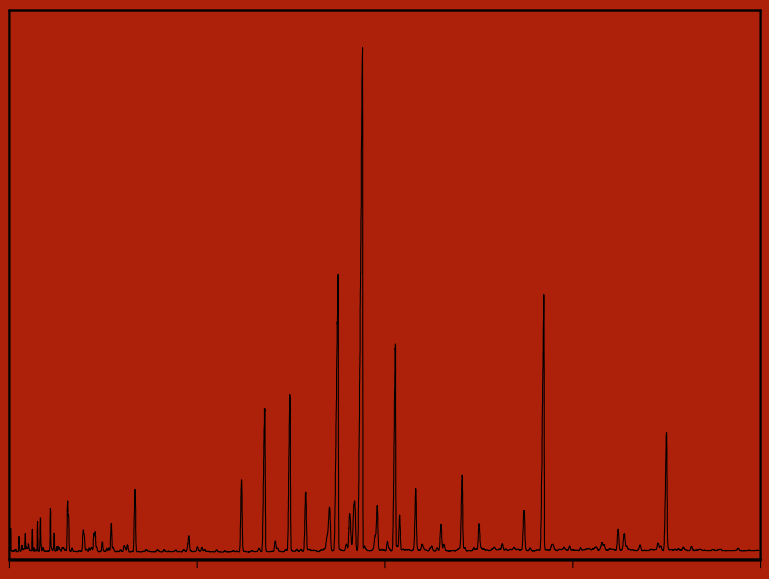
Katja Bringe

Surface characteristics of *Malus domestica* Borkh. leaves and fruits as influenced by ontogenesis and environmental factors



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Meinen Eltern

Meiner Schwester

Es gibt eine Weisheit des Kopfes
Und....eine Weisheit des Herzens.
C. Dickens

Surface characteristics of *Malus domestica* Borkh. leaves and fruits as influenced by ontogenesis and environmental factors

In this study surface of apple leaves and fruits (*M. domestica* Borkh., `Golden Delicious`, `Topaz`) were characterised chemically and physically, in this context the influence of ontogenesis as well as environmental factors were considered. UV-B radiation and water stress served as model factors, allegorising environmental factors. UV-B radiation was provided by UV-B lamps with an intensity of 0.22 kW m^{-2} during periods of 0, 90 and 150 min. Water stress was induced by withholding water for two weeks. Chemical composition of surface wax was examined by means of GC-MS, while physical characteristics were described by measurement of contact angle and SEM studies. Retention and rainfastness of the applied fungicide mancozeb after the exposure to artificial UV-B radiation, and the penetration of $CaCl_2$ through the isolated fruit cuticle were aspects investigated as well.

1 During the ontogenetic development of apple seedling leaves the surface wax mass tended to decrease, and their chemical composition changed, whereas for each developmental stage a specific pattern of compounds was studied. The detected chemical groups were displayed by fatty acids, alcohols, alkanes, triterpenes and esters. From the physical point of view the adaxial apple leaf surface was characterised by an amorphous film of epicuticular waxes; surface exhibited a hydrophobic character, the flattening process during the course of studied time may be attributed to cell area expansion. For the first time extracellular α-tocopherol was detected by means of HPLC in the epicuticular wax.

2 After influence of water deficit and UV-B irradiation, the chemical surface wax composition of adaxial apple leaves altered, as well as a changed morphology could be documented. Samples were studied 0, 24 and 48 h after irradiation with UV-B. The water deficit caused an increase in surface wax load of 30 %, being not exposed to UV-B. A statistical interaction could be detected only for the 24 h sampling time, therefor as well as UV-B as water deficit did excert an influence on cuticular wax mass. A slight rise in contact angle 48 h after the irradiation could be observed, signifying a decreased wettability compared to 0 and 24 h, sampling time influenced contact angle to a greater extent than tested environmental factors.

3 Retention and rainfastness (rain intensity 5 mm h^{-1}, rain amount 5 mm) of the applied fungicide mancozeb by means of adaxial apple surface were documented. UV-B radiation was applied. Within the first 24 h after radiation the retention increased significantly, not changing within the following 24 h, whereupon the rainfastness was not influenced significantly. So far no studies were accomplished, documenting retention of mancozeb after exposure to enhanced UV-B radiation. Further the chemical composition changed as well. A slight increase in surface wax mass could be demonstrated. Changed wettability after irradiation could not be shown, in addition a flattenting process of the cuticle after treatment with UV-B is possible, accompanied by feasible change in leaf epidermis thickness.

4 The apple fruits (`Topaz`), which were grown under environmental conditions, whereas half of the samples were adapted to usual radiation and half were wrapped in UV-B impermeable film from june until harvest. After harvest, fruits (adapted/not adapted) were exposed to UV-B radiation; the chemical composition of the surface wax of enzymatically isolated fruit cuticle altered slightly, whereas mass changed dependent on adaption, this adaption was decisive as well for the penetration of $CaCl_2$ through the cuticle, which changed. No correlation between was mass and Ca^{2+} - penetration could be established.

Oberflächencharakteristika von Apfelblatt und –frucht (*Malus domestica* Borkh.) unter Einfluss von Ontogenese und Umweltfaktoren

Im Rahmen dieser Studie wurde die Oberfläche von Apfelblatt und –frucht (*M. domestica* Borkh., `Golden Delicious`, `Topaz`) hinsichtlich chemischer und physikalischer Charakteristika untersucht. Der Einfluß von Ontogenese und Umweltfaktoren war dabei der Hauptaspekt. Als exemplarisch einwirkende Umweltfaktoren wurden UV-B Strahlung und Wasserdefizit betrachtet; wobei die UV-B Strahlung mit einer Intensität von 0,022 kW m^{-2} über einen Zeitraum von 0, 90 und 150 min appliziert wurde, Wasser wurde während der Anzucht zurückgehalten. Die chemische Komposition der Oberflächenwachse wurde mittels GC-MS Technologie untersucht, während die physikalischen Eigenschaften anhand von Kontaktwinkelmessungen und rasterelektronenmikroskopischen Studien analysiert wurden. Retention und Regenfestigkeit des applizierten Fungizids Mancozeb nach erfolgter UV-B Bestrahlung wurde ebenso untersucht wie die Penetration von $CaCl_2$ durch die isolierte Fruchtkutikel.

1 Während der ontogenetischen Entwicklung der Apfelblätter nahm die Oberflächenwachsmasse tendenziell ab, ebenfalls unterlag die chemische Zusammensetzung einer Veränderung, wobei für jedes Entwicklungsstadium eine typische Zusammensetzung der Hauptkomponenten Fettsäuren, Alkohole, Alkane, Triterpene und Ester gezeigt werden konnte. Die adaxiale Blattoberfläche war durch einen amorphen Film epikutikulärer Wachse gekennzeichnet, charakterisiert durch hydrophobe Eigenschaften. Ein Abflachen der Oberflächengestalt, welches im Laufe der Entwicklung einsetzte, kann mit einer Expansion der Zellen erklärt werden. Zum ersten Mal konnte extrazelluläres α-Tocopherol in der epikutikulären Wachsschicht mittels HPLC Untersuchungen nachgewiesen werden.

2 Unter Einfluss von Wasserdefizit und UV-B Strahlung veränderte sich die Zusammensetzung der kutikulären Wachse, zudem konnte eine veränderte Morphologie gezeigt werden. Die Probenahme erfolgte 0, 24 und 48 h nach erfolgter UV-B Bestrahlung. Die Wasserdefizitvariante (nicht UV-B bestrahlt) hatte eine Zunahme der Gesamtwachsmasse um 30 %. Statistische Wechselwirkungen hinsichtlich der Wachsmasse konnte nur 24 h nach UV-B Behandlung nachgewiesen werden. Einen leichten Anstieg des Kontaktwinkels als Zeichen für abnehmende Benetzbarkeit im Vergleich zu 0 und 24 h, konnte 48 h nach UV-B Bestrahlung gezeigt werden. Der Probenahmetermin übte einen stärkeren Einfluss im Vergleich zu den getesteten Umweltfaktoren aus.

3 Retention und Regenfestigkeit (Regenintensität 5 mm h^{-1}; Regenmenge 5 mm) des applizierten Fungizids Mancozeb wurden an der UV-B behandelten Apfelblattoberfläche untersucht. Die Retention des Mancozeb stieg signifikant 24 h nach UV-B Behandlung an, änderte sich aber nicht während der folgenden 24 h. Die Regenfestigkeit wurde nicht signifikant beeinflusst. Als Folge der UV-B Behandlung konnte eine Veränderung der chemischen Komposition der Oberflächenwachse dokumentiert werden, keine Veränderung des Kontaktwinkels wurde nachgewiesen. Von einem Zusammenhang zwischen UV-B Behandlung und veränderter Retention von Mancozeb ist auszugehen.

4 Apfelfrüchte, welche unter normalen Umwelt- und UV-Strahlungsbedingungen angezogen wurden, sind anschliessend in UV-B undurchlässige Folie eingewickelt worden. Als Kontrolle dienten adaptierte Früchte. Beide Varianten wurden nach der Ernte UV-B behandelt. Sowohl die chemische Komposition der Wachse als auch die Permeation von Ca^{2+} durch die isolierte Fruchtkutikel wurden untersucht. Die Oberflächenwachsmasse änderte sich in Abhängigkeit der Adaption, die auch entscheidend für die Penetration von Ca^{2+} war. Eine Korrelation zwischen Wachsmasse und Ca^{2+} - Penetration konnte nicht gezeigt werden.

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Abbreviations

a.i.	active ingredient
ANOVA	analysis of variance
B-LRS-2	laboratory rain simulator
BSTFA	N,O-bis (trimethylsilyl) trifluoroacetamide
$CHCl_3$	Chloroform
CLSM	Confocal Laser Scanning Microscope
cm	centimetre
cm^2	square centimetre
cv.	cultivar
d	days
e.g.	for example
ESEM	Environmental Scanning Electron Microscope
EW	epicuticular wax
Fig.	figure
g	gram
GC-FID	Gas Chromatography - Flame Ionization Detector
GC-MS	Gas Chromatography – Mass Spectroscopy
ha	hectare
HPLC	High Performance Liquid Chromatography
h	hours
i.e.	that is
km	kilometre
kPa	kilo Pascal
kV	kilo Volt
kW	kilo Watt
L.	Linné
l	litres
M. domestica	*Malus domestica*
m	metre
µm	mikrometre
mA	milliampere
ml	millilitre
mm	millimetre

min	minute
nm	nanometre
ng	nanogram
PAR	Photosynthetically Active Radiation
%	per cent
‰	one-tenth of a per cent
P. laurocerasus	*Prunus laurocerasus*
RGR	relative growth rate
RH	relative humidity
SEM	Scanning Electron Microscopy
s	seconds
spp.	species
SE	standard error
Tab.	table
UV-B	ultraviolet radiation-B
vs	versus
W	Watt
WG	wettable granule
°	degree
°C	degree Celsius

A Introduction

1 Plant surface

1.1 Cuticle and its function

Plant cuticles – as a continuous and extracellular membrane, synthesized by epidermal cells (Marga et al., 2001) with thickness between 0.1 to 20 µm (Schönherr and Baur, 1996) - represent the interface between plant and biotic and abiotic environment (Bargel et al., 2003) (Fig. 1). Primary aerial parts of plants like stems, leaves, fruits and petals are covered by the cuticle.

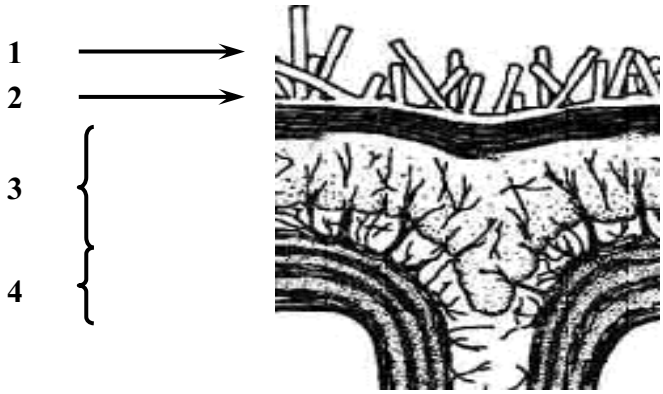

Figure 1: Schematic profile of plant cuticle (adapted from Jeffree, 1996); **1** epicuticular wax crystals, **2** epicuticular wax film, **3** intracuticular waxes and cutin, **4** cell wall.

The predominant structural model is a bilayer cuticular membrane, two layers distinguishable by their ontogeny, ultrastructure and chemical composition (Jeffree, 1996). The outermost part, which forms a layer outside the epidermal cell wall (Lee and Priestley, 1924), is composed predominantly of soluble and polymerized aliphatic lipids, while the inner layer, which is formed by impregnation of the cell wall, also contains substantial amounts of various embedded cell wall polysaccharides (Jeffree, 1996). During the early stages of its ontogeny, the cuticle is subtended by a layer of cell wall polysaccharides, which are characterised by a high pectin content. Therefore it is called pectin lamella. Later the cuticular membrane becomes more strong and cross-linked to the cell wall by embedment of cellulose microfibrils (Jeffree, 1996). The dominant structural polymer in the plant cuticle is cutin. Its detailed chemical composition has been comprehensively reviewed elsewhere (Baker, 1982; Holloway, 1982). It appears possible, since after saponification of cuticular membrane, insoluble residues cutan may present as well (Jeffree, 1996). The two polymers may occur in any ratio and differ in their abundance at different ontogenetic stages (Tegelaar, 1990). However, the structure has not yet been confirmed by conventional chemical analysis, and its biosynthetic origin is unknown. The cuticular membrane covering cells is often structured

with papillae or accomplished by folding. An expansion of the cuticular membrane can be an influencing factor for folding-appearance.

The functions attributed to the cuticle are the protection and waterproofing of the plant surface (Holloway, 1994). Almost all kind of interactions between plant and environment are depending on the chemical and physical structure of the cuticle. The cuticle has to accomplish multiple physiological and ecological functions. Thereby, for transport of chemical substances cuticle forms a barrier, whereas barrier properties can be influenced by many abiotic and biotic environmental factors, as well as by the existence of cuticular waxes (see 1.3). Figure 2 displays the influencing factors.

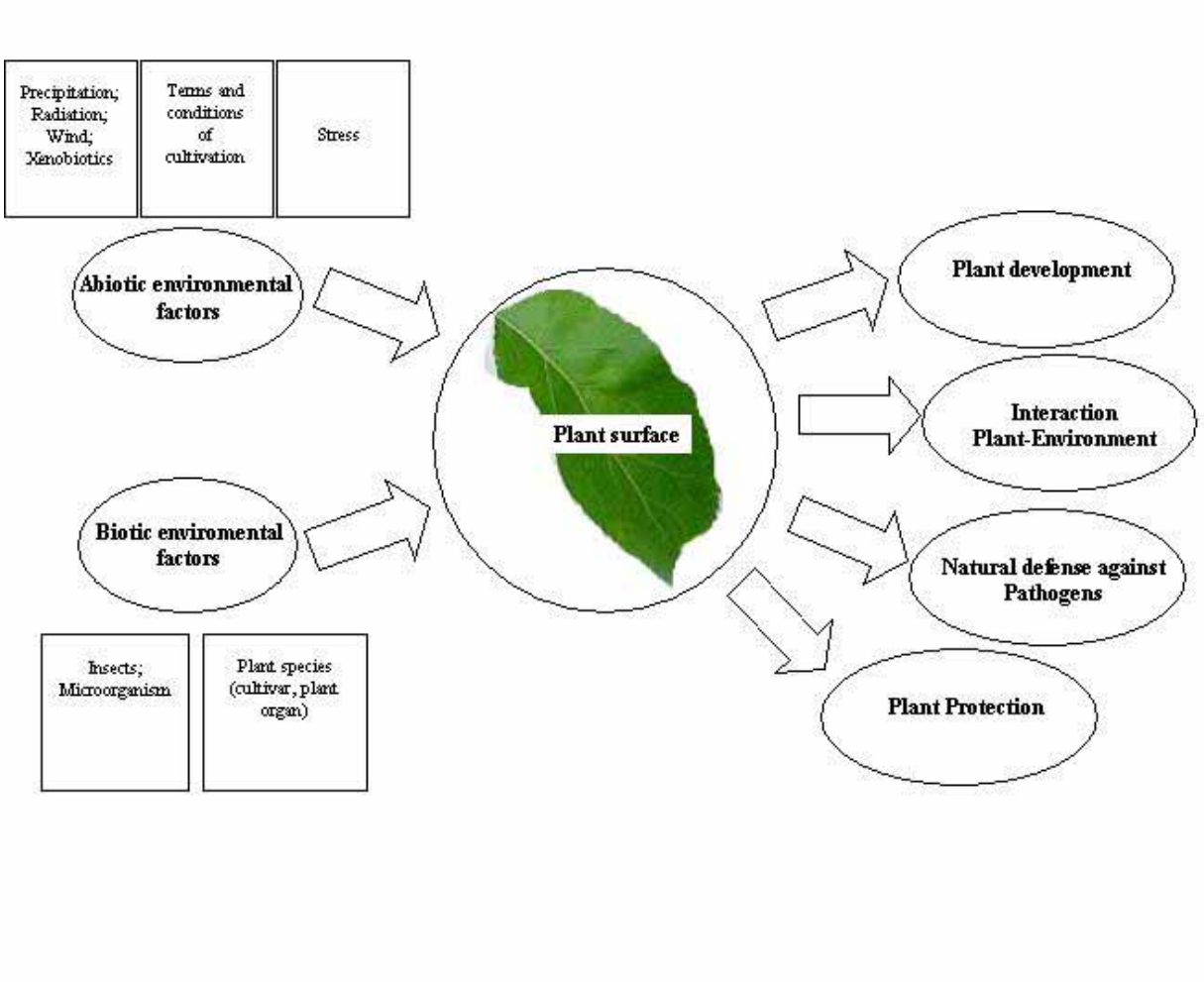

Figure 2: Influencing factors on chemical and physical properties of plant surface (left side) and effect on plants (right side).

The cuticle reduces leaching of ions and nutrients (Tyree at al., 1992; Niederl et al., 1998), and represents a major penetration barrier into leaf tissues of xenobiotics (Schönherr and Riederer, 1989; Schreiber and Schönherr, 1993). Bacterial and fungal attacks can be repelled by the existence of the cuticle and thus infection can be minimized (Barnes and Cardoso-

Wilhena 1996; Kerstiens 1996; Schreiber and Schönherr 1992). Besides, damage caused by harmful solar radiation (especially UV-B: 280-320 nm) can be impeded and alleviated, respectively (Kerstiens, 1996).

1.2 Cuticular waxes and their functions

Cuticular waxes are embedded within the cutin matrix of plant cuticular membranes and make up epicuticular films and aggregates (Riederer and Markstädter, 1996). Wax consists of various soluble lipids, being predominantly linear, long-chain and aliphatic molecules - particularly fatty acids and their derivates alcohols, esters, tritperenes, alkanes with addition of varying proportions of cyclic compounds including pentacyclic triterpenoids and hydroxycinnamic acid derivatives (Riederer and Markstädter, 1996). They are synthesized from C_{16} and C_{18}-precursors, produced in the plastids (Bird and Gray, 2003). The proportion of the single chemical compounds differs among plant species, and even the cultivar influences the chemical composition (Post-Beittenmiller, 1996). Cuticular waxes constitute between 1 to 10 % of the total cuticle (Walton, 1990).

Extrusion of wax to the surface via pores was first proposed by de Bary (1871, 1884), however, electron microscopy has failed to reveal transcuticular pores in sense of open transcuticular channels via which wax might pass freely. Recent research dealing with permeation of substances (wax monomers, proteins, sugar, ions) and water through cuticles, revealed no generally accepted mechanism for their penetration (Neinhuis et al., 2001). Thus, Neinhuis et al. (2001) suggested a co-transport mechanism of wax compounds and water.

The micromorphology of epicuticular waxes can be described as very complex in form and structure, shaped sometimes like crystals, or like an amorphous film; all in all 23 types have been classified (Barthlott et al., 1998), whereas the first attempt in classification was made by de Bary (1871). Using a light microscope he identified four main structural forms: needles, rods, granular layers and films. On his part, Baker (1982) divided the form of epicuticular waxes in plates, tubular waxes, ribbons, rodlets, filaments and dendrites.

An influence of endogenous and exogenous factors on amount and chemical composition of cuticular waxes can be stated; a pronounced effect is ontogenesis. Markstädter (1994) reported an increase of wax coverage from 20 up to 700 µg per leaf during leaf expansion (*Fagus sylvatica* L.).

In addition to genetic factors (Wissemann, 2000), environmental factors, e.g. light (Von Wettstein-Knowles et al., 1980; Letchamo and Gosselin, 1996), relative humidity, water stress (Sutter, 1984; Prior et al., 1997) and, ontogenetic development are reported to affect the chemical composition of epicuticular waxes (Rhee et al., 1998).

In spite of the low mass of cuticular wax compared to the mass of the cuticle, cuticular waxes are responsible for up to 99 % of the resistance of the cuticular membrane to water loss (Riederer and Schreiber, 1995).
Limiting diffusional flow of water and solutes are main functions of plant cuticles (Riederer and Schreiber, 1995; Schönherr, 1982). The wettability of plant surfaces is influenced by the chemical composition and micromorphology of epicuticular waxes, thus controlling leaching, epiphyllic microflora and foliar uptake of pesticides (Brunskill, 1956; Challen, 1962). Moreover, the cuticle makes a contribution to the attenuation of photosynthetically active radiation in order to avoid light inhibition and of ultraviolet radiation (reviewed by Riederer and Markstädter, 1996). However, wettability and light reflection may be affected to a much larger extent by the presence of trichomes and epidermal topography than by epicuticular waxes (Kerstiens, 1996).

1.3 Influence of environmental factors

Environmental factors, like global irradiance, water supply, temperature, acid rain, do influence growth and development of earth living plants. UV-radiation < 290 nm is completely absorbed by the atmosphere, while relevant radiation has a wavelength longer than 290 nm. Figure 3 shows a general overview of effects of UV-B radiation reaching earth's surface. In a variably manner plants do respond to environmental influences, like modified growth, changed efficiency of photosynthesis rate, changed thickness of the cuticle, physicochemical characteristics of the plant surface.

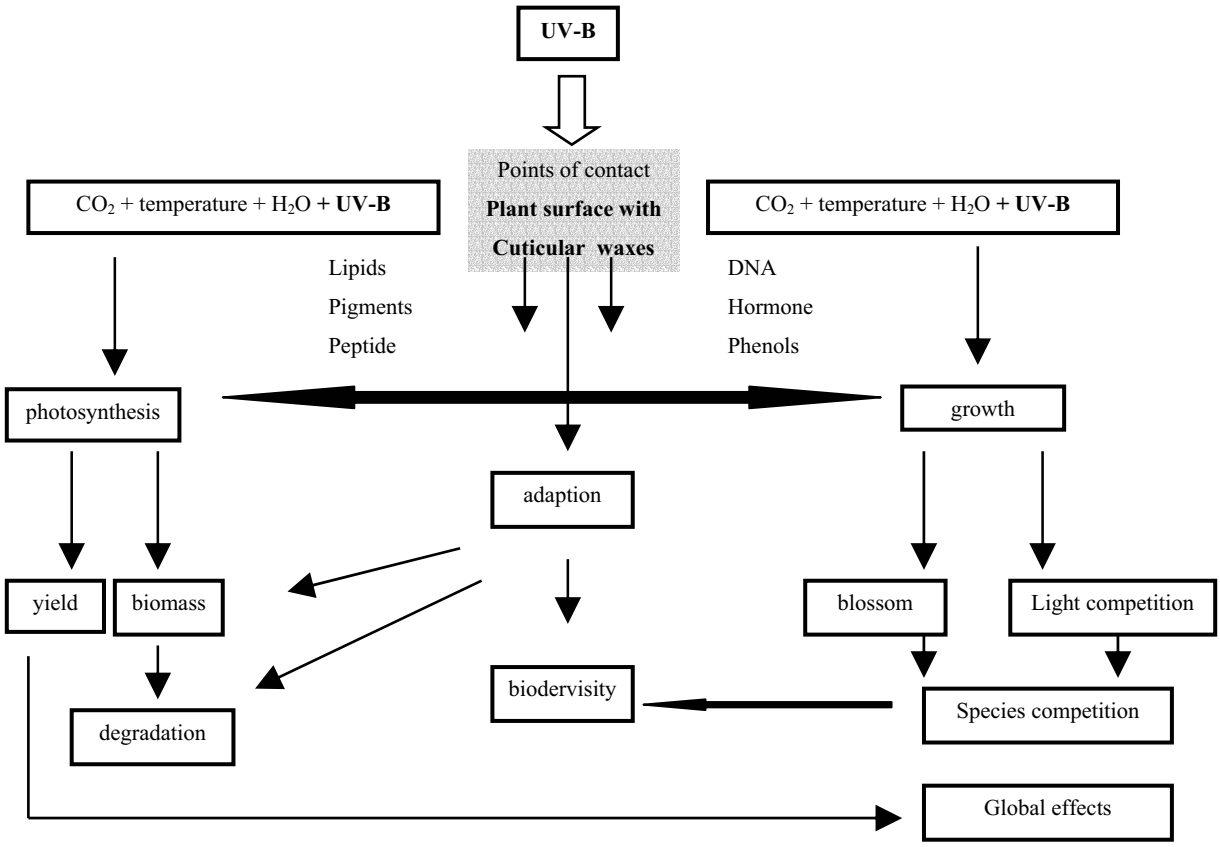

Figure 3: Overview on effects of UV-B radiation on earth living plants.

In the past decades few authors studied the influence of enhanced UV-B radiation inter alia as a consequence of an increasing global UV- radiation. Steinmüller and Tevini (1985) studied the adaxial and abaxial surface of the monokotyledon species barley (*Hordeum vulgare* L. cv. Villa) besides the two dikotyledones cucumber (*Cucumis sativus* L. cv. Delikatess) and bean (*Phaseolus vulgaris* L. cv. Favorit) in their reaction to enhanced UV-B radiation. Between the species there were differences in wax mass e.g. barley leaves had about five times more wax than from bean leaves. The irradiance caused an increase in total wax mass in all plants and a shift in chemical wax composition in different way depending on the studied plant. A significant change in the micromorphology of the studied leaves could not be stated.

The reaction in changing the physicochemical characteristics is depending on e.g. plant species and organ, age of the plant, UV-B dose. Gordon et al. (1998) studied the effects of UV-B radiation on epicuticular wax production and chemical composition of four *Picea* species. The authors stated that, the wax mass recovered from the needle surface did not vary with increasing UV-B dose after 35 d, whereas it changed between the species. No observations were reported concerning the micromorphology of the surface.

Kakani et al. (2003) observed the effects of UV-B (8 (ambient) and 16 kJ m^{-2} d^{-1} (high)) on cotton (*Gossypium hirsutum* L.) and found a reduction in plant height by 53 % over control shorter than plants not exposed to enhanced UV-B radiation. A changed density of stomata

after UV-B treatment was another observation of Kakani et al. (2003). An increase in UV-B irradiance can cause a change in chemical composition of surface waxes, as e.g. in *Pisum sativum* L., where a shift from alcohol to esters and hydrocarbons was analysed (Gonzalez et al., 1996) micromorphology was investigated as well, and for leaves no change after induction of water deficit was stated.

What are the causal reactions leading to changes of chemical composition of surface waxes? Steinmüller and Tevini (1985) stated that the changed wax mass can be a function of modified leaf area (irradiated leaves show a reduced leaf area), but distribution pattern within some wax classes is an indication that UV-B radiation affects wax biosynthesis. There is a dose-dependence, as effects on the adaxial side were much more pronounced than on the abaxial side.

Barnes et al. (1996) studied surface leaf wax of UV-B radiated tobacco (*Nicotiana tabacum* L.). They reasoned that there must be an impact on wax biosynthesis as branching of wax molecules upon UV-B irradiation. The mechanism involves effects on the microsomal-based elongases which are responsible for the addition of C_2 units within the elongase-decarboxylase pathway. The reaction to water deficit is besides others a function of species and organ.

Drought induces a large decrease in photosynthetic activity (Angelopoulos et al., 1996; Munné-Bosch et al., 1999). However, mechanistic bases of this inhibition of photosynthesis are not well understood. Inhibition of photosynthesis could be based on changes of electron transport as affected by oxidative processes in chloroplast after exposure of plants to drought. An insufficient water supply can cause a higher content of epicuticular wax compared to sufficient water supply (Letchamo and Gosselin, 1996). As well as the mass of epicuticular waxes as the chemical composition were affected. Water stressed organs of cotton (*Gossypium hirsutum* L.) had higher levels of long-chained alkanes in comparison with well watered plants (Bondada et al., 1996).

Leaf surface wax has been considered as an important component of drought tolerance (Premachandra et al., 1991), besides several other morphological and physiological adaptions are known to impart drought tolerance, like root structure, accumulation of osmotica, leaf folding, reduction in leaf area, regulation of transpiration rate are some of these mechanisms (Joshi et al., 1998; Subbarao et al., 1995; Blum, 1998).

Environmental factors involved in plant growth and development might not be separated from each other.

1.4 Implications of modified cuticula and surface waxes

Why there is importance for studying the possible modifications of plant surface caused by environmental factors?

A modified surface can influence the interaction plant:microorganism. The dense biofilm, which covers the cuticular surface, consists of epiphyllic microorganism, under changed environmental conditions the composition of microorganism is modified (Blakeman, 1982; Andrews and Harries, 2000; Herrera-Campos et al., 2004). These factors can impact the environmental behaviour of the plant. Changes in wettability caused by degradation of epicuticular wax crystals (Riederer, 1989) offer, in general, a more suitable microhabitat for most phyllosphere organism (Knoll and Schreiber, 1998), whereas surface wetness may hinder sporulation of others (Butler, 1996). Besides these aspects, pest management aspects are important. Deposition, retention and distribution of spray droplets are affected by amount and chemical composition of epicuticular wax and by leaf surface micro-roughness (McWorther, 1993). The surface exposure of wax chemical groups determine the adequate wetting of the leaf, which is an essential basis for pesticide efficiency (Challen, 1962; Holloway, 1970; Fogg, 1947; Furmidge, 1962). Changes in wax chemistry and physical properties may affect wettability and markedly alter spray retention and penetration, as documented for expanding peach leaves (Bukovac et al., 1979).

Besides the surface wax properties, density of trichomes are influenced as well (Barnes et al., 1996), which do impact the behaviour of water droplets on leaf surfaces (Brewer et al., 1991) and leaf optical properties (Ehleringer, 1984).

The change in physicochemical characteristics of the surface, evoked by abiotic and biotic environmental factors, can impact behaviour of the plant towards environment.

1.5 The objective target of the present study

Clearly the surface of plants – as the first point of contact between earth living plants and it's surrounding area - is subjected to impact of it`s surrounding environment.

The object of our study was *M. domestica* Borkh., particularly the leaves and fruits; whereas both are characterised by a different physical properties of the surface wax. The leaves do have amorphous wax film, whereupon the fruits do have crystals. The chemical composition is comparable. By means of these two different organs, the impact of environmental factors, like enhanced UV-B radiation and deficit in water supply on physicochemical characteristics

should be studied, because of possible variation of surface characteristics, which do impact plant-environmental-behaviour.

Moreover ontogenesis as a crucial factor impairing surface wax morphology and chemical composition was assayed. Former studies showed a definite content of tocopherols in surface wax layer of e.g. Rubus (Robertson et al., 1991). Because of it`s prevention of the plant from damage caused by a grat number of abiotic and biotic stressores, we tested the surface wax layer of apple leaves for tocopherol content.

A change in surface chemical and physical characteristics can have implications on agricultural aspects i.e. plant protection, therefore retention and rainfastness of a well established fungicide mancozeb on UV-B influenced leaf surfaces were studied. To what extent there is an interaction between two environmental factors concerning their influence on adaxial apple leaf surface, is to be examined as well.

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B Ontogenetic variation in chemical and physical characteristics of adaxial apple leaf surfaces

1 Introduction

For all terrestrial higher plants the cuticle forms a protective coating of aerial parts preventing the plant from desiccation due to uncontrolled non-stomatal water loss and the loss of organic and inorganic compounds by leaching. Moreover, the cuticle protects the plant against the infiltration of xenobiotics from the environment as well as from potentially harmful irradiance, like UV-B radiation. This interface between plant and environment, in particular the waxes forming its outermost surface, is relevant for the colonization by epiphytic microorganisms and the host recognition by pathogenic fungi as well as insects (Kolattukudy, 1985; Carver et al., 1990; van Loon et al., 1992; Podila et al., 1993; Eigenbrode and Espelie, 1995; Flaishman et al., 1995; Schoonhoven et al., 1998).

The highly lipohilic cuticle, varying from 0.1 - 20 µm in thickness depending on plant species and organ, consists of two main components, cutin and wax. The amount of cuticular wax ranges from 1 - 10 % of the total cuticle (Baker, 1982; Walton, 1990). Regarding the micromorphology of waxes, twenty three types have been classified in total (Barthlott et al., 1998). Thin wax films appear to be ubiquitous, while thick layers or crusts are rare.

The cuticular wax fraction can be differentiated into intra- and epicuticular waxes (EW). The chemical composition of cuticular waxes significantly varies among plant species and among cultivars within a species (Post-Beittenmiller, 1996). For apple fruits six classes of EW compounds have been described, including: triterpenoids; primary alcohols; ketones; aldehydes and secondary alcohols and acids (Holloway, 1982; Belding et al., 1998). Among apple cultivars ‘Golden B’, ‘Golden Delicious’, and ‘Ozark B’ the chemistry of cuticular waxes differed, e.g. in the nonacosane content (Belding et al., 1998; Verardo et al., 2003). The chemical composition of surface waxes largely determines the morphology of the surface. Studies by Jeffree et al. (1976) as well as Jetter and Riederer (1994) demonstrated that nonacosan-10-ol is essential for the formation of wax tubules.

In addition to the dominant constituents, cuticular wax may include also some minor or unusual constituents like sterols and phenolic compounds (Baker, 1982). Phlorizidin, phloretin, quercetin and quercetrin – phenols known for their antimicrobial properties – have been detected in the leaf waxes of *Malus* spp. (Richmond and Martin, 1959). The triterpenoid pathway and tocopherol synthesis are closely linked. Derived from isoprene, tocopherol is synthesized only by photosynthetic organisms in the envelope of plastids and consists of a

polar chromanol ring and a 15-carbon lipophilic prenyl chain derived from homogentisic acid and phytyl diphosphate (Collakova and DellaPenna, 2003). The lipid-soluble vitamin E is known to be an important intracellular antioxidant and scavenger of lipid peroxy radicals in plant tissue crucial for membrane stability (Munné-Bosch and Alegre, 2002). Tocopherols are not usually found in cuticular waxes of Rosaceae. They have been, however, detected in the wax of Gingko (Gülz et al., 1992) and *Rubus* (Robertson et al., 1991). The biological activity of tocopherol – the prevention of plants from damage induced by a great number of abiotic and biotic stress factors – has been well described (Schmitz and Noga, 2000a, b; Schmitz-Eiberger and Noga, 2001; Förschler et al., 2003).

In addition to genetic (Wissemann, 2000) and environmental factors, e.g. light (von Wettstein-Knowles et al. 1980; Letchamo and Gosselin, 1996), relative humidity and water stress (Sutter, 1984; Prior et al., 1997), chilling (Nordby and McDonald, 1991), plant nutrition (Schwab et al., 1994) and seasonal variation (Gülz and Müller, 1992), the ontogenetic development of plants and plant parts, e.g. leaves, are reported to affect the composition of epicuticular waxes (Rhee et al., 1998; Riederer and Markstädter, 1996). In *Fagus sylvatica*, the bimodal distribution of aliphatic wax constituents with maxima in the range of C_{28} and C_{52} shifted within 20 d after bud stage to a large single maximum of C_{28} when the leaf reached final size (Riederer and Markstädter, 1996). During leaf expansion of *Prunus laurocerasus*, the average chain length of alcohols and fatty acids of epicuticular waxes increased from C_{24} to approximately C_{32} (Jetter and Schäfer, 2001). Information on changes in the composition of EW during ontogenesis is rare amongst the species of Rosaceae as reported by Shepherd et al. (1999).

The objective of this study was to characterize the dynamics in chemical composition and physical characteristics of the adaxial surfaces of apple leaves during early stages of ontogenesis. Under controlled conditions the influence of environmental factors were minimized in order to focus on the effect of ontogenetic leaf age. Major physical characteristics of the adaxial epidermal cuticle were assessed microscopically and goniometrically, the amount of apolar waxes and their chemical composition were investigated using GC techniques. The α–tocopherol content of the wax layer was quantified as this antioxidant is reported to be involved in the reaction of plants to abiotic stress.

2 Material and Methods

2.1 Plant material

Seeds of *M. domestica* Borkh., cv. Golden Delicious, were treated with 0.1 % Euparen® M WG 50 (Tolylfluanid, Bayer CropScience, Monheim, Germany) for five minutes and than stored at 4 °C in the dark for two weeks. After sowing in substrate for salt-sensitive plants (pH 5-6, salt content: 0.8 g l^{-1}, Klasmann-Deilmann GmbH, Geeste, Germany), seedlings were cultivated in a greenhouse at 18 – 20 °C and 18 h daylight. Two weeks later the seedlings were singularized and grown in plastic pots (8.5 cm x 8.5 cm x 7.5cm) filled with standard potting mixture (special mixture, Klasmann-Deilmann GmbH, Geeste, Germany), irrigated and fertilized (liquid fertilizer Flory 2 special, 16+9+22.4 ‰) as and when required. The leaves of seedlings were protected from dust, chemical substances and fungal infection by growing the plants in a specific cabinet covered with cellophane in order to allow for the exchange of air and humidity. After 8 to 10 weeks apple seedlings were used for the wax analysis and the other studies. The experiments were carried out using the adaxial side of the youngest completely unfolded leaf (leaf insertion 1) and leaves of insertion 3, 5 and 7, respectively.

2.2 Wax extraction by chloroform and analyses

The adaxial side of the leaf was immersed twice in chloroform (purity >99 %) for per 10 s in a glass petri dish. Previous tests had shown that cuticular waxes from leaf samples were extracted almost completely after 10 s and that longer extraction periods were associated with tissue damages from $CHCl_3$. It was assured that during extraction only the adaxial surface had contact with chloroform. After adding an internal standard (C_{24} alkane, tetracosan) the samples were evaporated under nitrogen atmosphere. The internal standard was ca. 10 % of the estimated total wax amount. By adding 20 µl pyridine (Merck, Darmstadt, Germany) and 20 µl of BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide, Macherey-Nagel, Dueren, Germany) the samples were derivatized for 40 min at 70 °C according to Hauke and Schreiber (1998).

The samples were diluted with 100 µl of chloroform before GC-MS analysis (5890 series II, Hewlett-Packard, Avondale, PA, with on-column injection and applying a high resolution gas chromatography column, Agilent Technologies, 30 m × 0.321 mm DB-1, phase thickness

0.1 µm, J&W, Folsom, CA). The temperature program was as followed: start at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, then 30 min at 320 °C. The carrier gas was hydrogen.

The pressure program was: injection at 50 kPa, 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, 39 min at 150 kPa.

For qualitative GC-MS analysis the same method was used but instead of hydrogen, helium was used as carrier gas; injection volume was 1 µl.

2.3 Wax extraction by the freeze - embedding method

For the extraction of α-tocopherol from the epicuticular wax layer of apple leaves another method for the isolation of epicuticular waxes was used, a freeze-embedding method according to Ensikat et al. (2000). A disk (Ø 16 mm) was punched from apple leaves, cv. Golden Delicious, avoiding leaf vein. The adaxial surface of the leaf segment was placed onto a drop of glycerol applied on a spoon and than immersed into liquid nitrogen until frozen. With the help of tweezers the sample was lifted accurately off the spoon. After defrosting of the sample the wax was extracted with chloroform and filtered through a special metal filter (Frintrup, Bonn, Germany) with 200 holes per mm^2 to hold back the glycerol.

In order to have a clean sample of extracted wax, the chloroform procedure was repeated twice. The samples were evaporated using nitrogen and re-dissolved in 1000 µl of n-hexane. The tocopherol content was determined by HPLC (Schmitz and Noga, 2000b). The following equipment was used: HPLC model 6000A (Waters Associated Chromatography, Langenfeld, Germany) with a pump Waters 510, Sunchrom Marathon autosampler, Waters Millenium software, precolumn: 5 cm x 3 mm Lichrospher 100 diole, 5 µm (CS Chromatography service, Langerwehe, Germany); the applied eluent was a mixture of n-hexane 96.4 %, ethylacetate 3.6 %; the flow rate was 1.2 ml min^{-1}. The fluorescence detector model RF 551 was used with an extinction of 285 nm and emission: 320 nm. The external standard was α, β, γ, δ-tocopherol with 100 µl injection volume. The analyses were carried out at 22 °C and every sample was kept at –80 °C until 15 min before injection to prevent degradation of α-tocopherol. The sample injection volume was 100 µl.

2.4 Determination of apple leaf area of the adaxial leaf side

The investigations were conducted with six apple seedlings which had been grown for 40 d after planting. The areas of all leaves per plant were measured five times with an interval of four days. At the end of these investigations the seedlings were 56 d (8 weeks) old. This age

corresponded to those plants used in other investigations of this study. Leaf area of the adiaxal leaf side was calculated from length and width of leaves using an additional factor of 0.71 which had been derived in preliminary destructive measurements of leaf area. The relative growth rate (RGR) of leaf area was used for the assessment of the growth depending on the insertion (= leaf age) of apple leaves. RGR was calculated according to the formula
RGR = (ln AL2 - ln AL1)/(t2 - t1), where AL2 is the leaf area at time t2 and AL1 the leaf area at time t1, respectively, modified from Hunt (1982).

2.5 Goniometry

The hydrophobicity of apple leaves was assessed quantitatively by a drop shape analysis system (Contact Angle System OCA 30-2, Software SCA 202, DataPhysics Instruments GmbH, Filderstadt, Germany). Samples cut from the central area of the leaf lamina were affixed to glass slides by double-sided adhesive tape (TesaFix, Beiersdorf, Hamburg, Germany) to ensure an even surface. The sessile drop method was used. Contact angles were calculated by using the Laplace-Young-Fitting method. The volume of water drops (distilled water) amounted to 10 µl. Measurements were made on the adaxial side of four leaf insertions, representing different leaf ages. Six seedlings with up to ten leaves were investigated. For every developmental stage of leaves three measurements were made resulting in 18 values per leaf insertion.

2.6 Microscopy

For examination of the surface relief, samples of about 1 cm² cut from the middle of leaf lamina were prepared. For high-resolution SEM (Leo 440, Leica, Bensheim, Germany) fresh leaf samples were affixed to aluminum stubs and air dried. All samples were sputtered with gold (65 mA for 30 s, Sputter SCD 040, Balzers Union), and were examined at 15 kV and a beam diameter of 20 nm under high vacuum conditions. For ESEM (XL 30 ESEM, Philips Electron Optics, Eindhoven, The Netherlands) fresh samples were affixed on a polycarbonate adhesive tape and examined uncoated at 4 °C with a gaseous secondary electron detector (GSED) within a water vaporous environment under low vacuum conditions (4.5 Torr) in the chamber.

The area and height of epidermal cells of the adaxial surface of apple leaves were assessed with a CLSM (LSM 310, Zeiss, Oberkochem, Germany) equipped with a neon-helium laser (543 nm). Samples totally cleared in chloral hydrate (Sigma-Aldrich, Steinheim, Germany)

were observed with a 40x oil immersion objective lens. The topography of periclinal cell walls was analyzed over series of optical xy slices in the reflection mode. Five measurements on leaves of six different plants were made resulting in 30 values per leaf insertion.

2.7 Statistical analysis

Experimental data were analyzed with the statistic program SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The data were tested for normal distribution and variance homogeneity and compared by Tukey-HSD multiple range test or Duncan's multiple range test. Modifications in the amount of wax components were tested for significance using the non-parametric Kruskal-Wallis test. A 5 % probability level was accepted to indicate significant differences. All experiments were carried out at least twice.

3 Results

3.1 Growth rate of apple leaves depending on leaf insertion and developmental stage

The development of *M. domestica* leaf surfaces was monitored for apple plants, grown under greenhouse conditions for 40 d after planting. The area of all leaves - with leaf 1 being the youngest leaf at the end of the experiments - was calculated from measurements carried out on day 0, 4, 8, 12, and 16, respectively (Table 1). Day 0 being the first day of measurements. During the experiment, apple seedlings produced a new leaf every four to five days. For young leaves, the leaf area almost doubled within four days. Subsequently, the growth rate decreased rapidly and approached 0 about 20 d after first leaf appearance. Low leaf insertions, i.e. the oldest leaves produced by the seedlings, remained smaller than higher leaf insertions formed at later stages of leaf development. The latter`s fully expanded leaves reached a leaf area of almost 18 cm.

Table 1: Growth rate of apple leaves depending on leaf insertion and leaf age. Data indicate absolute leaf area [cm²] for the first and the final measurement and relative growth rate per day for all subsequent measurements. Leaf insertion 1 was the youngest, leaf insertion 12 the oldest leaf of apple seedlings (cv. Golden Delicious, means ± SE, n = 6).

Day	**Leaf area ± SE** [cm^2] and *relative growth rate per day* [$cm^2\ d^{-1}$], respectively											
	Leaf insertion											
	1	2	3	4	5	6	7	8	9	10	11	12
0						**3±0,5**	**11±1,9**	**17±0,7**	**16±2,9**	**17±1,8**	**14±0,8**	**13±2,3**
4				**2±0,1**	**4±1,2**	*0.38*	*0.11*	*0.04*	*0.01*	*0.00*	*0.00*	*0.00*
8			**3±0,3**	*0.41*	*0.25*	*0.11*	*0.03*	*0.01*	*0.01*	*0.00*	*0.00*	*0.00*
12		**3±0,4**	*0.3*	*0.16*	*0.12*	*0.03*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
16	**4±1,1**	*0.2*	*0.1*	*0.04*	*0.04*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
final size	**4±1,1**	**7±2,9**	**16±4,3**	**18±4,5**	**20±2,4**	**23±1,9**	**20±2,4**	**21±1,2**	**17±3,0**	**17±1,8**	**14±0,7**	**13±2,3**

3.2 Structure of leaf surfaces

Microscopic investigations using SEM displayed the typical pattern of puzzle-like epidermal cells forming the leaf surface of dicots. The adaxial leaf side did not show any stomata. Leaf age had an influence on the surface structures like following described. Epidermal cells of the upper side of young leaves (leaf insertion 1) showed a distinctive curvature of the periclinal cell walls resulting in a noticeably undulated surface of the leaf cuticle (Fig. 1, left side).

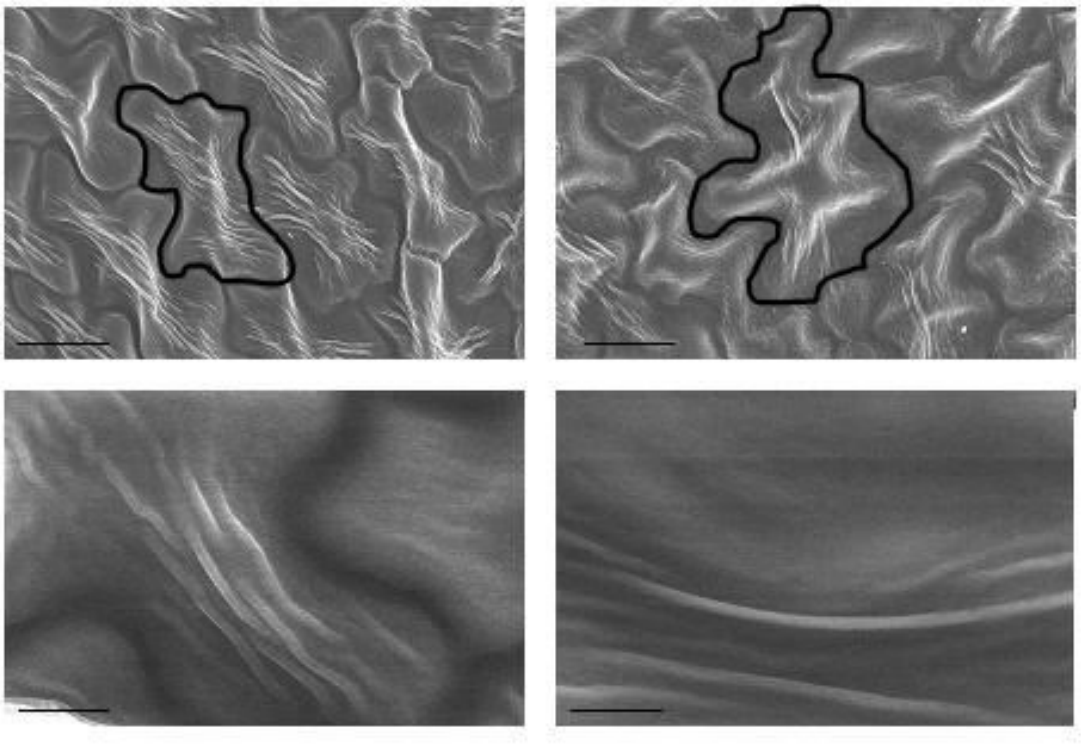

Figure 1: Adaxial surface of the epidermal cell layer of apple leaves, cv. Golden Delicious. Left: youngest, completely unfolded leaf; right: seventh leaf from the top, (top: SEM, 1000x, bars represent 20µm; bottom: ESEM, 4000x).

Pronounced lamellae of the cuticle showed highest density at the centre of the epidermal cells (on the top). Crystals of epicuticular waxes were detected neither by scanning electron microscopy nor by the ESEM technique without preparation of cuticles. During ontogenesis, the epidermal surface of leaves became more even, showing only minor surface sculpturing. The number of cuticular lamellae per cell was lower and lamellae were less pronounced (Fig. 1, right side).

The leveling of the leaf surface probably resulted from the expansion of epidermal cells (Table 2). The surface area of cells within the epidermal layer increased from about 630 μm^2 (leaf insertion 1) to approximately 2220 μm^2 (leaf insertion 5). For the oldest leaves investigated (leaf insertion 7) the cell area was smaller (about 1975 μm^2), largely corresponding to the smaller total leaf area. The number of epidermal cells per leaf calculated

from this data varied from 1.4 x 106 (leaf insertion 7) to 1.9 x 106 (leaf insertion 3 + 32%), with the three upper leaf insertions investigated showing a variation of only 7%. The height of epidermal cells decreased during ontogenetic development, with differences of up to almost 7 µm between the youngest and the oldest leaf insertion measured (Table 2). Therefore, the oldest leaf insertion had a lower number of epidermal cells, and also a smaller volume, than mature younger leaves.

Table 2: Area and height of epidermal cells from the upper side of apple leaves depending on the leaf insertion (cv. Golden Delicious, means ± SE, n = 30).

	Leaf insertion			
	Leaf 1	Leaf 3	Leaf 5	Leaf 7
Cell area [µm^2]	634 ± 20 ^{d}	1566 ± 52 ^{c}	2224 ± 82 ^{a}	1976 ± 51 ^{b}
Cell height [µm]	27 ± 1 ^{a}	26 ± 1 ^{a}	23 ± 0 ^{b}	20 ± 1 ^{c}

Figures with different letters are significantly different, Tukey test, $p \leq 0.05$

3.3 Hydrophobicity of leaf surfaces

The contact angle of water on the adaxial surface of young apple leaves (leaf insertion 1) was almost 110 °, indicating a pronounced hydrophobicity of the cuticle (Fig. 2). The wettability of artificial surfaces such Parafilm® and glass slides was measured as references for a hydrophobic and a hydrophilic surface, respectively. The hydrophobicity of apple leaf surfaces decreased with increasing leaf age. The largest increase in wettability was identified between leaf insertion 3 and 5 (contact angle > 100 ° and 80 °, respectively), with the wettability showing higher variability for older leaves. The contact angle for leaves of insertion 7 averaged 76.4 ° - well above the level of the hydrophilic glass surface.

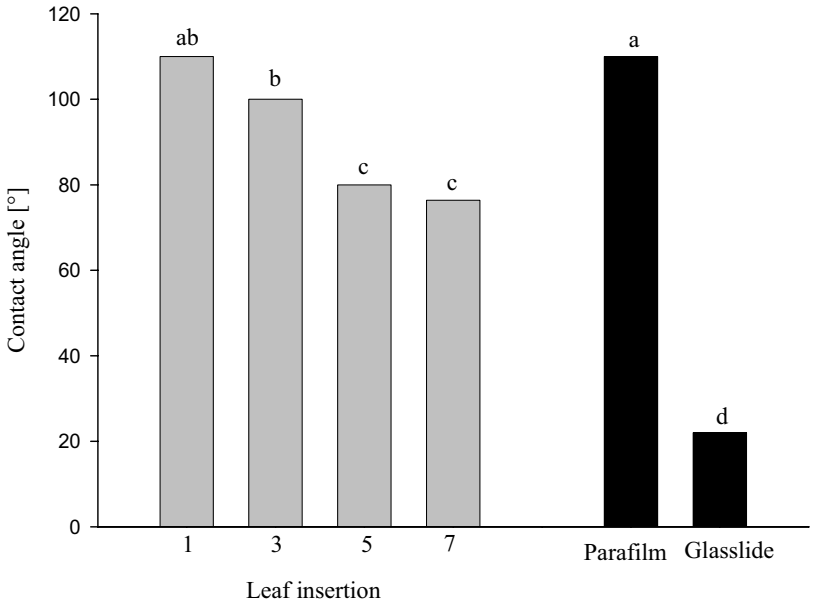

Figure 2: Effect of ontogenesis of apple leaves (cv. Golden Delicious) on the wettability of cuticular surfaces as measured by goniometry; artificial surfaces were measured for comparison (n = 18, Parafilm® n = 12, glass slides n = 6). Columns with different letters are significantly different, Tukey test, $p \leq 0.05$.

3.4 Characterization of the wax layer

The chemical composition of apolar wax compounds, extracted from the upper leaf surface layer of apple seedlings cultivar `Golden Delicious` was analyzed in relation to the leaf insertion (Fig. 3).

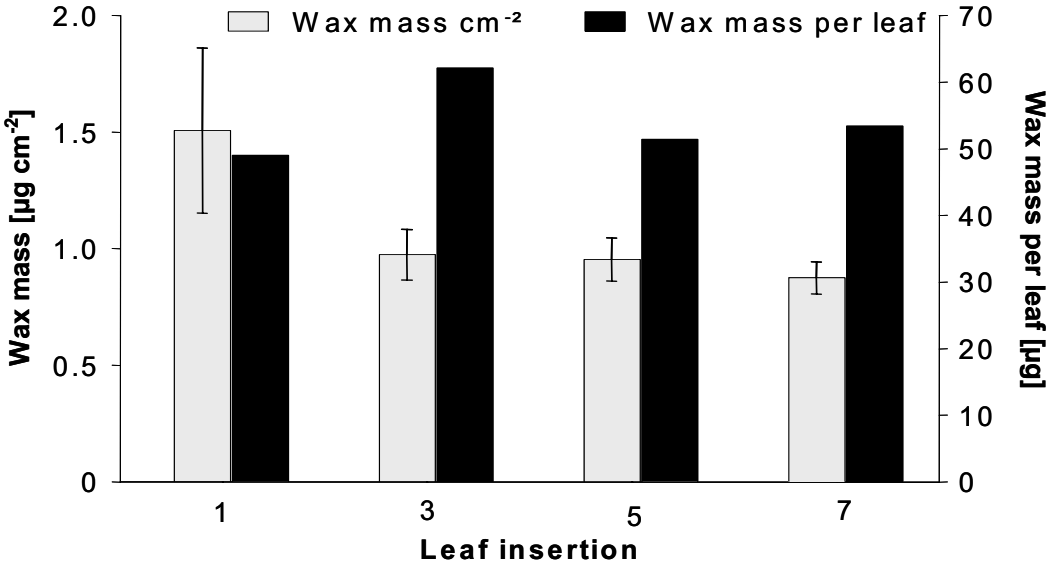

Figure 3: Effect of ontogenesis of apple leaves on the apolar wax mass per leaf area and total apolar wax mass per leaf (cv. Golden Delicious, n=5).

The amount of wax per unit of area ranged from 1.5 µg cm^{-2} for leaf insertion 1 to 0.9 µg cm^{-2} for leaf insertion 7. The area of young leaves increased during ontogenesis, while the wax amount per unit of leaf area decreased; the correlation coefficient between wax mass per cm^2 and the area of total leaf surface was -0.833 ($p \leq 0.05$). Differences in the total amount of waxes per leaf were not significant among leaf insertions 3 to 7.

Wax components were identified as primary alcohols, fatty acids, esters, triterpenes and alkanes. For young leaves, triterpenes (ursolic acid and oleanolic acid), esters and alcohols were the main wax components (Fig. 4). The triterpenes represented up to 32.3 % (leaf insertion 1) of the total amount of apolar waxes and decreased during ontogenetic development to 22.6 % (leaf insertion 7). Esters slightly increased from 23.2 % for leaf insertion 1 to about 25 % for the oldest leaves. The content of wax alcohols increased considerably during ontogenetic development from 19 % to 28.8 %. The amount of C_{22} up to C_{30} acids was always below 10 %; it was halved during the expansion of leaf area and did not alter in later stages of development. The amount of alkanes remained largely constant during leaf ontogenesis varying only from 16.6 – 19 %.

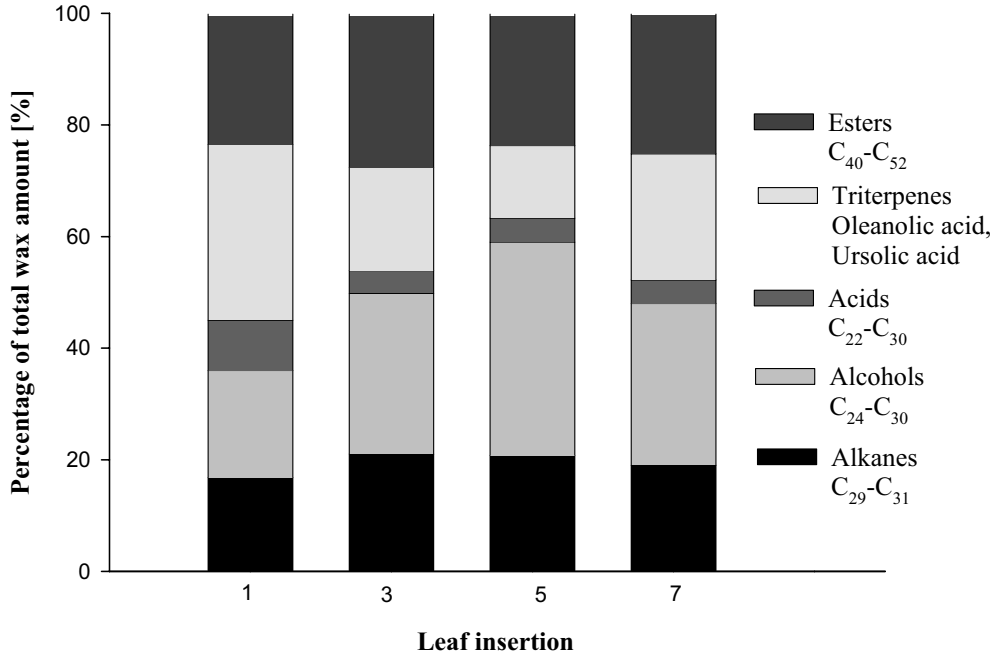

Figure 4: Effect of ontogenesis of apple leaves on the percentage of apolar wax compound (% of total apolar waxes, n = 5).

Data on the main constituents of the chemical classes detected are summarized in Table 3. In young leaves C_{29} alkane represented almost 5 % of total waxes. It decreased noticeably to less than 3 % in older leaves. C_{31} alkane was the main constituent of this chemical class

representing about 9.0 % and 13.5 % in young (leaf insertion 1) and old (leaf insertion 7) leaves, respectively. In absolute terms, however, the content of C_{31} alkane as well as of C_{33} alkane did not alter. The absolute amount of C_{24} alcohol significantly decreased during leaf development. The amount of the other alcohols largely remained constant among leaf insertions. At a very low range, long-chain acids formed a fraction of about 0.3 % (C_{22} acid) to 4.7 % (C_{26} acid) in young leaves. With a decline in absolute amounts - especially for C_{24}, C_{26}, and C_{30} acids - of more than 70 % the percentage of these acids in the total amount of apolar wax constituents decreased by about 50 % during leaf ontogenesis. The chain length for the C_{28} acid remained at a level of about 0.3 % during the ontogenetic development.

Table 3: Effect of ontogenesis of apple leaves on the composition of apolar wax components; bold numbers indicate the percentage of the total extracted and identified wax compounds (cv. Golden Delicious, n = 5), Olean. Acid = Oleanolic acid.

Compound	Leaf 1		Leaf 3		Leaf 5		Leaf 7	
	[ng cm^{-2}]		[ng cm^{-2}]		[ng cm^{-2}]		[ng cm^{-2}]	
	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%
C_{29} alkane	71 ± 28	**4.7**	26 ± 3	**2.7**	23 ± 4	**2.4**	22 ± 3	**2.5**
C_{31} alkane	135 ± 23	**9.0**	133 ± 45	**13.9**	125 ± 40	**13.1**	118 ± 34	**13.5**
C_{33} alkane	44 ± 19	**2.9**	33 ± 13	**3.4**	32 ± 8	**3.3**	24 ± 8	**2.7**
C_{24} alcohol *	35 ± 15	**2.3**	5 ± 1	**0.6**	4 ± 1	**0.4**	4 ± 0	**0.4**
C_{26} alcohol	88 ± 15	**5.9**	110 ± 33	**11.4**	166 ± 26	**17.4**	100 ± 12	**11.5**
C_{28} alcohol	86 ± 16	**5.7**	69 ± 7	**7.1**	85 ± 7	**8.9**	79 ± 7	**9.0**
C_{30} alcohol	77 ± 16	**5.1**	72 ± 12	**7.5**	76 ± 13	**8.0**	69 ± 9	**7.9**
C_{22} acid	5 ± 2	**0.3**	3 ± 1	**0.3**	2 ± 0	**0.2**	2 ± 1	**0.2**
C_{24} acid	31 ± 22	**2.1**	5 ± 1	**0.5**	4 ± 0	**0.5**	3 ± 1	**0.4**
C_{26} acid	70 ± 36	**4.7**	21 ± 4	**2.1**	22 ± 6	**2.3**	22 ± 6	**2.5**
C_{28} acid	3 ± 2	**0.2**	2 ± 1	**0.2**	4 ± 1	**0.4**	3 ± 1	**0.3**
C_{30} acid	24 ± 15	**1.6**	7 ± 1	**0.7**	5 ± 1	**0.6**	6 ± 1	**0.7**
Olean. acid *	97 ± 37	**6.4**	30 ± 5	**3.1**	21 ± 1	**2.2**	20 ± 1	**2.3**
Ursolic acid	390 ±184	**25.9**	192 ± 40	**20.0**	179 ± 24	**18.8**	177 ± 29	**20.3**
C_{40} ester *	40 ± 13	**2.6**	19 ± 3	**1.9**	9 ± 2	**1.0**	9 ± 1	**1.0**
C_{42} ester •	44 ± 10	**2.9**	26 ± 5	**2.7**	20 ± 6	**2.1**	18 ± 4	**1.0**
C_{44} ester	96 ± 24	**6.4**	58 ± 9	**6.0**	42 ± 6	**4.4**	48 ± 18	**5.5**
C_{46} ester •	15 ± 4	**1.0**	27 ± 4	**2.8**	42 ± 4	**4.5**	37 ± 5	**4.4**
C_{48} ester	118 ± 33	**7.9**	89 ± 18	**9.2**	54 ± 12	**5.7**	59 ± 11	**6.5**
C_{52} ester	37 ± 9	**2.4**	34 ± 3	**3.6**	38 ± 10	**4.0**	57 ± 8	**6.5**

* significant difference according to Tukey test, $p \leq 0.05$

• significant difference according to Kruskal-Wallis test, $p \leq 0.05$

In young apple leaves the main triterpene compound was ursolic acid, which represented up to 25.9 % of the total wax mass (leaf insertion 1). Oleanolic acid amounted to 6.4 % for leaf insertion one. During the ontogenetic development of leaves the amount of both triterpenes

decreased, whereas the percentage of ursolic acid and oleanolic acid in the total wax amount was reduced to 20.3 % and 2.3 %, respectively. The total content of esters with chain length of $C_{40\text{-}48}$ and C_{50} in the cuticular wax did not change. The C_{40} ester decreased significantly, whereas the concentration of the others remained largely constant. In contrast, the amount of C_{46} and C_{52} esters increased in both absolute terms and in the percentage of total wax amount from 1.0 % to 4.4 % for C_{46} esters, and from 2.4 % to 6.5 % for C_{52} esters) with increased leaf age.

3.5 Content of α-tocopherol in the surface wax layer

In the epicuticular wax fraction prepared with the freeze-embedding method, α-tocopherol was detected by HPLC measurements during all developmental stages. The amount of α-tocopherol increased significantly from 0.38 ng cm^{-2} for leaf insertion 1 to 1.5 ng cm^{-2} for leaf insertion 5 (Fig. 5). The content in the oldest leaves remained at an increased level. Neither γ- nor δ-tocopherol was found in the epicuticular wax layer.

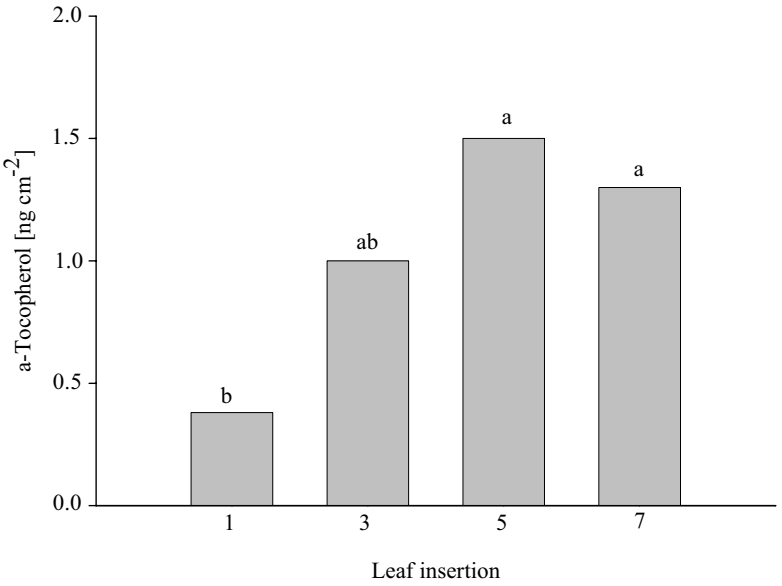

Figure 5: Effect of ontogenesis of apple leaves (cv. Golden Delicious) on the α-tocopherol content in the epicuticular waxes of adaxial apple leaves (n = 5). Columns with different letters are significantly different according to Duncan's multiple range test, $p \leq 0.05$.

4 Discussion

The dynamic process of the leaf development in relation to the surface chemical and physical characteristics was studied by means of apple leaves cultivar Golden Delicious. The plants were grown under controlled and particle-free conditions in order to exclude any environmental factors, which may have an effect on physical and chemical properties of the cuticle. The investigations focused on the effect of ontogenetic development and leaf age.

The adaxial side of apple leaves had an amorphous surface at the SEM level. The arrangement of wax in platelets or as an amorphous structure has an impact on the micro-morphology of leaves and fruits, for example wettability and water repellence. Wettability of cuticles differs depending on the presence of epicuticular waxes, their chemical composition and the micro-structure of waxes. The cuticle of young apple leaves exhibited at the upper side cuticular ridges or wrinkles about 0.8 – 1.0 µm in height, especially above the lumen of epidermal cells. The flattening out and partial disappearance of these wrinkles on older leaves may be attributed to the expansion of cell area. The increase in epidermal leaf area largely depended on the expansion of cells. The cuticle of organs which undergo rapid area expansion may form pronounced folds in juvenile stages as described for grape ovaries (Considine and Knox, 1979; Rosenquist and Morrison, 1988). The folding may increase the surface area of cuticles by a factor of two to three (Jeffree, 1996) and could largely accommodate the expansion of epidermal area during later stages of leaf development. Wrinkles are also supposed to be the sites of transportation and incorporation of new material – waxes and cutin – for cuticle growth.

For about 20 d the area of epidermal cells increased with leaf age, resulting in a flattening of cuticular lamellae as well as of total cells. The cuticle forms only a thin film above the cutin matrix known to increase during leaf expansion and the ontogenetic development of leaves, respectively (Hellmann and Stösser, 1992; Rhee et al., 1998). Hellmann and Stösser (1992) reported that the development of cuticle thickness was faster than leaf expansion and enlargement of epidermal cells reaching 80 % of final thickness within one week as compared to two weeks for final leaf size. All results point to a change in the wax arrangement and not to an increase in the wax amount. During all stages of leaf development the wax mass of adaxial cuticles remained at a low level (10 – 15 µg cm^{-2}), as compared to about 280 µg cm^{-2} (total wax mass) and 76 µg cm^{-2} (epicuticular waxes) for apple leaves in the field (Hellmann, 1992), and only 0.1 – 0.4 % of the amount of apple fruits as reported by Belding et al. (1998). According to calculations by Jetter and Schäffer (2001) this wax yield corresponds to a thickness of 10 – 15 nm. For *P. laurocerasus*, the total amount of chloroform-extracted cuticular waxes differed for the adaxial (280 µg cm^{-2}) and the abaxial (830 µg cm^{-2}) leaf

surface (Jetter and Schäffer, 2001), well above the amounts detected for *M. domestica*. In addition to differences between plant parts, the growth under high relative humidity at ambient temperature without UV light, conditions preventing the induction of cuticular wax production described for various environmental stress conditions (Maier and Post-Beittenmiller, 1998; Jenks et al., 2001; Gordon et al., 1998) are likely to contribute to the overall low wax mass per unit of area.

During ontogenetic development of apple leaves, the leaf area increased and the wax mass per unit of area tended to decrease. This was especially true during early leaf ontogenesis. During later stages of development the expansion of epidermal cell areas converged to zero and the amount of wax per unit of area remained constant. Examining leaves and fruits of citrus, Freeman et al. (1979) described the same phenomenon of expanding leaf area and a corresponding decline in wax concentration per unit of leaf area. They discussed, that a rapid leaf expansion exceeded the rate of wax production.

Under experimental conditions the formation of a new leaf position took about 4 to 5 d, and according to leaf area development and changes in chemical composition of cuticle`s waxes, the maturation of leaves took about 20 to 25 d. For older leaves with growth rates approaching zero, both the wax mass per unit of area and the contact angle remained largely constant. Changes in the total wax mass and the chemical composition of the surface wax layer have been well documented. During ontogenesis of peach leaves the individual wax mass as well as the composition of major components – e.g. triterpenes and alkanes – varied (Bukovac et al., 1979). Changes in chemical composition of surface wax of *Fagus sylvatica* over a three years period demonstrated that the appearance (=synthesis and secretion) and disappearance (=loss) of wax constituents results from dynamic processes and every developmental stage has a specific pattern in the composition of wax compounds (Markstädter, 1994).

This could also apply to cuticles of apple leaves, as the triterpene content amounted to 30 % (leaf insertion 1). Ursolic acid is especially prominent in the leaf and fruit waxes of *Malus* and *Prunus* species (Baker, 1982). However, the role and localization of the triterpenoid acids in the wax layer of cuticles is not clear. In grape berry cuticle, oleanolic acid can constitute up to 60% of total wax mass and seems to be present also in epicuticular waxes to a high percentage, but may be not present in leaf waxes (Comménil et al., 1997; Casado and Heredia, 1999). In *P. laurocerasus*, triterpenoids accounted for a high percentage of intracuticular waxes, but were absent in EWs (Jetter and Schäffer, 2001). In apple leaves from the field, triterpenoids were the prominent constituent of intracuticular waxes, whereas the

content in EWs was minor (Hellmann, 1992). The thin wax layer of apple leaves grown under protected conditions is likely to favour the extraction not only of epicuticular but also of intracuticular wax constituents resulting in the presence of oleanolic acid and ursolic acid in lipid extracts.

The hydrophobic character of the adaxial side of leaf surfaces is caused by the chemical composition of the epicuticular waxes. This correlation is supported by the results demonstrating a high apolarity of alkanes and primary alcohols resulting from long and very long alkyl chains of hydrocarbons in the cuticular waxes. C_{31} alkane was the most prevalent homologue. C_{26} and C_{28} were the predominant chain lengths for alcohols whereas C_{26} was predominant amongst acid for juvenile leaves. Amongst alcohols, the average chain lengths increased with leaf ontogenesis, while absolute amounts of alcohols remained largely constant with a tendency to increase. The diversification in chain length is a modification of cuticular waxes often reported for the process of leaf maturation (Hellmann, 1992). For apple leaves, the chain length of alkanes increased with leaf ontogenesis. A same effect was detected for the ester fraction; the C_{40} : C_{52} ratio was approximately 1 : 1.1 for the youngest leaf, and changed to 1 : 5 for the oldest one. C_{48} ester was the predominant compound.

Hydrophobicity of upper leaf surfaces decreased during the ontogenetic development of apple leaves. The increase in hydrophily was associated with a decrease in the total amount of extractable surface waxes as well as with modifications in the composition of wax compounds. Non-regarding differences in chain length, the content of alcohols significantly increased with leaf age, while triterpenes decreased. The accumulation of the OH – functional group plays an important role concerning the wettability; during the ontogenesis the leaf surface becomes more wettable. Hellmann (1992) studied the surface wax of different varieties of *M. domestica* depending on age and variety in field experiments. Leaf age had no effect on total wax mass, the proportion of alkanes and esters decreased during leaf ontogenesis, while primary alcohols increased. For grape berry, cuticular waxes became progressively enriched in wax esters and hydrocarbons from bloom to veraison, while the proportion of primary alcohols steadily decreased during fruit development (Comménil et al., 1997). These results point to diametrically opposed developments in the modification of cuticles from leaves and fruits, respectively, during ontogenesis.

The α-tocopherol concentration of upper epicuticular waxes of apple leaves was 0.5 up to 1.5 ng cm^{-2}, equivalent to about 130 - 400 µg g^{-1} leaf dry weight, compared to < 1 µg g^{-1} to > 1 mg g^{-1} dry weight for the tocopherol content of plant tissue according to Munné-Bosch and Alegre (2002). The α-tocopherol content increased during ontogenesis of leaves, very similar to the significant increase in the intracellular α-tocopherol content associated with aging of

plants reported for other species (Rise et al., 1989; Molina-Torres and Martinez, 1991; Tramontano et al., 1992). Gülz et al. (1992) reported high amounts of γ-tocopherol in the cuticle of *Gingko biloba* leaves. Shepherd et al. (1999) detected high levels of δ- and γ-tocopherol, and additionally low levels of α-tocopherol in the wax layer of red raspberry (*Rubus idaeus* L.). While γ-tocopherol is predominantly found in seeds, α-tocopherol is the main tocopherol in leaves (Bramley et al., 2000; Franzen and Haas, 1991; Shintani and DellaPenna, 1998; Sircelj et al., 2005).

The role of extracellular α-tocopherol in the epicuticular wax of apple leaves, described here for the first time, may be the protection of biomolecules from peroxidation processes incited by singlet oxygen radicals, thus maintaining the chemical composition and the physical function of epicuticular wax films. Herbicide stress of apple leaves induced by paraquat causing the formation of radicals, was significantly reduced by a treatment with α-tocopherol (Schmitz-Eiberger and Noga, 2001). A formation of radicals by unsaturated fatty acids having a long chain length could be assumed. In apple leaves subjected to drought stress, increased levels of α-tocopherol were reported to be involved in the adaptation to oxidative stress (Sircelj et al., 2005). According to Holloway (1970) damages in superficial waxes are the cause of the increased wettability of older leaves, however, this factor, which may be of great impact under field conditions, could not be held responsible in our experiments. In addition to modifications in chemical composition due to tissue age, changes in hydrophobicity of plant surfaces in the field may result from environmental conditions altering wax composition, mechanical strain or injury, and the colonization of surfaces by saprophytic and pathogenic bacteria and fungi which alter physical properties of the surface towards hydrophily (Knoll and Schreiber, 2000; Schreiber et al., 2004). All these factors were largely excluded in our experiments demonstrating that changes in the physical properties during ontogenetic development were associated with modifications in the chemical composition of cuticular waxes. Nevertheless, it seems very likely, that ontogenetic modifications are superimposed by modifications due to environmental factors. The impact of individual compounds and chemical groups for structure and function of the cuticle have to be elucidated in further experiments in more detail.

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C The chemical surface wax composition, morphology and wettability of apple leaves as affected by water deficit and ultraviolet radiation

1 Introduction

The aerial organs are covered by the cuticle - having crucial autoecological functions - with incubent epicuticular waxes and embedded intracuticular waxes. Epicuticular waxes of leaves contribute to functions such as conservation of water, minimization of leaching losses, and morever protection from injury due to various environmental factors (Johnson et al., 1983). The cuticle is primarily composed by cutin, a polyester matrix of hydroxy and hydroxy epoxy C_{16} and C_{18} fatty acids (Kolattukudy, 1996), which is overlaid and embedded with long chain hydrocarbon waxes such as primary and secondary alcohols, aldehydes, alkanes, ketones and fatty acids (Kunst and Samuels, 2003). Leaf surface wax contributes to the ability of a plant to reduce evaporation water loss and is particularly important when stomata are closed in response to reduced turgor (Weete et al., 1978). This epicuticular waxes are covering aerial surfaces of higher plants, mainly leaves and fruits (Hemmers and Gulz, 1986) and being part of the cuticle.

The survival of plants subjected to severe environmental factors like e.g. water stress and enhanced UV-B radiation depends on the ability of their aerial organs to prevent harmful adversity. Several studies dealed with the influence of environmental factors on particular plant properties like transpiration, surface wax composition, and cuticle composition (Bondada et al., 1996; Oosterhuis et al., 1991; Geyer and Schönherr, 1990; Latimer and Severson, 1997). The epicuticular wax load may contribute to drought tolerance of peanut in two ways: first because of the high initial level of wax the loss of water remains low because of epicuticular transpiration even under drought conditions; second plants having low epicuticular wax load enhance their epicuticular wax load caused by soil water deficit (Samdur et al., 2003). In contrast many investigations evidenced that thickness of leaf wax deposits had little influence on cuticular transpiration (Riederer and Schreiber, 2001), although increases of epicuticular waxes on leaves of plants under hydric stress have been observed (Bondada et al., 1996).

Besides water supply, enhanced UV-B radiation (280-320 nm) was assayed in terms of their influence on chemical wax composition. Depending on pea line (*Pisum sativum*), UV-B radiation caused increased total adaxial wax mass of leaves, moreover a shift in chemical wax compostion from alcohols to esters and hydrocarbons (Gonzalez et al., 1996). The influence of UV-B radiation is a complex process depending on other factors such as UV-B dose, species and age of plant.

As water deficit and UV-B radiation have an impact on physicochemical characteristics of adaxial apple leaf surfaces, we studied the influence of these two environmental factors (on the one hand enhanced UV-B radiation and on the other hand induced water deficit) on adaxial apple leaves of *M. domestica* Borkh. Interactions between this factors we tested as well. The experiments were conducted at three different sampling times after UV-B pretreatment.

2 Material and Methods

2.1 Plant material and growth conditions

Seeds of *M. domestica* Borkh. were sown in square pots (70 mm x 70 mm x 65 mm; Pöppelmann, Germany) using substrate with 3 parts loam and 1 part sand. Plants were raised in a growth chamber at constant temperature of 20 °C ± 1 °C, a relative humidity of 70 % ± 5 %, fertilized as to their needs. Water supply amounted to 5 ml d^{-1}. PAR (photosynthetically active radiation) was provided at a plant level for 16 h with 180 µmol s^{-1} m^{-2}. After 6 weeks the seedlings were used for the experiments.

The induction of water deficit was achieved by supplying 5 ml water every third day for a time of two weeks, whereas 5 ml were provided to control plants continously. The missing turgescence was assessed as symptoms of water stress. The dry weight as well fresh weight were measured with the scale BP210S (‘Sartorius’, Göttingen, Germany).

2.2 UV-B exposure

Waterstress induced seedlings as well as normal watered seedlings were taken for the irradiation with enhanced UV-B radiation. The seedlings were arranged having enough distance from neighbour plants preventing shade. UV-B radiation was provided by 9x100 W tubes of UV-B lamps (‘Philips’, Germany), having an emission spectrum of 280-320 nm, controlling the level of irradiation by a precalibrated spectroradiometer (‘Gröbel’, Germany). The dose of irradiance amounted to 0.022 kW m^{-2} applied for 150 min under room temperature of 20 °C ± 1 °C. Leaves were sampled 0 h, 24 h and 48 h after exposure to enhanced UV-B. Plants not exposed to UV-B served as control.

2.3 Wax extraction

The whole adaxial leaf surface of the second completely developed leaf (apical) was placed onto chloroform ($CHCl_3$, purity>99 %) for 20 s at room temperature. During preliminary studies the extraction time was between 5 and 20 s, and time of 20 s proved to be the optimal dipping duration to ensure that the majority of surface cuticular waxes were extracted without co-extraction of contaminating internal lipid compounds. The resulting extract was spiked with 20 µl of C_{24} alkane (199.72 mg l^{-1}), Tetracosane (internal standard) for quantifying individual wax compounds. The samples were evaporated under a stream of nitrogen. Subsequently 20 µl of pyridine (Merck, Darmstadt, Germany) and 20 µl BSTFA [(N,O-bis (trimethylsilyl) trifluoracetamide), Machery-Nagel, Düren, Germany] were added, and the samples were incubated for 40 min at 70 °C. Prior to analysis, the reaction mixture was diluted with 50 µl of chloroform after cooling down to room temperature. The cuticular wax compounds were quantified by GC-FID (5890 series II; HP, Avondale, PA) with on-column injection and applying high resolution gas chromatography column (Agilent Technologies, 30 m x 0.321 mm DB-1, phase thickness 0.1 µm, J&W, Folsom, CA). The temperature program was as followed: start at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to 200°C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, then 30 min at 320 °C. The carrier gas was hydrogen. The pressure program was: injection at 50 kPa, 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, 39 min at 150 kPa. An identification was accomplished by a combined GC and MS using an identical gas chromatograph equipped with Agilent 5973 N quadrupole mass spectrometer (Agilent Technologies, Böblingen, Germany). Temperature and columns were as described for GC-FID. The carrier gas was helium with a rate of 2 ml min^{-1}. The identification of the wax compounds was carried out from their EI-MS spectra (70eV, *m/z* 50-700) using a house created library.

2.4 Goniometry

The hydrophobicity of adaxial apple leaves was assessed by drop shape analysis (Krüss G10, Hamburg, Germany), cutting a part from the central area of the leaf lamina avoiding middle vein. Leaves were fixed to glass slides by double-sided adhesive tape. The contact angle of an applied drop of destilled water (droplet volume 1 µl) was measured.

2.5 Scanning electron microscopy (SEM)

The micromorphology of the adaxial leaf surface was studied with an environmental scanning electron microscope (XL-30-ESEM, FEI-Philips, Kassel, Germany; Microsoft control

software, version 5.90). Discs (diameter = 0.8 cm) of the adaxial leaf surface were punched out avoiding middle vein and scanned in the environmental mode. The samples were examined according to the different factors: sampling time from 0, 24 and 48 h; UV-B radiation and water deficit. As well for this study the second completely developed leaf (apical) was used.

2.6 Statistics

Conducted experiments studying chemical wax composition were repeated at least twice with three replications. The identified compounds were expressed as μg cm^{-2}. Data were analysed with the software SPSS 12.0 (SPSS Inc., Chicago, USA) for normal distribution and homogeneity of variances. Experiments were carried out in a bifactorial design (water deficit *vs* UV-B radiation) for 0, 24 and 48 h sampling time. After verifying interactions analysis of variance was accomplished and results were compared by Duncan $p \leq 0.05$. Results and graphs were illustrated with SigmaPlot 2001 (SPSS Inc, SigmaPlot, Chicago, USA).

3 Results

3.1 Chemical composition of surface wax

The identified apolar wax compounds were extracted according to standard methods and characterised as: alcohols (C_{26}-C_{30}), alkanes (C_{29}-C_{33}), fatty acids (C_{26}), triterpenes (oleanolic and ursolic acid) and esters (C_{44}-C_{48}), each group having a certain carbon chain length (data not shown). Concerning the total detected wax mass sampling time 0 h and 48 h displayed no interactions between water deficit and UV-B radiation whereas at sampling time of 24 h interactions between the mentioned factors could be shown (Fig. 1.). At closer examination of the single wax groups the following results were shown for sampling time 0 h: independent of an induced water deficit, irradiation caused a decline in the total alcohol and acid wax mass, whereas a slight increase was observed in the mass of triterpenes (Table 1). Induction of water stress independent of irradiation caused a slight but not significantly increase of the amounts of alcohols, fatty acids, and triterpenes mass. Dependent on water status and UV-B radiation, the mass of alkanes increased after UV-B pretreatment in combination with water deficit as well as without UV-B radiation and induction of water deficit (Table 1 C). The mass of esters

increased significantly in water stressed samples with 0.0 and 0.022 kW m^{-2} UV-B radiation dose (Table 1 C-D).

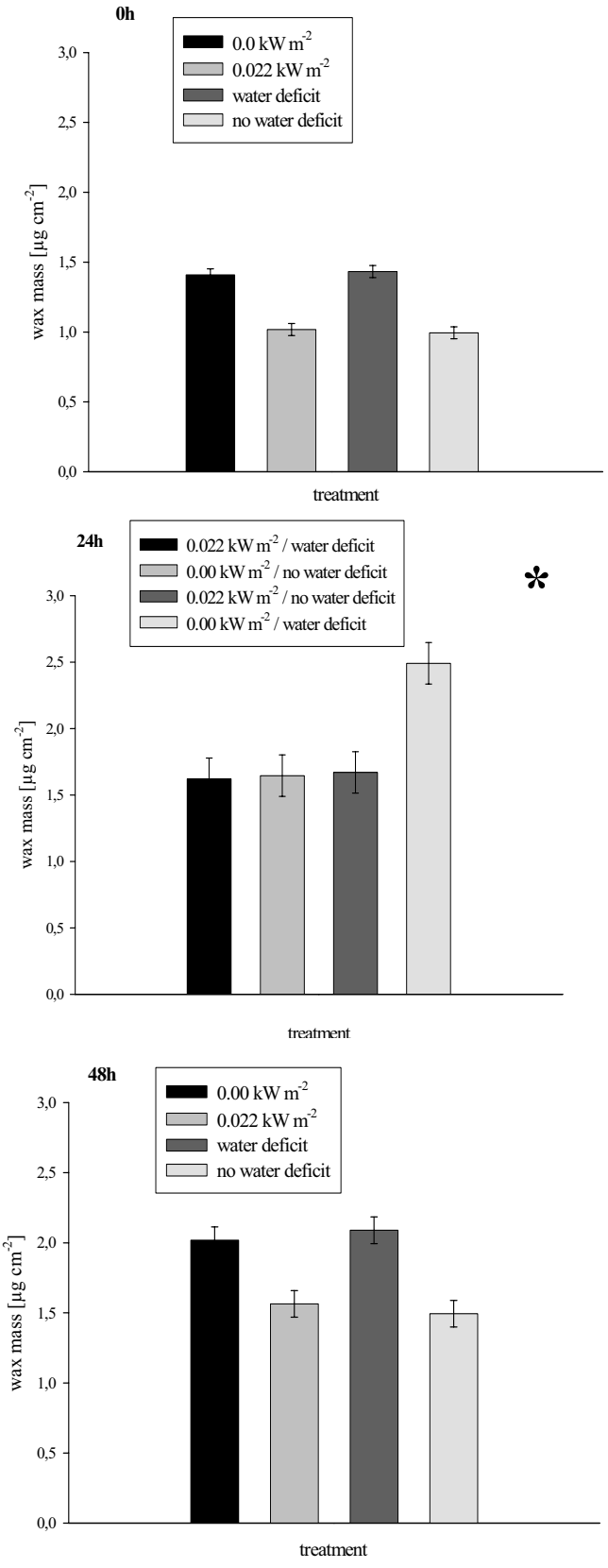

Figure 1: Effect of UV-B radiation [0.00 and 0.022 kW m^{-2}] and water supply [water deficit / normal water supply] on total wax mass of adaxial apple leaves at sampling times 0, 24 and 48 h after UV radiation; n=3, means±standard error. * Significant interaction between factors.

Table 1: Wax mass [µg cm^{-2}] of single wax groups after irradiation with UV-B [0.00 kW m^{-2}; 0.022 kW m^{-2} during 150 min] and water deficit; sampling time of 0 h; n=3, mean±standard error.

A	Wax mass after UV-B radiation [µg cm^{-2}]	
Compound	UV-B 0.00 kW m^{-2}	UV-B 0.022 kW m^{-2}
Alcohols (C_{26}-C_{30})	0.843±0.038	0.270±0.038
Fatty acids (C_{26})	0.090±0.007	0.038±0.007
Triterpenes	0.184±0.011	0.206±0.011

B	Wax mass after induce of water [µg cm^{-2}]	
Compound	No water deficit	Water deficit
Alcohols (C_{26}-C_{30})	0.516±0.038	0.596±0.038
Fatty acids (C_{26})	0.042±0.007	0.086±0.007
Triterpenes	0.156±0.011	0.234±0.011

C	Wax mass after 0.00 kW m^{-2} UV-B radiation [µg cm^{-2}]	
Compound	No water deficit	Water deficit
Alkanes (C_{29}-C_{33})	0.056±0.003	0.061±0.003
Ester (C_{44}-C_{48})	0.185±0.026	0.291±0.026

D	Wax mass after 0.022 kW m^{-2} UV-B radiation [µg cm^{-2}] and dependent on water supply	
Compound	No water deficit	Water deficit
Alkanes (C_{29}-C_{33})	0.049±0.003	0.134±0.003
Ester (C_{44}-C_{48})	0.254±0.026	0.477±0.026

Table 2: Wax mass [µg cm^{-2}] of single wax groups after an irradiation with UV-B [0.00 kW m^{-2}; 0.022 kW m^{-2} during 150 min] and water deficit; sampling time of 24h; n=3, mean±standard error.

A	Wax mass after UV-B radiation [µg cm^{-2}]	
Compound	UV-B 0.00 kW m^{-2}	UV-B 0.022 kW m^{-2}
Alkanes (C_{29}-C_{33})	0.070±0.006	0.093±0.006
Triterpenes	0.266±0.041	0.440±0.041
Ester (C_{44}-C_{48})	0.589±0.056	0.556±0.056

B	Wax mass after induce of water deficit [µg cm^{-2}]	
Compound	No water deficit	Water deficit
Alkanes (C_{29}-C_{33})	0.060±0.006	0.103±0.006
Triterpenes	0.419±0.041	0.287±0.041
Ester (C_{44}-C_{48})	0.552±0.056	0.593±0.056

C	Wax mass after 0.00 kW m^{-2} UV-B radiation [µg cm^{-2}]	
Compound	No water deficit	Water deficit
Alcohols (C_{26}-C_{30})	0.700±0.067	1.421±0.067
Fatty acid (C_{26})	0.076±0.013	0.146±0.013

D	Wax mass after 0.022 kW m^{-2} UV-B radiation [µg cm^{-2}] and dependent on water supply	
Compound	No water deficit	Water deficit
Alcohols (C_{26}-C_{30})	0.377±0.067	0.328±0.067
Fatty acid (C_{26})	0.097±0.013	0.311±0.013

Table 3: Wax mass [µg cm^{-2}] of single wax groups after an irradiation with UV-B [0.00 kW m^{-2}; 0.022 kW m^{-2} during 150 min] and water deficit; sampling time of 48 h; n=3, mean±standard error.

A	Wax mass after UV-B radiation [µg cm^{-2}]	
Compound	UV-B 0.00 kW m^{-2}	UV-B 0.022 kW m^{-2}
Fatty acid (C_{26})	0.160±0.018	0.140±0.018

B	Wax mass after induce of water deficit[µg cm^{-2}]	
Compound	No water deficit	Water deficit
Fatty acid (C_{26})	0.133±0.018	0.168±0.018

C	Wax mass after 0.00 kW m^{-2} UV-B radiation [µg cm^{-2}]	
Compound	No water deficit	Water deficit
Alcohols (C_{26}-C_{30})	0.967±0.102	1.489±0.102
Alkanes (C_{29}-C_{33})	0.104±0.012	0.070±0.012
Triterpenes	0.113±0.047	0.145±0.047
Ester (C_{44}-C_{48})	0.375±0.047	0.453±0.047

D	Wax mass after 0.022 kW m^{-2} UV-B radiation [µgcm^2] and dependent on water supply	
Compound	No water deficit	Water deficit
Alcohols (C_{26}-C_{30})	0.549±0.102	0.400±0.102
Alkanes (C_{29}-C_{33})	0.054±0.012	0.084±0.012
Triterpenes	0.294±0.047	0.502±0.047
Ester (C_{44}-C_{48})	0.311±0.047	0.699±0.047

At sampling time of 24 h: irradiation caused a significant increase of alkanes and triterpenes independent of water status, whereas esters did not change (Table 2 A). Independent of radiation with UV-B a decrease of water supply caused a significant rise in alkane content, whereas the mass per unit of area of triterpenes decreased (Table 2 B): mass of esters stayed at the same level. The group of alcohols and acids could not be considered independently because of interactions between UV-B radiation and water supply (Table 2 C-D). Here the highest amount of alcohols was measured in the treatment of 0.00 kW m^{-2} UV-B and water deficit (1.421 µg cm^{-2}) (Table 2 C). Fatty acids revealed the highest amount in the matter of water deficit without UV-B pretreatment (0.146 µg cm^{-2}): this mass was only outnumbered by combination treatment of UV-B and water deficit, amounting to 0.311 µg cm^{-2} (Table 2 C-D). At a sampling time of 48 h only the acids did not show any interaction between the mentioned factors, they decreased after UV-B pretreatment (Table 3 A), whereas a drought caused a rise in fatty acids (Table 3 B). The mass of alcoholes, triterpenes, alkanes and esters depended on both factors (Table 3 C-D). An increase in mass of alcohols, triterpenes and esters was observed in not UV-B treated seedlings and water deficit, whereas the mass of alkanes decreased (Table 3 C); comparing UV-B radiated leaves, only mass of alcohols decreased after water deficit, alkanes, triterpenes and esters increased (Table 3 D).

At a closer examination on masses (wax mass per area) of single wax compounds, a significant interaction between water supply and UV-B radiation did not exist in all components and during all sampling times (Fig. 2). At sampling time of 0 h C_{31} alkane, oleanolic and ursolic acid did not change after enhanced UV-B radiation, while C_{26} alcohol and C_{26} acid decreased in contrast to the increasing mass of C_{30} alcohol. The consequence of a shortage of water was an increase in amount of C_{26} acid, C_{31} alkane and ursolic acid. C_{26} and C_{30} alcohol did not change significantly, detailed informations are presented in Table 4.

Recapitulating, sampling times of 0 and 48 h, the impact of water supply and UV-B radiation are equal concerning total detected wax mass; 24 h revealed an interaction.

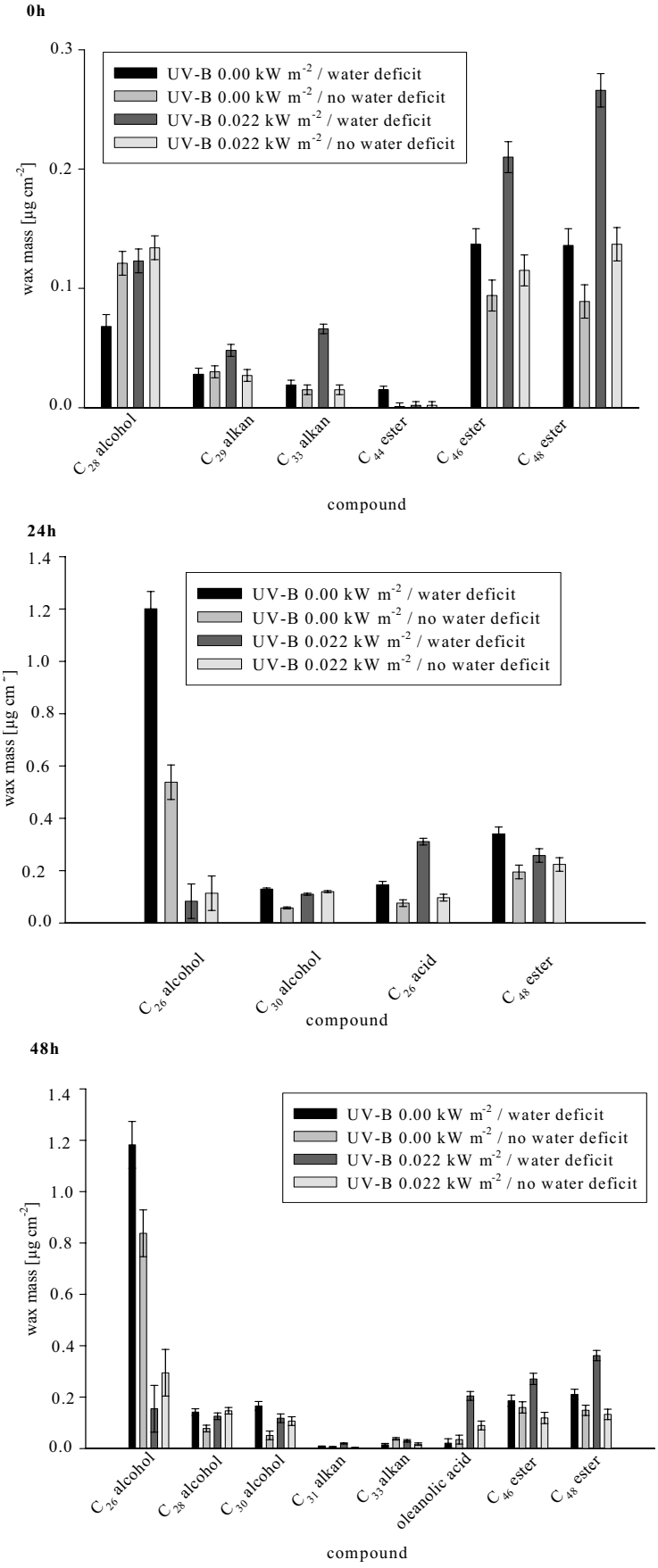

Figure 2: Effect of UV-B radiation (0.022 kW m^{-2} during 150 min) and water deficit on wax mass of single wax compounds of adaxial apple *M. domestica* Borkh. leaf surface at sampling times of 0, 24 and 48 h after UV-B pretreatment; n=3, mean±standard error.

Table 4: Effect of UV-B radiation[1] [0.022 kW m^{-2} during 150 min] and water supply[2] [water deficit -, or no water deficit +] on total detected wax mass of single wax compounds [µg cm^2], no significant interaction between [1] and [2] , n=3, mean±standard error. 0, 24 and 48 h are displaying sampling times.

0h	UV-B radiation for 150min		Water supply	
Compound	0.00 kW m^{-2}	0.022 kW m^{-2}	+	-
C_{26} alcohol	0.677±0.04	0.035±0.04	0.312±0.04	0.400±0.04
C_{30} alcohol	0.085±0.01	0.105±0.01	0.096±0.01	0.094±0.01
C_{26} acid	0.090±0.01	0.038±0.01	0.043±0.01	0.086±0.01
C_{31} alkane	0.012±0.00	0.003±0.00	0.007±0.00	0.017±0.00
Oleanolic acid	0.039±0.00	0.042±0.00	0.044±0.00	0.038±0.00
Ursolic acid	0.145±0.01	0.163±0.01	0.112±0.01	0.195±0.01

24h	UV-B radiation for 150min		Water supply	
Compound	0.00 kW m^{-2}	0.022 kW m^{-2}	+	-
C_{28} alcohol	0.097±0.01	0.139±0.01	0.124±0.01	0.113±0.01
C_{29} alkane	0.038±0.01	0.050±0.01	0.046±0.01	0.042±0.01
C_{31} alkane	0.008±0.00	0.010±0.00	0.002±0.00	0.012±0.00
C_{33} alkane	0.025±0.00	0.037±0.00	0.012±0.00	0.050±0.00
Oleanolic acid	0.045±0.02	0.146±0.02	0.122±0.02	0.069±0.02
Ursolic acid	0.221±0.03	0.295±0.03	0.299±0.03	0.217±0.03
C_{44} ester	0.105±0.02	0.097±0.02	0.121±0.02	0.082±0.02
C_{46} ester	0.216±0.02	0.217±0.02	0.222±0.02	0.211±0.02

48h	UV-B radiation for 150min		Water supply	
Compound	0.00 kW m^{-2}	0.022 kW m^{-2}	+	-
C_{26} acid	0.160±0.02	0.140±0.02	0.133±0.02	0.168±0.02
C_{29} alkane	0.053±0.01	0.034±0.01	0.046±0.01	0.040±0.01
Ursolic acid	0.101±0.03	0.228±0.03	0.118±0.03	0.210±0.03
C_{44} ester	0.061±0.01	0.063±0.01	0.063±0.01	0.061±0.01

3.2 Goniometry

The contact angle of applied water on surface of *M. domestica* Borkh. leaves ranged between 98 and 120 °. By univariate analyse of variance, significant interactions at sampling times of 0, 24 and 48 h between the studied factors water supply and UV-B radiation were shown. At sampling time of 0 h not irradiated and grown under water deficit plants exhibited 99 °, being the lowest contact angle among all variances, whereas the highest amounted to 119 ° (UV-B radiated and water deficit) – an increase could be shown. (Table 5). At sampling time 24 h remote shift from 103 ° up to 109 ° (not exposed to UV-B and water deficit; not exposed to UV-B and no water deficit, respectively) could be ascertained. Although a significant

interaction among evaluated factors existed, the differences in the contact angle were moderate. The level of contact angle after 48 h stayed at a high level of 115 ° – 120 °.

Table 5: Effect of enhanced UV-B radiation [0.022 kW m^{-2} during 150 min] and water supply [water deficit] on contact angle of applied water droplet (1 µl), significant nteraction. n= 10, mean±standard error. Different sampling times after UV-B pretreatment; A: 0 h; B: 24 h; C: 48 h.

A	0.00 kW m^{-2} / water deficit	0.00 kW m^{-2} / no water deficit	0.022 kW m^{-2} / water deficit	0.022 kW m^{-2} / no water deficit
Contact angle [°]	99±2	103±2	119±3	113±3

B	0.00 kW m^{-2} / water deficit	0.00 kW m^{-2} / no water deficit	0.022 kW m^{-2} / water deficit	0.022 kW m^{-2} / no water deficit
Contact angle [°]	103±2	109±2	105±2	108±2

C	0.00 kW m^{-2} / water deficit	0.00 kW m^{-2} / no water deficit	0.022 kW m^{-2} / water deficit	0.022 kW m^{-2} / no water deficit
Contact angle [°]	115±3	119±2	120±3	118±2

3.3 Surface wax morphology

Selected results are shown in Fig. 3 A-D – sampling time of 0 h. The micromorphology of the epicuticular wax on the adaxial side of leaf blade was investigated by SEM. The astomatous surface of apple leaves was made up by undulated epidermal cells with irregular polygonal outlines, adaxial wax layer was present as an amorphous film. No platelets were detected above this amorphous layer. Under high magnification of 500x, well watered samples and non-irradiated samples displayed a high density of lamellae, orientated on every single cell layer (Fig. 3 A), whereas irradiated leaves appeared smoother and and reaped (Fig. 3 B). The same observation concerning a flattening of the cuticle could be made in water deficit and UV-B treated samples (Fig. 3 D).

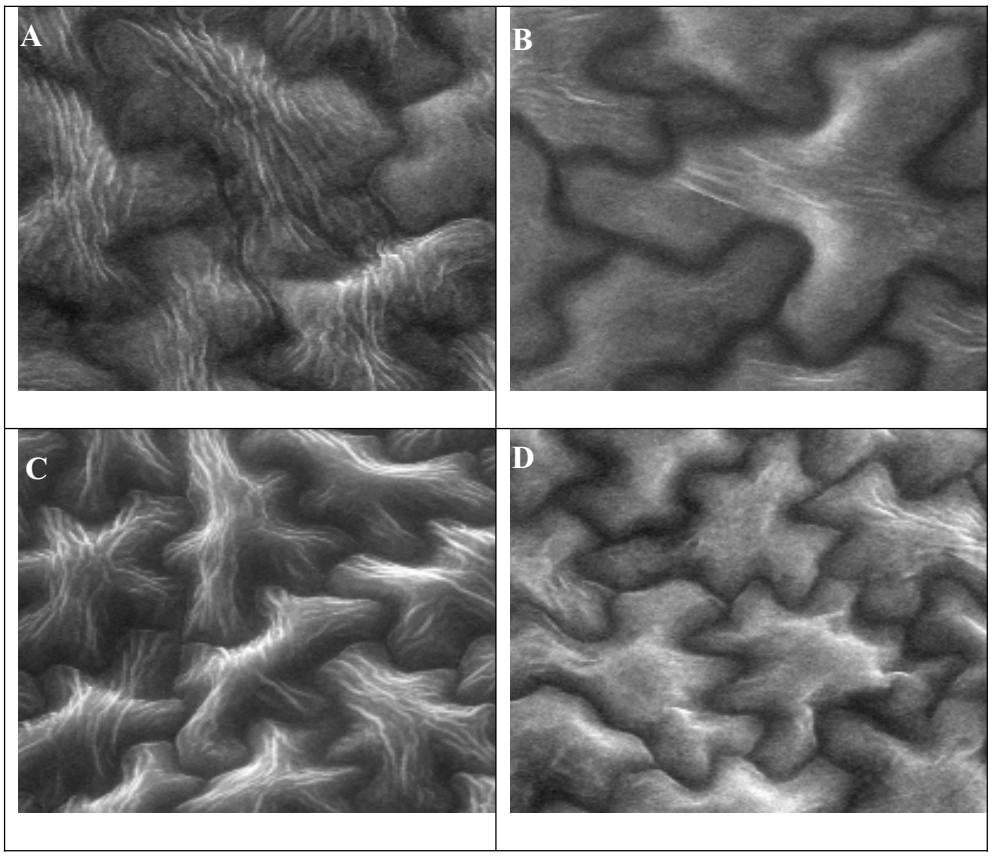

Figure 3: Electron scanning micrographs of adaxial apple leaf surface (*M. domestica* Borkh.). Sampling time 0 h; Detector: Gaseous Secundary Electron; Spot Size: 3.9; Magnification: 500x; A: no water deficit, no UV-B radiation; B: no water deficit, UV-B raditation [0.022 kW m^{-2} during150 min]; C: water deficit, no UV-B radiation; D: water deficit, UV-B raditation [0.022 kW m^{-2} during 150 min].

Water supply had no significant effects on the microphorphological structure of the cuticle. Fig. 3 C demonstrated adaxial apple leaf surface grown under water deficit and non-irradiated; having distinctive curvature of cells and cuticular wrinkles, consistent arranged over cells.

4 Discussion

4.1 Surface wax chemistry

The composition of leaf surface wax layer was found to be organ and species-dependent, and to vary with age, light conditions, season and different growth terms (Prasad and Gülz, 1990). Our study addressed physicochemical surface characteristics of adaxial apple leaf surface (*M. domestica* Borkh.) after being influenced by two changed environmental factors. Field-grown plants are exposed to a multitude of environmental factors interacting in their impact on terrestrial existence. Two of these factors - UV-B radiation and drought stress - were in the

focus of our study because they are of great importance for plant growth, metabolism, development, and for physicochemical surface characteristics and in this connection possible consequences on e.g. foliar-applied chemicals (Holloway, 1969; Hunsche et al., 2006; Bringe et al., 2005). It has been shown that epicuticular waxes are crucial for plant photoprotection (Barnes and Cardoso-Vilhena, 1996).

Samples were studied 0, 24 and 48 h after UV-irradiation in order to take a closer glance on possible dynamic effects after radiation on leaf surface. So far impairment of two stressores in terms of their possible disturbance on apple leaf surface characteristics - as well as physical as chemical - have not been studied thoroughfully.

Water as a pre-requisite for plant growth and development can influence plant growth and development by deficit as well as by affluence. During stomatal closure cuticular water loss is predominant (Hall and Jones, 1961). Former studies on the effets of water deficit revealed an increase in wax load in *Sorghum bicolor* (Premachandra et al., 1992). Tree tobacco as a drought–tolerant species, has a surface wax being remarkable simple in its chemical composition The plant responds to dehydration stress by dramatically up-regulating wax production (Cameron et al., 2006). Two week drying period caused an increase in apple leaf wax load of 30 % (not exposed to UV-B) noticed for 0 and 48 h. After 24 h there was a statistical significant interaction between UV-B and water deficit on wax mass. Not only the wax load, but also the thickness of the cuticle plays a crucial role in avoiding dehydration. De Lucia and Berelyn (1983) reported an inverse relationship between cuticle thickness and rate of cuticular transpiration. Riederer and Schreiber (2001) provided evidence that the amount of cuticular wax and cuticle permeance for water do not correlate. For 23 plant species with cuticular wax layers ranging in thickness between 0.1 and 5 µm no correlation was found between cuticular wax thickness and permeability of the cuticle. Weete et al. (1978) studied cotton leaves (*Gossypium hirsutum* L.`Deltapine`) under water deficit, where wax synthesis was inhibited by water stress. Ristic and Jenks (2002) studied two maize lines and found the more drought tolerant line having a cuticle being thicker by 48 % compared to a water stress sensitive maize line. Here as well, wax mass between this two lines differed, and in some case in chemical composition. There was more dotriacontanol (C_{32} primary alcohol) in the water stress sensitive line. Synthesis of the major components occurs in epidermal cells beginning with chain elongation of preformed fatty acids (C_{16}). In contrast to that, after 0 h fatty acids were doubled per leaf area grown under water deficit independent of UV-B radiation. This suggests that either synthesis of fatty acid was advanced or the excretion of surface wax compounds, confirming the fact of an increased wax mass per leaf area under water deficit.

Gordon et al. (1998) studied *Picea* species and did not ascertain any significant change in epicuticular wax production, when exposed to UV-B, suggesting that *de novo* wax synthesis might not have been a primary target of UV-B radiation, but in some species the chemical composition altered, indicating that the radiation influences specific enzymes involved in wax biosynthesis. The variant 48 h reveals having more wax during drought stress than having in relation to the surface wax load after 0 h, illustrating the possible adjustment of plants to decreased water supply. Besides, mass of fatty acid increased by about 20 %.. The present datas (sampling time 0 h) did not show any variation concerning alcohol mass between the water deficit and the control treatment, whereas triterpenes increased by about 30 % under reduced water supply. As opposed to 24 h variant, triterpenes were reduced by about 30 %, concerning the group of fatty acids and alcoholes as well as UV-B radiation as water supply did execute influence on their mass per leaf area. Oliveira et al. (2003) showed that the increase in wax mass per unit of leaf area (species of caatinga and cerrado) did not reduce water permeability significantly. Esters were hypothesised as being of importance for reaction to water deficit.

With proceeding plant development and increasing time interval after the UV-B radiation, water supply and radiation interact stronger compared to the immediate study of chemical composition of surface wax layer. A precise statement according the effect basing on present study can not be made.

Weete et al. (1978) suggested the same and concluded a feasible resistance to cuticular transpiration. The increased wax mass is not due to ontogenesis. In our studies on changes of surface wax mass per unit area in terms of ontogenetic development, we stated a decreased wax mass with increasing leaf growth (Bringe et al., 2006). Contrary to expectations wax mass and water loss did not correlate positively, the line having more wax per unit leaf area had a higher rate of epidermal water loss, showing that relationship between amount of cuticular wax and water loss might be very complex. Since 1990, the depletion of the stratosphere ozone layer due to anthropogenic and natural destruction led and is leading to increasing levels of solar UV-B radiation reaching the surface of the earth (Shiu and Lee, 2005). Amount and intensity of increased UV-B radiation, because of stratospheric O_3 layer depletion, depend upon atmospheric and geographic factors (Madronich et al., 1998) and upon plant species.

The role of epicuticular wax load in impeding cuticular water loss is very complex. Besides the quantity of wax play a role, moreover the chemical composition and physical micromorphology influence cuticular water loss (Hadley, 1981).

4.2 Goniometry and surface wax morphology

Micromorphological properties of adaxial apple leaf surface are of descisive relevance for its wettability and thus the retention af applied fungicides. Both investigated factors did not significantly influence the contact angle. Time lag after UV-B irradiation influenced the contact angle to a greater extent than the tested environmental factors.

Nothing could be stated concerning surface topology, influencing wettability as well (Wagner et al., 2003; Yoshimitsu et al., 2002). A decrease in wettability can be caused by an increased wax mass. Koch et al. (2006) stated that an increase in wettability of *Brassica oleracea* leaves could be caused by a reduced amount of wax. Increased contact angle in our results could not be explained with changed crystal density, as no crystals were formed on the adaxial apple leaf surface. Compensatory effect of both factors could be assumed.

This study revealed an interaction between enhanced UV-B radiation and water deficit pertained apple surface characteristics, studied for the first time. The complexity of environmental factors influencing terrestrial existence should not be underestimated, because especially cuticle as a primary target surface serves as a protection layer and plays a decisive role within application processes. A successful application of pesticides in terms of effective plant protection depends on several factors like chemical and physical surface characteristics of the target as well as on characteristics of the solution, for instance an increase.

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D Retention and rainfastness of mancozeb as affected by physicochemical characteristics of adaxial apple leaf surface after enhanced UV-B radiation

1 Introduction

Leaves and especially the cuticle as the outermost surface serve as a primary target and absorbing organ for foliar-applied plant growth substances and pesticides (Bukovac et al., 1973; 1976; Kirkwood, 1972). The cuticle consists of a polymeric cutin matrix, cuticular layer and soluble cuticular wax (Schönherr and Baur, 1996; Jeffree, 1996). From the chemical point of view cuticular wax is a highly complex mixture of homologous series of long chain aliphatics as well as varying proportions of cyclic compounds (Riederer and Markstädter, 1996). From the physical point of view the cuticle is characterised by a more or less rough conformation, including crystalline aggregates of epicuticular wax, whereas intracuticular wax is embedded in the polymer matrix (Barthlott, 1990). Chemical composition as well as micromorphology of surface waxes are dynamic during the whole life cycle of a plant, and may be influenced by anthropogenic as well as by ontogenetic factors and also affected by the environment (Hauke and Schreiber, 1998). The chemical composition of *Prunus laurocerasus* leaf surface was studied and it has been noted that chain length distribution of epicuticular wax compounds changed significantly during leaf development, e.g. increasing for alcohols and fatty acids. Particularly during the early stages epicuticular waxes can be regenerated, which can be a critical factor for pesticide application (Jetter and Schäffer, 2001). The ultrastructure and chemistry of leaf surface wax play an important role determining in retention of spray volume on leaf surfaces (Brunskill, 1956; Challen, 1962).

In addition to natural variations in physicochemical characteristics of surface wax, surface fine structure elements are susceptible to physical abrasion due to wind. Moreover, environmental factors such as temperature, rain, and UV-B radiation can alter cuticular waxes. However, waxes may regenerate in some plants within 48 h only (Hall and Jones, 1961; Amsden and Lewins, 1966; Hallam, 1967; Dewey et al., 1956).

It has been documented, that changed chemical wax composition of tobacco (*Nicotiana tabacum* L.) adaxial leaf surface can be influenced by UV-B radiation (e.g. 0, 4.54 and 5.66 kJ m^{-2} for 7 h d^{-1}), however, this seems to be a highly complicated process (Gordon et al., 1998; Barnes et al., 1996).

Deposition, retention and distribution of spray droplets are affected by amount and chemical composition of epicuticular wax and by leaf surface micro-roughness (McWorther, 1993). An adequate wetting of the leaf, which is an essential basis for pesticide efficiency depends on the chemical groups of surface exposed waxes (Fogg, 1947; Challen, 1962; Furmidge, 1962;

Holloway, 1970). Changes in wax chemistry and physical properties may affect wettability and markedly alter spray retention and penetration, as documented for expanding peach leaves (Bukovac et al., 1979). Surface wax quantity correlate positively with contact angle, and highest contact angles generelly coincide with the presence of crystalline wax formation (Stevens and Baker, 1987). For penetration of agrochemicals the surface wax is not the limiting factor concerning transcuticular transport of organic solutes (Armbost et al., 2001). Furthermore it was hypothesised that the cuticle as well as surface waxes play decisive role (e.g. retention, spreading, distribution, and penetration) in the performance of foliar applied chemicals (Brunskill, 1956; Bukovac et al., 1971; Norris and Bukovac, 1972; Watanabe and Yamaguchi, 1991; Neinhuis et al., 1992; Falk, 1994; Kirwood, 1999). The altered nature of leaf surface is too often ignored and the complex relationships oversimplified in determining pesticide application strategies (Baker et al.,1979). Only in case of sufficient retention the applied solution can be effective.

UV-B treatment impact micromorphology as well as chemical characteristics of adaxial apple leaf surface and thereby affects significantly retention and rainfastness of foliar applied agrochemicals. In this study the frequently used fungicide mancozeb was used as a model-pesticide.

2 Material and Methods

2.1 Plant material and growth conditions

Seeds of *M. domestica* Borkh. were sown in square pots (70 mm x 70 mm x 65 mm; Pöppelmann, Lohne, Germany) filled with standard cultivation substrate (loam:sand, 3:1). Plants were raised in a growth chamber at constant temperature of 20 °C ± 1 °C, at a relative humidity of 70 % ± 5 %, fertilized and watered as to their needs. PAR (photosynthetically active radiation) was provided at plant level for 16 h a day with 180 µmol s-1 m^{-2}. After 8 weeks the seedlings were used for the experiments.

2.2 UV-B treatment

UV-B radiation was provided by 9x100 W tubes of UV-B lamps (‘Philips’, Hamburg, Germany) with an emission spectrum of 280 nm – 320 nm. The level of irradiation was controlled by a precalibrated spectroradiometer (‘Gröbel’, Karlsruhe, Germany). The dose of irradiance amounted to 0.022 kW m^{-2} applied for 150 min. The samples were studied 0, 24

and 48 h after UV-B treatment. Plants not exposed to UV-B served as control. However, UVB-irradiation caused a temperature increase of 5 ± 1 °C at plant surface level due to the relatively short plant-lamp distance (25 cm). Therefore temperature has been increased for the control plants accordingly during the treatment.

2.3 Wax extraction

For cuticular wax extraction, the second completely expanded leaf from the top of the plant was removed and the adaxial leaf side placed onto chloroform (purity > 99 %) for 20 s. It was assured that during extraction only the adaxial surface was in contact with chloroform. After adding 20 µl of an internal standard (C_{24} alkane, tetracosane), the sample was evaporated under a stream of nitrogen. Subsequently, 20 µl of pyridine (Merck, Darmstadt, Germany) and 20 µl BSTFA [(N,O-bis (trimethylsilyl) trifluoracetamide), Machery-Nagel, Düren, Germany] were added, and the sample incubated for 40 min at 70 °C according to a standardised method (Hauke and Schreiber, 1998). Subsequently specimens were cooled down to room temperature and analysed after adding 50 µl of chloroform. The measurements were conducted employing GC-MS (5890 series II; HP, Avondale, PA) with on-column injection and a high resolution gas chromatography column (Agilent Technologies, 30 m x 0.321 mm DB-1, phase thickness 0.1 µm, J&W, California, USA). The temperature program was as follows: start at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, then 30 min at 320 °C. The carrier gas was hydrogen. The pressure program was: injection at 50 kPa, 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, 39 min at 150 kPa.
For qualitative GC-MS analysis the same method was used; however, instead of hydrogen, helium was used as carrier gas. The injection volume was 1 µl.

2.4 Microscopy

Leaf discs (diameter = 0.8 cm) were excised out of the leaf and mounted to alumina stubs. The micromorphology of the adaxial leaf surface was studied with an environmental scanning electron microscope (XL-30-ESEM, FEI-Philips, Kassel, Germany; Microsoft control software, version 5.90).

2.5 Goniometry

The hydrophobicity of adaxial apple leaves was assessed by drop shape analysis (Krüss G10, Hamburg, Germany), cutting a part from the central area of the leaf lamina avoiding the

middle vein. Leaves were fixed to glass slides with double-sided adhesive tape. The contact angle of an applied drop of destilled water (volume 1 µl) was measured.

2.6 Fungicide application and rain simulation

Mancozeb [(manganese ethylene bis(dithiocarbamate)(polymeric)] complex with zinc salt] in the commercial formulation Dithane Ultra WG 80 % (Spiess-Urania Chemicals GmbH, Hamburg, Germany) was used for experiments. Fungicide concentration was 2.40 g litre^{-1} a.i., and fungicide was applied with a Laboratory Pesticide Sprayer, equipped with a hollow cone, 80 ° nozzle (Lechler GmbH, Metzingen, Germany) placed 45 cm above the plant level. At a speed of 6 km h^{-1} and a pressure of 3 x 105 Pascal the application was accomplished. The total volume of the applied fungicide solution was equivalent to 390 litres ha^{-1}. Exposure to 5 mm heavy rain (5 mm h^{-1}) was simulated 4 h after application by means of B-LRS-2 rain simulator (Institute of Agricultural Engineering, University of Bonn, Germany). Rain unexposed seedlings served as reference.

Rainfastness of mancozeb was evaluated by measuring manganese concentration (17 % of mancozeb molecular weight) employing Atomic Absorption Spectrometry (Perkin-Elmer Analyst 300 Wellesley, USA). The amount of fungicide residues was related to FW of leaves.

2.7 Statistics

The experiments studying chemical wax composition were repeated at least twice with 4 replications per treatment group. The identified wax compounds were expressed as µg cm^{-2}. Fungicide residues were expressed as µg g^{-1} FW and as % of initial fungicide concentration. The data were analysed with the software SPSS 12.0 (SPSS Inc., Chicago, USA) for normal distribution and homogeneity of variances. Experiments were carried out in a bifactorial design (sampling time vs. UV-B radiation). After verifying interactions analysis of variance was accomplished and results were compared by Duncan $p \leq 0.05$. The results and graphs were illustrated with SigmaPlot 2001 (SPSS Inc, SigmaPlot, Chicago, USA).

3 Results

3.1 Chemical and physical characteristics of adaxial leaf surface

Standard methods were used for extraction and analysis of the chemical surface wax composition of *M. domestica* (Hauke and Schreiber, 1998). The apolar wax compounds,

extracted from the adaxial leaf surface, were identified as: triterpenes (oleanolic acid, ursolic acid), primary alcohols, fatty acids, alkanes and esters, each chemical group characterized by a certain chain length ranging from C_{22} – C_{26} (fatty acids), C_{24} – C_{30} (primary alcohols), C_{29} – C_{31} (alkanes) and C_{48} - C_{52} (esters).

No significant interaction between sampling time and UV-B radiation could be established; main effects for sampling time are presented in Fig. 1. The total wax mass ranged from 0.38 µg cm^{-2} (0 h) up to 0.49 µg cm^{-2} (24 h) and decreased after 48 h down to 0.44 µg cm^{-2}.

When combining individual wax components to the corresponding chemical group, interactions between sampling time and UV-B radiation could only be established for alkanes (Table 1), whereas primary alcohols, fatty acids, triterpenes and esters did not show any interaction.

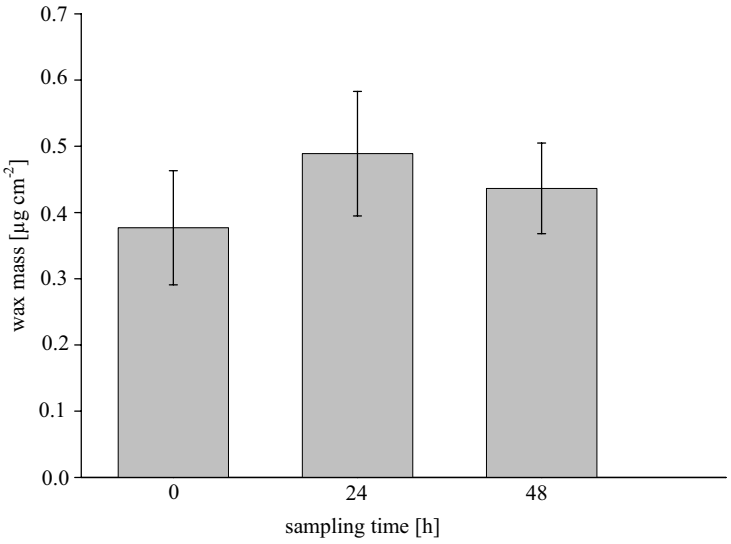


Figure 1: Effect of sampling time (0, 24 and 48 h) and exposition to UV-B radiation (0.022 kW m^{-2} for 150 min) on total detected wax mass of adaxial apple leaf surface (*M. domestica*). n=4, mean ± standard error.

However, individual components, such as C_{31} alkan, oleanolic acid and C_{48} ester, revealed significant interactions (Table 1). Twenty-four hours after UV-B treatment, C_{31} alkan tended to increase from 0.048 µg cm^{-2} up to 0.067 µg cm^{-2}; however, thereafter a significant decrease to a distinctly lower wax level was noted (0.030 µg cm^{-2}). In contrast non-irradiated plants C_{31} alkan wax mass differed significantly at 24 h and 48 h sampling time. Oleanolic acid increased significantly between 0 h and 24 h, but 48 h after UV-B radiation a decrease was noted.

Table 1: Main effects for UV-B radiation and sampling time on wax mass of alkanes and single wax compounds [µg cm^{-2}], n=4,means±standard error, ns=not signifcant by ANOVA.

	UV-B 0 kW m^{-2}			UV-B 0.022 kW m^{-2}		
Compound	Sampling time			Sampling time		
	0	24	48	0	24	48
Alkanes	0.039^{b} ±0.014	0.047^{b} ±0.014	0.088^{a} ±0.014	0.069^{a} ±0.014	0.083^{a} ±0.014	0.040^{b} ±0.014
C_{31} alkan	0.031^{b} ±0.012	0.033^{b} ±0.012	0.070^{a} ±0.012	0.048^{ab} ±0.012	0.067^{a} ±0.012	0.030^{b} ±0.012
Oleanolic acid	0.033^{b} ±0.010	0.080^{a} ±0.010	0.036^{b} ±0.010	0.038^{b} ±0.010	0.089^{a} ±0.010	0.092^{a} ±0.010
C_{48} ester	0.006^{ab} ±0.001	0.007^{a} ±0.001	0.002^{b} ±0.001	0.005^{ns} ±0.001	0.007^{ns} ±0.001	0.008^{ns} ±0.001

Means of wax compounds within UV-B treatment followed by the same letters do not differ by Duncan $p \leq 0.05$

Fig. 2 is displaying wax mass per leaf area unit for detected groups (acids, alcohols, triterpenes, esters), which did not show any interactions between afore-mentioned factors. Alcohols and triterpenes increased depending on sampling time due to UV-B radiation; interestingly, the 0 h - 24 h interval was the more sensitive period compared to the following sampling time. Alcohols tended to increase from 0.15 µg cm^{-2} up to 0.20 µg cm^{-2} whereas triterpene level raised from 0.09 µg cm^{-2} up to 0.14 µg cm^{-2}. Esters as well as the acids group did not show any significant changes in wax mass per leaf area unit. The other detected individual wax components are given in Fig. 3. The increase of C_{26} alcohol from 0.07 µg cm^{-2} at 0h up to 0.1 µg cm^{-2} and at 24 h up to 0.11 µg cm^{-2} is remarkable. Wax mass of all other components remained unchanged.

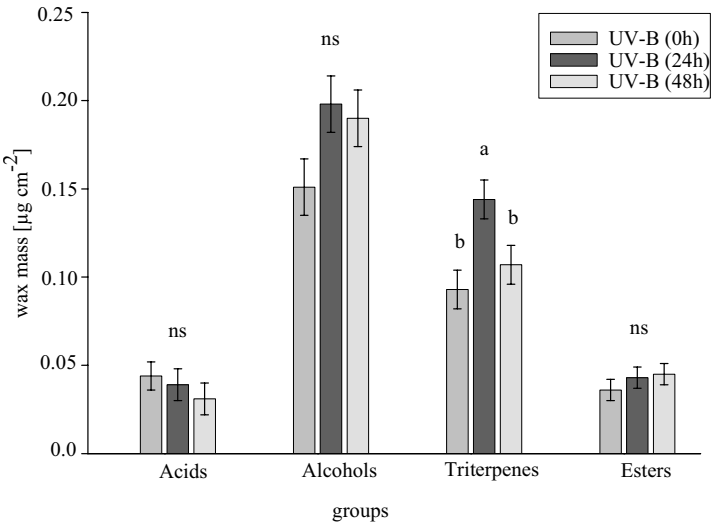
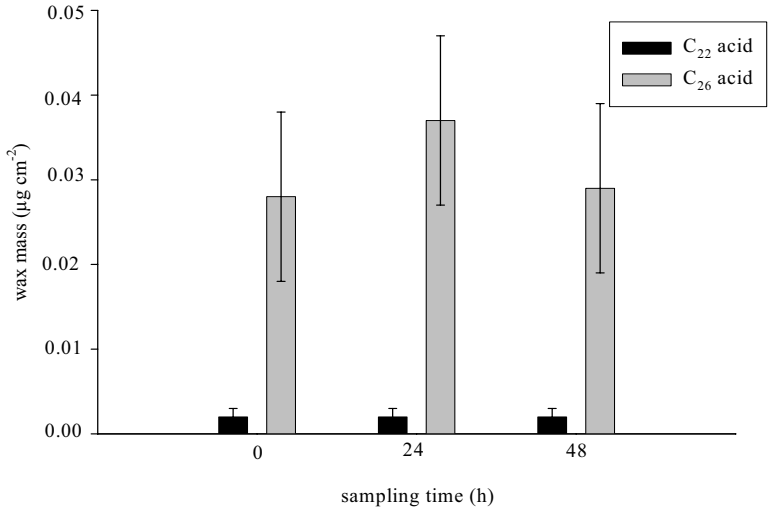
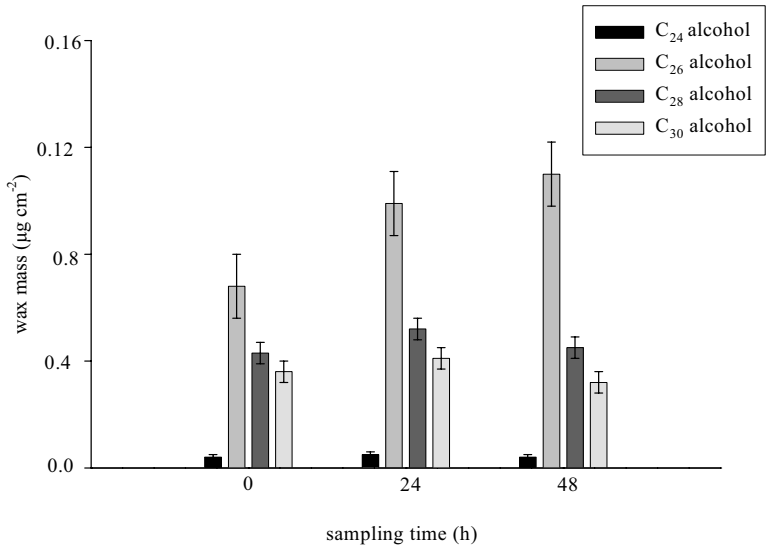


Figure 2: Effect of enhanced UV-B radiation (0.022 kW m^{-2} for 150 min) and sampling time (0, 24 and 48 h) on mass of wax fractions, main effects for sampling time, n=4, means±standard error, ns= not significant by ANOVA.

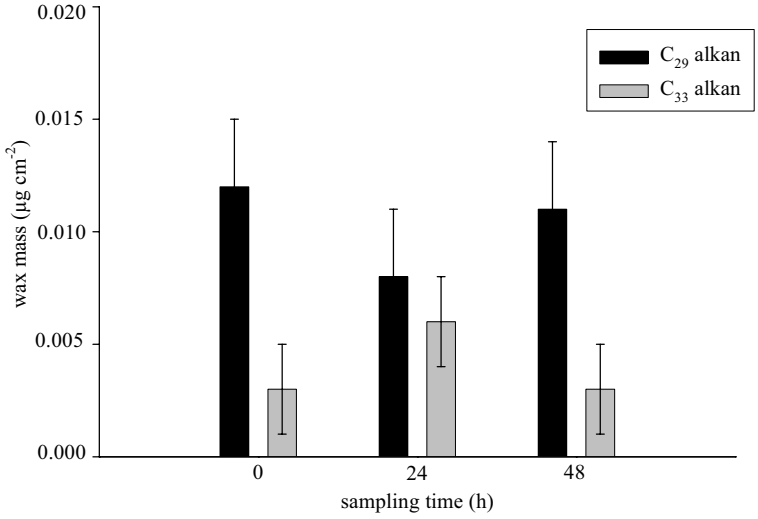
A


0.05
0.04
0.03
0.02
0.01
0.00
wax mass (µg cm-2)
C22 acid
C26 acid
0
24
48
sampling time (h)

B


0.16
0.12
0.8
0.4
0.0
wax mass (µg cm-2)
C24 alcohol
C26 alcohol
C28 alcohol
C30 alcohol
0
24
48
sampling time (h)

C


0.020
0.015
0.010
0.005
0.000
wax mass (µg cm-2)
C29 alkan
C33 alkan
0
24
48
sampling time (h)

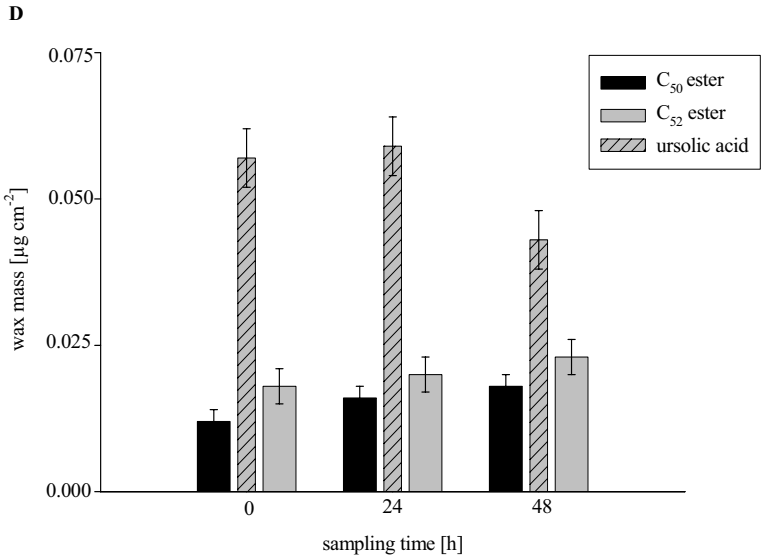


Figure 3: Effect of sampling time (0, 24 and 48 h) on single wax compounds, n=4. No significant interaction between sampling time and UV-B radiation could be studied within the wax mass of these compounds; A: acids; B: alcohols; C: alkanes; D: ursolic acid and esters.

3.2 Micromorphology

SEM investigations on morphological surface characteristics of adaxial apple leaf surface displayed the typical puzzle-like epidermal cell contours with a distinctive curvature of the periclinal cell walls. Stomata were only detected at the abaxial leaf surface (micrographs not shown).

Control plants showed a high density of fine cuticular foldings (Fig. 4 A), whereas the radiated surfaces seemed to have less crinkles (Fig. 4 B-D). Crystalloid microstructures could not be detected. The most conspicuous alterations, e.g. transient disappearance of foldings, were detected immediately after exposure to UV-B exposure (0 h). However, shrinkling of the cuticle re-established, as documented 24 h and 48 h after UV-B treatment. Whereas in the control fine foldings were un-oriented, when comparing among individula cells, shrinkles realigned in parallel over neighbouring cells (Fig. 4 D). The curvature of the periclinal cells remained unchanged (Fig. 4 A-D).

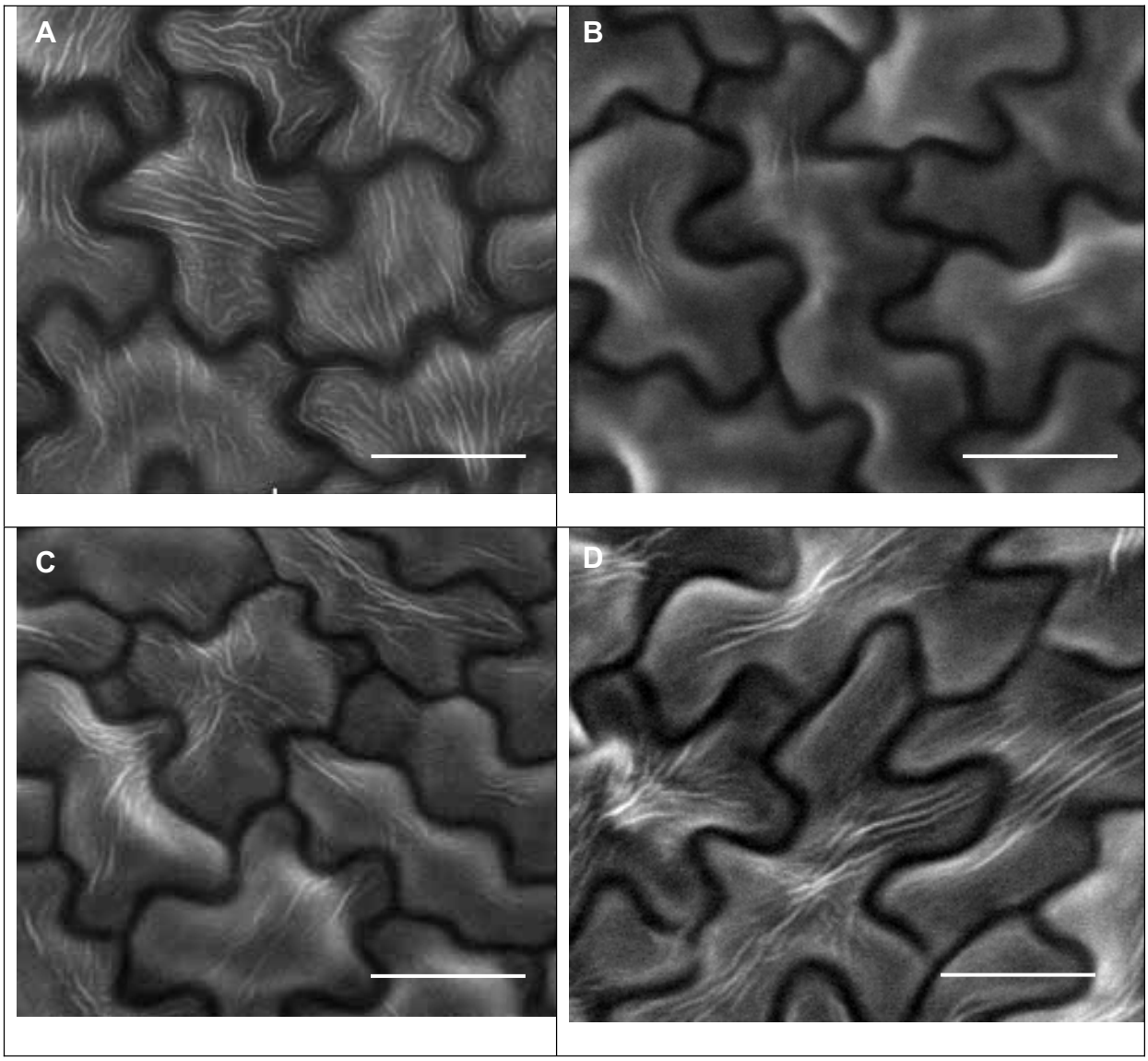


Figure 4: Surface micromorphology of adaxial apple leaf surface, SEM, bars represent 50 µm; **A**: control not exposed to enhanced UV-B radiation; **B**: 0 h after 0.022 kW m^{-2} UV-B radiation; **C**: 24 h after UV-B radiation; **D**: 48 h after UV-B radiation, n=5.

3.3 Goniometry

The contact angle of water droplets ranging from 100° up to 103° on adaxial apple leaf surface did not change significantly, depending on sampling time and UV-B treatment.

Table 2: Pearson`s correlation coefficient (r) for amount and chemical composition of surface wax compounds and microroughness, apple seedlings served as model plants, contact angle of water droplet (1 µl).

Surface wax fraction	Contact angle [°]
Total wax mass	0.39
Acids	-0.39
C_{22}	0.66*
C_{26}	-0.24
Alcohols	0.46
C_{24}	0.85**
C_{26}	0.50
C_{28}	-0.10
C_{30}	-0.19
Alkanes	0.49
C_{29}	0.23
C_{31}	0.43
C_{33}	0.54
Triterpenes	0.26
Oleanolic acid	0.39
Ursolic acid	-0.39
Esters	-0.11
C_{48}	-0.10
C_{50}	-0.11
C_{52}	-0.18

Significance level: * = $p \leq 0.05$; ** = $p \leq 0.01$, Pearson

The bidirectional correlation analysis revealed a highly significant correlation between the surface wax component C_{24} alcohol and leaf surface roughness and a significant correlation for C_{22} acid, respectively (Table 2).

3.4 Retention and rainfastness of mancozeb

Retention of applied mancozeb was influenced by time elapsed since UV-B treatment. UV-B - unexposed apple seedlings revealed an initial mancozeb concentration of 355 µg g^{-1} FW. No significant changes could be detected immediately after UV-B treatment (0 h, Fig. 5). However, there was a highly significant increase of mancozeb retention (720 µg g^{-1} FW) when applied 24 h after UV-B exposure. Only little more a.i. was retained on apple leaf surface (800 µg g^{-1}FW), when treatment was following 48 h after UV-B irradiation. Simulated rainfall (5 mm) affected the amount of deposited mancozeb.. UV-B pretreatment of plants did not affect the rainfastness of mancozeb significantly. Mancozeb concentration decreased to 55 µg g^{-1}FW (control), 64 µg g^{-1}FW (0 h), 81 µg g^{-1}FW (24 h), and 88 µg g^{-1}FW (48 h), respectively. The rainfastness amounted to 15 % of the initial a.i. concentration (control), to 16 % (0 h), 11 % (24 h) and 10 % (48 h), respectively, in the UV-B time course study.

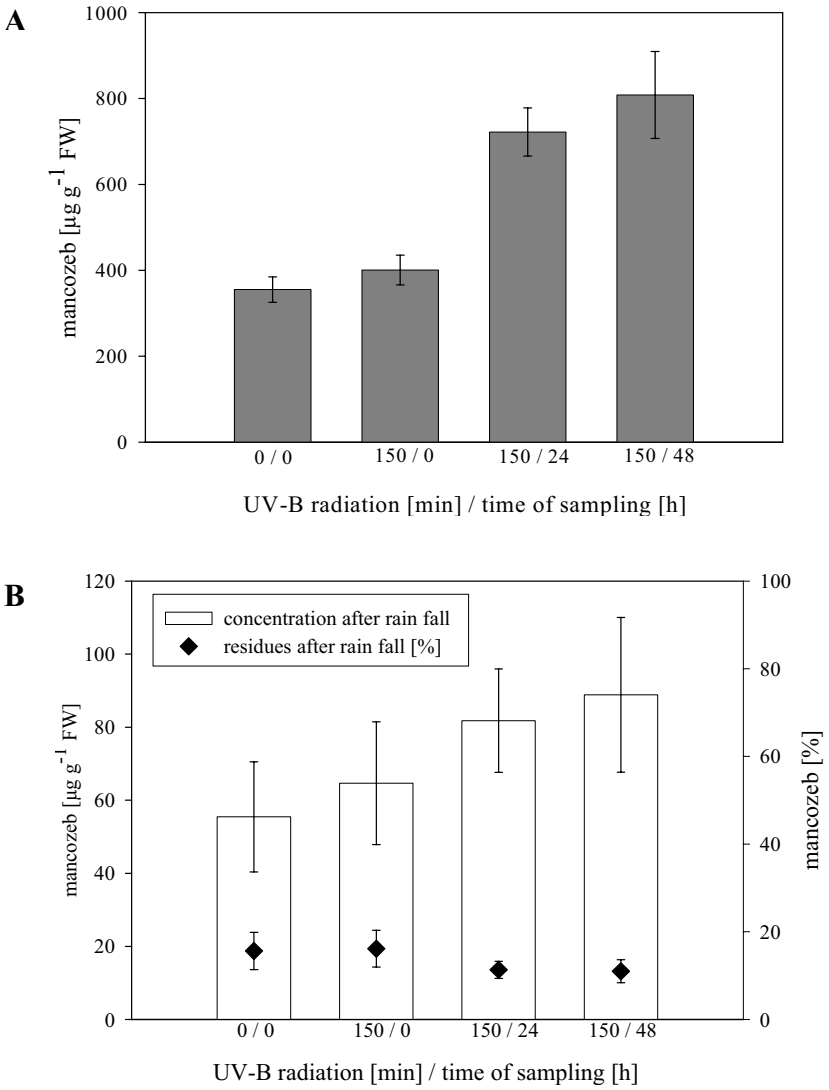


Figure 5: Effect of UV-B radiation [0.022 kW m^{-2} for 150 min] and sampling time on **A** retention and **B** rainfastness of mancozeb, mean±standard error.

4 Discussion

The results obtained in this study clearly confirm the hypothesis that UV-B treatment significantly affects micromorphology as well as chemical characteristics of adaxial apple leaf surface wax. Retention of mancozeb increased significantly 24 h after UV-B pretreatment, and did not change within the following 24 hours. Rainfastness was not influenced significantly, however, it tended to increase 24 h as well as 48 h after UV-B radiation. For the first time it could be shown, that a change in retention of mancozeb in adaxial apple leaves could be a consequence of UV-B radiation.

An influence of UV-B radiation on chemical composition of epicuticular wax has been reported by several authors. The total wax mass of leaf surface of Picea species did not change significantly after enhanced UV-B radiation (Gordon et al., 1998). The capability of

absorbing properties of surface wax layer of maize was studied. Results by Long et al., 2003 reveal that ultraviolet radiation below a wavelength of 300 nm is absorbed by surface wax layer probably due to some unsaturated bonds or other compounds like flavonoids in surface wax layer. Leaves of other species such as tobacco (*Nicotiana tabacum* L.) were studied concerning their response to UV-B radiation, showing that reaction to UV-B treatment is a function of leaf side, genotypes, and UV-B dose, even though reduction of wax amount on adaxial leaf sides of UV-B sensitive genotypes was observed (Barnes et al., 1996). The UV-B influence on wax biosynthesis is highly specific and direct (Steinmüller and Tevini, 1985; Tevini and Steinmüller, 1987). Our studies revealed neither a change in wax mass on adaxial leaf side exposed or unexposed to UV-B nor an influence of sampling time (0, 24 and 48 h after radiation). However a tendency could be noticed, namely sampling time after 24 h showed the highest wax mass, possibly as a short-term response to increased UV-B radiation. In addition, a long-term reaction could be accompanied by e.g. formation of a thicker epidermis (Laakso et al., 1996). Penetration of UV-B radiation due to increased reflection from leaf surface could be assumed, although reflection for most leaves is usually not more than 10 % (Robberecht et al., 1980; Laakso et al, 1996; Jordon, 1996; Rozema et al, 1997; Long et al., 2003). Finally it is to point out, that the whole system of cuticle with epi- and intracuticular waxes is decisive in consideration of hold off incoming ultraviolet radiation.

The group of primary alcohols decreased immediately after UV-B exposure followed by an increase after 24 h as well as after 48 h sampling time, and then leveling off to the mass of control plants.

The same phenomenon was studied with triterpenes. For instance Poa species were studied in their response to elevated ultraviolet radiation; the lower amount of surface wax after enhanced UV-B radiation was due to lower absolute amount of alcohols per unit leaf mass Pilon et al., 1999). Twenty-four hours thereafter both a slight increase in absolute wax amount and in the amount of alcohols were noted. The small increase of the wax amount could be attributed to a change in amount of alcohols and triterpenes. In contrast, levels of fatty acids as esters stayed more or less at a constant level. Whereas all the above mentioned compounds did not show any significant interaction between UV-B radiation and time of sampling, it applied for alkanes. The same increasing effect was studied by Pilon et al. (1999) examining *Picea trivialis*. In general, alterations of the chemical composition are very specific and complex. C_{26} alcohol was the main component found in apple leaf surface wax layer with an increasing tendency in time series of sampling, independent of ultraviolet radiation. Supposing that this compound is highly correlated with surface roughness, other factors like plant species, accompanied with different surface attributes in terms of morphology, leaf and

plant age besides others could play a role as well (Pilon et al., 1999). An increase in alkane content (e.g. nonacosane C_{29} alkane) is documented, whereas in this study the C_{31} alkane increased, postulating alkanes being important as a reaction and adaptive process to enhanced UV-B radiation (Gordon et al., 1998).

The physical character of the upper surface of leaves is besides others a function of the chemical composition. Hence, we studied micro-roughness as well as micromorphology and were not able to document changes in wettability after the exposure to enhanced UV-B radiation and different sampling time. Micromorphological studies revealed reduction in lamellae of the cuticle (0 h) for the first time. However, a general statement can not be made, because cell size and topography were not measured. In addition, a flattening process of the cuticle is possible, accompanied by feasible change in leaf epidermis thickness. Whereas UV-B untreated plants exhibited lamellae on every single cell, not overlapping each other, 24 and 48 h after UV-B treatment a modified lamellae structure could be demonstrated, namely wrinkles across cells. The apparent levelling of the cuticle may be explained with changes in water content of cells accompanied by altered cell turgor. UV-B treatment displays a stress factor, which can cause loss of water, ending up in new organisation of the upper cuticle, explaining the altered arrangement of the cuticular lamellae.

Spray retention process is an extremely complex series of events (Taylor et al., 2001). Since it is known that the processes of spray interaction with the plant surface are complex and not well defined, depending on e.g. droplet reflection or retention, the age of the plant in terms of wax abrasion and reciprocative rubbing of the leaves, plant surface characteristics and deposit formation, it is extraordinary important to study this closer (Furmidge, 1962; Bukovac et al., 1995; Boize et al., 1976). Holloway (1970) evaluated chemical composition of isolated cuticular wax in relation to its water repellency and found that the orientation of molecules at the wax surface affects wettability, even more, variation in type and number of chemical groupings exposed on the surface alter repellency. We investigated the influence of UV-B radiation on wax mass and composition accompanied by experiments of fungicide application and documented a significant increase of the initial mancozeb concentration 24 h after UV-B exposure; however, rainfastness was not significantly altered, though there was a similar trend, e.g. increasing rainfastness 24 h after UV-B pretreatment. It seems that there is no relation between increasing alkane content in surface wax mass after 24 h and retention of mancozeb. Nevertheless, proportion of single wax components to each other and the orientation of molecules at leaf surface could be figured.

Wash-off of fungicides by rain depends on duration of exposure and on interval between application of a fungicide and onset of rain (Bruhn and Fry, 1982; Spadafora et al.,1984). It

was shown for example for chlorothalonil, that small amounts of rain removed a large proportion of the original chlorothalonil, but the remaining deposit was difficult to remove with more rain (Fife and Nokes, 2002). The remaining chlorothalonil residue is probably held within the leaf matrix (Fife and Nokes, 2002). Mancozeb is known to be easily washed off by rain independent of the drying time (Kudsk et al., 1991; Hunsche, 2006). Our studies revealed a low rainfastness independent of UV-B radiation. However, parallels between increased retention and enhanced, but statistically not significant rainfastness after UV-B treatment could be stated. The residues [% of initial concentration] did not alter, indicating maybe the same phenomenon as assessed by Fife and Nokes (2002). Relationship between wax mass, changing wax composition and retention could be assumed, because the 24 h period was a very dynamic phase particularly considering retention of applied fungicide. We did not study the cell height, which can be a decisive factor regarding altered retention, expecting mancozeb in-between cells, in furrows between cells. In terms of correlation between all mentioned factors it is necessary to conduct further experiments, especially on rainfastness of applied fungicides in changed leaf surfaces, evoked by modified environmental conditions. Here, ontogenetically caused changes in surface wax composition, as outlined by several authors (Jetter and Schäffer, 2001; Gülz et al., 1991; Hellmann and Stösser, 1992; Bringe et al., 2006) should be taken into consideration.

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E Influence of ultraviolet-B radiation on chemical composition of cuticular wax of apple fruits and effect on penetration of $CaCl_2$ through isolated fruit cuticles

1 Introduction

Controlling and bewaring, respectively disadvantageous environmental conditions, plants have developed diverse physiological, anatomical and morphological adaptions, whereupon one of the basic adaptions is allegorised by the cuticle. The main function of the plant cuticle is the protection from non-stomatous water loss, and therefore it is the major ecological interest (Riederer and Schreiber, 2001). In agricultural plants, leaf and fruit cuticles are the primary surface leaf applied agrochemicals act with. Moreover, its permeability to ionic compounds such as calcium, is important, once an unbalance can cause bitter pit and other physiological diseases in apple fruits. Riederer and Schreiber (1995) stated about analysing the permeation of solutes and water molecules across the plant cuticle, it can be treated as a homogeneous solubility/mobility membrane, whereas the transport across cuticle is simply occuring along the chemical potential, caused by the difference of the concentrations of permeating molecules between inside and outside leaf.

The penetrability through the lipohilic cuticle has been analysed. It could be shown that charged molecules can penetrate isolated cuticles (Schönherr, 2000, 2001, 2002; Schönherr and Luber, 2001; Schönherr and Schreiber, 2004; Schlegel et al., 2005). Eichert et al. (1998) provided evidence for stomatal uptake of solutes by leaves without application of surfactants.

The underlying process of foliar penetration consists of two phases; surface adsorption (initial phase) and cuticular penetration (Schönherr and Riederer, 1989); occuring mainly through aqueous polar pores, and is affected among others by relative humidity (Schönherr, 2000, 2001, 2002). In contrast to ionic compounds, lipophilic molecules diffuse along lipophilic wax and cutin domains (Schreiber, 2005). Considering the apple fruit skin, rate of $CaCl_2$ penetration is greatly affected by the stage of fruit development (Schlegel and Schönherr, 2002). Highest rate is registered during early stages of fruit development, when approximately 100 % of applied calcium chloride penetrates within 24 h. After June drop, when trichomes had vanished and most stomata developed into lenticels, penetration rates decreased rapidly and large variability among the samples developed.

An increase in penetration of 3-chlorophenoxy-α-propionic acid through astomatous *Prunus persica* L. cuticles caused by re-wetting was related to the swelling of the cuticle, which increased their permeability (Bukovac, 1965). Differences among cultivars concerning the penetration rate can be great. In addition, the presence and density of lenticels on apple fruit surface must be considered. Chamel (1989), who studied calcium penetration through isolated

apple fruit cuticles (cv. Golden Delicious) having lenticels, could not figure a correlation between lenticels number and permeability values. The lenticels did not appear, therefore, to consitute preferential pathways allowing rapid diffusion across the cuticle.

Santier and Chamel (1998) studied transfers of hydrophilic and lipophilic compounds through isolated cuticles of five different species as well as leaves (*Ilex aquifolium, Hedera helix, Ficus elastica)* and fruits (*Lycopersicon esculentum, Capsicum annuum*). Results show that waxes are not always the main barrier to the penetration of chemicals through plant cuticles, and that it is necessary to consider the complete picture of cuticle organization with the polymer matrix and waxes.

The cuticle membrane consists of biopolymer cutin - depolymerizable - (Kolattukudy, 2001), the polymer cutan - non-depolymerizable - (Tegelaar et al., 1993), and is associated by soluble cuticular lipids - the cuticular waxes (Jenks and Ashworth, 2003). Waxes are differentiated between intracuticular - embedded in the cuticle -, and epicuticular - extruded on surface -, consisting of long chain aliphatic molecules with different functionalities. After the chemical extraction of surface wax using a solvent, the permeability of the cuticle to water and organic compounds increased by factors between 10 and 1000 (Schönherr, 1976). Wax extraction increased the diffusion rate of a hydrophilic and a lipohilic compound slightly, demonstrated with cuticles selected from leaves of *Ilex aquifolium, Hedera helix* and *Ficus elastica* and fruits of *Lycopersicum esculentum* and *Capsicum annum* (Santier and Chamel, 1998).

However, it will obviously depend on the physicochemical properties of the specific compound (Eichert and Burkhardt, 2001).

In recent years, the stratospheric ozone depletion caused a dramatic increase in transmission of solar UV-B radiation reaching the earth's surface, with reflects also in changes in plant development. For example, sunburn on apples can result in large yield losses, damages take place under conditions of both high temperature and light (Rabinowitch et al., 1974). In addition, it is known that enhanced UV-B radiation causes a change in chemical composition of surface waxes of leaves, furthermore in wax mass as well (Barnes et al., 1996; Gonzalez et al., 1996; Gordon et al., 1998).

Our hypothesis is that increased UV-B radiation and the ontogenetic development cause a change in permeability of $CaCl_2$ through isolated fruit cuticles of *M. domestica* as a possible consequence of an alteration in chemical composition of surface wax composition.

2 Material and Methods

2.1 Growth conditions

Apple trees *(M. domestica* Borkh.) cv. Topaz were grown in the experimental station 'Klein Altendorf', University of Bonn, Germany (longitude 6°59`32"E, latitude 50°37`51"N). They were planted in spring 2002, grafted on rootstock M9; predominant soil is Luvisol. The average of annual rainfall was 594 mm (2002-2005), 350 mm during the vegetation period from May until October; 1534 h of sunshine duration per year; mean temperature of the day amounted to 9.3 °C, whereas the ground temperature doused to 10.5 °C (20 cm depth).

In June 2004, after the June drop fall, apple fruits were wrapped into an UV-B absorbing film retained until harvest (Solovchenko and Schmitz-Eiberger, 2003). Figure 1 displays transmittance spectrum of the film used. The film is not absorbing PAR (Solovchenko et al., 2005). Adequate ventilation as well as the consecutive growth of fruits was considered. After harvest, these fruits were considered as non-adapted to solar radiation. On the other hand, fruits grown under natural radiation conditions served as control (adapted). After harvest all samples were submitted to UV-B treatments as described below.

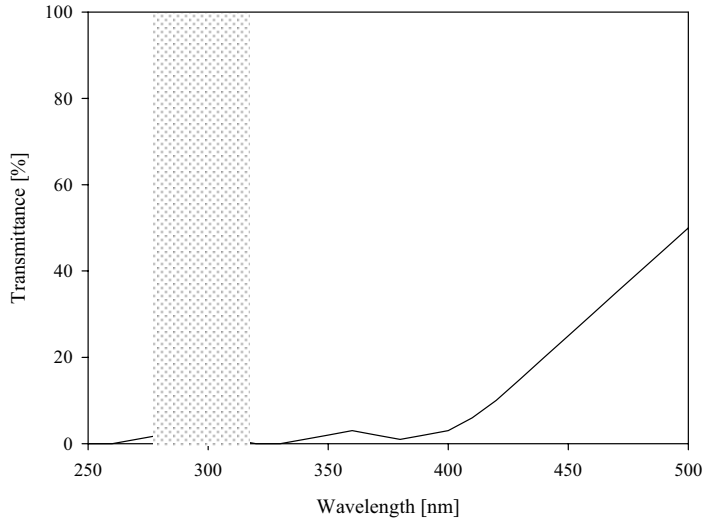

Figure 1: Transmittance spectrum of UV-B absorbing film UV-B-spectrum: 280-320 nm.

2.2 UV-B radiation

Adapted and not adapted fruits were irradiated as follows. UV-B radiation was provided by 9x100 W tubes of UV-B lamps ('Philips', Germany), having an emission spectrum of 280 nm – 320 nm. The level of irradiation was controlled by a precalibrated spectroradiometer (Gröbel, Germany). The intensity of irradiance amounted 0.022 kW m^{-2} applied for 90 and 150 min. Fruits not exposed to UV-B radiation served as control. However, these were

exposed to the same temperature like the irradiated fruits. This was assured by putting the control variant under the same tubes but covered with UV-B absorbing foil.

Zero, 6, and 48 h after finishing radiation, samples were punched for cuticles extraction and $CaCl_2$ penetration experiments. The apple cuticles were used without chemical wax extraction.

2.3 Cuticle isolation

The cuticles of apple fruits were isolated as described elsewhere (Schönherr and Riederer, 1986) modified by us. Samples punched (diameter 17 mm) from the central equatorial area of the fruit were put into an isolation enzyme solution (pH = 4.0) containing 14.7 g l^{-1} (50 mM) *tri*-sodium citrate (KMF, Germany), 0.09 g l^{-1} (1 mM) sodium azide (Merck, Germany), 40 g l^{-1} pectinase (Fluka, Germany) and 8 g l^{-1} cellulase (Sigma, Germany). The samples were stored in the dark at room temperature and the enzyme solution was replaced every 8 d. The isolated cuticles were separated from the debris after several weeks, thereafter they were washed two times in deionized water. A borax buffer (pH = 9; Merck, Germany) served as phenol cleaning medium, wherein cuticles were stored for 3 days; afterwards cuticles were cleaned with deionized water, dried at room temperature and stored in petri-dishes.

2.4 Wax extraction

In order to extract cuticular wax, cuticles were immersed into chloroform (purity > 99 %) for 30 min. It was assured that wax was extracted completely by testing different immersion times, 10 min until 120 min. (data not shown). After adding 100 µl of an internal standard (199.72 mg l^{-1} of C_{24} alkane, tetracosane) dissolved in chloroform, the samples were evaporated under a stream of nitrogen. Subsequently 20 µl of pyridine (Merck, Darmstadt, Germany) and 20 µl BSTFA [(N,O-bis (trimethylsilyl) trifluoracetamide), Machery-Nagel, Düren, Germany] were added, and the samples were incubated for 40 min at 70 °C according to standardised method (Hauke and Schreiber, 1998) and modified by us. Thereafter, specimens were cooled down to room temperature and analysed, after adding 50 µl of chloroform. The measurements were conducted by GC-MS (5890 series II; HP, Avondale, PA) with on-column injection and applying high resolution gas chromatography column (Agilent Technologies, 30 m x 0.321 mm DB-1, phase thickness 0.1 µm, J&W, California, USA). The temperature program was as follows: start at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, then 30 min at 320 °C. The carrier gas was hydrogen. The pressure program was: injection at 50 kPa, 5 min at 50 kPa, 3 kPa min^{-1} to 150

kPa, 39 min at 150 kPa. For qualitative GC-MS analysis the same method was used but instead of hydrogen, helium was employed as carrier gas. The injection volume was 1 µl. The identified wax compounds were expressed as µg cm^{-2}, whereas three replications served for statistical analysis.

2.5 Penetration studies of $CaCl_2$

The isolated apple fruit cuticles were mounted on small stainless steel boxes filled with 1.35 * 10^{-3} l of receiver solution containing 2 g l^{-1} citric acid, pH = 4. It was assured that the physiological abaxial side of cuticle was constantly in close contact with the receiver solution. A metal ring was put on the cuticle/box system to fix the cuticle. The whole system was weighed (Satorius BP 210 S, Germany), placed for 24 h up side down and weighed again, to ensure the cuticle is in intact and did not loose buffer. Afterwards 10 single droplets (volume 1 µl) of calcium chloride ($CaCl_2*2H_2O$) in a concentration of 29.8 g l^{-1} were applied and the system, which was stored for 24 h at 22 °C and 75 % RH. The calcium concentration in the receiver solution was measured using Atomic Absorption Spectrometry (AAS; Perkin-Elmer Analyst 300 Wellesley, USA). The penetration rate was couched as the percentage of initial Ca concentration applied on cuticles, as well as mg l^{-1} detected in receiver solution. 10 replications for permeations studies served for statistical analysis.

2.6 Statistics

The data were analysed with the software SPSS 12.0 (SPSS Inc., Chicago, USA) for normal distribution and homogeneity of variances. The results were compared by Duncan $p \leq 0.05$. In addition, a Pearson`s correlation analysis among surface wax mass and $CaCl_2$ penetration was carried out. Results and graphs were illustrated with SigmaPlot 2001 (SPSS Inc, SigmaPlot, Chicago, USA).

3 Results

3.1 Cuticular wax mass and chemical composition

In our evaluations, 70 to 80 % of the chromatogram peaks were identified as wax components. In control fruits (UV-B: 0 min), total cuticular wax mass differed significantly between adapted (44 µg cm^{-2}) and not adapted fruits (64 µg cm^{-2}) to natural radiation (Fig. 2). Irradiation of non-adapted fruits with UV-B light during 90 min decreased total wax mass to

46 µg cm^{-2}, whereas wax mass after UV-B exposure for 150 min did not show differences compared to the control (63 µg cm^{-2}). Between the adapted samples no significant differences in wax mass were observed, ranging from 44 µg cm^{-2} (not irradiated) to 50 µg cm^{-2} (exposure for 90 or 150 min to enhanced UV-B radiation).

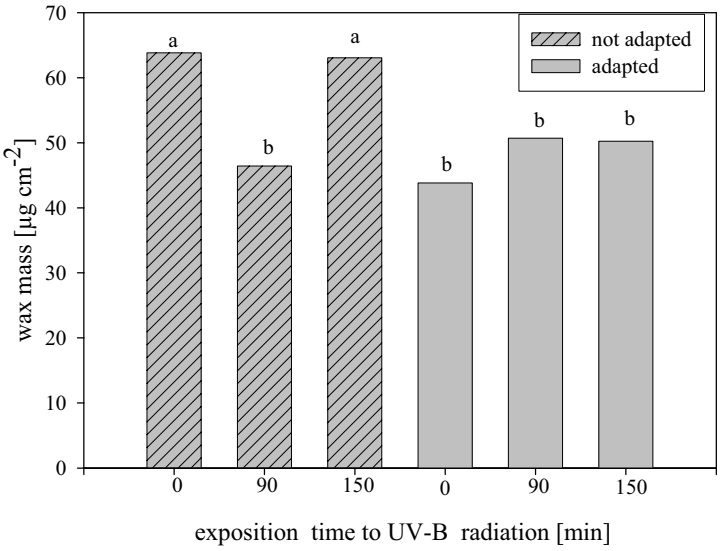

Figure 2: Impact of adaption and non-adaption to natural radiation as well as exposure to enhanced UV-B radiation [0.022 kW m^{-2}] on total wax mass of apple fruit cuticles (cv. Topaz). Means (n = 3) followed by the same letter are not significantly different by Duncan $p \leq 0.05$.

The main cuticular wax components extracted from isolated cuticles were identified as C_{22}-C_{30} alcohols, C_{27}-C_{31} alkanes, C_{24}-C_{26} acids, oleanolic and ursolic acids as triterpenes, and C_{48}-C_{54} esters (Fig. 3). Irrespective of treatment, each chemical group was detected in each variant, whereas acids and esters comprised less than 1 µg cm^{-2}. Wrapping fruits in the UV-B impervious film did not reveal changes in C_{22}-C_{30} alcohols and C_{27}-C_{31} alkanes wax mass. Mass of alcohols comprised between 1 and 2 µg cm^{-2} and alkanes between 15 and 20 µg cm^{-2}, respectively.

A significant decrease in wax mass of triterpenes could be established between non-irradiated and UV-B treatment for 90 min. However, a longer irradiation time (150 min) does not reduce the mass of triterpenes (Fig. 3 A). A growth under natural conditions without wrapping into foil displayed lower wax mass at all, neither triterpenes, nor C_{22}-C_{30} alcohols nor C_{27}-C_{31} alkanes had changed wax masses per area of fruit cuticle. Mass of alkanes was 17 µg cm^{-2} in non-irradiated, 19 µg cm^{-2} after 90 min UV-B treatment, and 17 µg cm^{-2} in 150 min UV-B treatment, respectively. Mass of alcohols ranged between 0 and 1 µg cm^{-2}, while mass of triterpenes amounted to 23 µg cm^{-2} (non-irradiated), 30 µg cm^{-2} (for 90 as well as 150 min UV-B treatment) (Fig. 3 B).

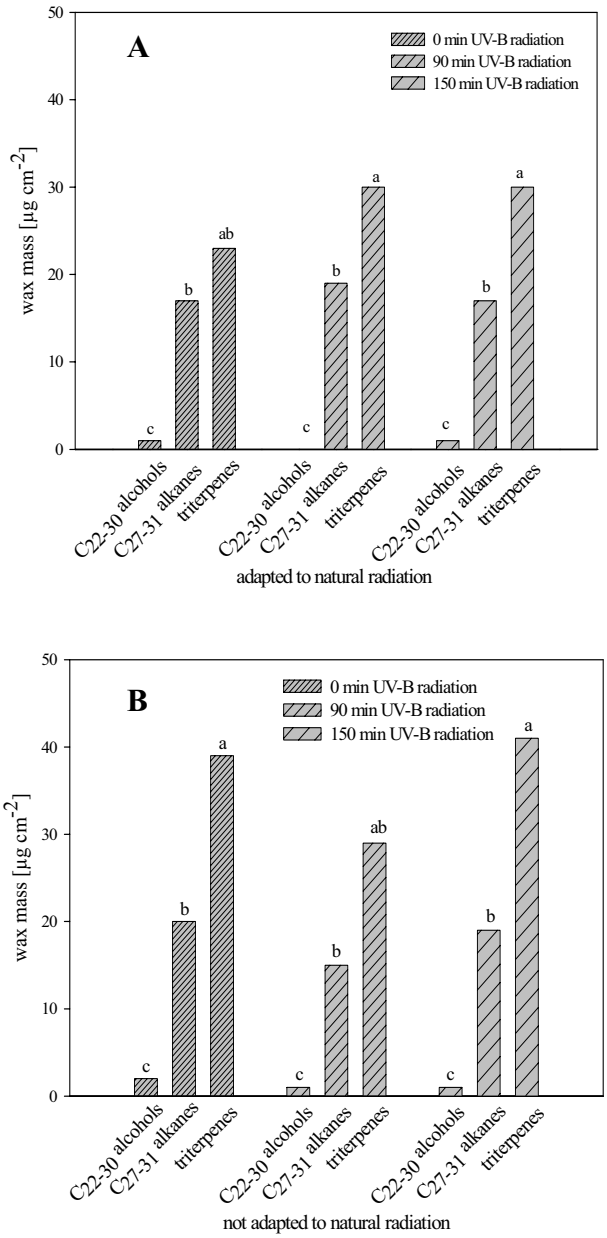

Figure 3: Impact of adaption and non-adaption to natural radiation as well as exposure to enhanced UV-B radiation [0.022 kW m^{-2}] on wax groups of isolated apple fruits cuticles (cv. Topaz). Means (n = 3) followed by the same letter are not significantly different by Duncan $p \leq 0.05$. Mass of acids and esters were less than 1 µg cm^{-2}. A: adapted to natural radiation; B: not adapted to natural radiation.

Considering single compounds, no differences depending on adaption and UV-B treatment could be established within C_{27} and C_{31} alkanes, whereas C_{29} alkane showed the highest wax mass on not-adapted and non-irradiated samples (19.5 µg cm^{-2}). The lowest level was found in not-adapted and UV-B irradiated for 90 min (12.8 µg cm^{-2}, Table 1). Considering each single component of the alcohol group, C_{30} alcohol was the most prevalent component through all different treatments, with 1.6 µg cm^{-2} in not adpated and non-UV-B treated fruits.

This component showed a significant decrease after radiation to below 1 µg cm^{-2}, in both under UV-B impervious film as under normal solar radiation grown fruits (Table 1).

Table1: Impact of adaption and non-adaption to natural solar radiation as well as exposure to UV-B radiation on mass of single wax compounds [µg cm^{-2}] of isolated apple fruit cuticles (cv. Topaz).

Compound	Duration of UV-B radiation (adapted fruits)			Duration of UV-B radiation (non-adapted fruits)		
	0 min	90 min	150 min	0 min	90 min	150 min
alcohol						
C_{22}	0.061^{b}	0.090^{a}	0.054^{b}	0.094^{a}	0.035^{b}	0.044^{b}
C_{24}	0.048^{b}	0.033^{b}	0.068^{a}	0.180^{a}	0.037^{c}	0.095^{b}
C_{26}	0.292^{b}	0.247^{b}	0.393^{a}	0.646^{a}	0.227^{c}	0.399^{b}
C_{28}	0.073^{b}	0.067^{b}	0.113^{a}	0.125^{a}	0.064^{c}	0.108^{b}
C_{30}	1.008^{a}	0.216^{c}	0.791^{b}	1.577^{a}	0.784^{c}	1.050^{b}
alkane						
C_{27}	0.720^{b}	0.856^{a}	0.318^{c}	1.004^{a}	0.518^{b}	0.603^{b}
C_{29}	15.030^{ab}	16.725^{a}	14.990^{b}	19.460^{a}	12.835^{c}	16.262^{b}
C_{31}	1.932^{a}	1.632^{b}	1.877^{a}	0.106^{c}	1.877^{b}	2.604^{a}
acid						
C_{24}	0.157^{b}	0.195^{a}	0.131^{c}	0.281^{a}	0.129^{c}	0.152^{b}
C_{26}	0.288^{a}	0.210^{b}	0.189^{c}	0.461^{a}	0.178^{c}	0.229^{b}
ester						
C_{48}	0.019^{b}	0.016^{b}	0.029^{a}	0.083^{a}	0.054^{b}	0.044^{b}
C_{50}	0.077^{b}	0.068^{b}	0.113^{a}	0.207^{a}	0.099^{b}	0.105^{b}
C_{52}	0.089^{b}	0.036^{c}	0.171^{a}	0.215^{a}	0.056^{c}	0.110^{b}
C_{54}	0.073^{b}	0.078^{b}	0.106^{a}	0.167^{a}	0^{c}	0.106^{b}
triterpenes						
Oleanolic acid	4.357^{b}	5.778^{a}	4.887^{b}	5.900^{a}	4.820^{b}	4.203^{b}
Ursolic acid	19.611^{c}	24.464^{b}	25.997^{a}	33.320^{b}	24.690^{c}	36.930^{a}

Figures with different letters are significantly different, Duncan test, $p \leq 0.05$.

3.2 Penetration of $CaCl_2$

The rate of penetration of calcium ranged between 3.2 % (6 h sampling time; 0 min UV-B treatment) and 28.1 % (6 h sampling time; not UV-B irradiated). Detailed results are

presented in Table 2. The solar-adapted fruits showed higher Ca^{2+} - penetration with increasing time of UV-B treatment concerning the 0 h sampling time from 8.3 % up to 17.3 %. In contrast, after 48 h sampling time Ca^{2+}-content in the receiver solution decreased from 20.9 down to 11.8 % (Table 2). Within 6 h sampling time highest Ca^{2+}-content could be found after 90 min UV-B treatment.

Table 2: Effect of sampling time [0. 6 and 48 h] and UV-B radiation on $CaCl_2$ penetration [24 h] through isolated cuticles of *M. domestica* cv. Topaz.

Sampling time [h]	UV-B radiation [min]	Ca^{2+}-penetration (adapted fruits)		Ca^{2+}-penetration (non-adapted fruits)	
		[mg l^{-1}]	[%] of applied	[mg l^{-1}]	[%] of applied
0	0	24.6±8.4	8.3	70.6±3.2	23.7
0	90	44.5±8.1	14.9	58.5±4.0	19.6
0	150	51.6±2.6	17.3	22.7±2.0	7.6
6	0	9.4±2.0	3.2	83.7±8.0	28.1
6	90	78.2±1.7	26.2	73.8±7.9	24.8
6	150	50.4±6.8	16.9	57.5±5.8	19.3
48	0	62.3±4.3	20.9	48.6±8.5	16.3
48	90	58.3±1.4	19.6	50.3±10.1	16.9
48	150	35.3±0.4	11.8	30.2±1.2	10.1

Within the non-adapted fruits different observations could be made: in sampling times of 0 and 6 h, a decrease of Ca^{2+} - content in the receiver solution in response to duration of UV-B treatment was stated. Sampling immediately after UV-B treatment 23.7 % of Ca could be detected, whereupon only 7.6 % after 150 min UV-B irradiation. Slight comparable decrease was found 48 h after UV-B treatment (28.1 down to 19.3 %; Table 2).

3.3 Pearson`s correlation analysis

Correlation analysis for surface wax mass and Ca^{2+} - content in receiver solution were carried out irrespective of UV-B treatment and wrapping into UV-B impermeable film or not. The Pearson`s correlation analysis revealed a very weak correlation between wax mass and Ca^{2+} - penetration. It amounted to 0.15.

4 Discussion

4.1 Chemical composition of surface wax

For the first time, naturally grown apple fruits (cv. Topaz) were wrapped in UV-B absorbing foil during the growing season, and thereafter the adjacent effect of artifical UV-B radiation on chemical composition of surface wax was studied. Apple fruits grown without this foil served as a control. Our hypothesis of an altered chemical composition of surface wax of apple fruits could be confirmed, whereas in non-adapted samples the UV-B irradiation caused more intense change in chemical wax composition.

The results of decreased wax mass of apple fruits grown under normal environmental conditions in comparison to apples protected against UV-B radiation by an absorbing film were not expected. A change of the microclimate under the film can be excluded, as an adequate aeration was ensured during the entire experiment. Remarkably, if leaves of apple seedlings, cv. Golden Delicious, (as demonstrated in chapter C) were irradiated with artificial UV-B, they did have less wax mass compared to control (not-radiated with UV-B). Independent of the cultivar, plant organ and irradiation with artificial or environmental UV-B radiation, it was shown that UV-B caused a decrease of total detected wax mass.

If the fruits are “adapted”, additional UV-B treatment after the harvest did not have any impact on total mass of surface wax. Actually, the plant is adapted to natural radiation, whereas the UV-B radiation reaching the earth’s surface increased because of a decrease of the ozone layer (Blumthaler and Amback, 1990). An adaption to sunlight can induce a different pattern of pigments (e.g. anthocyans, carotenoids) content and composition as compared to fruits not exposed to sunlight (Solovchenko and Schmitz-Eiberger, 2003). Therefore, flavonoids accumulated in the skin of apple fruits are able to serve as efficient sunscreens, which filter outmost of the solar radiation (Solovchenko and Schmitz-Eiberger, 2003). In consequence, possibly an additional UV-B radiation did not cause an increase in cuticular wax mass. The absorbing property of surface wax layer is presumably due to compounds like flavonoids (Long et al., 2003).

The distribution pattern within some wax classes indicates that wax biosynthesis was influenced by UV-B radiation, impacting more those plants/fruits grown under not adapted conditions. An UV-B effect on alkane elongation at the C_{29} to C_{31} stage, as stated by Barnes et al. (1996), could not be studied neither in the adapted nor in the not adapted fruit samples in our experiments. A possible assertion is the plant species, we in contrast to Barnes et al. (1996), studied the apple fruit cuticle grown in natural environment, they examined leaves of tobacco plants, which were grown in environmental controlled chambers. A point mutation

(Holloway et al., 1977), which describes the specific effect of UV-B radiation, here on n-alkane elongation at the C_{29} to C_{31} stage, here it can be excluded for these special chemical compounds.

No significant change of total mass of group of alcohols, alkanes and triterpenes could be measured, but the mass within the single groups did change, and dose-dependence could be shown. Gordon et al (1998) did not state a dose-dependence of changed wax mass and, moreover they observed a species-dependence.

4.2 $CaCl_2$ penetration

The percentage of Ca-content in the receiver solution decreased in not adapted samples, admittedly dependent on sampling time in different degrees, however, in adapted samples dependent on sampling time and UV-B irradiation intensity. Ca^{2+} - content in reiceiver solution fluctuated. A direct interrelationship between cuticular wax mass and penetration of $CaCl_2$ could not be stated, wax mass in not-adapted samples was 64 µg cm^{-2} (not radiated), 46 µg cm^{-2} (90 min UV-B) and 63 µg cm^{-2} (150min). The Ca content in the receiver solution ranged from 24 via 20 down to 8 %. Considering that permeability of cuticles to water and organic compounds increases upon wax extraction by factors ranging between 10 and 1000, it must be concluded that the barrier for the cuticular transport is formed by cuticular waxes (Schönherr, 1976). Accounting the immediate point of time after the UV-B radiation, a coherence neither between the mass of alcohols, nor of alkanes (in adapted and not-adapted samples) with penetration rate of calcium could not be revealed, only the mass of triterpenes seems to play a role.

Permeability of ions like Ca^{2+} plays a major role in agriculture, particularly in foliar nutrition. Polar domains in the cuticles, potentially serving as polar paths of diffusion for polar charged molecules like organic salts, can be blocked by precipitation of $AgCl_2$ crystallites in cuticular membranes (Elshatshat, 2004). Inorganic ions penetrate isolated cuticular membranes independently of temperature (Schönherr, 2001; Schönherr and Luber, 2001) and plasticizers (Schönherr, 2000) and are only weakly affected by wax extraction (Schönherr, 2000). Riederer and Schreiber (2001) have not observed a correlation between cuticular water permeability and wax amount. A correlation analysis (Pearson) between whole wax mass and Ca content in the receiver solution (for sampling time 0 h) revealed a very weak correlation of 0.15. For isolated apple skins (cv. Topaz) the permeability of Ca^{2+} is not dependent on wax mass. We did not tested wax extracted cuticles.

Wax is more hydrophobic than cutin, charged molecules such as ions will not be absorbed to cuticular waxes and thus the effect of cuticular wax mass on cuticular water permeability can not be stated (Elshatshat, 2004). Some studies did show that ion permeability through cuticles is rarely affected by wax extraction (Tyree et al., 1992; Schönherr, 2000), as opposed to the permeability of lipohilic molecules, a strong increase in their penetration can be studied after the wax extraction of the cuticle (Schönherr and Riederer, 1989; Baur et al., 1997; Schreiber, 2002).

Schreiber (2005) suggested in his work the penetration of ionic compounds is characterised by polar paths of diffusion, whereas chemical nature of these paths is still unsolved. Attributes of polar paths and chemical characteristics must decrypted for different plants and organs.

The diffusion of Ca through apple cuticle is beside others a function of the pH value (Chamel, 1989); whereas the effect of surfactants on Ca transport through cuticles is likely to be independent on pH level (Harker and Ferguson, 1991). The size of the molecules plays an important role as well, a 4-fold increase in molecular weight resulted in a decrease of the mobility by a factor of > 1000 (Schreiber, 2005).

More factors, like stomata etc. play a role regarding penetration of chemical compounds, at which the involvement of trichomes, stomata, and lenticels as preferential sites of penetration of calcium chloride across the apple fruit cuticles is discussed. Using a model calculation, it is argued that ten or more spray applications are needed to significantly increase calcium contents of apples. This has two causes: Only a small fraction of the spray liquid is intercepted by the fruits, and penetration can be rather slow (Schlegel and Schönherr, 2002).

UV-B treatment affect chemical wax composition and wax mass, besides a change of Ca^{2+} - permeation through the apple fruit cuticle. Like so far demonstrated, a clear explanation about changed permeability rates of $CaCl_2$ can not be given. Whether the group of triterpenes plays an important role in the not-adapted samples is still unknown, being the only group of chemical substances changing significantly after UV-B radiation. The group of alcohols and alkanes seems not to play a major role.

We did not evaluate a possible UV-B effect on polar paths, which are responsible for penetration of ionic compounds. A detailed assay concerning the influence of increasing UV-B radiation on polar paths should be accomplished in the near future. Ca as a foliar nutrition element and UV-B as an environmental factor becoming important in it’s influence on plant growth and development.

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F Summary

The surface of apple leaves and fruits as the interface between plant and it`s environment was in the focus of this study.

The chemical composition of the cuticular wax of apple leaves and fruits (*M. domestica*) were examined by GC-MS and the micromorphology of epicuticular waxes with SEM. The influence of ontogenesis was studied as well as the content of α-tocopherol in epicuticular wax layer. Besides that, two environmental factors - water deficit and treatment with UV-B radiation - were evaluated regarding an impact on wax layer of apple seedlings. Since the surface of plants plays the major role within the application process of pesticides, the effect of a changed surface, evoked by changed environmental conditions, on retention and rainfastness of applied fungicide mancozeb were tested. As the study dealed so far with apple leaves, apple fruits were studied as well. The fruits were wrapped into UV-B absorbing foil during the growing season, and afterwards irradiated with artificial UV-B irradiation. The effect of this treatment on chemical wax composition and permeation of $CaCl_2$ through the isolated fruit cuticles was tested.

1 The influence of ontogenesis on chemical and physical surface characteristics of leaves of apple seedlings (`Golden Delicious`) was studied. The relative growth rate (RGR) was examined; the older the leaf the RGR tended to amount to zero. The total detected wax mass decreased in the course of the studied time, besides that chemical composition changed, accompanied by specific pattern in composition of wax compounds in every developmental stage. The hydrophobic character of the adaxial side of apple leaf surfaces is caused by the chemical composition of the epicuticular waxes. SEM micrographs of the adaxial leaf surface displayed an amorphous film of epicuticular waxes, being typical for adaxial apple leaves. A process of flattening out and partial disappearance of the wrinkles on older leaves - in young leaves being 0.8 – 1.0 µm in height - may be here attributed to the expansion of cell area. The increase in hydrophily was associated with a decrease in the total amount of extractable surface waxes as well as with modifications in the composition of wax compounds.

The α-tocopherol concentration was measured – using HPLC - as well, indicating an increase during the ontogenetic development. Described here for the first time, extracellular α-tocopherol being in the epicuticular wax of leaves of apple seedlings.

By excluding the influence of environmental factors, it was provided an evidence that ontogenesis did impact chemical and physical surface characteristics of adaxial apple leaves.

2 Environmental factors like water deficit and UV-B radiation, evaluated here, did affect chemical surface wax composition, morphology and wettability of apple leaves (`Golden Delicious`), grown in a climate chamber. This can be attributed to a consequence for e.g. foliar applied chemicals. Admitting a closer glance on possible dynamic effect after UV-B treatment, samples were studied 0, 24 and 48 h thereafter. As well seedlings grown under sufficient water supply as well as seedlings with an induce of water deficit were UV-B radiated. A two week drying period caused an increase in apple leaf wax load of 30 % (not exposed to UV-B) noticed for 0 and 48 h compared to the control. The sampling time after 24 h showed statistical interaction, therefore as well as UV-B as water deficit did excert an influence on cuticular wax mass.

The present data – sampling time 0 h - did not show any variation concerning alcohol mass between the water deficit and the control variant, whereas triterpenes increased by about 30 % after interfere of reduced water supply; as opposed to 24 h variant, triterpenes were reduced by about 30 %, concerning the group of fatty acids and alcoholes as well as UV-B radiation as water supply did execute influence on their mass per leaf area.

Both studied factors did not have a distinguished meaning for measured contact angle. A slight rise in contact angle level 48 h after enhanced UV-B radiation in all variants coul be studied. Sampling time influences height of contact angle to a greater extent than tested environmental factors.

This study revealed an interaction between enhanced UV-B radiation and water deficit pertained apple surface characteristics, studied for the first time.

3 Studies on retention and rainfastness on adaxial leaves of apple seedlings (`Golden Delicious`) of frequently used fungicide mancozeb were accomplished. Retention of mancozeb increased significantly 24 h after UV-B pretreatment, and did not change within the following 24 hours. Rainfastness was not influenced significantly, however it tended to increase 24 h as well as 48 h after UV-B radiation. For the first time, a change in retention of mancozeb in adaxial apple leaves could be shown as a consequence of UV-B radiation.

As a further consequence chemical wax composition changed. The slight increase of wax amount could be attributed to a change in amount of alcohols and triterpenes. Whereas alcohols, triterpenes, acids and esters did not show any significant interaction between UV-B radiation and time of sampling, it applied for alkanes. In general, alterations of the chemical composition are very specific and complex.

The physical character of the upper surface of leaves is besides others a function of the chemical composition; hence, we studied micro-roughness as well as micromorphology and were not able to document changes in wettability after the exposure to enhanced UV-B radiation and different sampling time. In addition, a flattening process of the cuticle after treatment with UV-B radiation is possible, accompanied by feasible change in leaf epidermis thickness.

Relationship between wax mass, changing wax composition and retention could be assumed, because the 24 h period was a very dynamic phase particularly considering retention of applied fungicide.

4 Apple fruits (`Topaz`), which were grown in and adapted to normal environmental radiation and conditions served in this study as control, unlike fruits wrapped from june until harvest in UV-B impermeable film. After the harvest both variants were irradiated with artificial UV-B radiation, the control variant was not radiated. The fruit cuticle was enzymatically isolated. The surface wax of the cuticle as well as penetration of $CaCl_2$ through isolated cuticles were evaluated. Dependent on adaption and non-adaption to solar radiation the total surface wax mass differed, ranging from 44 µg cm^{-2} (adapted) up o 64 µg cm^{-2} (not adapted). An artificial UV-B irradiation of 90 min caused a decrease down to 46 µg cm^{-2} (not adapted) and within the adapted samples a slight increase up to 50 µg cm^{-2}, irrespective of irradiation time. A radiation of the not adapted apple fruits caused an increase. The chemical composition of surface wax altered slightly, whereas wrapping into UV-B impermeabel film played a decisive role besides the irradiation.

No correlation between wax mass and Ca penetration could be established. The permeability for the salt $CaCl_2$ changed, within the adapted fruits it increased with an increase of duration of UV-B treatment (0 h sampling time), and in contrast it decreased looking at the not-adapted fruits (0 and 6 h sampling time).

UV-B treatment affect chemical wax composition and wax mass, besides a change of Ca-permeation through the apple fruit cuticle. Like so far demonstrated, a clear explanation about changed permeance rates of $CaCl_2$ can not be given. Whether the group of triterpenes play an important role in the not-adapted samples is still unknown, being the only group of chemical substances changing significantly after UV-B radiation. The group of alcohols and alkanes seems not to play a major role. Detailed studied should be accomplished in the future.

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