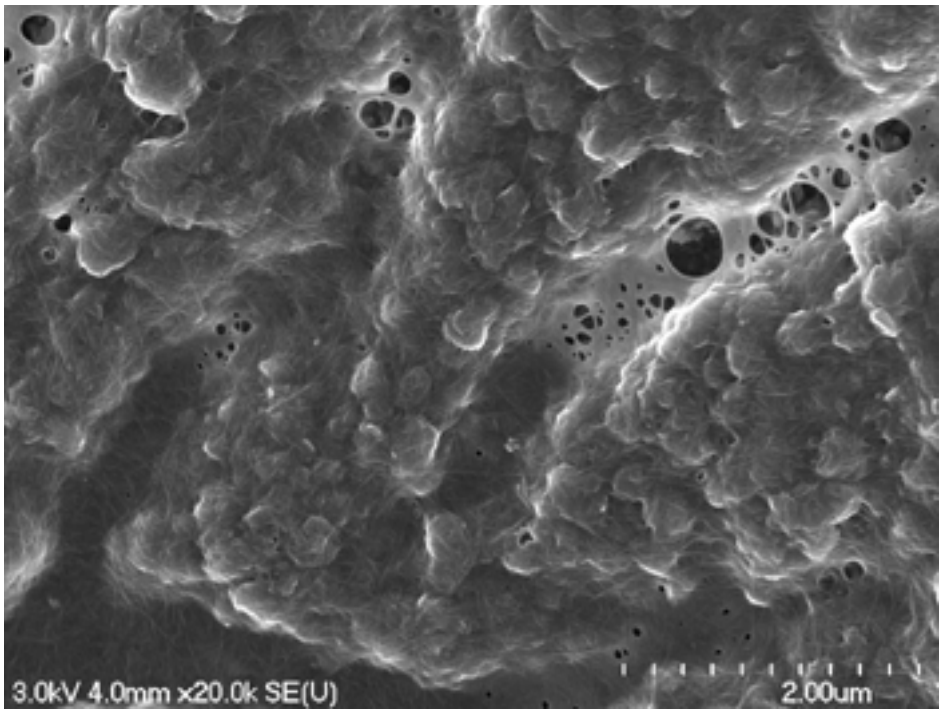


Figure 4.9 Lower side of the isolated grapevine cuticle. Focus on the structure. Fibrils are observable, highly presumably made up of polysaccharides or cutin. Magnification ca. 51,500x.

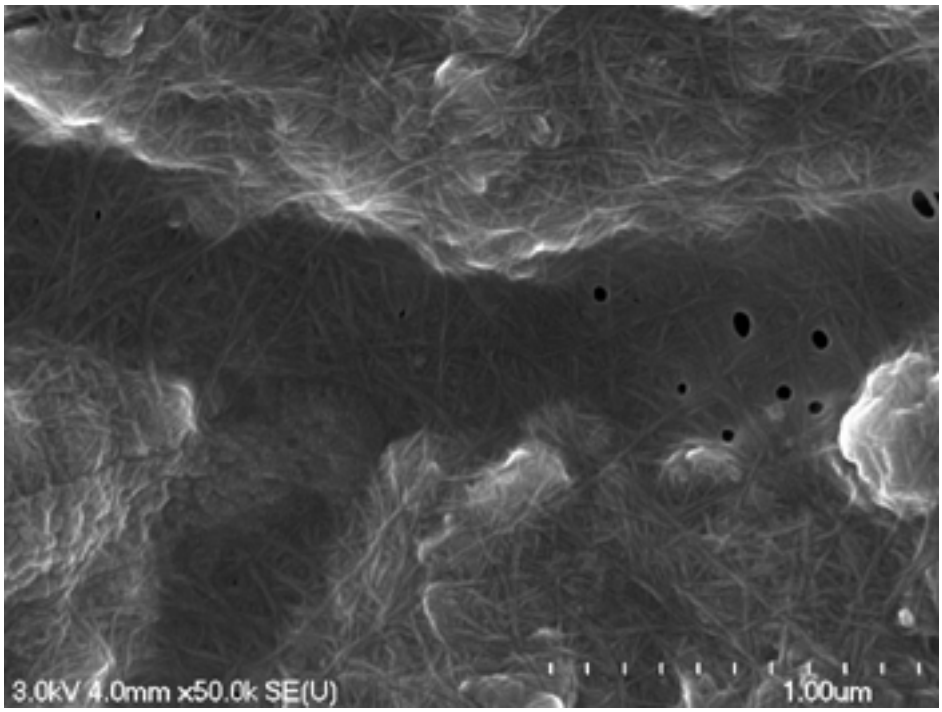


Figure 4.10 Lower side of the isolated grapevine cuticle. Putative polysaccharide fibrils are observable. These fibrils should be originated from the epidermal cell wall. Magnification ca. 130,000x.

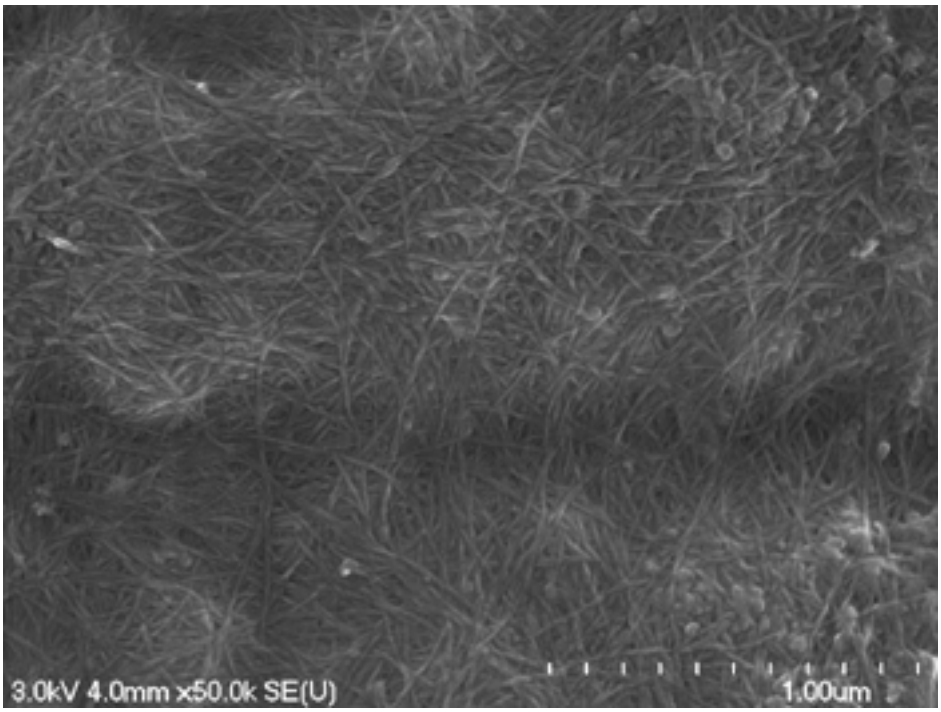


Figure 4.11 Lower side of the isolated grapevine cuticle. Fibrilar network, highly presumable originated from the epidermal cell wall. Magnification ca. 130,000x.

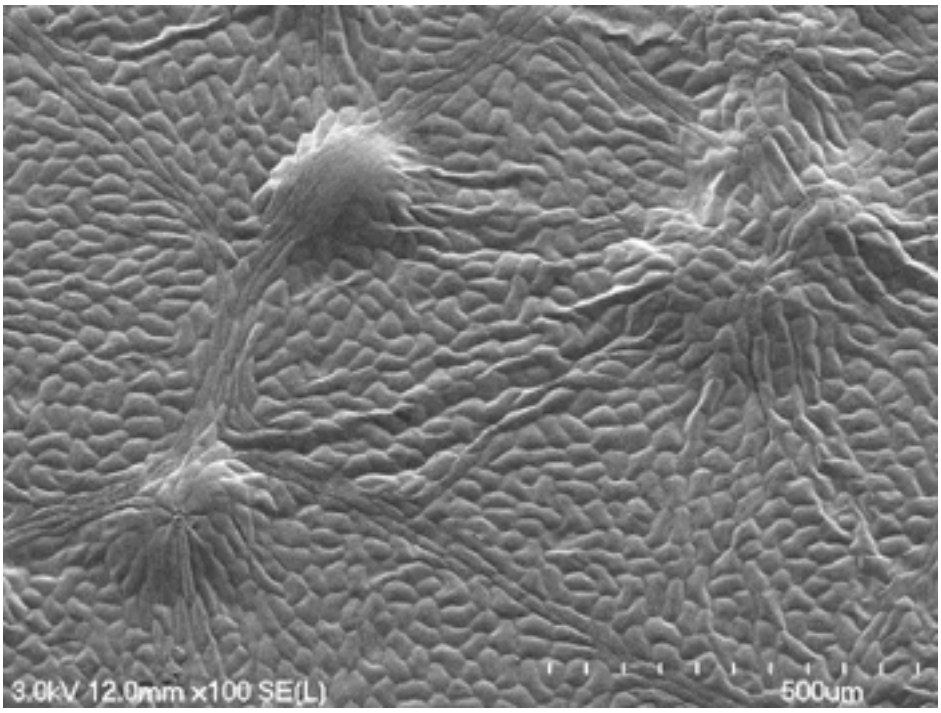


Figure 4.12 Upper side of the isolated grapevine cuticle. Clearly observable are imprints of epidermis cells and of leaf veins. The risings are caused by not fully digested cell material on the under side as shown from figure 4.3 and 4.4. Magnification ca. 260x.

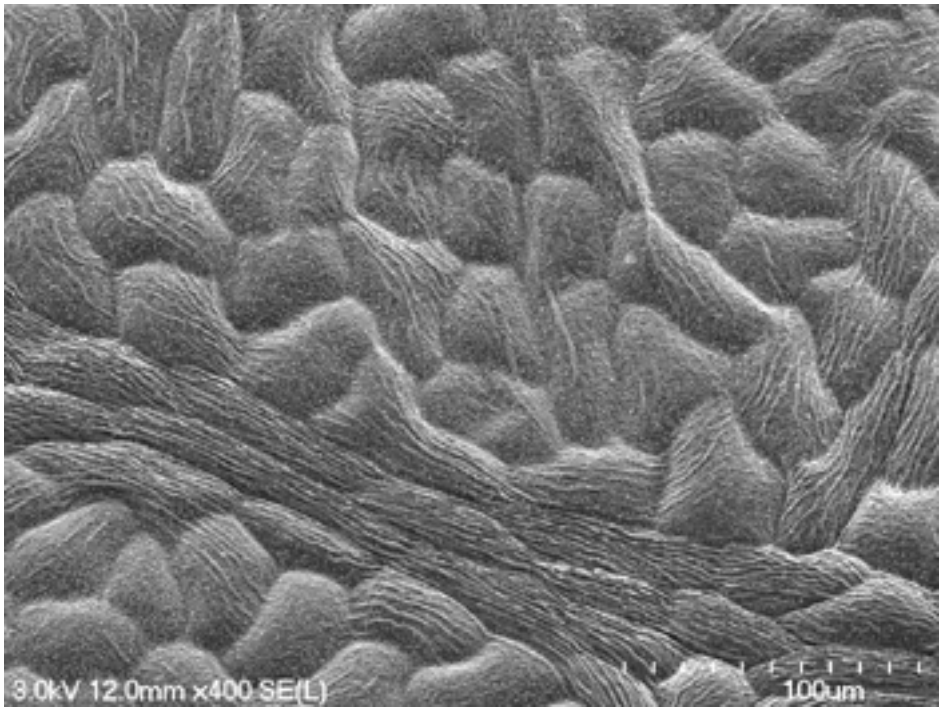


Figure 4.13 Upper side of the isolated grapevine cuticle. Clearly observable are imprints of epidermis cells and of leaf veins. Magnification ca. 1,050x.

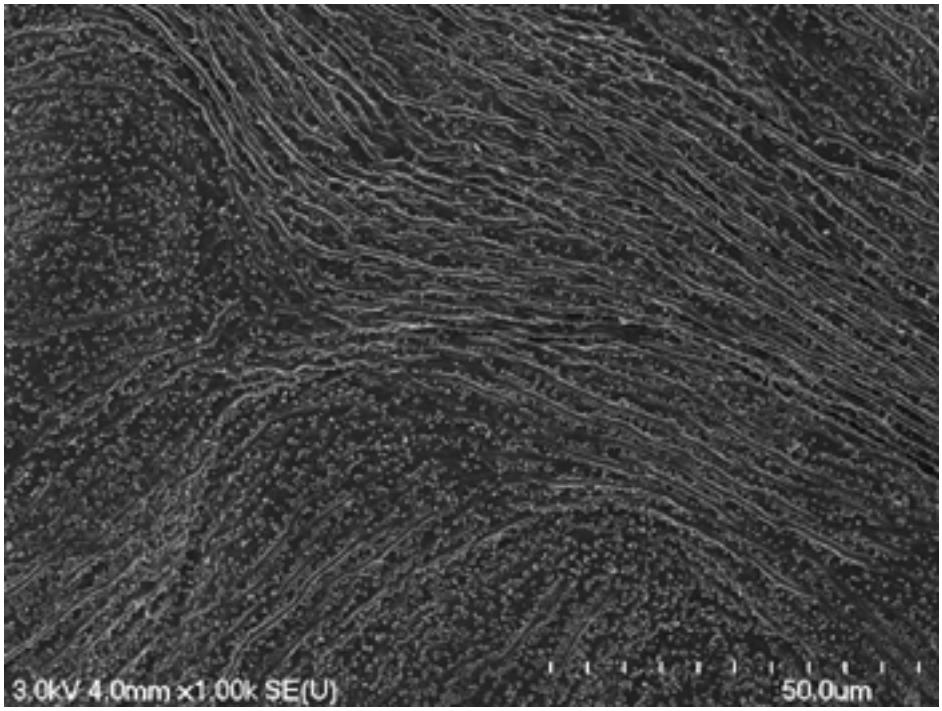


Figure 4.14 Upper side of the isolated grapevine cuticle. The cuticular ledges are clearly observable. Magnification ca. 2,600x.

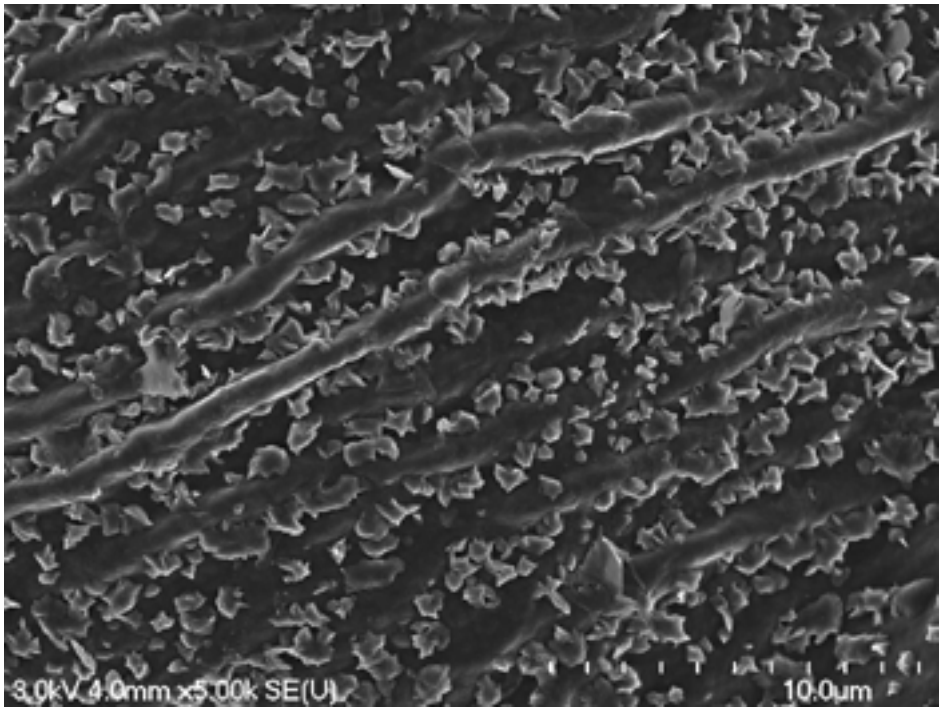


Figure 4.15 Upper side of the isolated grapevine cuticle. The cuticular ledges are clearly observable. Magnification ca. 13,000x.

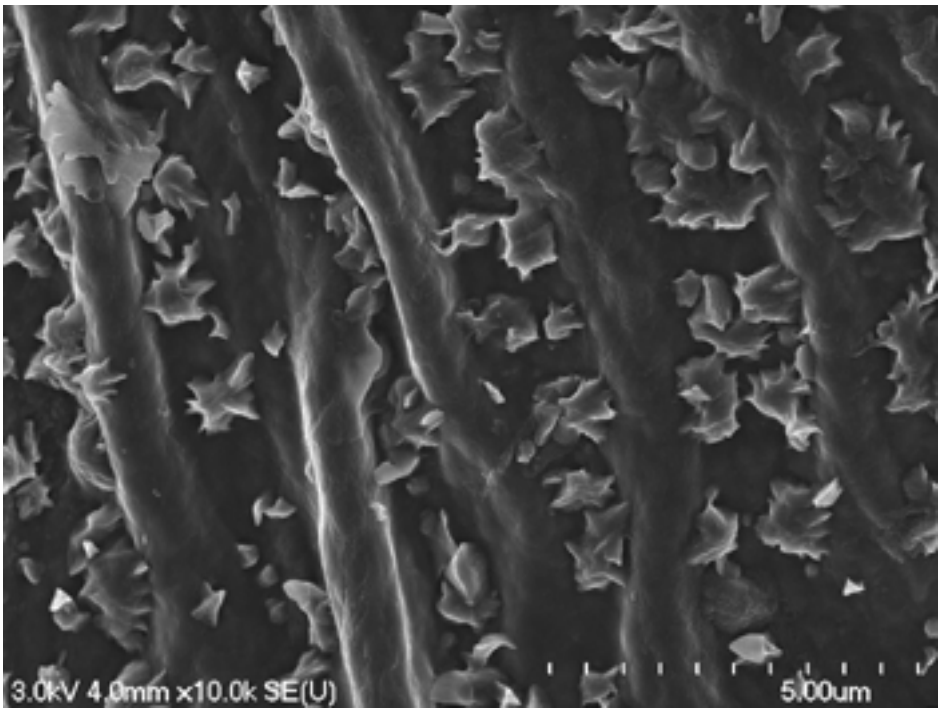


Figure 4.16 Upper side of the isolated grapevine cuticle. Clearly observable are the cuticular ledges and also wax crystals. Magnification ca. 26,000x.

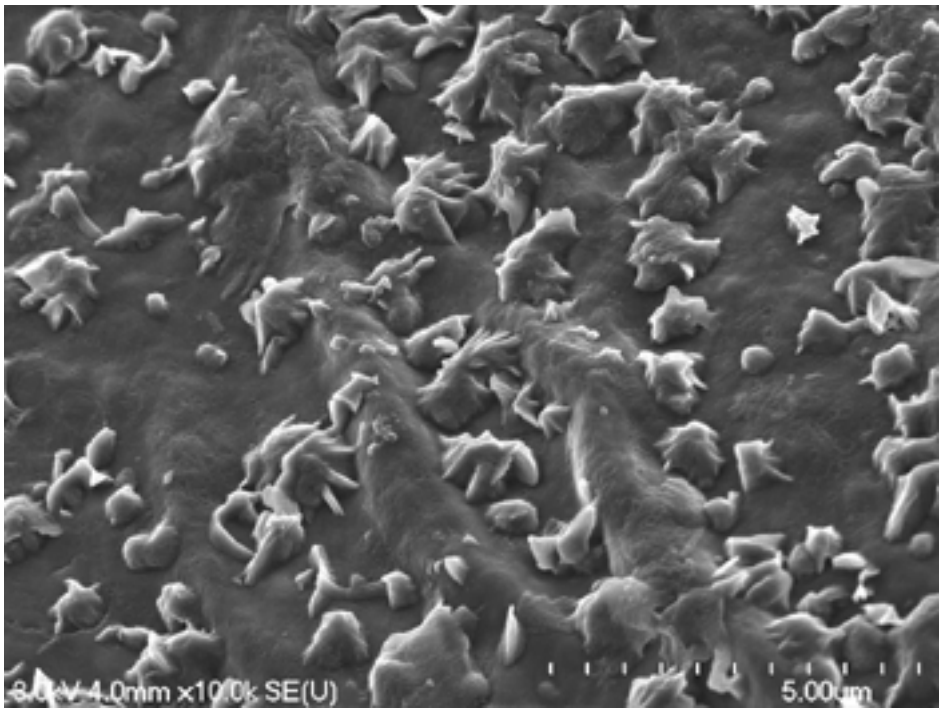


Figure 4.17 Upper side of the isolated grapevine cuticle. Clearly observable are the cuticular ledges and also wax crystals. Magnification ca. 26,000x.

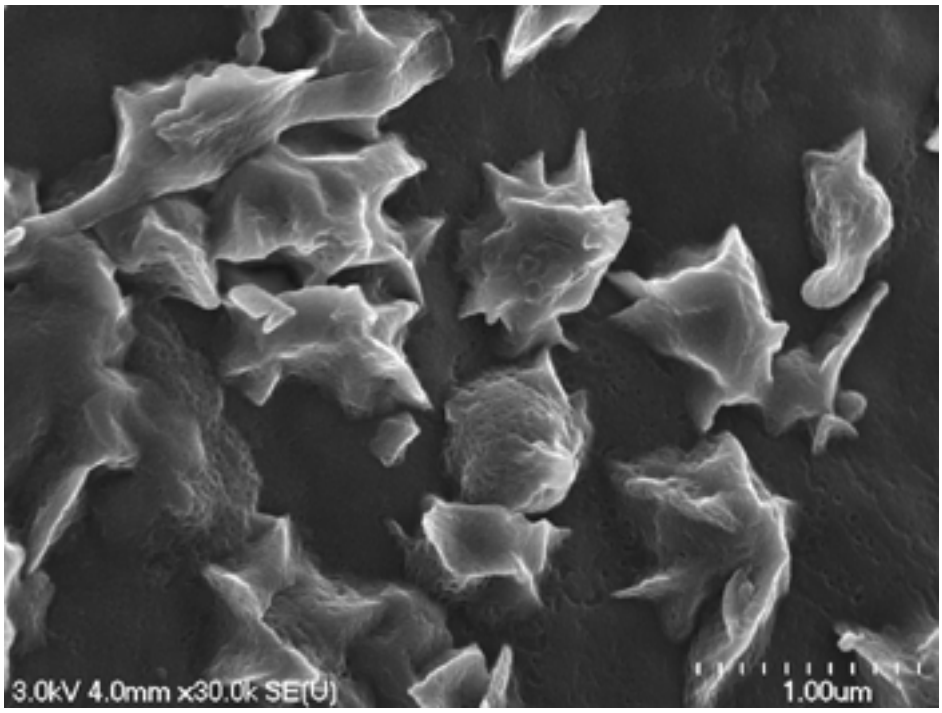


Figure 4.18 Upper side of the isolated grapevine cuticle. Clearly observable are the wax crystals. Magnification ca. 80,000x.

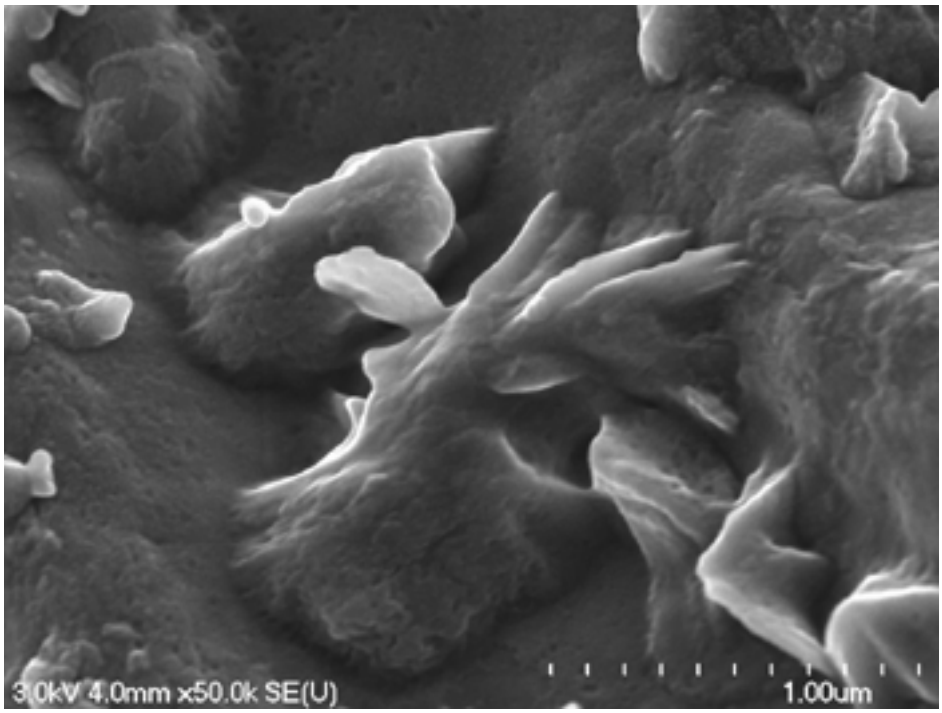


Figure 4.19 Upper side of the isolated grapevine cuticle. Clearly observable are the wax crystals. Magnification ca. 130,000x.

4.2 Non-steady state experiments with focus on paraquat

4.2.1 Experimental conditions

Where no further details on experimental conditions are given, all experiments described in this chapter have been conducted with isolated grapevine cuticles *(V. vinifera)* at moderate relative humidity (50%) and 23 °C. Droplet volume was 5 µl each time and paraquat concentration 0.02 g l^{-1}. All results are summarised in table 4.1 and 4.2 at the end of this chapter.

4.2.2 Long-term cuticular penetration of paraquat dichloride

The line-course of the penetration of paraquat dichloride across isolated grapevine cuticles is shown in figure 4.20A. It is a representative curve as obtained in most experiments done. The curve in figure 4.20A is hyperbolic which is largely due to limited water availability in the progress of the experiment.

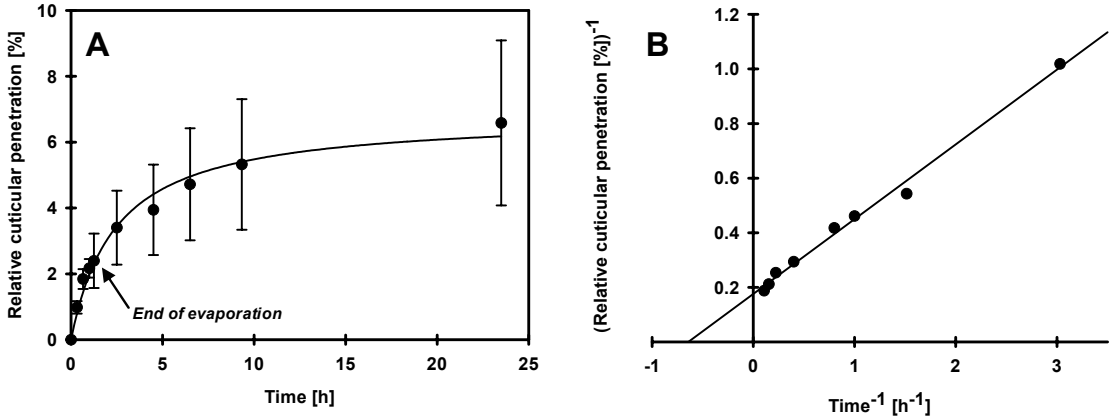


Figure 4.20 Penetration of paraquat dichloride across isolated grapevine cuticles. **A:** Relative cuticular penetration plotted versus time results in an asymptotic curve. The arrow indicates the end of water evaporation. All data are given as means with standard errors (SE) **B:** Double reciprocal presentation of cuticular penetration. Equation of the regression line is y = 0.277x + 0.169 (R^2 = 0.99).

From the equation of the regression line found with the reciprocal presentation of the data in figure 4.20B it is possible to calculate the maximum percentage cuticular penetration ($\%_{max}$) of paraquat from the y-intercept of the regression line. The y-intercept in figure 4.20B is 0.169 and it is the reciprocal number of the maximum percentage cuticular penetration which is 5.9% (± 0.3%). This calculated value fits very well to the experimental value as seen from figure 4.20A. The time needed for half of the maximum percentage cuticular penetration ($t_{max}/2$) is obtainable from the x-intercept. The x-intercept is -0.61 and the corresponding time is 1.6 h. It is also apparent, that the time interval whilst water is evaporating is the most important phase during the total penetration period. The end of evaporation is indicated by the arrow (Figure 4.20A). After that time, penetration velocity decreases as seen from the regression line. This is due to recrystallisation of the active ingredient on the surface of the cuticle. After about 24 hours the penetration across the cuticle is negligible and the maximum amount penetrated is reached. It is the only measurement over a period of 24 hours. This time-consuming experiment was done to support this mode of data-analysis.

4.2.3 Rewetting of paraquat dichloride after 24 hours

The importance of water for cuticular penetration processes from a drying droplet is shown in figure 4.21.

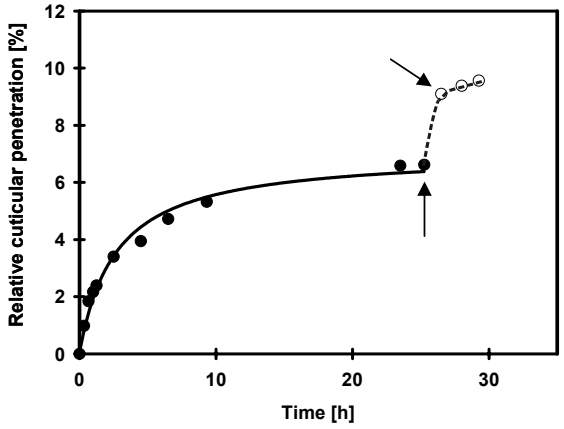


Figure 4.21 Relative cuticular penetration of paraquat dichloride across isolated grapevine cuticles. It is the same experiment as shown in figure 4.20A. After 24 hours a 5 µl droplet of deionised water was applied on the same position as the droplet before ('rewetting'). The arrows display the application of the droplets and the end of evaporation. For clarification no error bars were included.

As mentioned before, paraquat penetration across grapevine cuticles was measured for 24 hours and penetration process was negligible after that time. So a 5 µl droplet of pure water was applied on the same position as the droplet at the beginning of the experiment which included the active ingredient. Rewetting dissolved paraquat on the surface of the cuticle immediately and cuticular penetration rose noticeable. After another 75 minutes water was totally evaporated again and penetration decelerates dramatically.

4.2.4 Initial phase of paraquat penetration

It has been mentioned above, that penetration of paraquat demands for the availability of water.

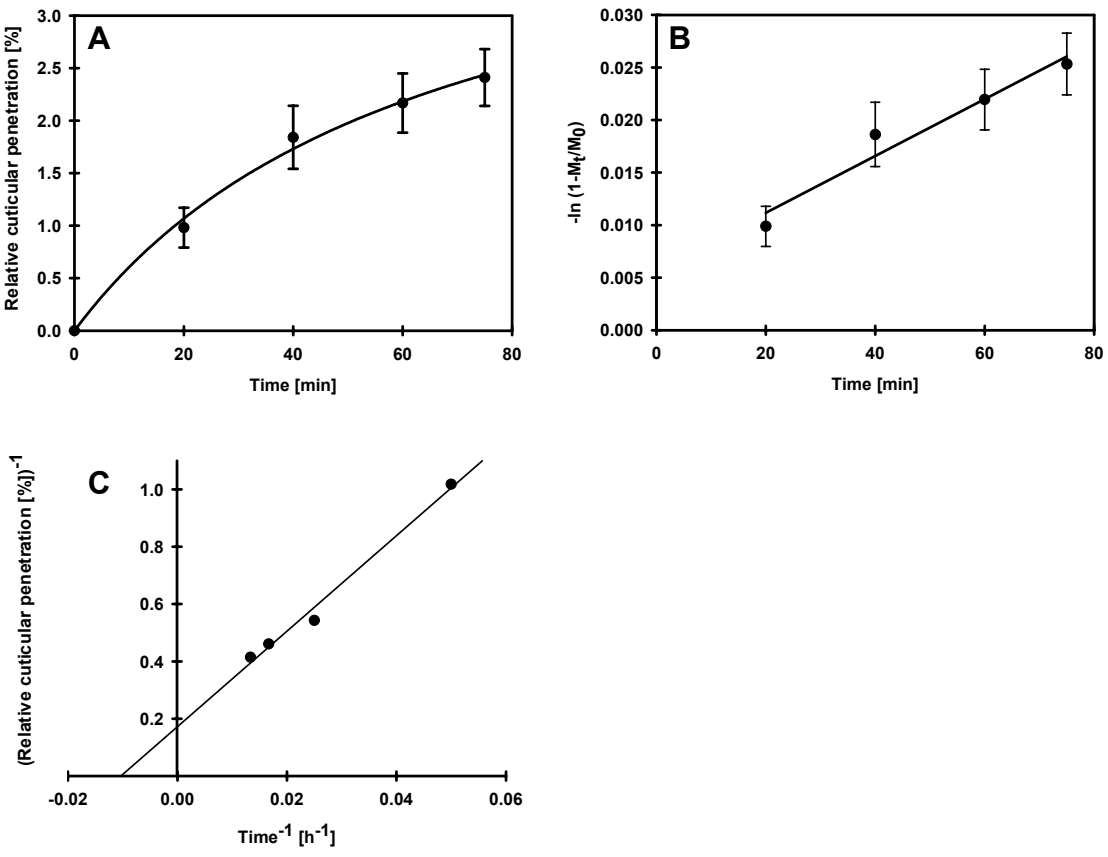


Figure 4.22 Penetration of paraquat dichloride across isolated grapevine cuticles during the evaporation of water within the first 75 minutes. **A:** Relative cuticular penetration plotted versus time results in an asymptotic curve. **B:** Logarithmic presentation of the initial phase. **C:** Double reciprocal presentation. Equation of the regression line is $y = 16.7x + 0.172$ ($R^2 = 0.99$).

From that finding it is obvious that the initial phase within the evaporation of water is a very important phase during the whole uptake phase, because 41% of the total maximum paraquat penetration occurs within the evaporation time of the water. Figure 4.22A focuses on the first 75 minutes of paraquat penetration. This data are included in figures 4.20 and 4.21. The initial phase also follows a hyperbola and the extrapolation of that curve fits very well with the long-term experiment as shown in figure 4.20A, because the calculated maximum cuticular penetration is 5.8% (± 0.2%). The rate constant during this initial phase varies, but a mean rate constant of 16.1 x 10^{-3} (± 2.6 x 10^{-3}) h^{-1} was calculated from the slope of figure 4.22B (R^2 = 0.95).

4.2.5 Paraquat penetration in comparison with other herbicides

To evaluate the penetration of paraquat, two further herbicides were examined. The lipophilic compound clodinafop-propargyl (log $K_{O/W}$ 3.9) and the highly hydrophilic compound glyphosate (log $K_{O/W}$ -4.0) were selected. Glyphosate was applied as potassium salt (K^+-Glyphosate). The maximum cuticular penetration of these three herbicides was calculated from the equation of the respective regression lines from figure 4.23C. Maximum penetration of K^+-glyphosate was only 1.3% (± 0.1%) which is significantly lower than that of paraquat (5.9%). Time for half of maximum uptake of glyphosate was 0.5 h (paraquat 1.6 h). In contrast to these hydrophilic compounds, the kinetics of the lipophilic compound clodinafop displays a less declining curve progression which results in a maximum penetration of 9.8% (± 0.8%).

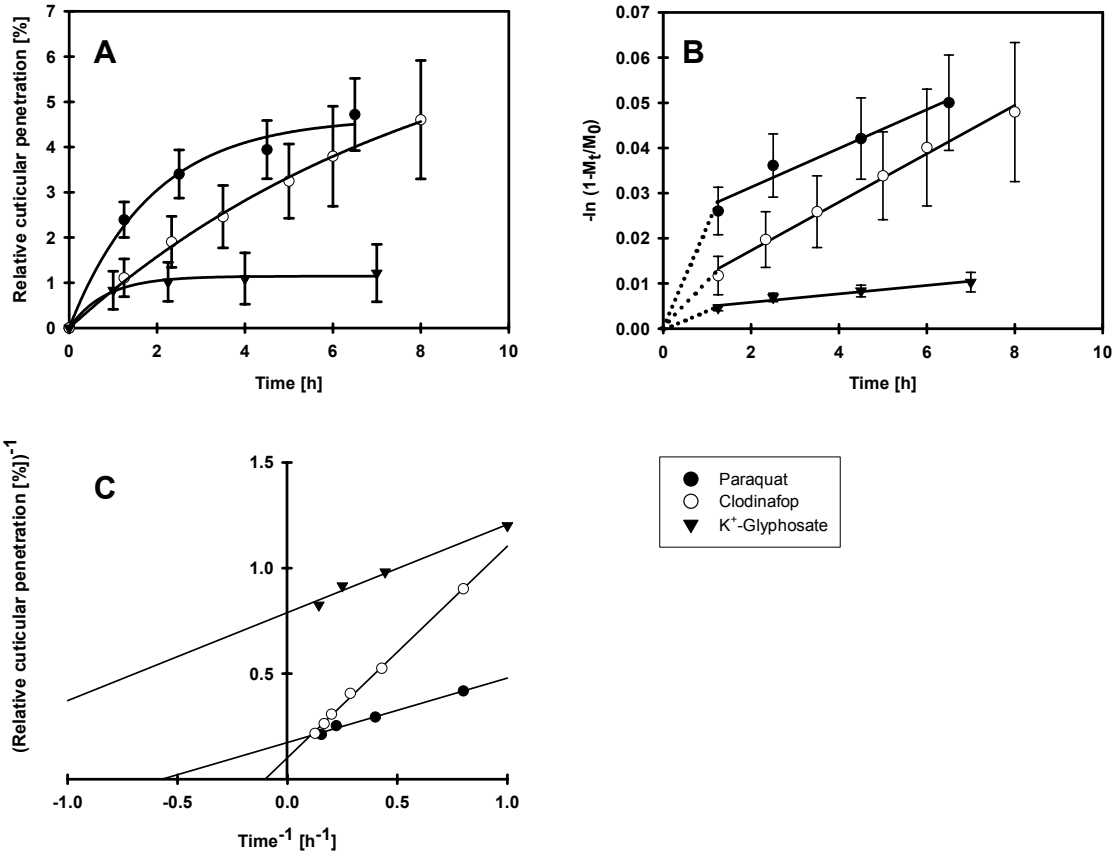


Figure 4.23 **A:** Relative cuticular penetration of paraquat dichloride across isolated grapevine cuticles in comparison with a lipophilic compound (clodinafop) and another hydrophilic compound (K^+-glyphosate). **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of cuticular penetration of the selected herbicides. Equations of the regression lines are y = 0.277x + 0.169 (R^2 = 0.99) (paraquat), y = 1.0x + 0.102 (R^2 = 0.99) (clodinafop) and y = 0.417x + 0.789 (R^2 = 0.98) (K^+-glyphosate). Time for half maximum uptake is one hour. Rate constants are 4.3 x 10^{-3} (± 0.6 x 10^{-3}) h^{-1} of paraquat (R^2 = 0.96), 0.9 x 10^{-3} (± 0.1 x 10^{-3}) h^{-1} of glyphosate (R^2 = 0.99) and 5.4 x 10^{-3} (± 0.2 x 10^{-3}) h^{-1} of clodinafop (R^2 = 0.96).

4.2.6 Variation of the relative humidity

Generally, all experiments were conducted at a moderate relative humidity of 50%. The experiment reported here was focused on the correlation between paraquat penetration and relative humidity. Humidity was varied between 25% and 75%. However, in the range of 25% to 70% there are no real differences in cuticular penetration. To the contrary, at 75% relative humidity uptake increased conspicuously.

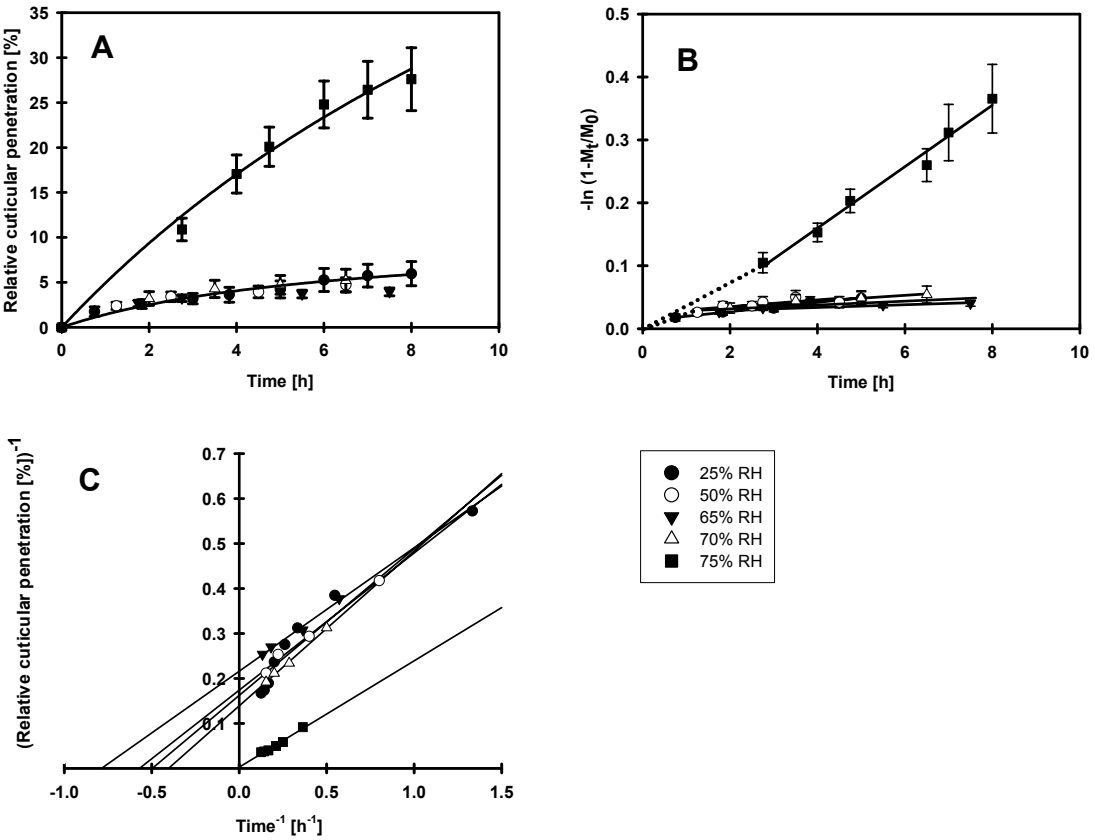


Figure 4.24 Variation of relative humidity and its effect on cuticular paraquat penetration. **A:** Plot of relative cuticular penetration of paraquat at 25%, 50%, 65%, 70% and 75% relative humidity. No differences were detected between 25% and 70% RH. At 75% a significant increase was found. **B:** Logarithmic presentation of that data for determination of the rate constants. **C:** Double reciprocal presentation of cuticular penetration of paraquat in dependence of different relative humidities. Regression lines of 25% to 70% RH are at close quarters while that of 75% RH differs clearly. $y = 0.326x + 0.162$ ($R^2 = 0.94$) (25% RH), $y = 0.277x + 0.169$ ($R^2 = 0.99$) (50% RH), $y = 0.275x + 0.216$ ($R^2 = 0.99$) (65% RH), $y = 0.344x + 0.139$ ($R^2 = 0.99$) (70% RH), $y = 0.236x + 0.0028$ ($R^2 = 0.98$) (75% RH).

While the curves between 25% and 70% relative humidity had a typically declining shape, the deceleration in uptake at 75% is relatively delayed – nevertheless it also has a hyperbolic curve progression. The maximum cuticular penetration of paraquat dichloride at 75% relative humidity is 100% as it was found from figure 4.24C. Visual extrapolation of figure 4.24A supports this finding. Results found from figure 4.24 are also supported by figure 4.25. Paraquat has hygroscopic properties itself. Water uptake of paraquat from the surrounding environment depends on the relative humidity as shown below. In the range of 67% to 75% relative humidity paraquat water content rises dramatically. This is in agreement with the results found from the transport

experiments. Rate constants are 7.4 x 10^{-3} (± 0.5 x 10^{-3}) h^{-1} at 25% RH (R^2 = 0.98), 4.3 x 10^{-3} (± 0.6 x 10^{-3}) h^{-1} at 50% RH (R^2 = 0.96), 2.1 x 10^{-3} (± 0.5 x 10^{-3}) h^{-1} at 65% RH (R^2 = 0.91), 4.6 x 10^{-3} (± 0.7 x 10^{-3}) h^{-1} at 70% RH (R^2 = 0.95) and 48.8 x 10^{-3} (± 3.1 x 10^{-3}) h^{-1} at 75% RH (R^2 = 0.99).

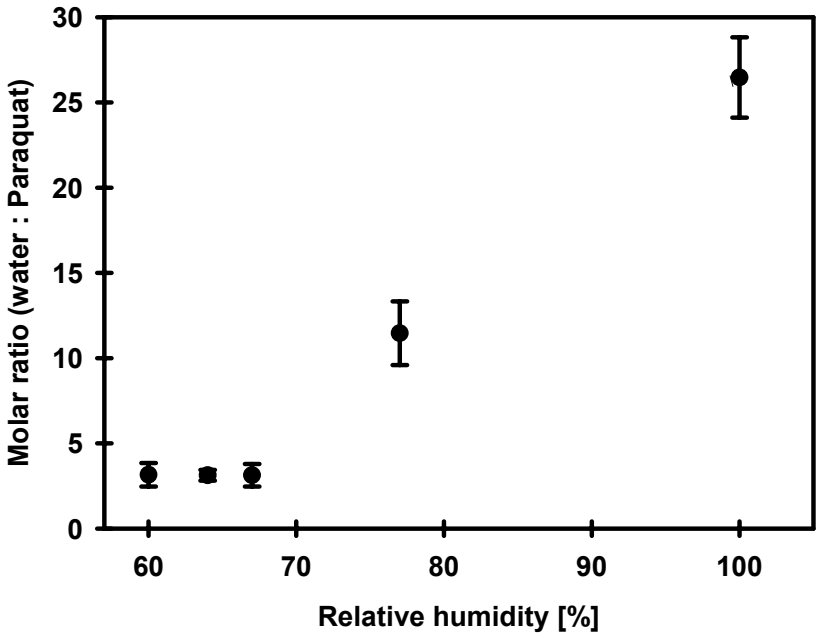


Figure 4.25 Water absorption of paraquat depends on the surrounding relative humidity. In the range of 67% to 75% relative humidity water uptake rises dramatically.

4.2.7 Variation of the paraquat concentration

Three different concentrations of paraquat were examined: 0.02 g l^{-1}, 0.2 g l^{-1} and 2.0 g l^{-1}. Increasing paraquat concentrations rise cuticular penetration of paraquat. A tenfold increase in concentration increases maximum cuticular penetration from 5.9% (± 0.3%) to 14.9% (± 0.5%). A hundredfold increase of concentration raises the maximum penetration to 17.4% (± 0.5%). The times needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) are 1.6 h (0.02 g l^{-1}), 3.3 h (0.2 g l^{-1}) and 1.9 h (2.0 g l^{-1}). Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (0.02 g l^{-1}) (R^2 = 0.96), 12.2 x 10^{-3} h^{-1} (± 0.9 x 10^{-3} h^{-1}) (0.2 g l^{-1}) (R^2 = 0.99) and 12.4 x 10^{-3} h^{-1} (± 3.2 x 10^{-3}) h^{-1} (2.0 g l^{-1}) (R^2 = 0.89).

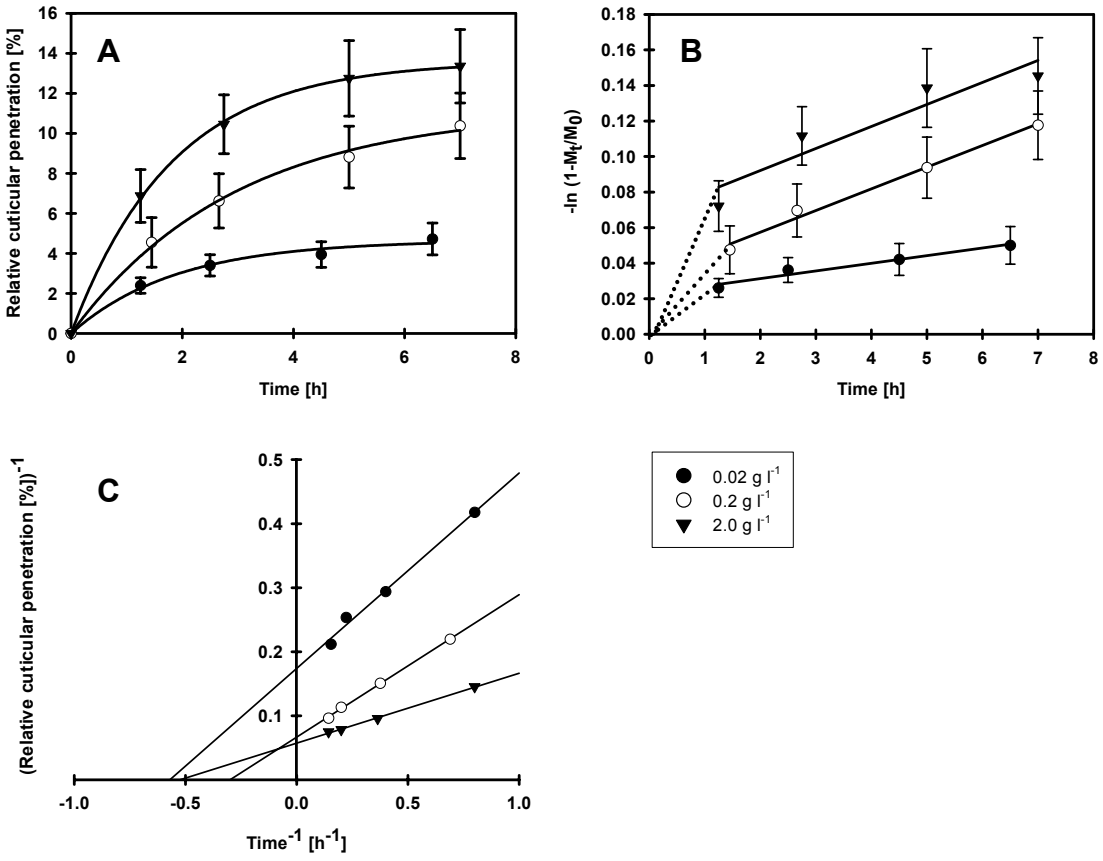


Figure 4.26 Variation of paraquat concentration and its effect on cuticular penetration. **A:** Plot of relative cuticular penetration of paraquat at three different concentrations versus time. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (0.02 g l^{-1}), y = 0.222x + 0.067 (R^2 = 0.99) (0.2 g l^{-1}), y = 0.109x + 0.058 (R^2 = 0.99) (2.0 g l^{-1}).

4.2.8 Effect of the cuticular waxes on paraquat penetration

Cuticular waxes inhibit the penetration of paraquat across grapevine cuticles dramatically. With dewaxed membranes a maximum cuticular penetration of 20% (± 1.1%) is reached. Time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 5 h. Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) for cuticular membranes and 15.8 x 10^{-3} h^{-1} (± 1.8 x 10^{-3} h^{-1}) for dewaxed membranes (R^2 = 0.96).

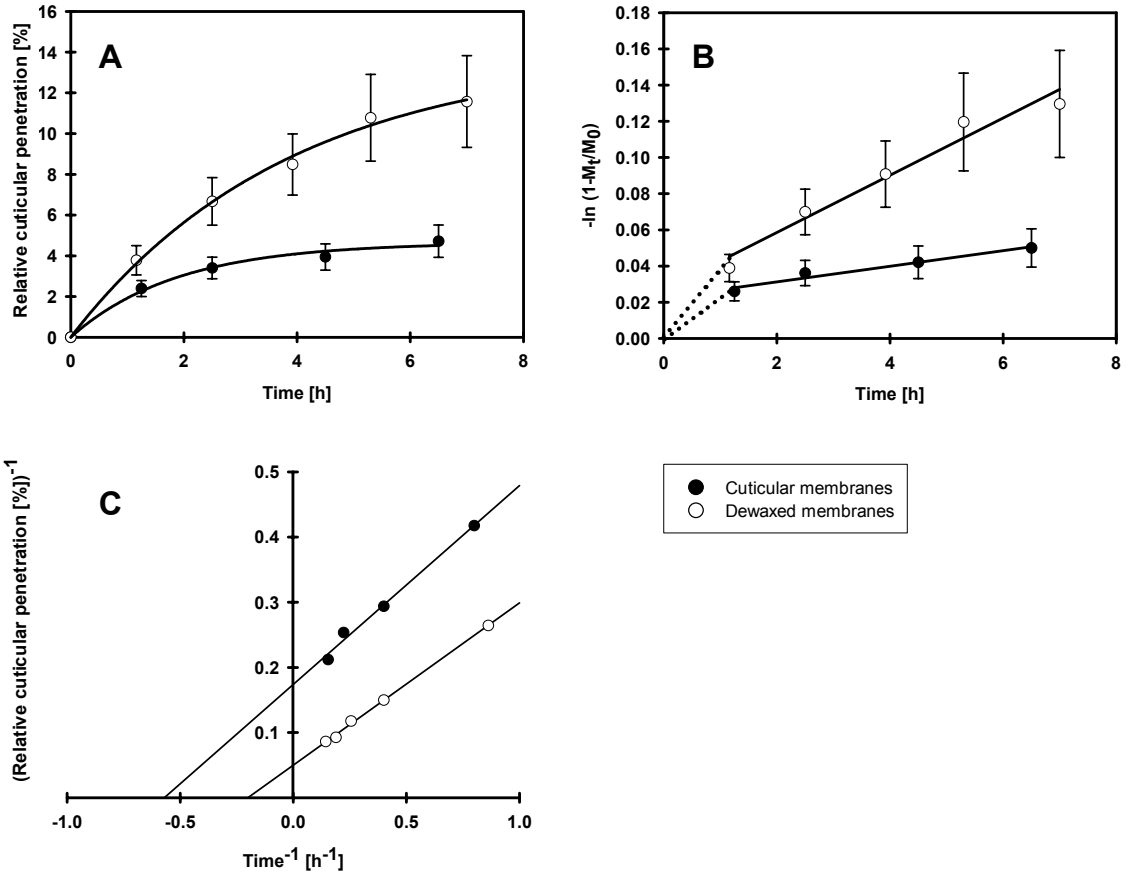


Figure 4.27 Effect of wax extraction on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes and dewaxed membranes. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (cuticular membrane), y = 0.249x + 0.050 (R^2 = 0.99) (dewaxed membrane).

4.2.9 Effect of droplet size on paraquat penetration

The influence of droplet size on cuticular penetration was examined. Total volume was normally 5 µl. In this experiment droplets were applied as one single droplet (1 x 5 µl), as two droplets (2 x 2.5 µl), five droplets (5 x 1 µl) or 10 droplets (10 x 0.5 µl). Droplet size influences the duration of evaporation. Partly, the velocity of uptake during the initial phase is influenced which also has an effect on the maximum cuticular penetration. Maximum cuticular penetration of paraquat: 5.9% (± 0.3%) (1 droplet), 2.5% (± 0.1%) (2 droplets), 3.2% (± 0.1%) (5 droplets) and 5.9% (± 0.4%) (10 droplets). Times needed for the half of the

maximum percentage cuticular penetration ($t_{max}/2$) were 1.6 h (1 droplet), 0.8 h (2 droplets), 0.2 h (5 droplets) and 0.9 h (10 droplets). Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (1 droplet), 1.3 x 10^{-3} h^{-1} (± 0.4 x 10^{-3} h^{-1}) (R^2 = 0.87) (2 droplets), 1.3 x 10^{-3} h^{-1} (± 0.5 x 10^{-3} h^{-1}) (R^2 = 0.70) (5 droplets) and 3.5 x 10^{-3} h^{-1} (± 0.5 x 10^{-3} h^{-1}) (R^2 = 0.95) (10 droplets).

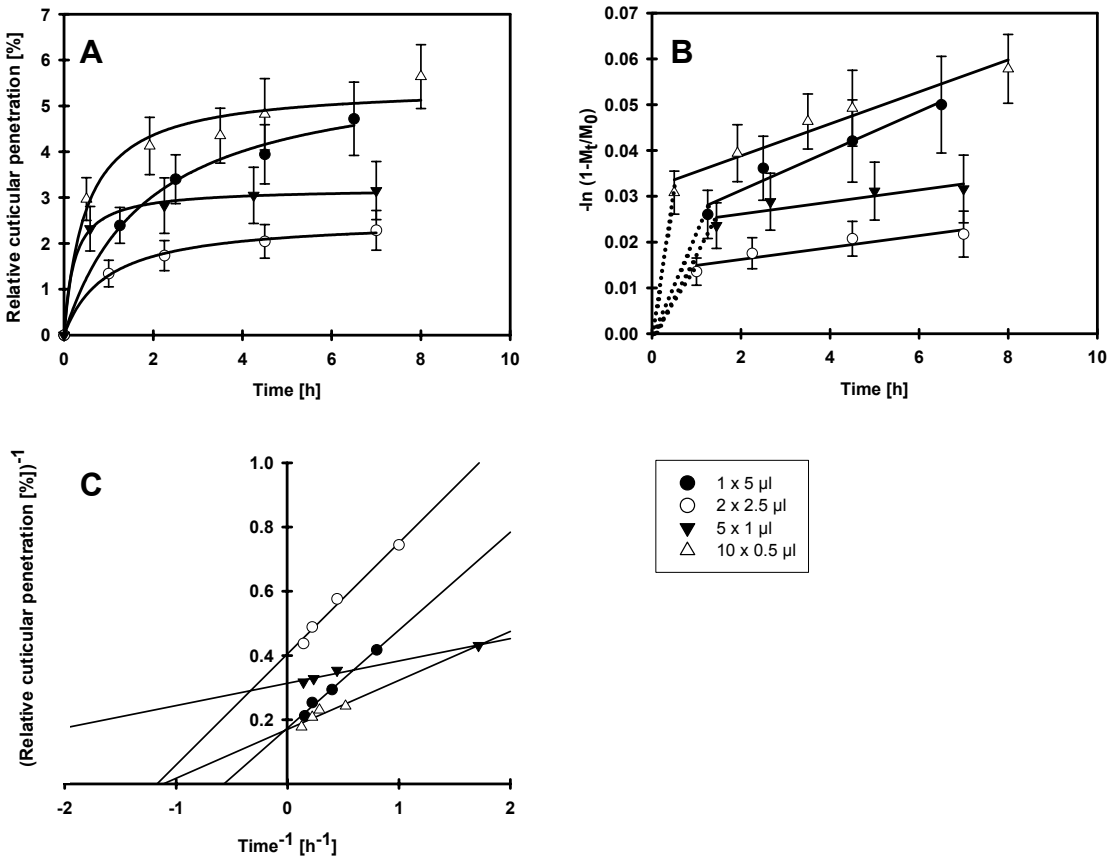


Figure 4.28 Effects of droplet size on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes using varying droplet sizes. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (1 droplet), y = 0.346x + 0.405 (R^2 = 0.99) (2 droplets), y = 0.070x + 0.313 (R^2 = 0.98) (5 droplets), y = 0.153x + 0.170 (R^2 = 0.82) (10 droplets).

4.2.10 Effect of additives on paraquat penetration

Accelerator for hydrophilic active ingredients. Ethomeen T/25 was used as a hydrochloride salt to measure acceleration effects on the cuticular penetration of paraquat. Ethomeen T/25 was used in concentrations of 0.2% (w/v) and 0.5% (w/v). All experiments were conducted at 50% relative humidity. Maximum cuticular penetration of paraquat was: 5.9% (± 0.3%) (without ethomeen T/25), 12.2% (± 1.4%) (+ 0.2% ethomeen T/25) and 30.3% (± 4.5%) (+ 0.5% ethomeen T/25).

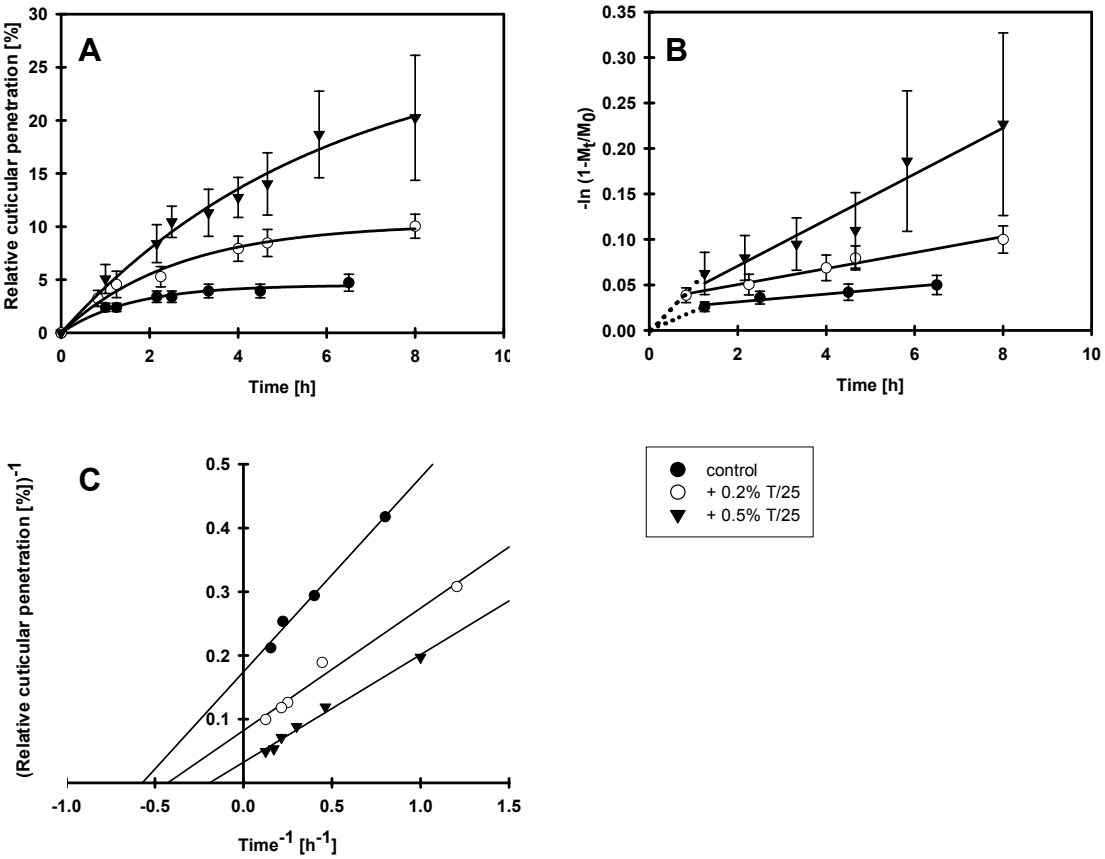


Figure 4.29 Effect of ethomeen T/25 on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes using varying concentrations of ethomeen T/25. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (without ethomeen T/25), y = 0.192x + 0.082 (R^2 = 0.98) (+ 0.2% ethomeen T/25), y = 0.169x + 0.033 (R^2 = 0.98) (+ 0.5% ethomeen T/25).

Time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 1.6 h (without ethomeen T/25), 2.4 h (+ 0.2% ethomeen T/25) and 5.1 h (0.5% ethomeen T/25). Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (without ethomeen T/25), 8.8 x 10^{-3} h^{-1} (± 0.8 x 10^{-3} h^{-1}) (R^2 = 0.98) (+ 0.2% ethomeen T/25) and 24.9 x 10^{-3} h^{-1} (± 3.3 x 10^{-3} h^{-1}) (R^2 = 0.97) (+ 0.5% ethomeen T/25).

Use of a humectant. Sorbitol was employed to measure humectant effects on cuticular penetration of paraquat. Sorbitol was used in concentrations of 2.0% (w/v) and 3.5% (w/v).

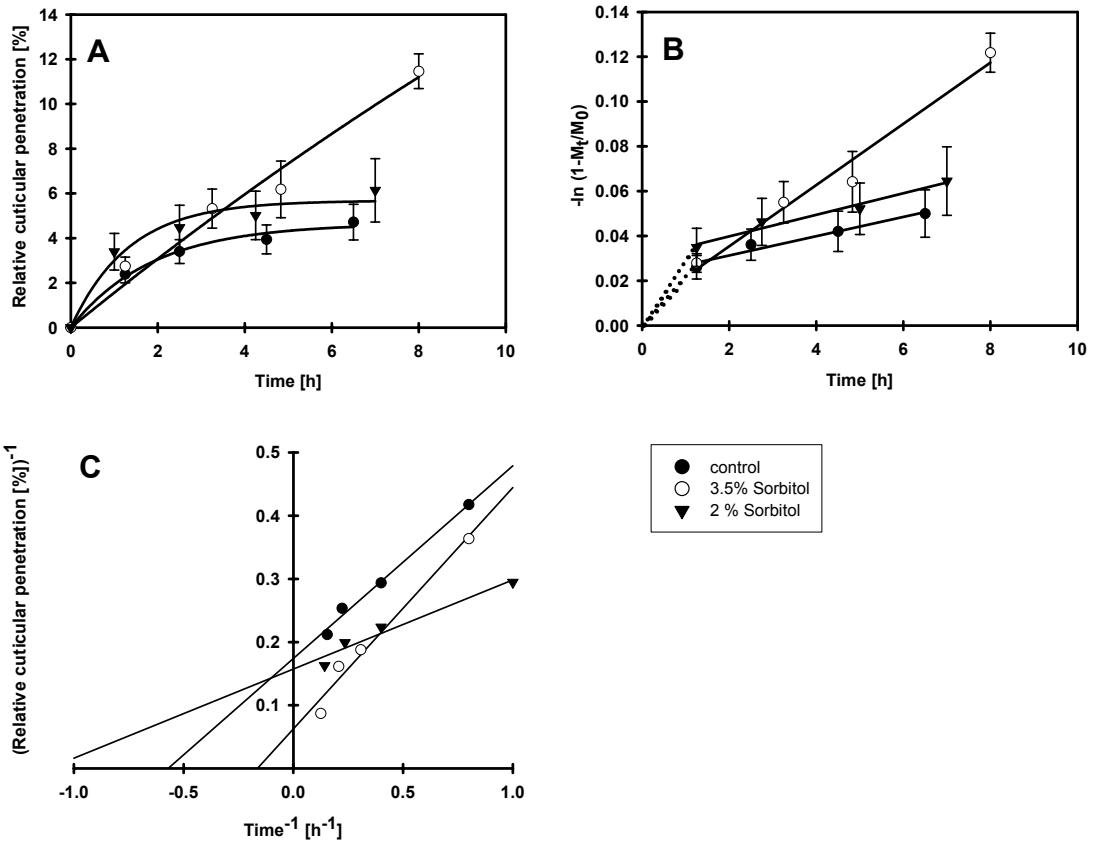


Figure 4.30 Effect of sorbitol on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes using varying concentrations of sorbitol. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (without sorbitol), y = 0.226x + 0.137 (R^2 = 0.93) (+ 2.0% sorbitol) and y = 0.382x + 0.063 (R^2 = 0.98) (+ 3.5% sorbitol).

Maximum cuticular penetration of paraquat: 5.9% (± 0.3%) (without sorbitol), 7.0% (± 0.7%) (+ 2.0% sorbitol) and 15.9% (± 5.3%) (+ 3.5% sorbitol). The time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 1.6 h (without sorbitol), 1.7 h (+ 2.0% sorbitol) and 6.0 h (+ 3.5% sorbitol). Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (without sorbitol), 4.8 x 10^{-3} h^{-1} (± 0.6 x 10^{-3} h^{-1}) (R^2 = 0.97) (+ 2.0% sorbitol) and 13.7 x 10^{-3} h^{-1} (± 1.6 x 10^{-3} h^{-1}) (R^2 = 0.97) (+ 3.5% sorbitol). Sorbitol is able to absorb water from the adjacent atmosphere. The amount of water absorbed depends on the surrounding relative humidity. With increasing relative humidity water sorption increases. Figure 4.31 displays the water sorption of sorbitol as a function of the relative humidity.

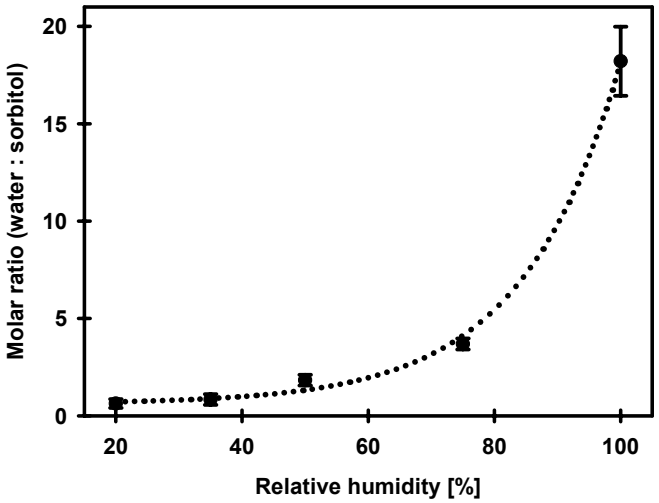


Figure 4.31 Hygroscopic effect of sorbitol plotted as molar ratio of water : sorbitol versus relative humidity.

Combination of accelerator and humectant. Both, ethomeen T/25 and sorbitol had favourable effects on cuticular penetration of paraquat. In this experiment both additives were combined in a concentration of 0.5% (w/v) ethomeen T/25 and 3.5% (w/v) sorbitol. Maximum cuticular penetration of paraquat: 5.9% (without additives) and 52.6% (+ 0.5% ethomeen T/25 and 3.5% sorbitol). The time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 1.6 h (without additives) and 1.3 h using ethomeen T/25 and sorbitol simultaneously. Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (without additive) and 63.5 x 10^{-3} h^{-1} (± 6.6 x 10^{-3} h^{-1}) (+ T/25 and sorbitol) (R^2 = 0.97).

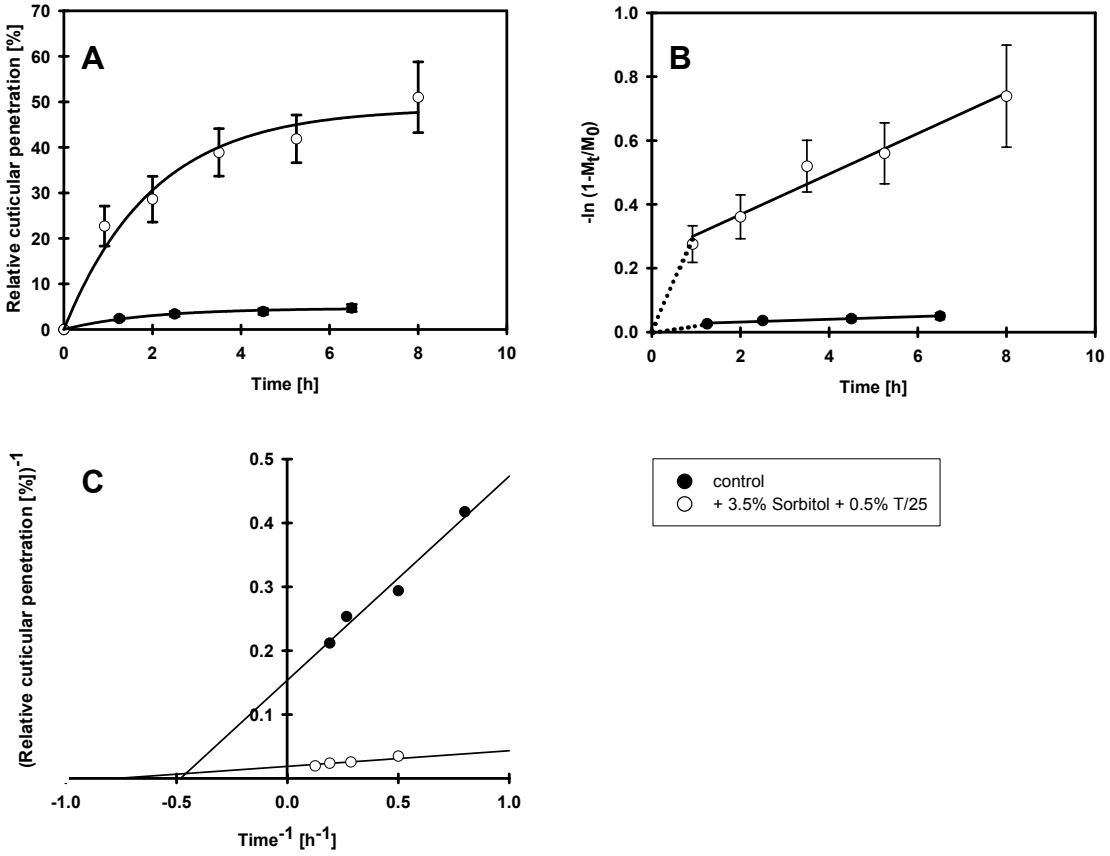


Figure 4.32 Effect of ethomeen T/25 and sorbitol on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (without additives) and y = 0.024x + 0.019 (R^2 = 0.94) (+ ethomeen T/25 and sorbitol).

Accelerator for lipophilic active ingredients. Methyl oleate is a very well suited accelerator for cuticular transport of lipophilic active ingredients like clodinafop-propargyl. Emulsified methyl oleate was used to examine its effect on cuticular penetration of hydrophilic paraquat. It was used in a concentration of 0.5% (w/v). Maximum cuticular penetration of paraquat: 5.9% (± 0.3) (without methyl oleate) and 2.0% (± 0.1) (+ 0.5% methyl oleate). The time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 1.6 h (without methyl oleate) and 1.1 h using methyl oleate. Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (without methyl oleate) and 1.6 x 10^{-3} h^{-1} (± 0.2 x 10^{-3} h^{-1}) (+ 0.5% methyl oleate) (R^2 = 0.94).

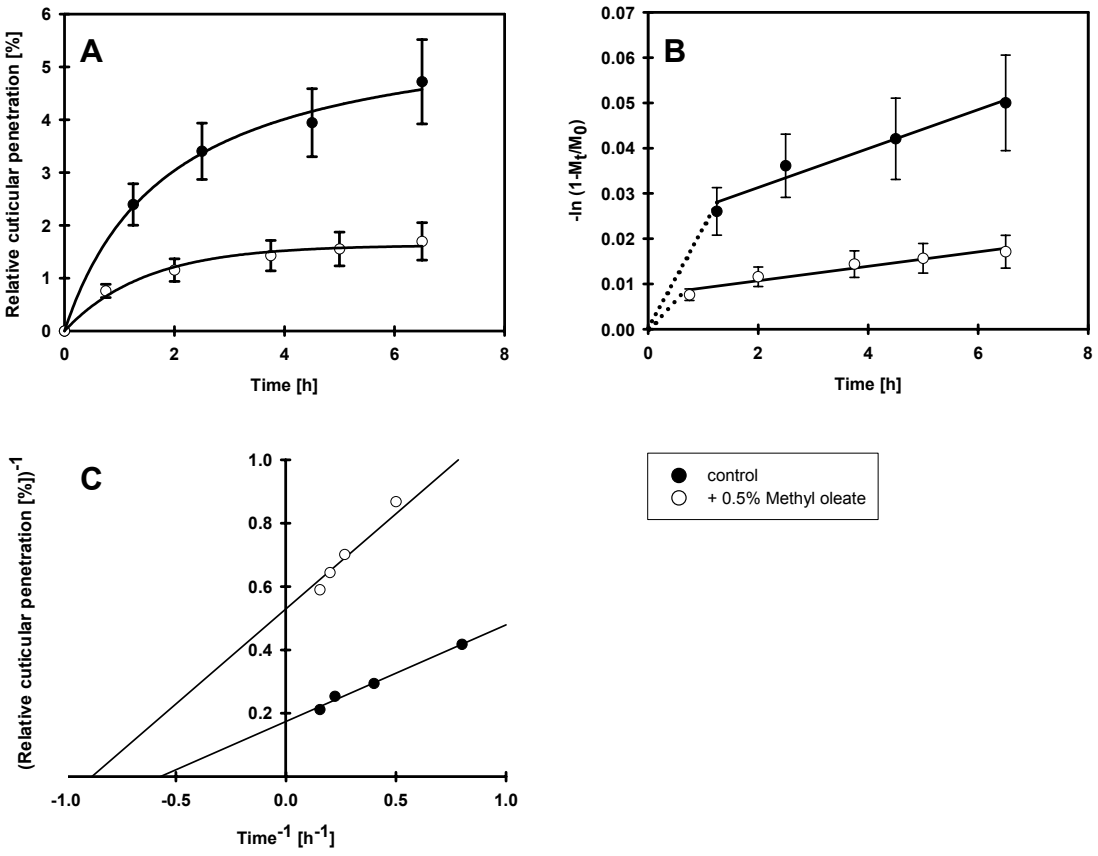


Figure 4.33 Effect of methyl oleate on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (without methyl oleate) and y = 0.600x + 0.530 (R^2 = 0.99) (+ methyl oleate).

Accelerator for human skin. Urea is a well known penetration enhancer for pharmaceuticals in dermatology. Its suitability as an additive for cuticular penetration of paraquat was examined. Urea was used in a concentration of 0.5% (w/v). Urea also was used in a concentration of 2.0% (w/v), but there were no differences in penetration compared with 0.5% urea so no data are shown. Maximum cuticular penetration of paraquat: 5.9% (± 0.3%) (without methyl oleate) and 3.9% (± 0.1) (+ 0.5% urea). The time needed for the half of the maximum percentage cuticular penetration ($t_{max}/2$) is 1.6 h (without urea) and 0.9 h using urea. Rate constants are 4.3 x 10^{-3} h^{-1} (± 0.3 x 10^{-3} h^{-1}) (R^2 = 0.96) (without urea) and 2.5 x 10^{-3} h^{-1} (± 1.0 x 10^{-3} h^{-1}) (+ 0.5% urea) (R^2 = 0.94).

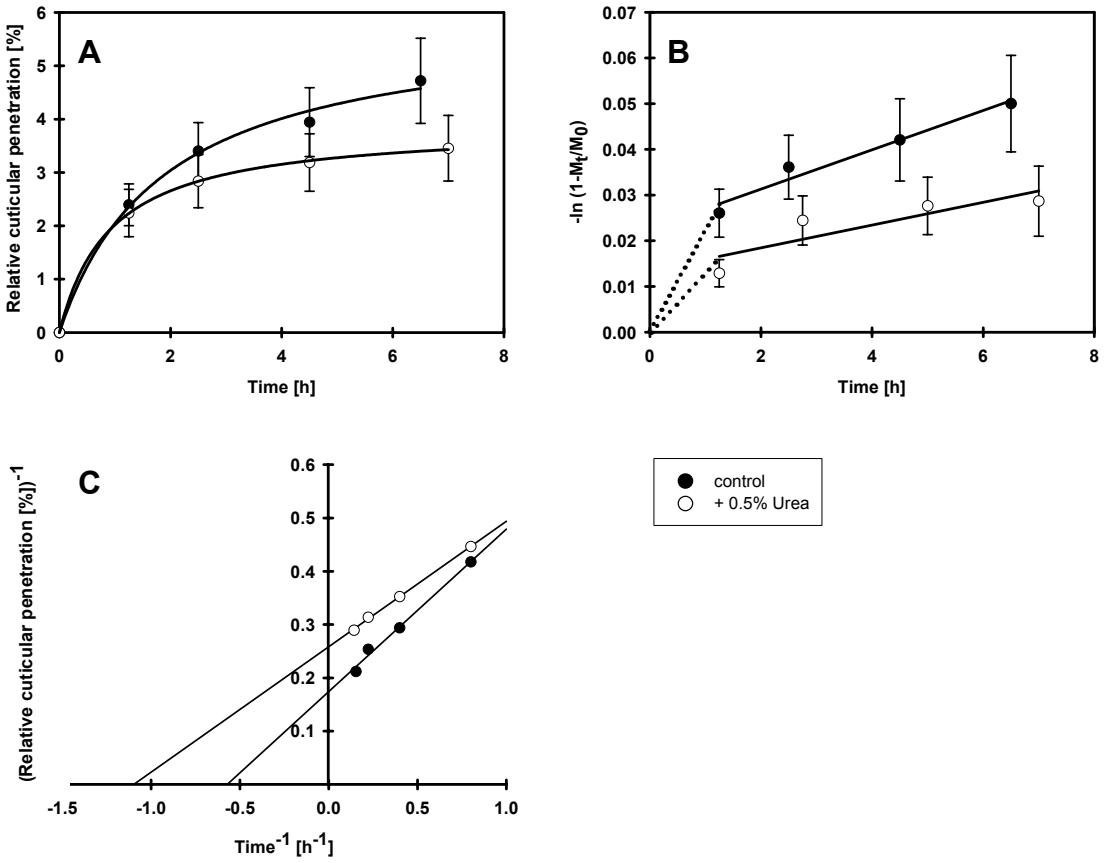


Figure 4.34 Effect of urea on paraquat penetration across isolated grapevine cuticles. **A:** Plot of relative cuticular penetration of paraquat across cuticular membranes. **B:** Logarithmic presentation of the data for determination of the rate constants. **C:** Double reciprocal presentation of figure A. y = 0.277x + 0.169 (R^2 = 0.99) (without urea) and y = 0.235x + 0.258 (R^2 = 0.99) (+ urea).

Evaporation of water from drying droplets. Additives change the behaviour of droplets; e. g. the duration of water evaporation is influenced. One experiment was done to obtain information about the effect of ethomeen T/25 and sorbitol on evaporation of applied droplets. This work was conducted at a relative humidity of 40%. Ethomeen T/25 increased the spread area of a droplet compared to pure water. This resulted in an increased water evaporation. Both measured concentrations of ethomeen T/25 (0.2% & 0.5%) did not display any differences. However, addition of sorbitol avoids total evaporation of water which resulted in a permanently wet residue of the droplet. This is an explanation for the high suitability of sorbitol in terms of cuticular penetration of paraquat. Combination of ethomeen T/25 and sorbitol resulted in a faster evaporation on the one hand and in a permanent wet residue on the other hand. Curves from figure 4.35 are mean values of 20 replicates.

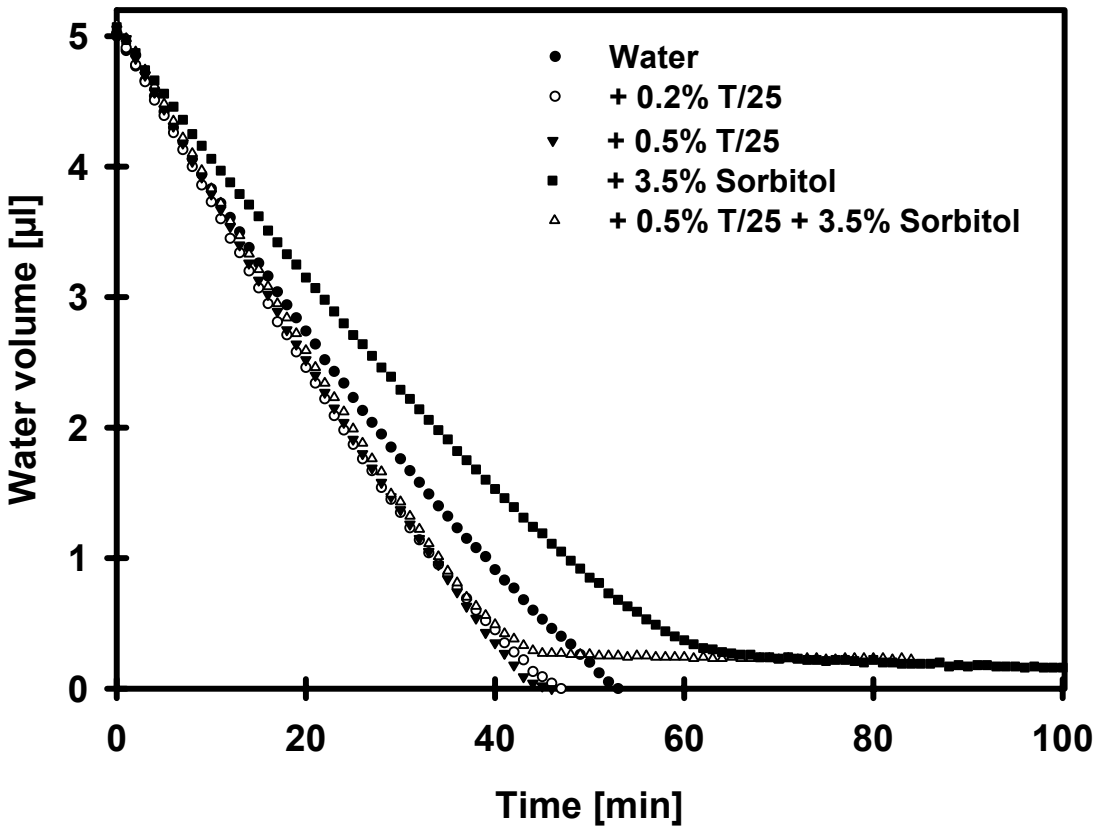


Figure 4.35 Evaporation of water from droplets on grapevine cuticles as a function of varying combination of additives. All curves show mean values of 20 replicates. Experiments were conducted at 40% relative humidity.

Table 4.3 Summary of all paraquat penetration experiments. Results were found from the double reciprocal presentation of the data. The number of replicates (n) is given in the last column.

Variable	Factor	Max. cuticular penetration [%]	Portion of initial phase [%]	Time needed for half max. penetration [h]	Slope	R^2	n
Paraquat	Control	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	Initial phase (mean value)	5.8 ± 0.2	-	-	16.66 ± 1.30	0.99	12
Active ingredients	Paraquat	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	Clodinafop	9.8 ± 0.8	11	1.0	1.002 ± 0.021	0.99	8
	K^+-Glyphosate	1.3 ± 0.1	62	0.5	0.417 ± 0.037	0.98	9
Relative humidity	25%	6.2 ± 0.7	27	2.0	0.326 ± 0.035	0.94	8
	50%	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	65%	4.6 ± 0.2	59	1.3	0.275 ± 0.021	0.99	9
	70%	7.2 ± 0.2	44	2.5	0.344 ± 0.014	0.99	9
	75%	-> 100	11*	-	0.237 ± 0.018	0.98	8
Paraquat concentration	0.02 g l^{-1}	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	0.2 g l^{-1}	14.9 ± 0.5	31	3.3	0.222 ± 0.005	0.98	11
	2.0 g l^{-1}	17.4 ± 0.5	40	1.9	0.109 ± 0.004	0.98	10
Wax	Cuticular membrane	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	Dewaxed membrane	20.0 ± 1.1	19	5.0	0.249 ± 0.006	0.99	8
Droplet size	1 droplet	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	2 droplets	2.5 ± 0.1	52	0.8	0.346 ± 0.003	0.99	11
	5 droplets	3.2 ± 0.1	72	0.2	0.070 ± 0.007	0.98	8
	10 droplets	5.9 ± 0.1	51	0.9	0.153 ± 0.052	0.81	8
Additives	Without additives	5.9 ± 0.3	41	1.6	0.305 ± 0.021	0.99	10
	Ethomeen T/25 (0.2%)	12.2 ± 1.4	26	2.4	0.192 ± 0.016	0.98	8
	Ethomeen T/25 (0.5%)	30.3 ± 4.5	17	5.1	0.169 ± 0.010	0.99	8
	Sorbitol (2%)	6.3 ± 0.7	49	1.1	0.141 ± 0.021	0.96	11
	Sorbitol (3.5%)	15.9 ± 5.3	17	6.0	0.382 ± 0.043	0.98	8
	Ethomeen T/25 (0.5%) + Sorbitol (3.5%)	52.6 ± 5.5	43	1.3	0.024 ± 0.004	0.94	8
	Methly oleate (0.5%)	2.0 ± 0.1	40	1.1	0.601 ± 0.031	0.99	8
	Urea (0.5%)	3.9 ± 0.1	56	0.9	0.236 ± 0.006	0.99	12

Table 4.4 Summary of all paraquat penetration experiments. Results were found from the logarithmic presentation of the data. The number of replicates (n) is given in table 4.1.

Variable	Factor	Rate constant x 10^{-3} [h^{-1}]	R^2 (m)	y-intercept	Rate constant initial phase x 10^{-3} [h^{-1}]
Paraquat	Control	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	Initial phase (mean value)	16.1 ± 2.6	0.95	0.0059 ± 0.0023	-
Active ingredients	Paraquat	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	Clodinafop	5.4 ± 0.2	0.99	0.0066 ± 0.0012	9.4
	K^+-Glyphosate	0.9 ± 0.1	0.96	0.004 ± 0.0006	3.7
Relative humidity	25%	7.4 ± 0.5	0.92	0.0123 ± 0.0018	23.6
	50%	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	65%	2.1 ± 0.5	0.91	0.0252 ± 0.0024	15.4
	70%	4.6 ± 0.7	0.95	0.0254 ± 0.0033	16.4
	75%	48.8 ± 3.1	0.99	-0.0395 ± 0.0178	38.2
Paraquat concentration	0.02 g l^{-1}	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	0.2 g l^{-1}	12.2 ± 0.9	0.99	0.033 ± 0.0040	32.7
	2.0 g l^{-1}	12.4 ± 3.2	0.89	0.0674 ± 0.0144	57.7
Wax	Cuticular membrane	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	Dewaxed membrane	15.8 ± 1.8	0.96	0.0269± 0.0079	33.5
Droplet size	1 droplet	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	2 droplets	1.3 ± 0.4	0.87	0.0136 ± 0.0016	32.7
	5 droplets	1.3 ± 0.5	0.7	0.0235 ± 0.0023	16.3
	10 droplets	3.5 ± 0.5	0.95	0.0319 ± 0.0021	61.6
Additives	Without additives	4.3 ± 0.6	0.96	0.0227 ± 0.0025	16.1
	Ethomeen T/25 (0.2%)	8.8 ± 0.8	0.98	0.0329 ± 0.0035	46.6
	Ethomeen T/25 (0.5%)	24.9 ± 3.3	0.97	0.020 ± 0.016	71.7
	Sorbitol (2%)	4.8 ± 0.6	0.97	0.03 ± 0.0029	27.9
	Sorbitol (3.5%)	13.7 ± 1.6	0.97	0.0081± 0.0081	22.4
	Ethomeen T/25 (0.5%) + Sorbitol (3.5%)	63.5 ± 6.6	0.97	0.2412 ± 0.0308	299.6
	Methly oleate (0.5%)	1.6 ± 0.2	0.94	0.0076 ± 0.0010	10.2
	Urea (0.5%)	2.5 ± 1.0	0.76	0.0135 ± 0.0046	18.2

4.3 Steady state experiments with focus on primary metabolites

4.3.1 Permeances obtained with *H. helix* membranes

Cuticular permeances (P_{CM}) of the used lipophilic compounds ranged from 1.75 x 10^{-11} (metribuzin) to 5.81 x 10^{-9} m s^{-1} (cloquintocet-mexyl). Permeances of the uncharged hydrophilic compounds ranged from 2.74 x 10^{-15} (maltotriose) to 4.70 x 10^{-8} m s^{-1} (erythrose). Permeances of the dissociated hydrophilic model compounds ranged from 4.97 x 10^{-12} m s^{-1} (valine) to 4.78 x 10^{-9} m s^{-1} (benzoic acid, pH 7). Any permeance of leucine, phenylalanine and paraquat dichloride was not detectable.

The effect of wax extraction, expressed as the quotient of the permeance of the polymer matrix membrane and the cuticular permeance (P_{MX} P_{CM}^{-1}), ranged for the lipophilic compounds from 222 (cloquintocet-mexyl) to 418 (metribuzin). Removal of waxes enhanced permeances of the uncharged hydrophilic compounds by factors between 1.8 (erythrose) and 993 (maltose).

All permeances of English ivy cuticular membranes and matrix membranes are summarised in table 4.6.

Temperatur effect. Cuticular permeances of the uncharged species of benzoic acid increased in the temperature range from 15 °C to 35 °C by a factor of 75. The activation energy of permeation obtained from the Arrhenius plot amounted to 164 (± 26) kJ mol^{-1}. No significant effect of the temperature on the permeance of xylose was detected. Activation energy was not significantly different from zero.

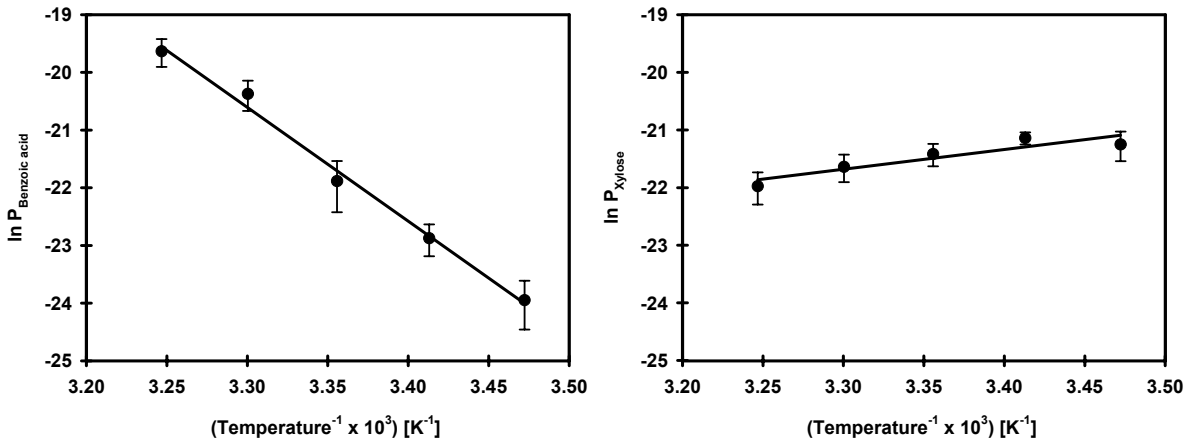


Figure 4.36 Temperature effect on the permeance of benzoic acid (R^2 = 0.99) and xylose (R^2 = 0.84) plotted as Arrhenius plots. Error bars represent 95% confidence interval.

Surfactant effect. The non-ionic surfactant triethylene glycol monododecyl ether ($C_{12}E_3$) (288 cm^3 mol^{-1}) accelerated the permeance of the lipophilic compounds benzoic acid and salicylic acid. Permeance of benzoic acid was enhanced by a factor of 4.0 ± 1.7 and that of salicylic acid by a factor of 6.7 ± 2.3. Both effects are statistically significant ($P = 4 \times 10^{-6}$ and $P = 3 \times 10^{-10}$ respectively). There was no significant acceleration effect of the surfactant on the permeance of the polar substance xylose (P = 0.12).

4.3.2 Permeances obtained with *V. vinifera* membranes

Cuticular permeances for *V. vinifera* of the uncharged hydrophilic compounds ranged from 3.00×10^{-09} m s^{-1} (ethanol) to 1.81×10^{-6} m s^{-1} (erythrose). Comparatively fewer experiments were conducted with grapevine cuticles (Table 4.5).

4.3.3 Partition coefficients obtained with *H. helix* membranes

Cuticle/water partition coefficients ranged from 26 (metribuzin) to 53,560 (cloquintocet-mexyl) for the lipophilic compounds. Cuticle/water partition coefficients ranged from 0.025 (water) to 99 (erythrose) for the uncharged

hydrophilic compounds and from 0.07 (glycine) to 12.4 (benzoic acid, pH 7) for the dissociated compounds. Matrix membrane/water partition coefficients ($K_{MX/W}$) were on the average 1.4-fold higher than cuticle/water partition coefficients (R^2 = 0.97). Sorption of the hydrophilic compounds into reconstituted cuticular waxes was not detectable. No partition coefficients were determined with grapevine cuticles. All cuticle/water partition coefficients and all matrix membrane/water partition coefficients are summarised in table 4.7.

Table 4.5 Summary of all measured permeances determined with grapevine cuticles. The number of replicates (n) is given in the last column.

Vitis vinifera			**P_{CM} ± 95% CI [m s^{-1}]**	**n**
Small hydrophilics	1	Water	3.02 (± 1.90) x 10^{-09}	9
	2	Ethanol	3.00 (± 2.11) x 10^{-09}	8
	3	Urea	-	-
Carbohydrates	4	D(-)-Erythrose	1.81 (± 0.86) x 10^{-06}	9
	5	D(+)-Xylose	2.57 (± 1.75) x 10^{-07}	9
	6	D(+)-Glucose	1.10 (± 0.65) x 10^{-08}	12
	7	D(+)-Maltose	1.50 (± 1.00) x 10^{-08}	7
	8	Maltotriose	-	-

Table 4.6 Summary of all measured permeances (P) determined with English ivy membranes. n is the number of replicates.

Hedera helix			**Cuticular membrane**		**Matrix membrane**		
			P_{CM} ± 95% CI [m s^{-1}	**n**	**P_{MX} ± 95% CI [m s^{-1}]**	**n**	**P_{MX} P_{CM}^{-1}**
Small hydrophilics	1	Water	7.20 (± 0.92) x 10^{-11}	22	2.56 (± 0.85) x 10^{-09}	8	36
	2	Ethanol	1.60 (± 0.07) x 10^{-10}	8	2.18 (± 0.76) x 10^{-09}	8	14
	3	Urea	6.48 (± 3.44) x 10^{-10}	8	-	-	-
Carbohydrates	4	D(-)-Erythrose	4.70 (± 1.48) x 10^{-08}	7	8.30 (± 2.81) x 10^{-08}	11	2
	5	D(+)-Xylose	2.30 (± 0.26) x 10^{-10}	9	1.94 (± 1.11) x 10^{-08}	7	84
	6	D(+)-Glucose	1.08 (± 0.30) x 10^{-11}	9	5.13 (± 1.60) x 10^{-10}	9	53
	7	D(+)-Maltose	8.31 (± 1.43) x 10^{-14}	8	8.25 (± 3.99) x 10^{-11}	8	993
	8	Maltotriose	2.74 (± 0.73) x 10^{-15}	8	9.19 (± 2.15) x 10^{-11}	10	33548
Amino acids	9	Glycine	1.84 (± 1.05) x 10^{-11}	16	-	-	-
	10	L-Alanine	3.74 (± 0.98) x 10^{-11}	9	-	-	-
	11	L-Serine	2.46 (± 0.48) x 10^{-11}	8	-	-	-
	12	L-Valine	4.97 (± 0.86) x 10^{-12}	8	-	-	-
	13	L-Threonine	6.24 (± 1.72) x 10^{-12}	11	-	-	-
	14	L-Leucine	*Not detectable*	8	-	-	-
	15	L-Phenylalanine	*Not detectable*	9	-	-	-
Polar AI.	16	Glyphosate	1.23 (± 0.65) x 10^{-11}	10	-	-	-
	17	Paraquat dichloride	*Not detectable*	11	-	-	-
Lipophilics	18a	Benzoic acid (pH 2)	1.06 (± 0.38) x 10^{-10}	8	3.57 (± 1.55) x 10^{-08}	10	337
	18b	Benzoic acid (pH 7)	4.78 (± 1.62) x 10^{-09}	9	-	-	-
	19	Salicylic acid	2.72 (± 0.87) x 10^{-11}	11	2.60 (± 1.30) x 10^{-08}	10	956
	20	2,4-D	3.23 (± 0.82) x 10^{-10}	9	2.59 (± 0.20) x 10^{-07}	12	803
	21	Metribuzin	1.75 (± 0.32) x 10^{-11}	9	7.32 (± 1.22) x 10^{-09}	8	418
	22	Cloquintocet-mexyl	5.81 (± 1.75) x 10^{-09}	10	1.29 (± 0.13) x 10^{-06}	10	222
	23	Bitertanol	5.15 (± 1.52) x 10^{-10}	8	1.63 (± 0.41) x 10^{-07}	8	317

Table 4.7 Summary of all measured partition coefficients (K) determined with English ivy membranes. n is the number of replicates.

Hedera helix			**Cuticular membrane**		**Matrix membrane**	
			$K_{C/W}$ ± 95% CI	**n**	**$K_{MX/W}$ ± 95% CI**	**n**
Small hydrophilics	1	Water	0.025 (± 0.007)	7	0.03 (± 0.008)	8
	2	Ethanol	0.113 (± 0.04)	7	0.043 (± 0.022)	7
	3	Urea	0.23 (± 0.13)	9	-	-
Carbohydrates	4	D(-)-Erythrose	99 (± 6)	19	76.7 (± 14.2)	13
	5	D(+)-Xylose	4.7 (± 0.5)	9	4.1 (± 1.1)	8
	6	D(+)-Glucose	6.7 (± 1.9)	9	12.2 (± 4.0)	11
	7	D(+)-Maltose	3.8 (± 1.5)	7	31.7 (± 16.0)	10
	8	Maltotriose	2.2*	-	17.3 (± 5.1)	7
Amino acids	9	Glycine	0.07 (± 0.03)	7	-	-
	10	L-Alanine	0.27 (± 0.19)	8	-	-
	11	L-Serine	0.18 (± 0.10)	7	-	-
	12	L-Valine	0.14 (± 0.07)	11	-	-
	13	L-Threonine	0.18 (± 0.09)	8	-	-
	14	L-Leucine	-	-	-	-
	15	L-Phenylalanine	-	-	-	-
Polar Al.	16	Glyphosate	5.0 (± 1.5)	10	-	-
	17	Paraquat dichloride	-	-	-	-
Lipophilics	18a	Benzoic acid (pH 2)	36.0 (± 7.3)	8	52.0 (± 6.1)	10
	18b	Benzoic acid (pH 7)	12.4 (± 3.6)	9	-	-
	19	Salicylic acid	29.1 (± 4.2)	12	99.0 (± 18.5)	11
	20	2,4-D	287.0 (± 50.6)	10	348.0 (± 112.0)	8
	21	Metribuzin	26.0 (± 5.8)	10	43.3 (± 8.0)	8
	22	Cloquintocet-mexyl	50,560 (± 2573)	8	58,084 (± 6,886)	7
	23	Bitertanol	8842 (± 1657)	11	10,341 (± 1,717)	9

* extrapolated from the partition coefficients of erythrose, xylose, glucose and maltose.

5. DISCUSSION

The mechanisms governing cuticular penetration of lipophilic molecules are pretty well known. In contrast to that, the mechanisms governing the cuticular transport of hydrophilic compounds are not well understood. However, hydrophilic molecules cross the cuticle as it was shown many times (see chapter 1.5). Understanding of these mechanisms provides an explanation for leaching of primary metabolites and for uptake of foliar applied hydrophilic agrochemicals. Therefore, this present study was focused on cuticular transport of primary metabolites and hydrophilic active ingredients.

5.1 Non-steady state experiments with focus on paraquat

In 1967 R. C. Brian published a paper about foliar uptake of paraquat and diquat. In spite of the high water solubility of these herbicides the author found a rapid uptake, even in the dark and without surfactants. From these findings the author concluded that both herbicides are taken up across the plant cuticle - in spite of the mentioned water solubility:

…As uptake in the dark exceeded that in the light, and was large even in the absence of surfactant, entry was not through open stomata but through the cuticle. Cuticular membranes appear to be highly permeable to aqueous solutions of monovalent and divalent cations and anions but their mode of entry through the cuticle is still not well understood. Pathways through the cuticle may consist of cracks and perforations although solutions of ions may enter through the unbroken cuticle (Yamada et al., 1964). Cuticular transpiration is evidence of such movement of water molecules (Crafts & Foy, 1962).

Taken from Brian (1967) p. 96

5.1.1 Cuticular penetration of paraquat dichloride at moderate relative humidity

The importance of water for cuticular transport of paraquat is shown in chapter 4.2. It is conspicuous that 41% of total paraquat uptake occurs within the first 75 minutes after droplet application. The remaining 59% are taken up in the following 23 hours. When the water is evaporated the active ingredient recrystallises which counteracts a continuously prolonged uptake. It is well-known that active ingredients must be either liquid or in solution to penetrate readily across the cuticular membrane (Briggs & Bromilow, 1994). The presence of a 'water bridge' between the active ingredient and the leaf is essential for herbicide uptake from a droplet (Douglas, 1968). This was confirmed by the rewetting experiment (Figure 4.21). At moderate relative humidity paraquat penetration was negligible after 24 hours. After application of water on the recrystallised active ingredient, uptake increased immediately. The duration of this improved paraquat transport was limited by the presence of water which keep paraquat ions dissolved. From this long-term experiment it was not possible to calculate a rate constant, since linearity failed. Therefore, rate constants between each measure-interval were calculated and plotted versus time resulting in figure 5.1. It is obvious that rate constants are the highest, when there is a wet residue on the cuticle. Immediately after application, rate constants were high. When water was evaporated, rate constants decreased continuously. The maximum rate constant was reached at the very beginning of the experiment (20 minutes). Within the duration of water evaporation, rate constants decreased again. This is in contrary to Baur and Schönherr (1997) where the authors found the highest rate constant when the residue was apparently dry. But this experiment was done using the highly lipophilic surfactant octaethylene glycol monododecyl ether ($C_{12}E_8$).

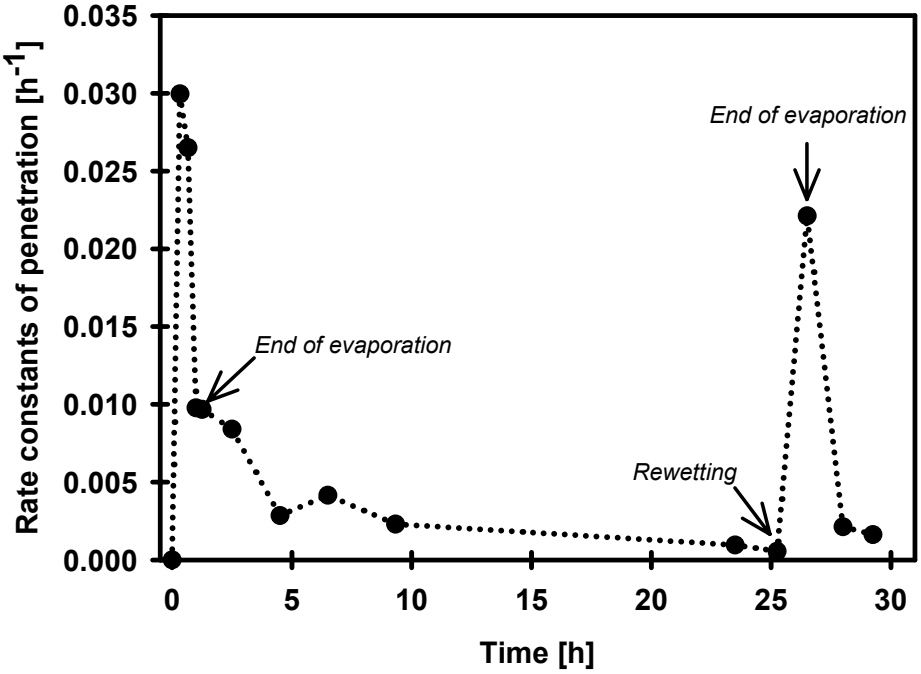


Figure 5.1 Variation of the rate constants of penetration of paraquat dichloride from the time of application (t_0). After 25 hours a droplet of pure water was applied on the dried residue on the top of the cuticle.

However, rate constants of paraquat and $C_{12}E_8$ decreased during the course of the measurement. After 25 hours the paraquat residue on the cuticle was redissolved which caused a large increase of the rate constant again. But this increase was limited to the duration of water evaporation. When the residue was dry again, rate constants were low again, too. Reason for such low rate constants are the decreasing driving forces during the course of the measurement. The driving force is defined as the cuticle/water partition coefficient multiplied by the concentration of the compound in the formulation residue ($K_{C/W}$ x c_{fr}) (Baur & Schönherr, 1997). When water is totally evaporated and the relative humidity is low, the driving force tends to zero, because the concentration in the residue tends to zero, too. This is caused by recrystallisation of the active ingredient in absence of water. Figure 5.2 displays the driving force of paraquat penetration during the initial phase when water is still present and droplet water is evaporating. Relative humidity was 50%.

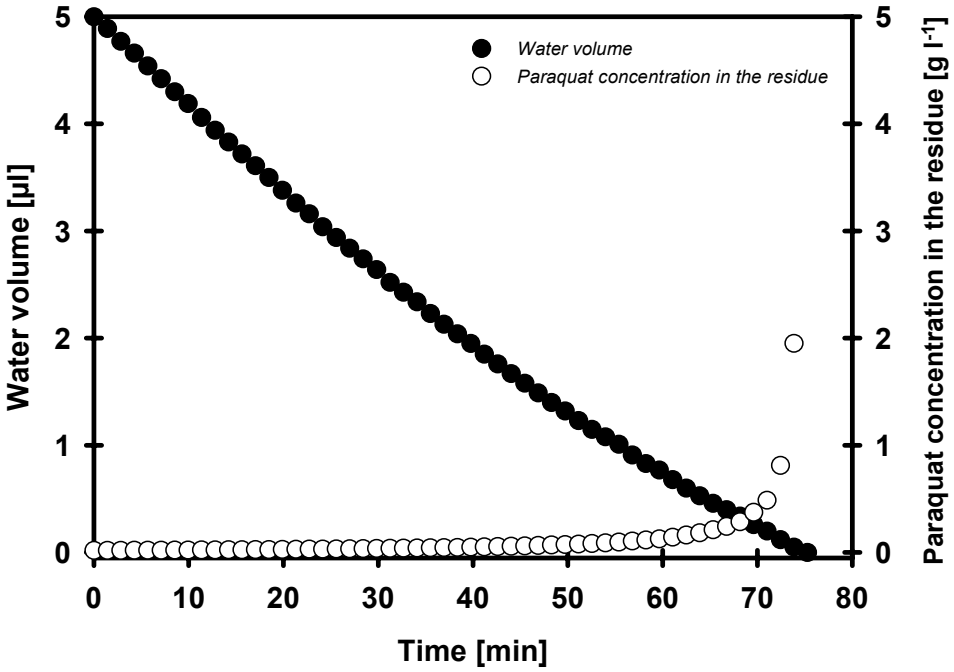


Figure 5.2 Variation of the water volume and the remaining paraquat concentration in a residue on the top of the cuticle as a function of time. Paraquat concentration is proportional to the driving force since the cuticle/water partition coefficient is assumed to be constant.

On the left part of the y-axis the volume of the evaporating water is displayed as a function of time. Because of the decreasing water volume, the concentration of paraquat in the residue is varying, too. Paraquat concentration of the residue is also decreased by continuous penetration across the membrane. On the right side of the y-axis, the concentration of paraquat in the remaining water is plotted as a function of time. Since the cuticle/water partition coefficient of paraquat is assumed to be constant, the paraquat penetration should be proportional to the driving force within the initial phase. Due to the high water solubility of paraquat (700 g l^{-1}) the recrystallisation during the whole evaporation-time does not take place which is contrary to experiments done with molecules of low water solubilities (e. g. lipophilic compounds). From figure 5.2 it is obvious, that driving forces are high when the water volume is very low. However, from figure 5.1 it is apparent that the maximum rate constants are reached at the beginning of the experiment and decrease with continuing evaporation.

The penetration of paraquat across isolated grapevine cuticles was compared to that of another highly hydrophilic herbicide. While approximately 6% of the

cationic herbicide paraquat penetrates the cuticle at 50% relative humidity, only 1.3% of the anionic herbicide glyphosate penetrates. It is assumed that 50% relative humidity is too low for a permanent dissolution of both herbicides. Regarding to figure 4.23A it is obvious that during the initial phase - when there is a wet residue on the cuticle - paraquat penetration is much higher than penetration of glyphosate. Application of glyphosate as potassium salt or as its acid did not vary under these conditions (data not shown). The examination of this low glyphosate penetration calls for further experiments. Very low cuticular penetration of glyphosate was also found with isolated leaf cuticles of *Ficus elastica, Hedera helix, Ilex aquifolium* and isolated fruit cuticles from *Lycopersicon esculentum* and *Capsicum annuum* at 75% relative humidity (Santier & Chamel, 1998). Different glyphosate salts at high relative humidities led to high penetration rates (Schönherr & Schreiber, 2004a). Penetration of lipophilic clodinafop results in a straight line; nevertheless, it also shows a declining curve progression. Clodinafop reaches a maximum cuticular penetration close to 10% which is higher than the maximum penetration of paraquat. Only 11% of the maximum cuticular penetration of clodinafop penetrated within the initial phase, while the proportions of paraquat (41%) and glyphosate (61%) are much higher. From this finding it is assumable that the need for moisture has not the same importance as with hydrophilic molecules. This might be caused by the favourable membrane solubility of clodinafop. The high driving force of clodinafop as a result of its much higher cuticle/water partition coefficient leads to higher absorption inside the cuticular membrane which is a prerequisite for traversing the cuticular membrane.

Paraquat penetration across isolated cuticles of English ivy under non-steady state conditions was not detectable, even after 24 hours. Under steady-state conditions, using transport chambers which consist of two aqueous compartments (Figure 3.7), no permeation was detectable, also. So, cuticles of English ivy seem to be impermeable for paraquat which might be due to their low permeability compared with grapevine cuticles, since the cuticle of *H. helix* has very low permeances for water compared with other species (Riederer & Schreiber, 2001). Another possible argument is given in chapter 5.2.5.

5.1.2 Influence of relative humidity on cuticular penetration of paraquat dichloride

Generally, all experiments were conducted at 50% relative humidity, since humidity is very often a limiting factor in practice, too. It is obvious that humidity did not influence the penetration of paraquat in the range of 25% to 70% relative humidity. However, at 75% RH paraquat penetration raised dramatically and it is shown in figure 4.24B that the regression line is straight instead of declining. One reason for this phenomenon the prolonged water evaporation, but the most important reason is the point of deliquescence (POD) of paraquat which is expected to be close to 75% RH. The POD corresponds to the humidity over a saturated salt solution containing undissolved salt. If ambient humidity is lower than the POD the salt crystallises on the cuticle and penetration stops (Schönherr, 2001). For a salt residue to deliquesce, the humidity above the salt must be higher than the point of deliquescence. So at 75% humidity paraquat is dissolved continuously and a permanent penetration is possible. In contrast to all other experiments, the initial rate constant is lower than the rate constant after the initial phase. In all other plots it is the reverse. This might be caused by high driving forces, since water volume is low, but paraquat concentration in the remaining aqueous residue is high. This is supported by its favourable water solubility. It is a matter of discussion whether a still higher relative humidity still increases paraquat uptake, since water absorption goes on as seen from figure 4.25. In enzyme kinetics this shape of a Lineweaver-Burk plot is typical for the presence of an inhibitor which inhibits an enzyme-catalysed reaction. From figure 4.24C one can assume that humidity below the POD inhibits paraquat penetration. It is postulated that effects of humidity are not restricted on the penetrating compound but also on the cuticle itself. Swelling of the cuticle affects size and number of aqueous pores (Schönherr, 1982). Swelling of the membrane depends on humidity, since it was shown for several times that the water content of the cuticle depends on the ambient humidity (Chamel *et al*., 1991; Schönherr, 2001; Schreiber *et al*., 2001). As ions are confined to the aqueous pathway (Schönherr, 2000; Schönherr & Schreiber, 2004a) their rate of diffusion depends on the relative humidity. The effect of the water content on the swelling of cellulose strains is displayed in figure 1.5.

The high importance of the relative humidity on paraquat uptake was also shown by Brian (1966). The author concluded that paraquat would be most effective in the field when sprayed under dry soil conditions in late afternoon or evening when increased humidity can follow soon after treatment. Allen (1960) showed that uptake of magnesium salts was affected by changes in relative humidity. This was also supported for paraquat (Douglas, 1968).

5.1.3 Influence of different concentrations on cuticular penetration of paraquat dichloride

The effect of the driving force was discussed above for several times. Since the cuticle/water partition coefficient is constant, the concentration in the aqueous residue is easily influenced. A tenfold increase in the concentration corresponds to a tenfold increase of the driving force. A hundredfold increase of the concentration corresponds to a hundredfold increase of the driving force. The upper limit of the driving force is the water solubility of the examined compound. As seen from figure 4.26, increased concentrations lead to increased paraquat penetration. The driving force of compounds with a low cuticle/water partition coefficient can be increased *via* the concentration. That is why compounds with low membrane solubility are characterised by high water solubility. This was shown with highly water soluble paraquat. Increased paraquat uptake from more concentrated droplets was also observed with experiments done on intact plants (Douglas, 1968; McKinlay *et al*., 1974).

5.1.4 Effect of cuticular waxes on cuticular penetration of paraquat dichloride

Cuticular waxes constitute the main barrier in plant cuticles and extracting the waxes increases the permeability of non-electrolytes up to three orders of magnitude (Schönherr & Riederer, 1989; Schönherr & Baur, 1994; Riederer & Schreiber, 1995; Baur *et al*., 1999). The effect of cuticular waxes on the cuticular penetration of paraquat was examined. The maximum cuticular

penetration increased 3.4-fold up to 20% and, correspondingly, rate constants rose also 3.7-fold. This is a significant increase in cuticular transport which is in contrast to findings by Schönherr (2000) where a very low increase in $CaCl_2$ penetration after wax removal was found using *Pyrus communis* membranes. For isolated cuticles of English ivy it was assumed, that extraction of the cuticular waxes uncovers additional polar pathways which results in an increased number of passable pathways leading to an increased penetration (see chapter 5.2.5). Figure 4.27C displays a parallel translation of the regression line of cuticular membranes compared to dewaxed membranes. In enzyme kinetics this shape of a Lineweaver-Burk plot is typical for the presence of an inhibitor which inhibits an enzyme-catalysed reaction. From that finding it is obvious that cuticular waxes of grapevine inhibit the penetration of paraquat. Taking the octanol/water partition coefficient of paraquat (log $K_{O/W}$ = -2.71) into account, it is clear, that a penetration *via* the lipophilic pathway is out of the question.

5.1.5 Influence of droplet size on cuticular penetration of paraquat dichloride

The effect of droplet size on cuticular penetration of paraquat was examined. While the concentration of paraquat was kept constant, the number of droplets was varied. Therefore, the driving forces were steady. Considering the smaller droplet sizes 0.5 µl, 1 µl and 2.5 µl there is a correlation between the volume and cuticular penetration. Small droplets showed higher cuticular penetration. However, the largest droplet (5 µl) did not fit to that correlation (figure 4.28). Actually, in the field, small droplets are favoured because of the reduced drift. Knoche (1994) reviewed the effect of droplet size on the performance of foliage-applied herbicides. Generally, a decreasing droplet size increased the performance of the herbicide. Knoche cited many publications about paraquat performance and droplet size. Although there is a tendency to the preference of droplets of smaller volume (McKinlay *et al*., 1974), some publications are inconsistent (Merrit, 1982; Douglas, 1968). It is important to mention, that all these results cited were found in experiments done with intact plants where the

performance of the herbicide was detected - not the absolute cuticular penetration of paraquat. From these results - which focus on the absolute cuticular penetration - a tendency in favour of small droplets is supported.

5.1.6 Influence of additives on cuticular penetration of paraquat dichloride

Addition of ethomeen T/25 had an pronounced influence on the cuticular penetration of paraquat. Absolute penetration was enhanced twofold (+ 0.2% T/25) or rather fivefold (+ 0.5% T/25). The proportion of the initial phase on the total cuticular penetration decreased with increasing T/25-concentration. Ethomeen increases the wetted area of the droplet conspicuously which results in a shortened evaporation of the water. Nevertheless, a wet residue was recognisable during the whole experiment which keeps paraquat in solution - at least partially. So it is assumed, that ethomeen T/25 has hygroscopic properties when it is used as a hydrochloride salt. Uncharged T/25 did not improve paraquat penetration (data not shown). An increased interface between droplet and cuticle increases the number of accessible polar pathways (chapter 5.2.5). Hence the higher number of polar pathways and the hygroscopic property of T/25 at pH 7 might be responsible for its favourable effect on the cuticular penetration of paraquat. Interestingly, a noticeable increase in the mobility of non-ionised 2,4-dichlorophenoxyacetic acid (2,4-D) caused by ethomeen T/25 has been shown with *Citrus* cuticles (Schönherr & Bauer, 1992; Baur & Schönherr, 1996). Since this was an 'UDOS' experiment, the wetting property of the additive has no relevance. Riederer & Schönherr (1990) found significant effects of non-ionic ethomeen T/25 on the water permeability of isolated cuticles of *Citrus aurantium* and *Pyrus communis*. The authors postulated structural changes of the cuticular waxes due to the surfactant. This could be caused by hydrated polyoxyethylene residues within the cuticle which originate from ethomeen T/25. These polyoxyethylene residues probably cause an increase in the water content of the transport limiting barrier and thus may further contribute to increased permeabilities (Riederer & Schönherr, 1990). For that reason, the beneficial effect of ethomeen T/25 is not restricted to improved wetting

properties but also affects the water content of the membrane. Since ions are excluded from the lipophilic pathway (Schönherr, 2002) and charged ethomeen T/25 was used in this study, structural changes of the cuticular waxes are improbable. In figure 4.29C it is shown that ethomeen T/25 can activate the cuticular penetration of paraquat.

'Humectant' is the name commonly used for a substance which can take up water from an under-saturated atmosphere. It can therefore cause a deposit to remain partly liquid in an atmosphere in which it would otherwise become completely solid. This may be of advantage where a liquid medium is necessary to provide a diffusion path between crystals and leaf surface (Hartley & Graham-Bryce, 1980). To examine the meaning of a humectant on cuticular paraquat penetration under moderate relative humidity, sorbitol was added in two different concentrations. Water absorption from the environment as a function of the relative humidity is shown in figure 4.31. The presence of sorbitol leads to increased paraquat penetration (figure 4.30). During the whole experiment a wet residue was visible on the top of the cuticle, keeping the active ingredient dissolved – at least partially. This phenomenon is visualised in figure 4.35. While the evaporation is delayed, the total water loss is prevented. Adding 3.5% sorbitol results in a straight line. This is a hint for sufficient water availability, so the herbicide is kept in solution continuously and an unhindered penetration is assured.

Both, ethomeen T/25 and sorbitol have a very beneficial effect on cuticular paraquat penetration. Combination of both additives results in a synergistic effect. Ethomeen increases the wetted area which accelerates the evaporation. Sorbitol keeps the active ingredient in a dissolved state (figure 4.35). The rate constant of the initial phase exceeds all other rate constants determined in this study very clearly. The rate constant after the initial phase was also the highest value found in this study. More than half of the amount of paraquat penetrated the membrane and the time needed for the half of maximum penetration was 1.3 hours only. So, combination of both additives resulted in a very rapid and quantitative cuticular penetration at unfavourable circumstances, since relative humidity was very clearly below the point of deliquescence.

Methyl oleate is a well known accelerator for lipophilic active ingredients (Santier & Chamel, 1996; Serre *et al*., 1996). One of its favourable properties seems to be the high lipophilicity (log $K_{O/W}$ = 8.0; Briggs & Bromilow, 1994) which allows a quantitative sorption of the adjuvant inside the cuticular waxes. The noticeable deceleration effect of methyl oleate on the cuticular penetration of paraquat (figure 4.33) supports the assumption that paraquat is excluded from the lipophilic pathway and restricted to the polar pathway. Another property of the methyl oleate emulsion is to increase the wetted area. Generally, this seems to be beneficial for paraquat penetration as discussed above. On the other hand, this highly lipophilic additive could partly cover polar pathways on the surface of the cuticle which would cause a reduction of the number of accessible pathways – comparable with cuticular waxes (see chapter 5.2.5). This might be an explanation for the deceleration effect of methyl oleate on the cuticular penetration of hydrophilic paraquat. Figure 4.33C displays the inhibiting effect of methyl oleate on the cuticular paraquat penetration.

Urea is used as an additive for pharmaceuticals in dermatology. It is used to enhance the water content of human skin. Therefore, it was studied, whether urea can also increase the water content of isolated plant cuticles which might result in an increased cuticular penetration of paraquat. Two different concentrations were added, but no improvement of paraquat penetration was observed. In dermatology a concentration of at least 10% is necessary to obtain an increase of the hydration of the human skin (Beastall *et al*., 1986). Additive concentrations like that are not usual in formulation of pesticides.

5.1.7 Insights about paraquat penetration from droplets

The most important parameter for cuticular paraquat penetration is the availability of water. Either the relative humidity is sufficient for a permanent wet residue which keeps the herbicide dissolved, or the water availability of the residue must be improved by additives, when the surrounding relative humidity is below the point of deliquescence. Figure 5.3 summarises the maximum cuticular penetration of all parameters examined in this study. Conspicuous

effects are highlighted. Black bars represent experimental conditions with sufficient water availability, due to surrounding humidity or improvement by additives. Grey bars represent favourable conditions in spite of limited humidity, e. g. improvement due to increased driving forces or due to increased number of accessible polar pathways.

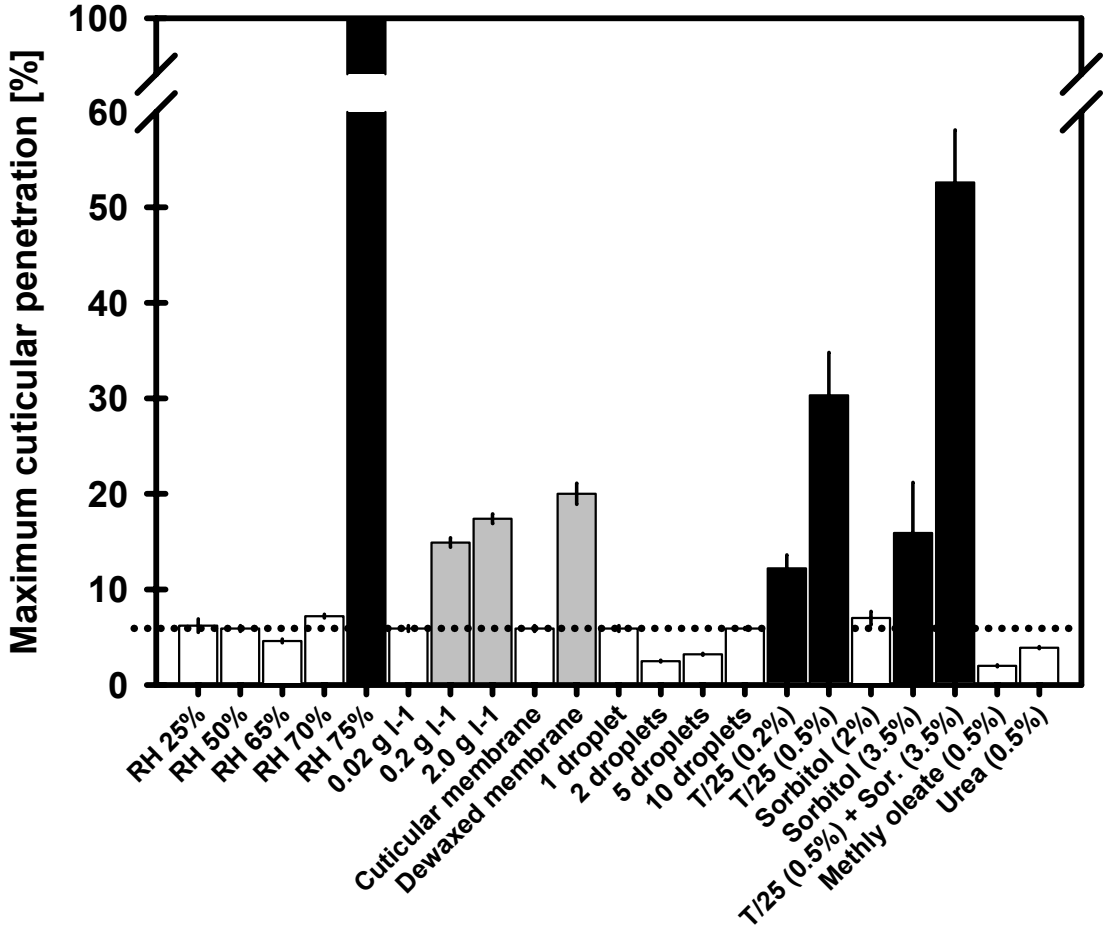


Figure 5.3 Maximum cuticular penetration of paraquat of different experiments. Effects caused by increased water availability are shown as black bars and positive effects reached in spite of humidity limitation are shown as grey bars. Error bars represent standard errors.

A very good correlation was found between the calculated rate constants and the maximum cuticular penetration of paraquat found from the double reciprocal plots ($R^2 = 0.98$) (Fig. 5.4). From the following equation the maximum cuticular paraquat penetration ($\%_{max}$) is predictable from the rate constants:

$$\%_{max} = 1.17\ (\pm\ 0.047) \times (k \times 10^3) + 1.13\ (\pm\ 0.42) \qquad \text{(Eq. 5.1)}$$

This correlation confirms the double reciprocal presentation of the data, since the determination of the rate constants is well established (see chapter 2.3).

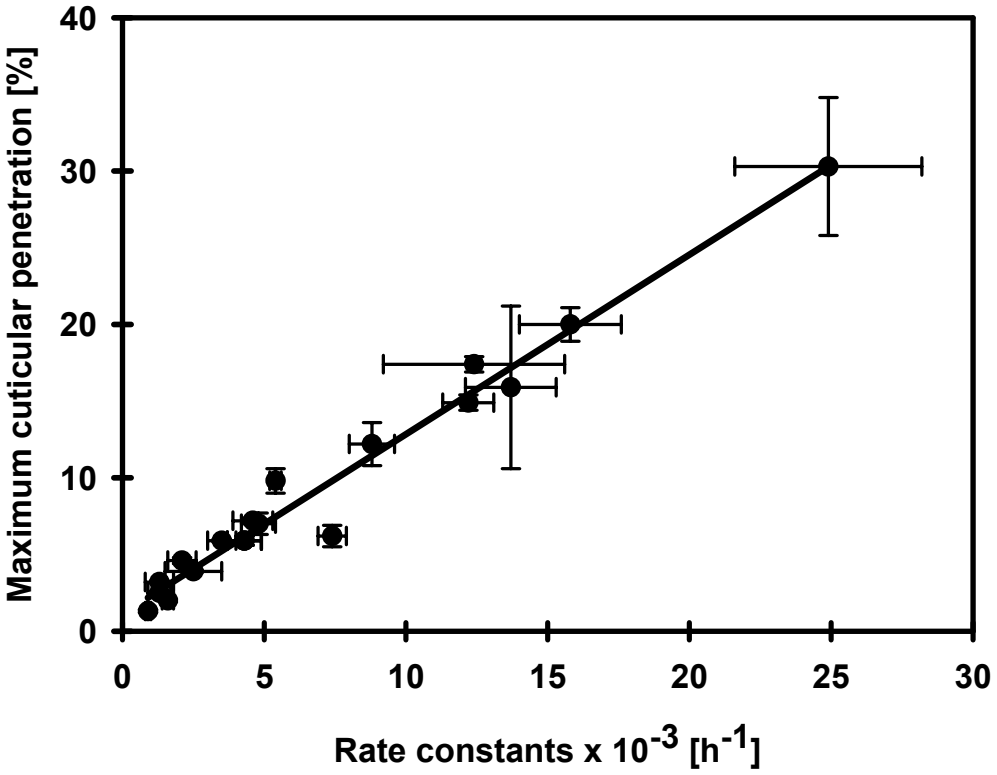


Figure 5.4 Maximum cuticular penetration plotted versus the rate constants of the conducted experiments ($R^2 = 0.98$).

5.2 Steady state experiments with focus on primary metabolites

5.2.1 Hints for the occurrence of water soluble primary metabolites on plant surfaces

Leaching is defined as the removal of substances from plants by the action of aqueous solutions, such as rain, dew, mist and fog (Tukey, 1970). Materials leached from foliage include - among others - carbohydrates and amino acids (Tukey *et al*., 1965). Actually, figure 5.5 presents a visual hint for the occurrence of water soluble reducing carbohydrates (A) and amino acids (B) on the astomatous plant surface of grapevine. While the staining method from figure 5.5A is specific to glucose and also to further reducing carbohydrates, the staining method for amino acids is unspecific. Therefore, it can not be excluded, that any by-products on leaf surfaces cause the staining. However, occurrence of amino acids on plant surfaces is well documented, so it is highly presumable that the purple dye is due to the presence of amino acids. The goal of this experiment was not to proof the occurrence of primary metabolites on plant surfaces – since this was done already - but to give a visual hint.

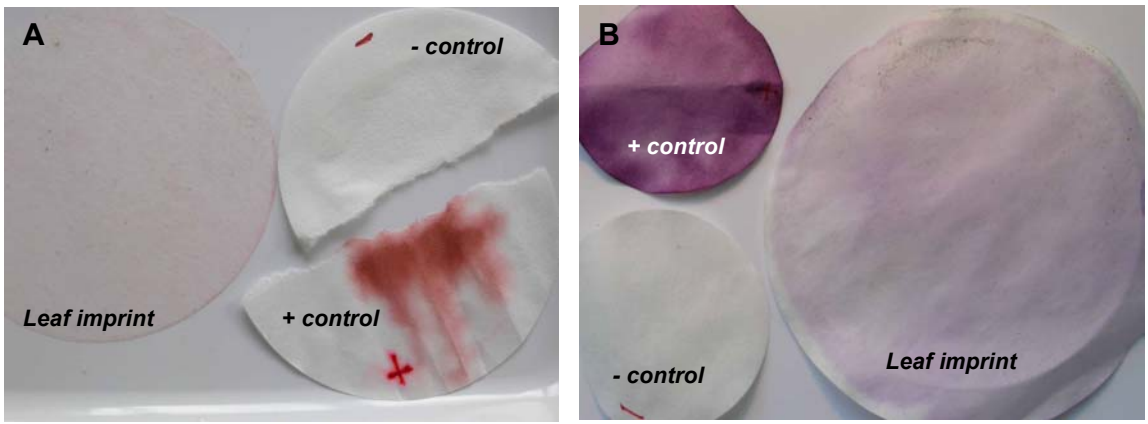


Figure 5.5 Leaf imprints made from astomatous grapevine leaves. **A** shows a clearly reddish staining on the filter paper which is a hint for the presence of reducing carbohydrates on the leaf surface. **B** shows a clearly purple dye on the filter. It is highly presumable that this staining is caused by amino acids.

5.2.2 Membrane solubility of the model compounds

Octanol/water partition coefficients ($K_{O/W}$) are widely used to describe lipophilicity of chemicals (Leo *et al*., 1971). According to the octanol/water partition coefficient, organic compounds can be classified as lipophilic when log $K_{O/W} > 0$ and as hydrophilic when log $K_{O/W} \leq 0$. A fundamental prerequisite for cuticular permeance is the solubility of the respective compound inside the cuticular membrane. That is why, in the simplest way, cuticular permeation can be described as a diffusion process from an aqueous donor compartment across the cuticular membrane into an aqueous receiver compartment (Schönherr & Riederer, 1989). The octanol/water partition coefficient provides a very useful tool to forecast the membrane solubility of lipophilic compounds. A very good correlation between octanol/water and cuticle/water partition coefficients was found by Kerler & Schönherr (1988a). There is a good correlation of the sorption properties of *1*-octanol and the cuticle which are very similar. This was confirmed for all six lipophilic model compounds in the present study (figure 5.6). The equation of the regression line fits exactly to that found by Kerler & Schönherr. From figure 5.6 it is also obvious that the sorption of all hydrophilic compounds is higher than expected. This implies that carbohydrates, which have been found in substantial amounts as components in cuticular membranes (Schreiber & Schönherr, 1990; Krüger *et al*., 1996; Dominguez & Heredia, 1999; Marga *et al*., 2001), are potential sorption sites for hydrophilic compounds. Therefore, it is explainable that sorption of the hydrophilic model compounds like carbohydrates, amino acids, water, urea and glyphosate is higher than expected.

Cuticular waxes form the transport limiting barrier of cuticular membranes as it was shown for lipophilic compounds (Riederer & Schreiber, 1995). Sorption of lipophilic compounds into cuticular waxes is up to one order of magnitude lower than sorption into cuticular membranes while sorption of the hydrophilic compounds into waxes was not detectable. This implies that for hydrophilic compounds transport across the cuticular wax barrier is not relevant. It has been suggested that wax/water partition coefficients should be used instead of cuticle/water partition coefficients in order to describe the permeation of

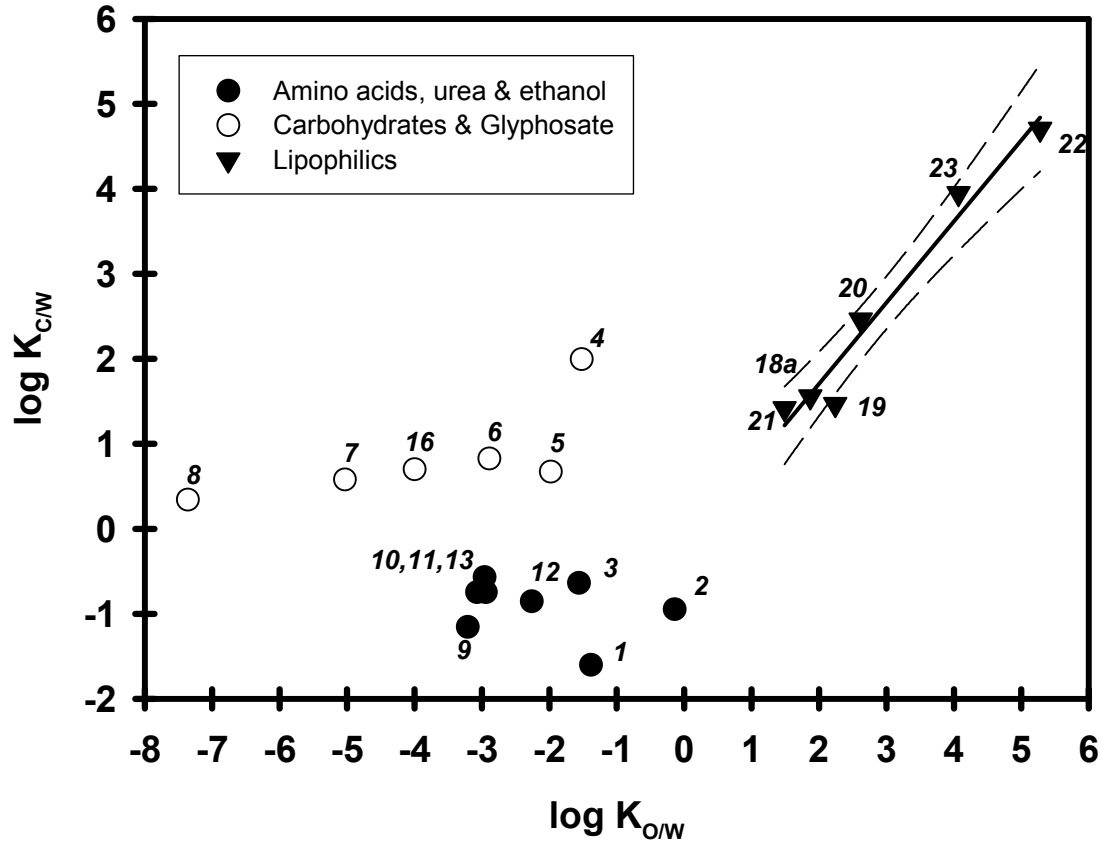


Figure 5.6 Experimentally determined cuticle/water partition coefficient of the model compounds plotted versus the estimated (EPIWIN v3.11) octanol/water partition coefficients. A correlation is restricted only for the lipophilic model compounds (▼) (R^2 = 0.96). The membrane solubilities of the hydrophilic compounds are higher as predicted which might be caused by polar regions inside the plant cuticle. The usage of error bars was renounced to simplify the diagram. Dashed lines represent 95% confidence interval of the regression line.

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1 water, 2 ethanol, 3 urea, 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 8 maltotriose, 9 glycine, 10 alanine, 11 serine, 12 valine, 13 threonine, 16 glyphosate, 18a benzoic acid (pH 2), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

lipophilic compounds through the transport-limiting barrier of cuticular membranes (Schönherr & Baur, 1994). However, cuticular membranes are of heterogeneous structure combining lipophilic and hydrophilic properties arising from the components cutin, cuticular waxes and polysaccharides. Cuticular permeances are related to the transport across the whole cuticular membrane. Therefore, in the present work cuticle/water partition coefficients are used for the analysis of cuticular transport in order to ensure a consistent treatment of the hydrophilic and lipophilic compounds.

5.2.3 Membrane permeability of the model compounds

As mentioned before, cuticular permeation can be described as a diffusion process from an aqueous donor compartment across the cuticular membrane into an aqueous compartment (Schönherr & Riederer, 1989). This implies dissolution of the permeating compound inside the plant cuticle. A correlation was found between the logarithm of the permeance and the logarithm of the octanol/water partition coefficient (Kerler & Schönherr, 1988b). This regression is restricted to compounds with an octanol/water partition coefficient beyond log 2. Unfortunately, this prediction partly leads to large errors. However, from this forecast cuticular permeances of hydrophilic compounds would be conspicuously underestimated. Schönherr & Baur (1996a) predicted very low membrane mobilities of hydrophilic amino acids and carbohydrates from their octanol/water partition coefficients. However, cuticular transport of polar compounds is measurable which implies sorption inside the cuticle. A somewhat better forecast can be obtained by predicting permeances from cuticle/water partition coefficients. This prediction is restricted to the lipophilic model compounds used in the present study (figure 5.7):

$$\text{Log } P_{CM} = 0.61 \log K_{C/W} - 11.29 \quad (R^2 = 0.87) \qquad \text{(Eq. 5.2)}$$

This equation fits very well to the prediction found by Kerler & Schönherr (1988b). Interestingly, all used carbohydrates (4-8) respond very sensitive to differences in their membrane solubility. While the cuticle/water partition coefficient varies very slightly by a factor of 45, the permeance varies up to eight orders of magnitude. In contrast to that finding the variation in the solubility and the permeability of the small hydrophilic molecules (1-3) and the amino acids (9-13) is limited.

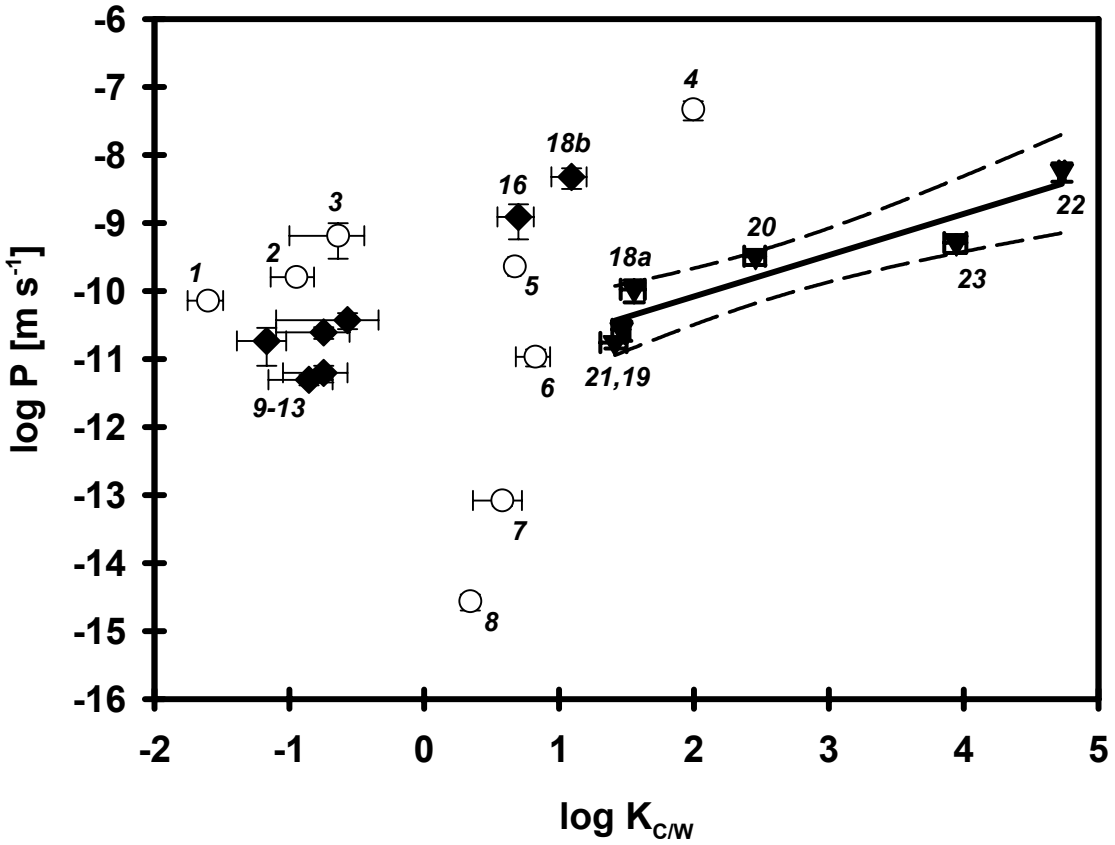

Figure 5.7 Permeance of the model compounds plotted versus the cuticle/water partition coefficient. The correlation is restricted to the lipophilic model compounds (▼) (R^2 = 0.87), not for the uncharged hydrophilic model compounds (○) or rather for the dissociable hydrophilic compounds (◆). Dashed lines represent 95% confidence interval of the regression line.

---

1 water, 2 ethanol, 3 urea, 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 8 maltotriose, 9 glycine, 10 alanine, 11 serine, 12 valine, 13 threonine, 16 glyphosate, 18a benzoic acid (pH 2), 18b benzoic acid (pH 7), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

5.2.4 Membrane mobility of the model compounds

The permeance [m s^{-1}] is a composite quantity consisting of the diffusion coefficient (D), the partition coefficient (K), the membrane thickness (l) and the tortuosity of the diffusional path length (τ):

$$P = \frac{D \times K}{\tau \times l} \qquad \text{(Eq. 5.3)}$$

In order to compare the transport properties of all hydrophilic and all lipophilic model compounds, the permeances were corrected by the respective membrane solubility. This quotient can be regarded as a mobility parameter m:

$$m = \frac{P}{K} = \frac{D}{\tau \times l} \qquad \text{(Eq. 5.4)}$$

Within one plant species, the variability of the mobility can be attributed directly to changes of the diffusion coefficient. Plotting the logarithm of the mobility versus the molar volume (MV) yields the relationship (Schönherr & Baur, 1994):

$$\log\ m = \beta' \times MV - \log m_0 \qquad \text{(Eq. 5.5)}$$

The slope of the regression line represents the size selectivity (ß') of diffusion. The y-intercept (m_0) is equal to the mobility of a molecule of zero molar volume which can be assumed to be a measure for the tortuosity of the diffusion path.

5.2.4.1 Membrane mobility of lipophilic compounds

The slope of the regression line obtained from figure 5.8 represents the size selectivity (ß') of the lipophilic pathway. Using an alternative experimental approach by measuring rate constants of solutes across the transport-limiting barrier of cuticular membranes it was found that the size selectivity varied only little between plant species. An average value of 0.0095 mol cm^{-3} was obtained (Buchholz *et al*., 1998). This value fits very well to the size selectivity of English ivy found in the present work (0.0083 mol cm^{-3}). The size selectivity of the lipophilic pathway can be interpreted by the 'free volume theory' claiming an exponential distribution of the free volume size (voids). The 'free volume theory' is a general theory to explain diffusion across polymers which is applicable for the plant cuticle, too. This theory states that diffusion of lipophilic compounds takes place in voids formed by thermal motion of the molecules making up the medium (see chapter 2.1). Diffusion of a given solute depends on the probability that there is free volume adjacent to it which is sufficiently large for

accommodating it (Crank & Park, 1968; Vieth, 1991). A mean free volume (V_f) of 52 cm³ mol^{-1} can be obtained directly from the size selectivity (ß') from the lipophilic compounds of figure 5.8:

$$V_f = (2.303 \times ß')^{-1} \qquad \text{(Eq. 5.6)}$$

There is no doubt that these six lipophilic model compounds must traverse the plant cuticle across the highly lipophilic cuticular waxes and the cutin pathway.

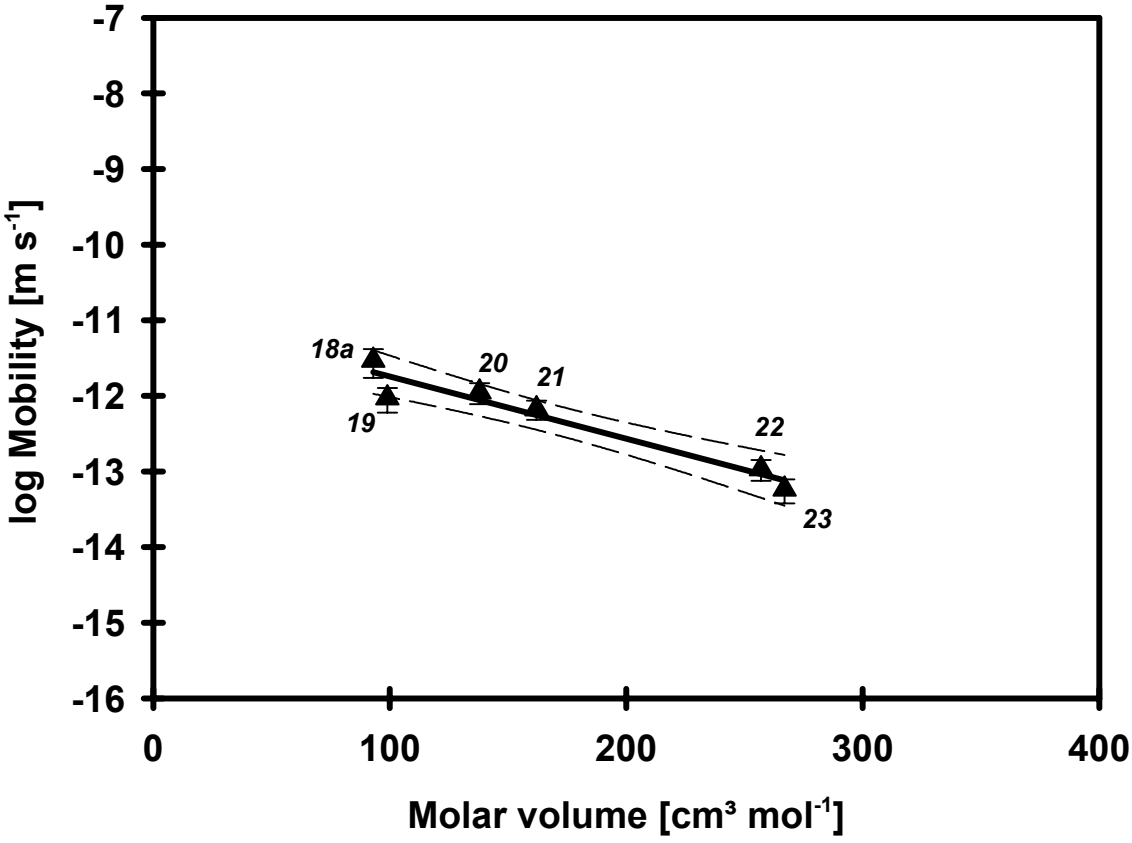

Figure 5.8 Mobility of the lipophilic model compounds plotted versus the molar volume. The regression line represents the lipophilic pathway (R^2 = 0.93). Dashed lines represent 95% confidence interval of the regression line.

18a benzoic acid (pH 2), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

5.2.4.2 Membrane mobility of uncharged hydrophilic compounds

Plotting the mobility of the lipophilic and the uncharged hydrophilic molecules versus the molar volume results in a separation of the lipophilic pathway and a second pathway, which is clearly differentiated from the lipophilic pathway (Fig. 5.9). All uncharged hydrophilic compounds in the range of 17 – 100 cm^3 mol^{-1} debunk a separate hydrophilic pathway traversing the plant cuticle of English ivy. Three carbohydrates of higher molar volume do not fit to the regression line of the hydrophilic pathway. Transport of these molecules across the lipophilic pathway is very improbable. This observation will be discussed in chapter 5.2.5.

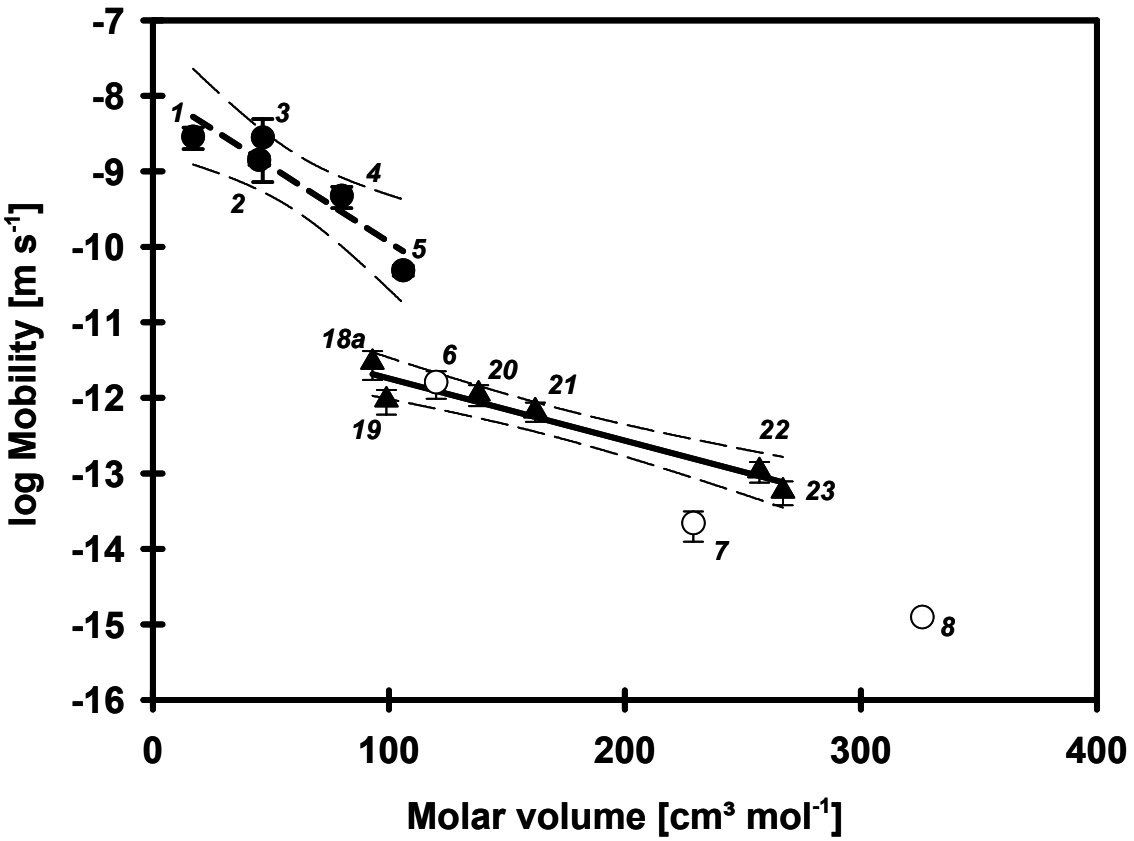

Figure 5.9 Mobility of the lipophilic (▲) and the uncharged hydrophilic model compounds < 110 cm^3 mol^{-1} (●) and > 110 cm^3 mol^{-1} (○) plotted versus the molar volume. Dashed regression line represents the hydrophilic pathway (R^2 = 0.87), solid regression line represents the lipophilic pathway (R^2 = 0.93). Dashed lines represent 95% confidence intervals of the regression lines.

---

1 water, 2 ethanol, 3 urea, 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 8 maltotriose, 18a benzoic acid (pH 2), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

Sorption of carbohydrates into reconstituted cuticular waxes of English ivy was not detectable. Additionally, this is not expectable since the octanol/water partition coefficient is a well suited tool to forecast solute sorption into lipophilic waxes which lack any polar compartments. Transport of hydrophilic molecules across the lipophilic compartments cutin and cuticular waxes is excluded for physicochemical reasons. Therefore, transport of all hydrophilic model compounds must occur *via* a hydrophilic pathway. The permeation of hydrated ionic calcium and glyphosate salts has been taken as evidence for the existence of aqueous pores in cuticular membranes (Schönherr, 2000; Schönherr, 2002). So far, a distinction between hydrophilic diffusion pathways and water-filled pores building up an aqueous continuum across the membrane is not possible.

There is some circumstantial evidence that microfibrils contribute to transport in cuticles where the polysaccharides extend right to the outer surface of the membrane (Kerstiens, 1994). A visual hint was found, since electron micrographs depicting strands of polysaccharide material stretching over the whole thickness of the cuticles. These strands were considered as evidence for the chemical nature of hydrophilic pathways (Jeffree, 1996).

Focusing on figure 5.9 in the range of approximately 100 $cm^3\ mol^{-1}$ molar volume supports the postulation of two distinguishable pathways across the cuticle of English ivy. Xylose (100 $cm^3\ mol^{-1}$), benzoic acid (93 $cm^3\ mol^{-1}$), and salicylic acid (99 $cm^3\ mol^{-1}$) are very similar in their molecular size but evidently penetrate *via* different cuticular pathways. In this part of the plot both pathways even overlap. To verify the penetration of xylose across the hydrophilic pathway and the restriction of benzoic acid and salicylic acid to the lipophilic pathway further evidences were collected. Initially, the fact of the much higher mobility of the polar carbohydrate in comparison to the two lipophilic organic acids is a very clear piece of evidence itself. The polar and the lipophilic pathway also differ in their sensitivity to lipophilic penetration accelerators. Surface active alcohol ethoxylates increase the mean free volume in the amorphous wax fraction which results in higher diffusion rates of lipophilic compounds (Burghardt *et al.*, 1998). Acceleration effects of triethylene glycol monododecylether ($C_{12}E_3$) on

lipophilic compounds were shown several times (Schönherr 1993b; Riederer *et al*., 1995; Burghardt *et al*., 1998). $C_{12}E_3$ accelerated the permeance of benzoic acid and salicylic acid significantly. In contrast to that, this alcohol ethoxylate had no significant acceleration effect on the permeation of xylose. This agrees with results reported previously (Schönherr, 2000; 2001; 2002). An increase of temperature decreases the size selectivity of cuticular waxes (Baur *et al*., 1997) which has a strong influence on the diffusion coefficient of lipophilic molecules (Riederer & Schreiber, 2001). Cuticular permeance of benzoic acid increased in the temperature range between 15 °C and 35 °C by a factor of 75. This results in an activation energy of 164 kJ mol^{-1}. Activation energies of lipophilic compounds penetrating across the cuticular wax are in the range of 75 – 189 kJ mol^{-1} (Baur *et al*., 1997). In contrast, no significant effect of temperature on the permeance of xylose was detected which indicates exclusion from diffusion *via* the lipophilic waxes. Comparable results have been reported for charged compounds (Schönherr, 2000; 2001; 2002). The mobilities of water (Becker *et al*., 1986) and 2,4-D (Riederer & Schönherr, 1985), measured independently with ivy cuticles, fit very well to these data. Finally, all these results clearly support the occurrence of a lipophilic and a hydrophilic pathway across the cuticular membrane of English ivy.

There is a long-standing debate, whether water penetrates across a polar pathway or a lipophilic pathway. Contradictory evidence was found supporting both possibilities. A correlation was found between the cuticular transpiration and the permeability of lipophilic molecules (Niederl *et al*., 1998; Schreiber, 2002). Furthermore, permeability of water is affected in the same way by accelerators (Riederer & Schönherr, 1990), wax extraction (Riederer & Schreiber, 2001) and increase in temperature (Schreiber, 2001), as it is known for the permeability of lipophilic molecules. All these findings support the assumption that water and lipophilic compounds are transported *via* the same path. However, it was also shown that cuticular transpiration was significantly affected by humidity (Schreiber, 2001). This was interpreted by the existence of polar domains inside the cuticle. The absence of a positive correlation between cuticular thickness and its resistance to the diffusion of water is well documented (Martin & Juniper, 1970; Becker *et al*., 1986). This present work is

the first study comparing membrane mobility of different compounds with a very broad spectrum of water solubilites in one single plot. From figure 5.9, there is no doubt that the bulk of water diffuses across the hydrophilic pathway. Nevertheless, because of the huge amount of contradictory literature it is assumed, that another fraction – but most likely a minor fraction - diffuses across the lipophilic pathway.

5.2.4.3 Membrane mobility of dissociable hydrophilic compounds

The cuticular mobility of another group of primary metabolites was examined additionally. Several zwitterionic amino acids of different molecular size were selected. A special feature of dissociable molecules are their ionisable functional groups which have a high affinity for water (Collins, 1997). This results in the creation of hydration shells, which cannot be shed. Therefore, charged molecules will be not soluble in the lipophilic cutin and wax domains of the cuticles. The tightly bound water increases their apparent molecular size (Kiriukhin & Collins, 2002). Hydration of a compound is described by the dimensionless hydration number ($mol_{H_2O}/mol_{compound}$). Hydration numbers of carbohydrates are relatively low: 3.5 for glucose (Beenackers *et al*., 1985) and 7 for sucrose (Engelsen & Perez, 1996). In contrast to that, hydration number of glycine at a concentration of 1 M at its isoelectric point is 17.7 (Tschapek & Wasowski, 1979). In this study, zwitterionic glycine was used in a concentration of 0.7 M. However, it is a matter of discussion to what extent hydration shells cause an increase of the absolute diffusing diameter. Therefore, in order to determine the molecular size, the molar volume was used for the dissociable molecules, too. An alternative measure for molecular proportions would be the molecular weight. Plotting the mobility versus the molecular weight results in a very similar shape of the graph (Fig. 5.11). Moreover, the overlap of the regression lines of the hydrophilic and the lipophilic pathway is clearer when the mobility is plotted versus the molecular weight.

From figure 5.10 it is obvious that the mobility of the amino acids is continuously lower than the mobility of uncharged hydrophilic compounds of similar molar

volume. As mentioned above, on the basis of the hydration numbers of glycine (17.7) and glucose (3.5) it is expected that the hydration shell of amino acids is larger than the hydration of uncharged hydrophilic compounds. Additionally, hydration shells of charged molecules can not easily be shed. From this assumption the calculated molar volume might be underestimated. This issue calls for closer examination.

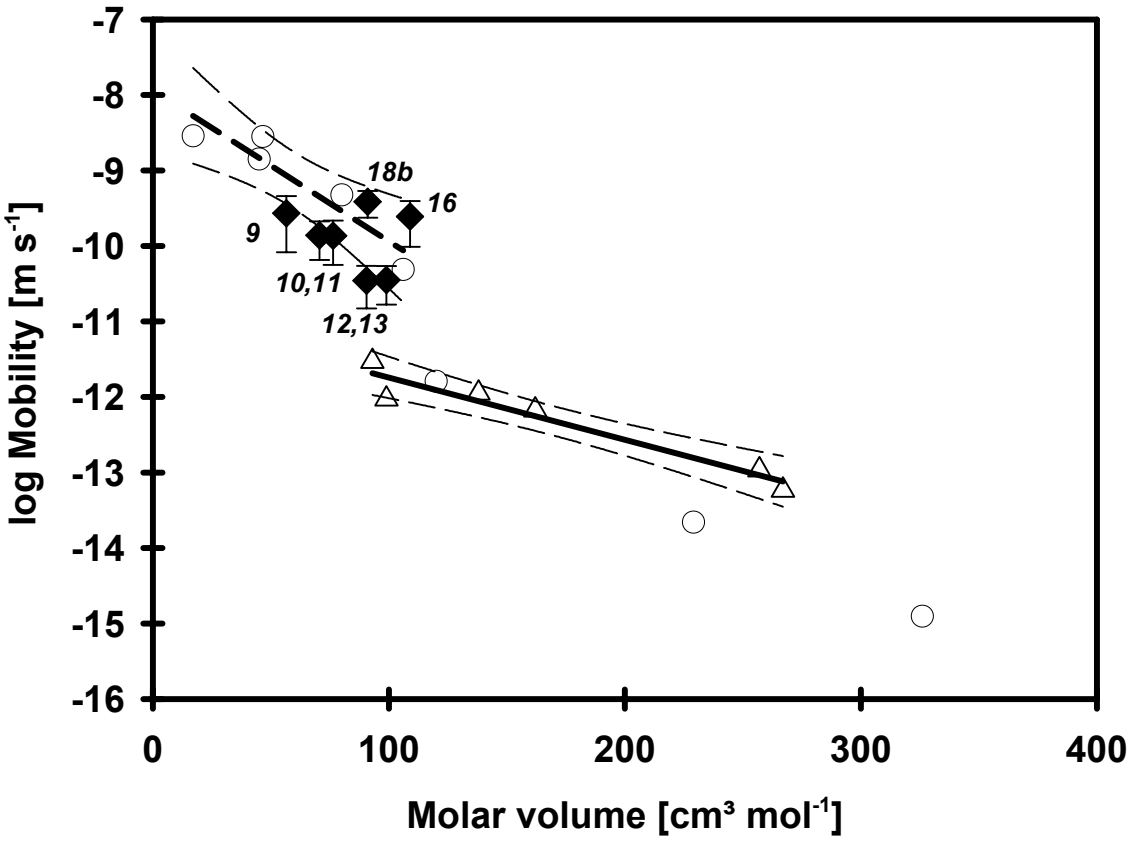

Figure 5.10 Mobility of the dissociable hydrophilic compounds (◆), the lipophilic (△) and the uncharged hydrophilic model compounds (O) plotted versus the molar volume. Dashed regression line represents the hydrophilic pathway (R^2 = 0.87), solid regression line represents the lipophilic pathway (R^2 = 0.93). Dashed lines represent 95% confidence intervals of the regression lines.

---

9 glycine, 10 alanine, 11, serine, 12 valine, 13, threonine, 16 glyphosate, 18b benzoic acid (pH 7)

However, a very sharp size limitation of the hydrophilic pathway was found for the penetration of dissociable molecules, which is contrary to the results found for the carbohydrates. Five amino acids in the range of 57 – 91 cm^3 mol^{-1} could penetrate the cuticular membrane of English ivy. Penetration of leucine (113 cm^3 mol^{-1}) and phenylalanine (131 cm^3 mol^{-1}) was not detectable. Transport of

paraquat dichloride (257 cm^3 mol^{-1}), which is a charged hydrophilic herbicide was also not detectable. These results allow the conclusion that the penetration of hydrophilic uncharged molecules is not size limited, but there is a strong size-selectivity. In contrast to that, dissociable hydrophilic compounds with a molar volume higher than 110 cm^3 mol^{-1} are excluded from any cuticular penetration.

In the following, two hypotheses are presented that might explain this phenomenon of ion exclusion beyond 110 cm^3 mol^{-1}. The first hypothesis states a significant underestimation of the molar volume of the dissociable hydrophilic compounds, caused by a large hydration shell which can not be shed. In order to verify this underestimation, it would be useful to try to determine apparent molecular sizes experimentally or to find hints in the literature. The second hypothesis is better documented. Polar transport paths within the lipophilic plant cuticle could be formed by carbohydrates extending from the outer epidermal cell walls into the cutin polymer, and moreover to the outer surface, as observed by Wattendorf and Holloway (1984). Cellulose and pectin are the widespread elements in plant cell walls (Cook & Stoddart, 1973). Consequently it is presumable that uncharged carbohydrates could be able to penetrate along a hydrophilic pathway made up of cellulose or pectin. Therefore, charged compounds could be restricted to hydrophilic pathways which are solely made of cellulose. This could support the observation of the higher mobility of carbohydrates in comparison to amino acids. However, one hypothesis does not exclude the other.

Dissociation of a molecule increases its water solubility dramatically. Since charged compounds carry hydration shells, they are excluded from the lipophilic pathway. For salicylic acid a decrease of the permeance was observed with increasing number of ionised molecules (Niederl *et al*., 1998). As seen in figure 5.9, benzoic acid (18a) has a molar volume which would allow the penetration across the hydrophilic pathway. Due to its lipophilicity, the penetration of uncharged benzoic acid is restricted to the lipophilic pathway. Therefore, the mobility of charged benzoic acid (pH 7) was examined, additionally. Surprisingly, it was observed for the first time, that dissociation of a lipophilic compound increases its permeance or rather its mobility. This very special case

is caused by the molar volume of benzoic acid which is sufficient for entrance of the hydrophilic pathway and due to the increased water solubility of the dissociated molecule. Since it is the same compound which is able to penetrate across both pathways, it is obvious that the hydrophilic pathway is faster than the lipophilic pathway. The ratio of the mobility of benzoic acid in a charged or rather in an uncharged state and the tortuosity of the lipophilic pathway are very similar. That is why it is expected, that the tortuosity of the hydrophilic pathway is close to one. The very high mobility of the dissociated benzoic acid is supported by the very high mobility of charged glyphosate. In terms of the high water solubility and its charged state, glyphosate is restricted to the hydrophilic pathway and excluded from the lipophilic pathway.

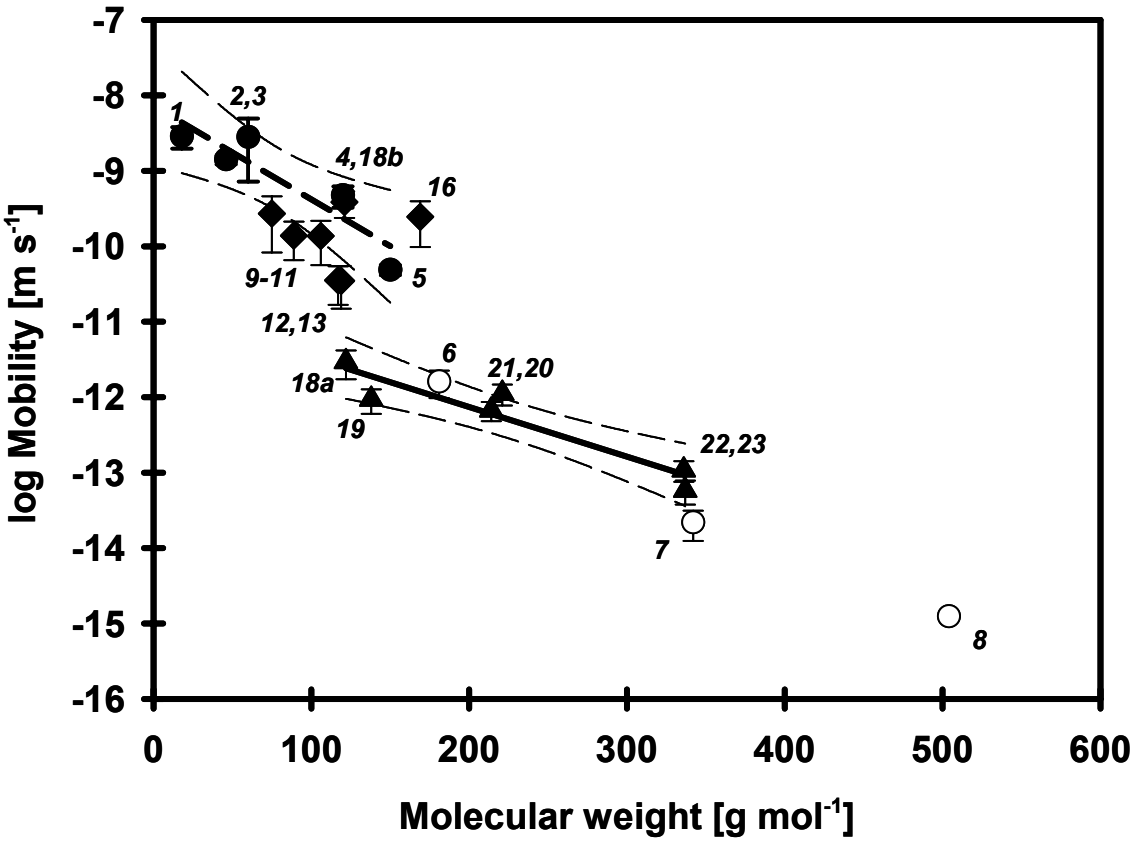

Figure 5.11 Mobility of the lipophilic (▲), the uncharged hydrophilic model compounds below 110 cm³ mol^{-1} (●) and above 110 cm³ mol^{-1} (○) and the dissociable hydrophilic compounds (◆) plotted versus the molecular weight. Dashed regression line represents the hydrophilic pathway (R^2 = 0.84), solid regression line represents the lipophilic pathway (R^2 = 0.88). Dashed lines represent 95% confidence intervals of the regression lines.

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1 water, 2 ethanol, 3 urea, 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 8 maltotriose, 9 glycine, 10 alanine, 11 serine, 12 valine, 13 threonine, 16 glyphosate, 18a benzoic acid (pH 2), 18b benzoic acid (pH 7), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

5.2.4.4 Matrix membrane mobility of the lipophilic and the uncharged hydrophilic compounds

Cuticular waxes form the transport-limiting barrier of cuticular membranes (Riederer & Schönherr, 1995). Extraction of cuticular waxes leads to increased permeances which result in a parallel translation of the regression line of the lipophilic pathway. Since removal of waxes does not affect the size selectivity but the y-intercept, it is argued that extraction reduces the path lengths (Baur *et al.*, 1999). This y-intercept reflects the mobility (m_0) of a compound with a molar volume of zero. The ratio of the y-intercept of the lipophilic compounds penetrating across the dewaxed membrane and the cuticular membrane is 318. This value is a measure for the tortuosity of the diffusion path of the lipophilic pathway. Baur *et al.* (1999) found a variation in the tortuosity between 28 and 759 for different species. Since the size selectivity of the lipophilic pathway differs only slightly between plant species (Buchholz *et al*., 1998), tortuosity of the diffusional path length can be considered as the main reason for a species-specific variability of cuticular permeances (Baur *et al*., 1999).

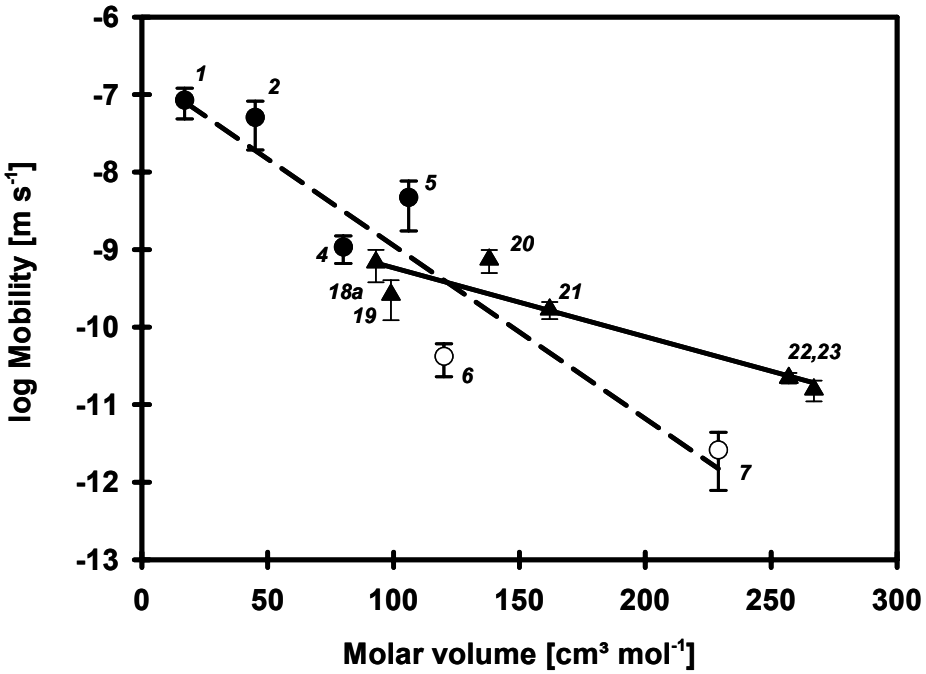

Figure 5.12 Mobility of the uncharged hydrophilic compounds < 110 cm³ mol^{-1} (●), the uncharged hydrophilic compounds > 110 cm³ mol^{-1} (○) and the lipophilic model compounds (▲) plotted versus the molar volume across dewaxed cuticular membranes (MX). Dashed regression line represents the hydrophilic pathway (R^2 = 0.87), solid regression line represents the lipophilic pathway (R^2 = 0.87).

1 water, 2 ethanol 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 18a benzoic acid (pH 2), 19 salicylic acid, 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

Wax extraction also causes a 16-fold increase of m_0 of the hydrophilic pathway. Since it is assumed that the hydrophilic pathway is made up of polysaccharide strains it is implausible that the tortuosity of the diffusion path is changed. In figure 5.17 it is shown that removal of cuticular waxes increases the absolute pore number (chapter 5.2.5). It is assumed that polysaccharide strains which do not reach the plant surface are excluded from any cuticular penetration. The uncovering of these strains increases the number of accessible pathways. Moreover, the increase in the mobility of the hydrophilic pathway corresponds to a tenfold increase in the absolute number of accessible polar pathways. Additionally, wax extraction does enlarge the mean pore size distribution which leads to a dramatic increase of accessible pores for molecules of higher volume (Figure 5.16). This is better reflected in the large extraction effects of hydrophilic compounds of higher molar volume compared to the effects for the hydrophilic compounds of lower molar volume (Figure 5.13).

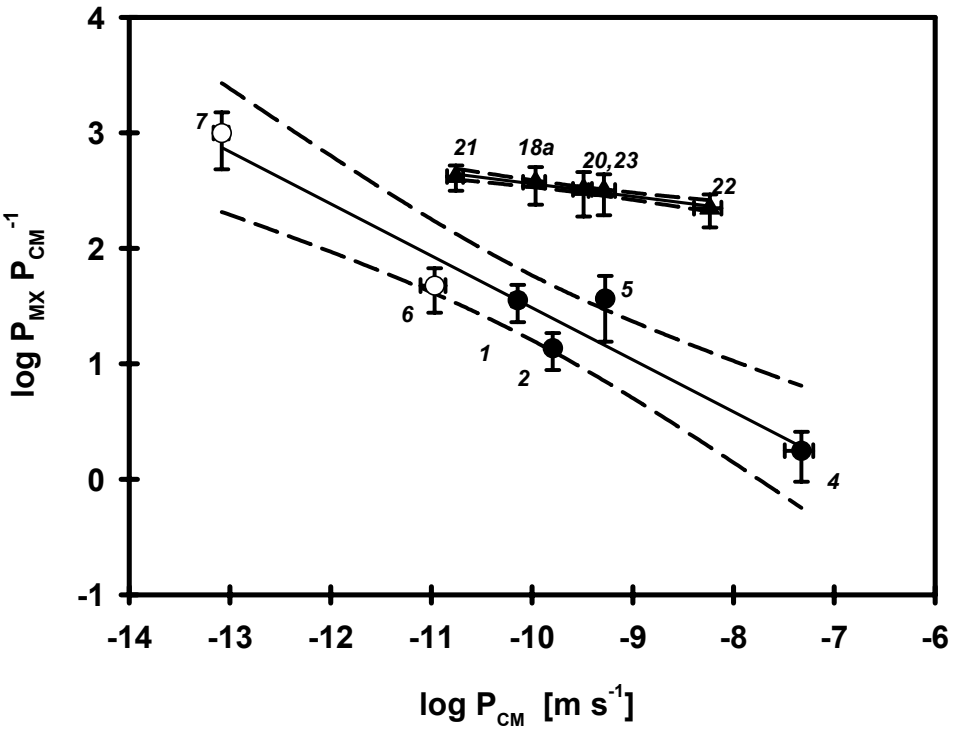

Figure 5.13 Effect of wax extraction (P_{MX} P_{CM}^{-1}) on the permeance as a function of the cuticular permeance P_{CM} for the uncharged hydrophilic compounds < 110 cm³ mol^{-1} (●), the uncharged hydrophilic compounds > 110 cm³ mol^{-1} (O) and the lipophilic model compounds (▲). Dashed lines represent 95% confidence intervals of the regression lines.

---

1 water, 2 ethanol 4 erythrose, 5 xylose, 6 glucose, 7 maltose, 18a benzoic acid (pH 2), 20 2,4-D, 21 metribuzin, 22 cloqintocet-mexyl, 23 bitertanol

5.2.5 Size selectivity of the hydrophilic pathway and pore model

Summarising all results previously shown, there is no doubt about the existence of a hydrophilic pathway traversing the plant cuticles of English ivy. The following chapter shall go a step ahead. The present data include information about proportions and properties of the hydrophilic pathway. In the literature it is frequently speculated, that the hydrophilic pathway is made up of polysaccharide strains traversing the lipophilic domains of the plant cuticle (see chapter 1.4). In contrast to transmembrane proteins which provide specific pathways for transmembrane movement of specific molecules and ions, this pathway in unspecific. Aquaporins are a well suited example for highly specific membrane transporters in plants. Aquaporins are integral membrane proteins which allow the transport of polar water molecules across the lipophilic lipid membrane (Chrispeels & Maurel, 1994; Henzler & Steudle, 1995). This transport-system is a specific transport, since up to 4×10^9 water molecules can permeate the bilayer *via* one channel per second (Strasburger, 1998). Contrary to this water transport, the flow rate of water across the hydrophilic pathway is about four orders of magnitude lower than the active transport across aquaporins. It is assumed, that the occurrence of these transmembrane proteins is essential to guarantee cellular water transport. It would be a matter of speculation, whether hydrophilic pathways traversing the plant cuticle are essential or not. It is assumed that the quality of plant cuticles is high enough to meet their demands. A further improved cuticle could be regarded as a waste of resources. However, it was shown by Singh *et al*. (2004), that epiphytic *Pseudomonas* species from *Malus domestica* have an antagonistic effect on the apple scab pathogen (*Venturia inaequalis*). For microbial colonisation of the leaf surface, carbon and nitrogen sources or essential inorganic components are required, which are leached out of the leaf interior.

It is not expectable that polysaccharide strains create a tube-shaped pore. It is more probable, that interfibrilar vacancies of molecular dimension create the hydrophilic pathway, since single cellulose strains are organised in a three-dimensional structure which is caused by creation of H-bonds. The fibre repeat distance of two cellulose molecules is approximately 0.8 nm (Fengel, 1985).

The pronounced size selectivity of diffusion of the hydrophilic compounds indicates that diffusion is hindered by narrow pores. In order to compare the present data with data for diffusion in water the diffusion coefficients of all model compounds in water were calculated according to equation 5.7. This equation was obtained by plotting several diffusion coefficients of different compounds in water (D_W) versus the molar volume. All data were taken from literature (Beck & Schultz, 1972; Tanaka, 1976).

$$D_W = 1.74 \times 10^{-9} \exp(-0.0046 \times MV) \qquad \text{(Eq. 5.7)}$$

D_W is expressed in $m^2\ s^{-1}$. All diffusion coefficients in water calculated were compared with the experimental data found in this study. Cuticular diffusion coefficients (D_{CM}) [$m^2\ s^{-1}$] in the membrane were obtained according to:

$$D_{CM} = \frac{P_{CM} \times l}{K_{C/W}} \qquad \text{(Eq. 5.8)}$$

$K_{C/W}$ is the solubility of the respective compound in the cuticular membrane and P_{CM} is the permeance in the cuticle. Thickness of the membrane (l) is 4.3 µm (*H. helix*) (Becker *et al*., 1986) and 0.7 µm (*V. vinifera*).

From figure 5.14 it is obvious that diffusion of hydrophilic compounds in the cuticular membrane does not agree with Stokesian diffusion since $D \times MV^{1/3}$ = constant is fulfilled only for diffusion in water but not for diffusion across the cuticle.

Size selectivity of the polar pathway is 0.019 $cm^3\ mol^{-1}$ which is one order of magnitude above that of diffusion in water according to Stokes-Einstein. D_0 is the y-intercept which corresponds with the diffusion coefficient of a fictive molecule having a molar volume of zero. Experimental D_0 is several orders of magnitudes higher than the one found for the model compounds.

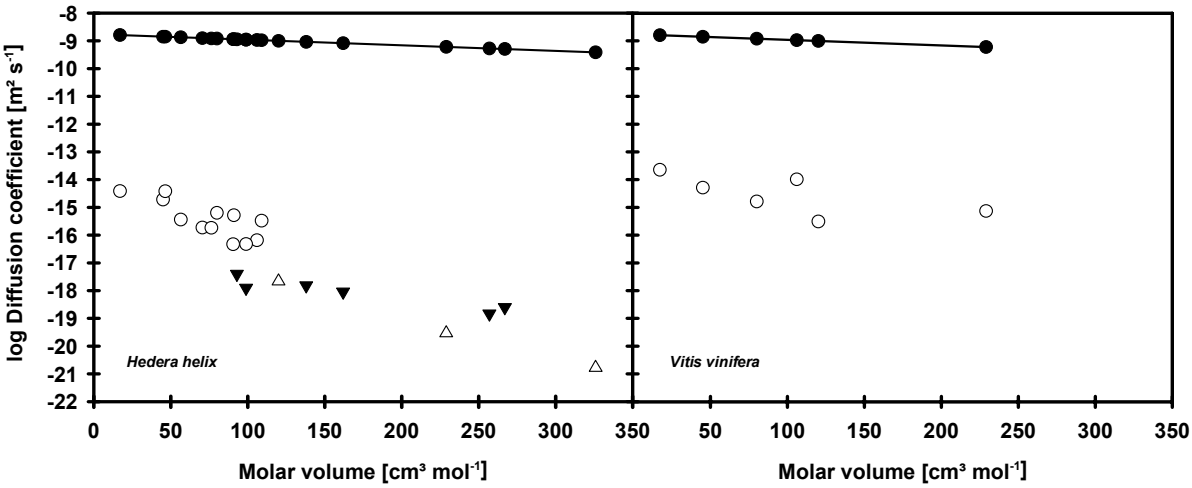

Figure 5.14 Diffusion coefficients of the model compounds in water (●) and in the cuticular membranes of *H. helix* (left) and *V. vinifera* (right) plotted versus the molar volume. The model compounds are the hydrophilic molecules with a molar volume below 110 cm^3 mol^{-1} (○), higher than 110 cm^3 mol^{-1} (△) and also the lipophilic molecules (▲). Diffusion coefficients in water fit to Stokesian diffusion, diffusion coefficients in the membranes do not fulfil Stokesian diffusion.

For diffusion in aqueous pores of molecular dimensions steric restriction at the pore entrance and friction at the pore wall is taken into account by the diffusional hindrance factor H(λ). For small molecules the hindrance factor is given by (Mitragotri, 2003):

$$H(\lambda) = (1-\lambda)^4 \qquad \text{(Eq. 5.9)}$$

λ is the ratio of the radius of the diffusing molecule (r_m), which is available from the molar volume assuming a spherical shape, and the effective pore radius of the membrane (r_p):

$$\lambda = \frac{r_m}{r_p} \qquad \text{(Eq. 5.10)}$$

Given equation 5.10, it follows that the hindrance factor is a function of the ratio of the radius of the diffusing molecule and the pore radius. For reasons mentioned above diffusion coefficient in the cuticular pore (D_{CM}) and the diffusion coefficient in an aqueous solution (D_W) are related as follows:

$D_{CM} = H(\lambda) \times D_W$ (Eq. 5.11)

The determination of the pore radius (r_p) is possible, since all other parameters which are essential to solve equation 5.11 are known, e. g. D_{CM}, D_W and r_m. This calculation was conducted for all hydrophilic compounds which are obviously located on the polar pathway (Fig. 5.11) (MV < 110 cm³ mol^{-1}). Therefore, it was possible to calculate a pore radius and the standard deviation of the radius. This calculation was also done for the data obtained with dewaxed membranes of English ivy and also for the data obtained with grapevine cuticles. For *H. helix* cuticular membranes a mean pore diameter of 0.31 ± 0.05 nm was determined. For dewaxed membranes of ivy a mean pore diameter of 0.35 ± 0.09 nm was found. This corresponds to a molar volume of 75 or rather 110 cm³ mol^{-1}. The mean pore radius of grapevine leaf cuticle is 0.34 ± 0.09 nm which corresponds to 100 cm³ mol^{-1}. This calculated pore radius fits very well to the fibre repeat distance of cellulose (~ 0.8 nm) molecules as discussed before.

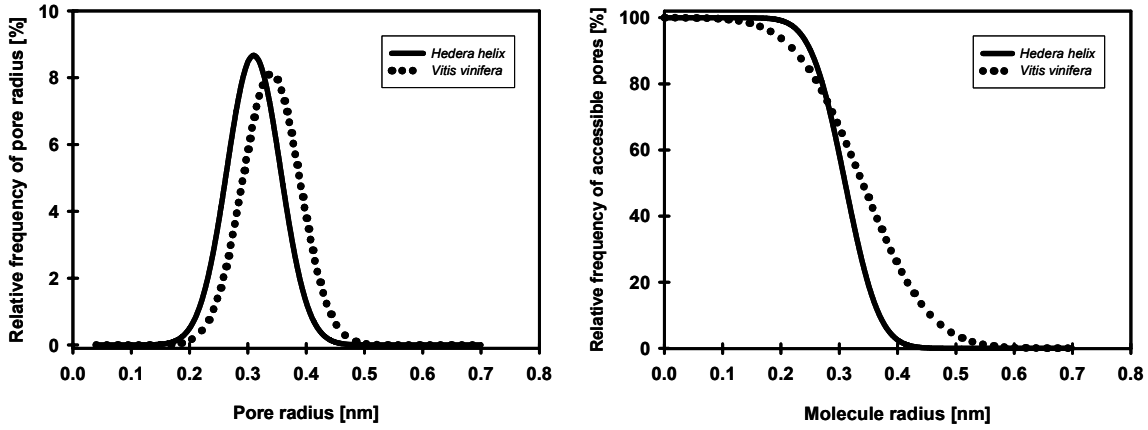

Figure 5.15 Relative frequency of the pore radius of cuticular membranes of *H. helix* and *V. vinifera* assuming a normal distribution of the pore size (left figure). Cumulative frequency of the accessible pores as a function of the molecular radius of the solute (right figure).

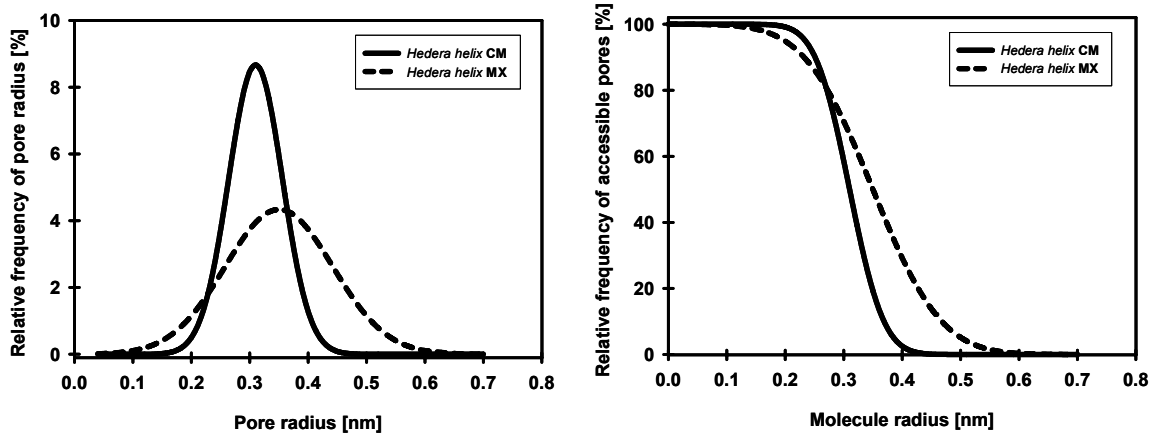

Figure 5.16 Frequency of pore radius of cuticular membranes of *H. helix* cuticular membranes and *H. helix* matrix membranes assuming a normal distribution of the pore sizes (left figure). Cumulative frequency of accessible pores as a function of the molecule radius (right figure).

As a result of the distinct size selectivity of the hydrophilic pathway it is assumed that the pore radius varies dramatically. As an approximation to the real pore size, a normal distribution was assumed. Given the mean pore radius and the standard deviation it was possible to determine the pore size distribution. It is shown in figure 5.15, that the mean pore width of grapevine is moved rightwards. The consequences for molecules of higher radius are enormous, since accessibility of pores in the range of 0.3 to 0.5 nm is increased dramatically. This might be a good explanation for the high permeability of grapevine cuticles for hydrophilic molecules of higher molar volume like paraquat dichloride in contrast to English ivy cuticles.

Dewaxed membranes of English ivy show similar properties as grapevine cuticles. The increased standard deviation of the mean pore radius of matrix membranes results in a broader spectrum of the pore size distribution. This might be the reason for an easy penetration of molecules of higher molar volume across dewaxed cuticles. From these results it is very likely that pore size distribution limits the permeation of large hydrophilic compounds. The probability (p) of a diffusing molecule with the radius r_m to find a passable pathway is equal to the cumulative frequency (f) of pores with the radius r_p of equal or larger size.

$$p(r_m) = \int_{r_m}^{\infty} f(r_p)\, dr_p \qquad \text{(Eq. 5.12)}$$

For a molecule with a radius r_m tending to zero, there is no limitation by pore size distribution and the cumulative frequency of passable pores tends to $f(r_p)$ = 100%. In contrast, for r_m -> ∞, suitable pores are not available and the cumulative frequency of passable pores reaches $f(r_p)$ = 0%. This might be an explanation for the low mobility of the carbohydrates with a molar volume above 110 cm³ mol^{-1} (Fig. 5.9). For hydrophilic compounds of large molar volumes the probability to find a pore of sufficient size is very low. That is the reason why there is a threshold in the range of 110 cm³ mol^{-1}. Below this threshold the hydrophilic pathway is faster than the lipophilic pathway. Above this threshold the lipophilic pathway is faster than the hydrophilic pathway. The most accurate description of the cuticular permeance of hydrophilic compounds inside a narrow pore can be described as follows (Mitragotri, 2003). This equation was modified by addition of the cuticle/water partition coefficient.

$$P_{CM} = K_{C/W} \times \frac{\varepsilon}{\tau \times l} \times D_W \times \int_{r_m}^{\infty} f(r_p) \times H(\lambda) dr_p \qquad \text{(Eq. 5.13)}$$

This relationship is similar to equation 5.3, with the exception that the diffusion coefficient in hydrophilic cuticular pores is expressed as the product of the porosity (ε: ratio of pore area and total area), the diffusion coefficient in an aqueous solution (D_W), the frequency of accessible pores (f) and the corresponding hindrance factor ($H(\lambda)$). Assuming that the polar pathway is a very straight way traversing the cuticle (tortuosity factor τ = 1), it is possible to calculate the porosity of the membrane since all further parameters are known. Given the porosity it is also possible to calculate the number of pores per area. The mean area of one pore can be calculated from the mean pore radius with the assumption that the cross-section of a pore is circular. The porosity of cuticular membranes of English ivy is 0.00034 and 0.0055 of dewaxed membranes. This corresponds to a mean pore number of 1.1 x 10^9 (CM) or

rather 1.5 x 10^{10} (MX) per cm². Porosity of grapevine cuticles is 0.0012 which corresponds to 3.3 x 10^{9} pores per cm².

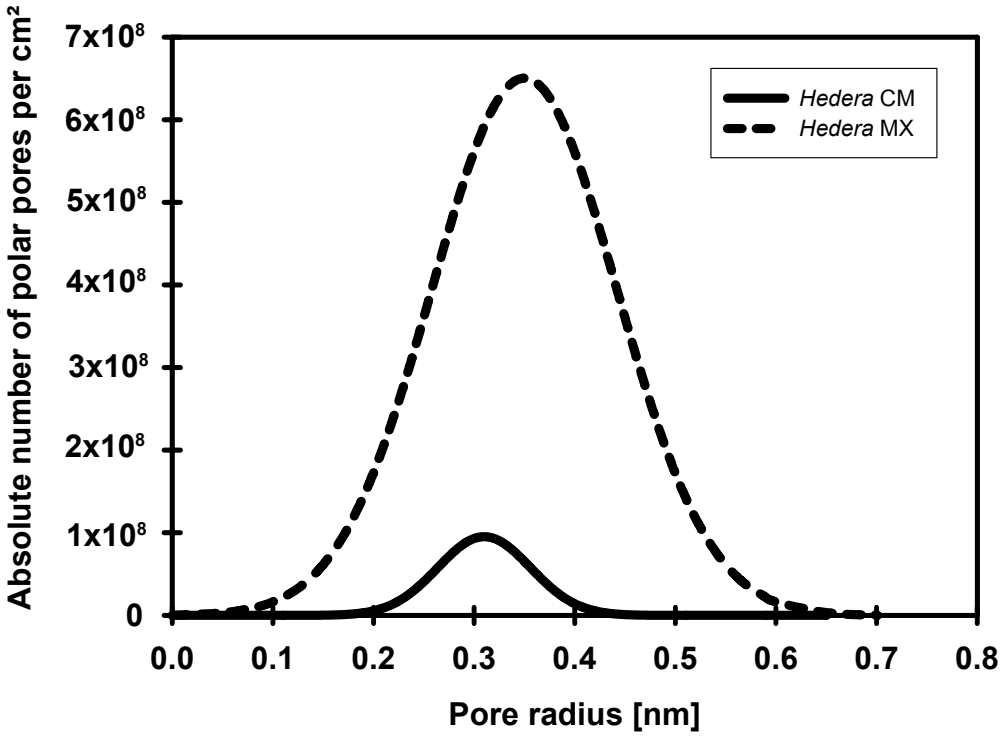

Figure 5.17 Absolute number of polar pores as a function of the pore radius. The absolute number of pores at matrix membranes of ivy is tenfold higher than that of cuticular membranes. So wax extraction causes an uncovering of embedded pores.

These numbers fit very well to the number of pores found with dewaxed membranes of *Citrus aurantium* (Schönherr, 1976b). From the absolute number of pores and the pore size distribution it is possible to determine the absolute frequency of pores as a function of the pore radius. Many polar pores traverse the plant cuticles of English ivy (1.1 x 10^{9} per cm²). Wax extraction leads to a tenfold increase in the pore number. It is suggested, that the increase in pore number after wax extraction can be explained with an uncovering of embedded pores which do not reach the outer surface of the cuticle. Such embedded pores are not accessible for transport of hydrophilic compounds across the cuticle. After wax extraction they are able to transport molecules resulting in an increased permeance, caused by the increased number of passable pathways. A correlation was found between the mobility of the hydrophilic molecules and the relative frequency of accessible pores. The smaller the radius of a diffusing

molecule, the higher the relative frequency of passable pathways and the higher the respective mobility.

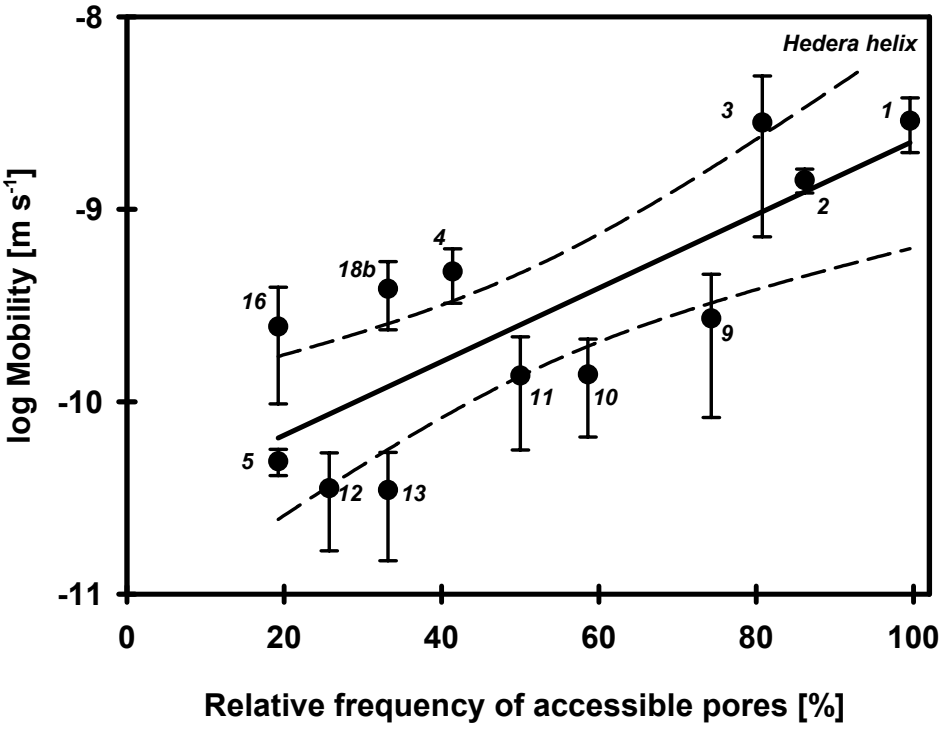

Figure 5.18 Relative frequency of accessible pores plotted versus the mobility of hydrophilic compounds with a molar volume lower than 110 cm³ mol^{-1}. The higher the frequency of passable pores the higher the mobility. Dashed lines represent 95% confidence intervals of the regression lines.

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1 water, 2 ethanol, 3 urea, 4 erythrose, 5 xylose, 9 glycine, 10 alanine, 11 serine, 12 valine, 13 threonine, 16 glyphosate, 18b benzoic acid (pH 7)

Although the number of pores per cm² seems to be very high, the porosity of the cuticle is very low. However, this hydrophilic routes across the lipophilic cuticle result in a higher mobility of the hydrophilic compounds of lower molar volume than 110 cm³ mol^{-1} than the mobility of lipophilic compounds of similar molar dimensions (Fig. 5.12).

6. SUMMARY

The plant cuticle as an interface between the plant interior and the adjoining atmosphere plays an important role in any interaction between the plant and its environment. Transport processes across the cuticles were the object of countless research since many decades. However, bulk of the work done was focused on transport of lipophilic molecules. It is highly plausible to examine the penetration of lipophilic compounds, since the cuticle is dominated by lipophilic compartments itself, and the most crop protection agents have lipophilic character. As a result of this research, cuticular transport of lipophilic compounds is relatively well understood. Since several years, examinations were expanded on transport of hydrophilic molecules. In the present study, a direct comparison was made between transport properties of lipophilic and hydrophilic compounds, which allows an objective assessment of the mechanism governing their penetration. The results of this present study debunked the existence of two different pathways across isolated cuticles of *Hedera helix* (English ivy), a lipophilic and a hydrophilic pathway. This finding was supported by examinations regarding to accelerator and temperature effects on the mobility of both pathways, because the hydrophilic path is insensitive to them - in contrary to the lipophilic one. The lipophilic pathway is rigorously restricted to lipophilic molecules and the hydrophilic pathway is only accessible for hydrophilic molecules. Uncharged hydrophilic compounds can cross the cuticle even the molecules are of relatively large dimensions. In contrast to that, dissociable compounds with a molar volume higher than 110 $cm^3\ mol^{-1}$ are excluded from cuticular penetration. Differences in the mobility of uncharged and dissociable molecules might be a hint towards the chemical nature of the polar pathways. It is assumed, that both, cellulose and pectin fibrils, traverse the cuticle which are originated from the epidermal cell wall. While uncharged carbohydrates might be able to penetrate across a pathway made up of cellulose and pectin, dissociated amino acids might be restricted to the cellulose path. This could be a plausible explanation for the higher mobility and the higher cuticle/water partition coefficients of the carbohydrates compared with the amino acids.

A hydrophilic pathway was found with isolated grapevine cuticles, too. The apparent size selectivity of the hydrophilic pathway implies transport *via* narrow pores. From the present data, a mean pore radius of 0.31 nm (*H. helix*) or rather 0.34 nm (*V. vinifera*) was calculated. The absolute number of pores per cm² is 1.1×10^9 for *H. helix* and 3.3×10^9 for *V. vinifera* cuticles. This finding and the enlarged pore size distribution of grapevine cuticles might be an explanation for the transport of uncharged and dissociable hydrophilic compounds of higher molar volume like paraquat dichloride - in contrast to ivy membranes

Wax extraction of ivy membranes uncovers additional pores, which explains the increased mobilities of the hydrophilic compounds across dewaxed membranes. From these extensive measurements it is very conspicuous, that the bulk of cuticular water transpiration occurs *via* the polar pathway. Since the work was focused on cuticular penetration of primary metabolites like amino acids and carbohydrates, a mechanistic explanation of leaching processes is obtained, simultaneously.

In cuticular research, an inconsistent terminology regarding the transport path of the hydrophilic compounds was used. The term ‘hydrophilic pathway’ is definitely correct, since it makes no statement with regard to the shape of this path. In contrast to that, the terms ‘polar pore’ or ‘aqueous pore’ could imply that there is a tube or rather a water-filled tube traversing the cuticle. However - at this point of time – the imagination about the shape of this path is a pathway across interfibrilar gaps within polysaccharide strains. The proposed diameter of these interfibrilar gaps fits very well to the diameter determined in this study. Therefore, the imagination of a pore is not unfounded, but it is a very narrow pore, definitely. Additionally, this pathway is a very straight pathway which corresponds to this simplified imagination.

An expanded study was done with paraquat dichloride, which was applied as aqueous droplets on grapevine cuticles. It is assumed that these model membranes reflect transport properties which are very close to that of relevant crops and weeds. The predominating parameter for paraquat penetration is the moisture, either originated from a relative humidity of at least 75% or provided

by added chemicals. There is a tendency for good suitability of hygroscopic additives. Increased paraquat penetration was also obtained by raised concentrations and removal of the cuticular waxes.

All examinations done, shall improve the knowledge about cuticular transport of hydrophilic molecules. Additional work with further active ingredients and further additives would result in a still better understanding. Besides that, variation of the charge of amino acids could be very informative, since these molecules provide the possibility of different charge states. Because of the well-documented cuticular transport of primary metabolites it would be of interest, whether the size selectivity of the hydrophilic pathway is reflected in a size-distribution of leachates found on plant surfaces, or not. The increased membrane mobility of charged benzoic acid in comparison with uncharged, lipophilic benzoic acid calls for further examination, because this is contradictory to the literature, so far. Finally, in spite of many hints in terms of the chemical nature of the hydrophilic pathway a thorough chemical analysis would be desirable.

6. ZUSAMMENFASSUNG

Kutikulärer Transport von hydrophilen Primärmetaboliten und Aktivsubstanzen

Die pflanzliche Kutikula als Grenzfläche zwischen der Pflanze und ihrer Umgebung nimmt eine wichtige Aufgabe hinsichtlich der Interaktion Pflanze/Umwelt ein. Kutikuläre Transportprozesse sind bereits seit Jahrzehnten Gegenstand zahlreicher Forschungen. Das Hauptaugenmerk war dabei jedoch größtenteils auf lipophile Verbindungen gerichtet, was auch plausibel ist, da die Kutikula an sich eine lipophile Membran darstellt und nicht zuletzt auch viele Aktivsubstanzen aus dem Bereich des Pflanzenschutzes lipophil sind. Daraus resultiert ein sehr gutes Verständnis hinsichtlich der Transportmechanismen lipophiler Moleküle. In den letzten Jahren wurde die Forschung jedoch auf hydrophile Modellverbindungen ausgeweitet. Die vorliegende Studie beruht auf einem direkten Vergleich der Transporteigenschaften von lipophilen und hydrophilen Verbindungen, der Unterschiede in den jeweiligen Mechanismen aufzeigen soll. Die Ergebnisse dieser Messungen erbrachten den Nachweis für die Existenz zweier klar differenzierbarer Transportwege in isolierten Kutikularmembranen von Efeu (*H. helix*); einen lipophilen und einen hydrophilen Pfad. Diese Unterscheidung wurde durch weitere Experimente untermauert, da der lipophile Weg - im Gegensatz zum hydrophilen Weg - tensid- und temperatursensitiv ist. Lipophile Moleküle können ausschließlich über den lipophilen Weg permeieren, während der hydrophile Weg ausschließlich für hydrophile Verbindungen zur Verfügung steht. Eine Besonderheit hinsichtlich der Ladung hydrophiler Verbindungen wurde gefunden: Ungeladene hydrophile Moleküle können auch bei größerem Molvolumen noch durch die Membran transportiert werden. Im Gegensatz dazu wurde für hydrophile dissoziierbare Moleküle mit einem Molvolumen größer als 110 cm^3 mol^{-1} ein Transportausschluss festgestellt. Unterschiede in der Mobilität geladener und dissoziierbarer hydrophiler Verbindungen könnten ein Hinweis in Richtung der Chemie des hydrophilen Weges sein. Es wird angenommen, dass sowohl Cellulose- als auch Pektinfibrillen die Kutikula durchziehen. Ursprung dieser Fibrillen ist die epidermale Zellwand. Eine höhere Mobilität der Kohlenhydrate

gegenüber den Aminosäuren könnte dadurch erklärt werden. Ungeladene Kohlenhydrate können demnach sowohl über Cellulose- als auch Pektinfibrillen transportiert werden, während dissoziierte Aminosäuren möglicherweise nur über Cellulosefibrillen permeieren können. Diese Hypothese würde auch die im Vergleich zu den Aminosäuren höheren Verteilungskoeffizienten der Kohlenhydrate erklären. Auch für Blattkutikeln von Wein (*V. vinifera* cv. Nelly) wurde ein hydrophiler Weg gefunden.

Die ausgeprägte Größenselektivität des hydrophilen Weges impliziert einen Transport durch enge Poren. Aus den gemessenen Daten ließ sich ein mittlerer Porenradius von 0.31 nm für Efeu und 0.34 nm für Wein bestimmen. Die absolute Porenanzahl pro Quadratzentimeter beträgt bei Efeu 1.1 x 10^9 und bei Wein 3.3 x 10^9. Diese Ergebnisse und die breitere Verteilung des mittleren Porenradius' bei Wein können den Transport von Paraquat durch Weinmembranen und den Transportausschluss bei Efeumembranen erklären. Eine Extraktion der kutikulären Wachse bei Efeukutikeln legt weitere Poren frei, die ansonsten blind in der Wachsschicht enden und für Transportprozesse daher nicht zur Verfügung stehen. Aus der Auftragung aller Daten ergibt sich, daß Wasser hauptsächlich über den polaren Weg transportiert wird. Die vorliegenden Daten liefern gleichzeitig eine mechanistische Erklärung für das Auswaschen von Primärmetaboliten aus Blättern, was bereits vor vielen Jahrzehnten beobachtet wurde.

Im Verlauf der Arbeit wurden verschiedene Begriffe für den Transportweg der hydrophilen Verbindungen verwendet. Der Ausdruck ‚hydrophiler Weg' (hydrophilic pathway) ist günstig, da er keinerlei Aussagen über die Beschaffenheit dieses Weges macht. Im Gegensatz dazu beinhalten die in der Literatur präferierten Begriffe ‚polare Pore' (polar pore) oder ‚wässrige Pore' (aqueous pore) scheinbar Informationen hinsichtlich der Gestalt dieser Wege. Aus den bisherigen Erkenntnissen lässt sich dennoch ein Vorschlag für die Gestalt des hydrophilen Weges machen. Die Tatsache dass zu Fibrillen aggregierte einzelne Cellulosestränge einen Abstand von ca. 0.8 nm aufweisen, was dem postulierten Porenradius sehr nahe kommt, könnten interfibrilläre

Zwischenräume den hydrophilen Weg darstellen. Der Begriff einer ‚Pore' ist daher möglicherweise gar nicht abwegig. Jedenfalls ist der hydrophile Weg, im Gegensatz zum lipophilen Weg, ein ungewundener Weg, was der Vorstellung einer ‚Pore' entgegen kommt.

Eine ausführliche Studie zum kutikulären Transport des geladenen Herbizides Paraquat-Dichlorid wurde angefertigt. Diese sehr stark wasserlösliche Verbindung wurde in wässrigen Tropfen auf isolierte Weinkutikeln appliziert. Wein als Modellpflanze hat die Vorteile, dass diese Spezies einerseits bedeutsam für den Pflanzenschutz ist und außerdem Transporteigenschaften der Kutikula aufweist, die relevanten Kulturpflanzen oder Kräutern sehr nahe kommen dürfte. Der für den Paraquat-Transport wichtigste Parameter ist die Verfügbarkeit von Wasser. Ausreichend Feuchtigkeit wird einerseits durch eine relative Luftfeuchte ab 75% geliefert, oder, wenn dies nicht erreicht wird, durch zugesetzte Hilfsmittel. Hygroskopische Additive scheinen hierfür besonders günstige Eigenschaften zu haben. Eine gesteigerte Transportrate wurde auch durch Extraktion der Wachse oder eine Erhöhung der Aufwandsmenge erreicht.

Die vorgestellten Ergebnisse zu kutikulären Transporteigenschaften hydrophiler Verbindungen sollen helfen, die zugrunde liegenden Mechanismen besser zu verstehen. Messungen mit weiteren Aktivsubstanzen sind erforderlich, um die Transportmechanismen von Paraquat besser einordnen zu können. Eine Veränderung der Ladung bei Aminosäuren könnte ebenfalls sehr informativ sein, da die Bedeutung der Ladung am selben Molekül studiert werden kann. Es wäre auch von Interesse, die Größenverteilung ausgewaschener Primärmetabolite auf der Blattoberfläche zu untersuchen, da der hydrophile Weg selbst eine ausgeprägte Größenselektivität aufweist. Am Beispiel der Benzoesäure wurde erstmals gezeigt, dass eine geladene Verbindung eine höhere Mobilität aufweist als eine ungeladene Verbindung. Dieses Phänomen war bislang in der Literatur nicht beschrieben und bietet sich daher als Gegenstand weiterer Forschungen an. Schließlich wäre eine genauere Untersuchung der Gestalt und der Chemie des hydrophilen Weges wünschenswert.

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Curriculum vitae

Christian Popp

Hauptstraße 35

97999 Igersheim/Harthausen

Personal information

Date of birth: 08 August 1974

Place of birth: Bad Mergentheim

Education

2002 – 2005	PhD thesis in the group of Prof. Markus Riederer at the University of Würzburg. Thesis*:* 'Cuticular transport of hydrophilic molecules with special focus on primary metabolites and active ingredients'
1996 – 2002	Study of Biology at the Universities of Heidelberg and Würzburg, Diploma in Biology
1995 – 1996	Alternative civilian service in the Hospital 'St. Rochusstift', Bad Mergentheim
1992 – 1995	Secondary school, Wirtschaftsgymnasium Bad Mergentheim, A-levels ('Abitur')
1990 – 1992	Secondary school, Kaufmännische Wirtschaftschule Bad Mergentheim, O-level ('Mittlere Reife')
1985 – 1990	Secondary school, Hauptschule Igersheim, CSE ('Hauptschulabschluss')
1981 – 1985	Primary school, Grundschule Igersheim

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Erklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die Dissertation wurde in gleicher oder ähnlicher Form noch zu keinem anderen Prüfungsverfahren vorgelegt.

Ich erkläre hiermit, dass ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, der 28. Juli 2005