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**Adjuvant-induced alterations of cuticular waxes
and their consequences for
mechanisms of drought stress resistance**

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Summary

Adjuvant-induced alterations of cuticular waxes and their consequences for mechanisms of drought stress resistance

The most important function of the cuticular waxes is the shielding of the plant from uncontrolled water loss as well as from pathogens, UV radiation and pollutants. Penetrating agrochemicals are also effectively shielded against by the waxes, which often necessitates the addition of adjuvants. While the mechanisms of adjuvant actions on the cuticular waxes for improving spray delivery are well understood, the consequences of these actions for the functionality of the cuticular waxes and the subsequent integrity of the plant organism have hardly been studied. The present work aimed to contribute to this understanding with an emphasis on the consequences for mechanisms of drought stress resistance.

The objective of the first chapter is to thoroughly review the available research on the possible modifications that adjuvants may impose on the cuticular waxes and to summarize the literature on the consequences of these alterations for the barrier functionality. Wax plasticization was found to be widely accepted as the main modification that adjuvants cause in the waxes and the main reason for the improved penetration of adjuvant-containing sprays. The extent of plasticization is known to depend on the solubility of the adjuvant in the specific wax. Plasticizing adjuvants were long assumed to exclusively influence amorphous waxes, but more recent findings call for a reassessment of this assumption. Thus, a more comprehensive explanation of the permeability-increasing mechanism of adjuvants is proposed in which effects on crystalline waxes and effects of surfactant-admicelles adsorbed into wax crevices are also considered. Adjuvants can also change the appearance of epicuticular waxes. This is usually attributed to the solubilizing effect of surfactants. In this work, loss of integrity due to plasticization of the epicuticular structures and coverage of the wax structures by adsorbed adjuvants are proposed as alternative reasons. There is evidence that adjuvant-induced changes may affect susceptibility to pathogen infection, water repellency, and water loss. However, the available literature is sparse.

The most important function of the waxes embedded in the cuticle is the restriction of water loss. As plasticizing adjuvants enhance the permeability of the waxes for incoming agrochemicals, the hypothesis was made here that as a consequence, the exiting of water through the cuticular barrier of living plants might also be facilitated. Therefore, the second part of the present work investigates the effect of adjuvants on cuticular transpiration *in vivo*. This question has not been addressed so far in the available literature. *Brassica oleracea* and *Malus domestica* were chosen as model organisms due to their very different epicuticular wax characteristics, and were treated with adjuvants with and without surface activity and with differing solubility. In accordance with the hypotheses made in the first chapter of this thesis, the potential to increase minimum transpiration and to alter the epicuticular waxes was not determined by the presence of surface activity, but instead related to the plasticization capability of the adjuvant, as estimated by its solubility. Minimum transpiration increased up to 10.4-fold immediately after application of rapeseed methyl ester in *Brassica oleracea*. This increase was associated with a delayed stomatal closure and a strong increase of leaf wettability. Most effects reverted within the measurement period of 14 days. The minimum conductance of *Malus domestica*, whose epicuticular waxes were amorphous as opposed to the crystalline epicuticular waxes of *B. oleracea*, was considerably less affected by the adjuvants.

On a physiological level, drought stress is best avoided by low cuticular water loss, but increased cuticular water loss does not necessarily equal impaired drought resistance. Drought resistance of plants is established through a complex interplay between different mechanisms. Plants may effectively counteract increased water loss on a physiological level by adjusting osmotic potential or elasticity of the cell walls for the goal of maintaining turgidity and thereby functionality. Thus, to fully understand the effect of adjuvants on physiological drought resistance, cuticular conductance must be evaluated in conjunction with turgor-related parameters. The pressure-volume curve is widely accepted to be the most thorough method for understanding the dynamics of turgor, but reliable analysis of pressure volume curves is difficult due to a lack of objective and reproducible analysis techniques. Therefore, in the third chapter of this thesis, an R-package ('pvcurveanalysis') was developed and published which meets the need for standardization of the analysis by detecting the turgor loss point via a modeling approach. Reliable detection of the turgor loss point determines the quality of all other pressure volume parameters because the turgor loss point is used to separate between the influence of osmotic and pressure potential on the overall water potential. The package eliminates the need for subjective assessments of curve characteristics and improves the reliability of the analysis when curves consist of few data points or contain random noise.

The package was developed using data derived from apple and tested on data from an experiment on *B. oleracea* showing the plasticity of pressure volume curve parameters in response to drought

Kurzfassung

Adjuvantien-induzierte Veränderungen von kutikulären Wachsen und deren Konsequenzen für die Mechanismen der Trockenstressresistenz

Die wichtigsten Funktionen der kutikuläre Wachse sind die Abschirmung der Pflanze vor unkontrolliertem Wasserverlust sowie vor Krankheitserregern, UV-Strahlung und Schadstoffen. Auch eindringende Agrochemikalien werden von den Wachsen aufgehalten, was häufig den Zusatz von Adjuvantien erforderlich macht. Während die Wirkungsmechanismen der Adjuvantien zur Verbesserung der Effizienz von Spritzungen gut verstanden sind, sind die Folgen auf die Funktionalität der kutikuläre Wachse und die Unversehrtheit des Pflanzenorganismus derzeit kaum untersucht. Die vorliegende Arbeit soll zu diesem Verständnis beitragen, wobei der Schwerpunkt auf den Folgen für die Mechanismen der Trockenstressresistenz liegt.

Im ersten Kapitel werden die verfügbaren Forschungsergebnisse zu den möglichen Veränderungen, die Adjuvantien an den Wachsen der Kutikula bewirken können, und deren Folgen für die Barrierefunktion zusammengefasst. Adjuvantien können zur Plastifizierung der Wachse führen, wodurch Agrochemikalien deutlich besser penetrieren können. Das Ausmaß der Plastifizierung hängt von der Löslichkeit der Adjuvantien in dem jeweiligen Wachs ab. Lange Zeit ging man davon aus, dass weichmachende Adjuvantien ausschließlich amorphe Wachse beeinflussen, aber neuere Erkenntnisse erfordern eine Neubewertung dieser Annahme. In dieser Arbeit wird daher eine umfassendere Erklärung für den Mechanismus der Verbesserung der Permeabilität durch Adjuvantien vorgeschlagen. Diese berücksichtigt auch die Auswirkungen auf kristalline Wachse und von in Wachsspalten adsorbierten Tensid-Admicellen. Adjuvantien können außerdem auch das Aussehen der epikutikulären Wachse verändern. Das wird meist auf die solubilisierende Wirkung von Tensiden zurückgeführt. In dieser Arbeit werden als alternative Gründe Integritätsverlust durch Plastifizierung der epikutikulären Strukturen und Bedeckung der Wachsstrukturen durch adsorbierte Adjuvantien vorgeschlagen. Es gibt Hinweise, dass die durch Adjuvantien hervorgerufenen Veränderungen sich auf die Anfälligkeit für Pathogeninfektionen, auf die Wasserabweisung und auf den Wasserverlust auswirken könnten. Die verfügbare Literatur ist jedoch gering.

Die wichtigste Funktion der in die Kutikula eingebetteten Wachse ist die Begrenzung des Wasserverlustes. Da weichmachende Adjuvantien die Durchlässigkeit der Wachse für eindringende Agrochemikalien erhöhen, wurde hier die Hypothese aufgestellt, dass infolgedessen auch der Wasseraustritt durch die Kutikularbarriere lebender Pflanzen erleichtert werden könnte. Daher wird im zweiten Teil der vorliegenden Arbeit die Wirkung von Adjuvantien auf die kutikuläre Transpiration *in vivo* untersucht. Diese Frage wurde in der Literatur bisher noch nicht behandelt. *Brassica oleracea* und *Malus domestica* wurden aufgrund ihrer sehr unterschiedlichen epikutikulären Wachseigenschaften als Modellorganismen ausgewählt und mit Adjuvantien mit und ohne Oberflächenaktivität und mit unterschiedlicher Löslichkeit behandelt. In Übereinstimmung mit den im ersten Kapitel dieser Arbeit aufgestellten Hypothesen wurde das Potenzial zur Erhöhung der minimalen Transpiration und zur Veränderung der epikutikulären Wachse nicht durch das Vorhandensein von Oberflächenaktivität bestimmt, sondern hing mit der Plastifizierungsfähigkeit des Adjuvans zusammen, geschätzt durch dessen Löslichkeit. Die minimale Transpiration stieg unmittelbar nach der Anwendung von Rapsmethylester bei *Brassica oleracea* um das 10,4-fache. Dieser Anstieg ging mit einem verzögerten Schließen der Stomata und einer starken Zunahme der Blattbenetzbarkeit einher. Die Effekte bildeten sich innerhalb des Messzeitraums von 14 Tagen größtenteils zurück. Die minimale Transpiration von *Malus domestica*, dessen epikutikuläre Wachse im Gegensatz zu den kristallinen epikutikulären Wachsen von *B. oleracea* amorph sind, wurde durch die Adjuvantien deutlich weniger beeinflusst.

Auf physiologischer Ebene wird Trockenstress am besten durch einen geringen kutikulären Wasserverlust vermieden, aber ein erhöhter kutikulärer Wasserverlust ist nicht unbedingt gleichbedeutend mit einer verminderten Trockenheitsresistenz. Die Trockenheitsresistenz von Pflanzen wird durch ein komplexes Zusammenspiel verschiedener Mechanismen erreicht. Pflanzen können einem erhöhten Wasserverlust wirksam auf physiologischer Ebene entgegenwirken, indem sie das osmotische Potenzial oder die Elastizität der Zellwände anpassen, um die Turgidität und damit die Funktionalität aufrechtzuerhalten. Um die Wirkung von Adjuvantien auf die physiologische Trockenheitsresistenz vollständig zu verstehen, muss die kutikuläre Transpiration in Verbindung mit turgorbezogenen Parametern bewertet werden. Die Druck-Volumen-Kurve gilt weithin als die gründlichste Methode zum Verständnis der Dynamik des Turgors, aber eine zuverlässige Analyse von Druck-Volumen-Kurven ist aufgrund des Mangels an objektiven und reproduzierbaren Analyseverfahren schwierig. Daher wurde im dritten Kapitel dieser Arbeit ein R-Paket ("pvcurveanalysis") entwickelt und veröffentlicht, das dem Bedürfnis nach Standardisierung der Analyse nachkommt, indem es den Turgorverlustpunkt über einen Modellierungsansatz ermittelt. Die zuverlässige Erkennung des Turgorverlustpunktes bestimmt die Qualität aller anderen Parameter der

Druck-Volumen-Kurve, da mit dem Turgorverlustpunkt zwischen dem Einfluss des osmotischen Potentials und des Druckpotentials auf das Gesamtwasserpotential unterschieden wird. Das R-Paket macht damit subjektive Beurteilungen der Kurvenmerkmale überflüssig und verbessert die Zuverlässigkeit der Analyse, wenn die Kurven aus wenigen Datenpunkten bestehen oder zufälliges Rauschen enthalten. Das Paket wurde anhand von Daten aus Äpfeln entwickelt und an Daten aus einem Experiment mit *B. oleracea* getestet, das die Plastizität von Druck-Volumen-Kurvenparametern als Reaktion auf Trockenheit zeigte.

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INTRODUCTION

1 Impact of drought on agricultural plants

The intent to keep the global temperature increase below 2 °C was reinforced at the 2021 United Nations Climate Change Conference, but escalating emissions of greenhouse gases are raising concerns about the attainability of this goal. The severity of drought is predicted to increase at the same time as the severity of climate change (Pokhrel et al. 2021), which will challenge global food security further.

Drought threatens the productivity of agricultural ecosystems more than any other environmental stress (Seleiman et al. 2021). Both accumulation of vegetative and reproductive biomass can be impaired under drought conditions, and farmers can experience large yield losses. Early drought during the germination of the crop may inhibit sufficient sprouting and cause reduced plant density (Taiz and Zeiger 2010). Drought in the later phases of plant development impairs cell division and cell enlargement, ultimately leading to impaired plant development and growth (Ilyas et al. 2021). Before growth is affected, drought already manifests in changes in the plant's water relations. A reduced water uptake from the soil will lead to reduced relative water content or water potential in the tissue before the plant is affected in any other way (Kirkham 2005). If dehydration continues, many crops close their stomata to avoid further water loss. However, reducing transpiration involves downsides since transpiration is associated with nutrient uptake and translocation, and most importantly with CO₂ influx through the stomata (Aroca 2012). Downregulation of stomatal aperture is the main cause for lowered photosynthesis under mild to moderate drought stress. At elevated drought stress, photosynthesis might additionally be impaired by further physiological mechanisms, e.g. altered activities of enzymes and photosynthetic pigments (Farooq et al. 2009).

2 Mechanisms of drought resistance

Plants undergo different morphological and physiological adaptations to better resist drought stress. On a morphological level, the most important drought resistance mechanisms are related to the roots (Ilyas et al. 2021). High rooting depth, density or root expansion improves the ability to fetch water from the soil, and some plants may change their rooting pattern during drought to maintain plant water status (Wang and Yamauchi 2006). On a physiological level, plants may resist drought by improving the turgor loss point. The turgor loss point marks the plant water status where wilting occurs, and where metabolic function and stomatal conductance are impaired (Bartlett et al. 2016). Plants may improve their turgor loss point by increasing osmotic solutes in the cells, which leads to an improvement of osmotic potential, or by adjusting their cell wall elasticity (Bartlett et al. 2012). Osmotic adjustment is considered the key strategy to resist drought on a cellular level (Aroca 2012).

Plants may further avoid drought by reducing transpirational water loss. They may do so by reducing the transpiring leaf area or by reducing stomatal or cuticular transpiration (Wang and Yamauchi 2006). In contrast to stomatal transpiration, transpiration through the cuticle does not fuel photosynthesis. It comes as no surprise that the cuticle of terrestrial plants is built to be a nearly perfect barrier to water loss, and the ratio of cuticular to stomatal transpiration is low under normal circumstances (Riederer and Schreiber 2001). However, under conditions of drought, the stomatal water exchange is downregulated and the share of cuticular transpiration in total transpiration increases. Eventually, the amount of transpiration through the cuticle and through imperfectly-closed stomata (i.e. minimum transpiration) might even forecast the duration of survival under severe drought stress (Duursma et al. 2019). The cuticular waxes are the most important component of the cuticle in preventing water molecules from passing (Riederer and Schreiber 2001).

3 Cuticular waxes: structure and chemical composition

The cuticle is the outer barrier of plants and comprises two major hydrophobic components: the insoluble cutin and the soluble lipids, i.e. the cuticular waxes (Zeisler-Diehl et al. 2021). Waxes occur on all aerial plant organs in an astonishing diversity of shape, size, and arrange-

ment. The most common wax morphologies encountered on plant leaves using high-resolution microscopy are films and three-dimensional crystals shaped as granules, plates, filaments, rodlets and tubules, but the description of the morphological diversity is more limited by our vocabulary than by the structures themselves (Barthlott et al. 1998, Jeffree 2008). Epicuticular wax crystals reside on an underlying wax film (Koch and Ensikat 2008). Subjacent to the epicuticular wax layer, intracuticular waxes are embedded in the cutin polymer, forming a distinctive section termed the “cuticle proper” in some species, while being integrated into the whole cuticle in other species (Fernández et al. 2016).

The cuticular waxes are dominated by two major substance groups: linear long-chain aliphatic components and cyclic terpenoids. Cyclic terpenoids occur only in some species and mostly in the waxes of fruits, e.g. in *Rosaceae*, where they preferentially accumulate in the intracuticular areas, while the epicuticular areas are dominated by long-chain aliphatic wax components (Buschhaus and Jetter 2011). The long-chain aliphatics are mostly derivatives of n-alkyl alkanes with chain lengths in the range of C16-C35 or C36-C70 resulting from differing degrees of esterification (Schreiber and Schönherr 2009b). Substitutions by functional groups broaden the spectrum to fatty acids, ketones, primary and secondary alcohols, aldehydes and β -diketones (Bargel et al. 2006). In most plants, one or two of these molecules dominate and confer the morphology of the epicuticular wax crystals. Platelet-shaped crystals often coincide with primary alcohols or ketones, while tubules correlate with nonacosyl-10-ol or β -diketones (Koch and Ensikat 2008, Jeffree 2008). However, there is no mandatory relationship between the main wax component and their morphology. For example, *Brassica oleracea* and *Arabidopsis thaliana* have a similar wax composition but the crystals of *B. oleracea* are shaped as dendrites and the crystals of *A. thaliana* as rodlets (Jetter et al. 2006).

Epicuticular waxes are assumed to be ordered molecularly in a mostly crystalline way, whereas intracuticular waxes exhibit both crystalline and amorphous regions (Jetter et al. 2000). According to the most widely-accepted model for the spatial arrangement of cuticular waxes, the linear aliphatic components of waxes organize themselves in a crystalline structure by a regular alignment of the hydrocarbon chains. The crystalline structures exhibit a solid-state polymorphism across a temperature scan leading to orthorhombic, triclinic or hexagonal phases (Reynhardt and Riederer 1991, Ensikat et al. 2006). These crystalline aggregates form laterally-extended layers on the cuticle which may be stacked to many sheets (Koch et al.

2004). Disorder is introduced into this system through, for example, a mixture of phases or by varying chain lengths and functional groups which cannot be accommodated completely within the crystalline region, and a volume fraction consisting of chain-ends and associated functional groups fills the space between two adjacent crystalline regions, forming an amorphous phase (Reynhardt 1997). The amorphous phase of the intracuticular regions is further broadened in some species by the presence of cyclic terpenoids (Ensikat et al. 2006). Adjacent crystalline layers may be bridged by intercalation of longer chains such as alkyl esters, or by secondary alcohols and ketones (Riederer and Schreiber 1995).

Whereas the morphology and composition of cuticular waxes are species-specific, the same species may develop drastically different characteristics depending on factors like age and environment. Firstly, plant cuticular wax biosynthesis strictly follows a preset ontogenetic program which determines changes in wax composition during the development of the plant organ (Hauke and Schreiber 1998, Jetter and Schäffer 2001). Besides these ontogenetic adjustments, changes in the structure or composition of waxes may indicate either adaptations or impairments due to environmental conditions. In general, higher wax depositions are an adaptive response to water or salinity stress, increasing irradiation and temperature and decreasing humidity in many plants (Shepherd and Griffiths 2006). Drought may further change wax composition (Bueno et al. 2020, Zhang et al. 2021). Damages to the internal structure of the waxes occur, for example, as a response to increasing temperatures which lead to a transformation from crystalline to amorphous structures depending on the melting point of the mixture (Riederer and Schneider 1990). The wax fine structure may be further impaired by mechanical abrasion, acid rain or epiphyllic microorganisms colonizing the leaf surface (Latimer and Severson 1997, Haines et al. 1985, Zeisler-Diehl et al. 2020). Airborne pollutants have long been theorized to result the degrading of waxes, but Burkhardt (2010) attributed this observation to the formation of thin water layers on affected plants due to the hygroscopicity of some aerosols.

4 Cuticular waxes: the multifunctional interface

4.1 Protection against water loss

Being the direct barrier to the atmosphere, cuticular waxes have developed a multitude of functionalities which enabled terrestrial life. The most important of these functions is the restriction of water loss (Schreiber and Schönherr 2009d). Waxes are the main cuticular components which establish the transpiration barrier (Isaacson et al. 2009, Leide et al. 2007), thereby shielding the underlying cells from uncontrolled water losses. Water restriction capability does not necessarily correlate with the thickness of the wax layer or the amount of wax as had been assumed in early studies on this subject (Riederer and Schreiber 2001). However, there is good evidence that the ratio between crystalline and amorphous regions correlates with the efficiency of the cuticular transport barrier (Merk et al. 1998), and it is even thought that a single crystalline monolayer on the cuticle surface composed of C30-C40 aliphatics would be sufficient to establish a transpiration barrier (Schreiber and Schönherr 2009d). In contrast, it was shown by Jetter and Riederer (2016) that the cuticular transpiration barrier in species with waxes composed only of long-chain aliphatics is mainly formed by the intracuticular section, whereas the transpiration barrier in species with high amounts of intracuticular terpenoids is also located in the epicuticular section. More recent studies found that the removal of terpenoids did not influence water restriction, but the removal of long-chain aliphatics had a significant influence on cuticular water permeability (Seufert et al. 2022). Further, several studies on mutants and backcross populations with altering amounts of terpenoids have shown that water permeability in general is negatively correlated with the ratio of n-alkanes to cyclic terpenoids (Vogg et al. 2004, Parsons et al. 2012). However, information on the relationship between restriction of water loss and chemical or structural properties is sparse and remains an objective of ongoing research.

4.2 Mechanical stability

Terpenoids might not contribute to the transpiration barrier, but recent studies indicate their importance for the physical toughness of fruits. Whereas aliphatic waxes are known to improve the elasticity of fruit cuticular membranes and hence their susceptibility to fracture (e.g., Petracek and Bukovac 1995), terpenoids acted as even better composites of the cutin matrix in the fruits of *Diospyros kaki* (Tsubaki et al. 2013). The terpenoid-rich fruits lost a considerable amount of their mechanical strengths after wax extraction. The role of terpenoids

as a rheological strengthener in the fruit cuticle was also emphasized by the finding of correlated mechanical properties and terpenoid contents in ripening *Vitis vinifera* fruit (Pensec et al. 2014) and in many other fruit cuticles (e.g., Peschel et al. 2007, Bargel et al.)

4.3 Water repellency, self-cleaning, and defence against pathogen infections

The hierarchical structuring of the leaf surfaces enables some striking features for the interaction of plants with their environment. Epicuticular waxes are the smallest scale in the hierarchical sculpturing of a plant surface, which is additionally structured by trichomes and cuticular folds (Koch and Barthlott 2009). Well-structured leaves exhibit a roughness which renders wetting very difficult and thus leads to hydrophobicity. The self-cleaning mechanism termed the “lotus effect” is correlated with a non-wettable, superhydrophobic surface associated with a hierarchical structure comprising a very dense layer of tubular crystals superimposed to the papillate epidermal cells (Barthlott and Neinhuis 1997, Barthlott et al. 2016). The abundant wax crystals prevent water droplets from reaching subjacent layers by minimizing the contact area of the droplet and forming a layer of air beneath the droplets. This establishes a tendency for water droplets to roll at low tilt angles and collect deposited substances on their way while rolling off the leaf. Superhydrophobic properties are exhibited by many plants, including important agricultural crops such as wheat, rice, and cabbage (Neinhuis and Barthlott 1997). The anti-adhesive property helps to prevent the deposition of contaminants like dust, pollutants, and microbial spores. A feature displayed by leaves of varying degrees of hydrophobicity is water-repellency, which impedes the application of aqueous agrochemicals but is beneficial in terms of a decrease in leaf wetness duration and hence reduces the germination and attachment of many fungal spores and insects (Butler 1996). However, the interaction of the cuticle with invading pathogens is not just restricted to hydrophobicity. It is a highly complex process due to the largely varying characteristics of the involved organisms (Whitney and Federle 2013, Müller and Riederer 2005), and only some aspects of their interaction will be emphasized here. Whereas cutin presents the main mechanical barrier, surface roughness introduced by abundant wax crystals (“wax blooms”) can prevent the attachment of phytopathogenic fungi and phytophagous insects just as it may impair the attachment of their predators and parasitoids (Duetting et al. 2003, Bullock and Federle 2011, Eigenbrode 2004). The chemical composition of the surface waxes and metabolites present in the waxes

provide chemical and physical cues that are necessary for the infestation of fungi or the sensing of a potential host by insects (Yeats and Rose 2013, Müller and Riederer 2005, Hansjakob et al. 2011). Once infested, the cuticular lipids contribute to the signaling of defense responses and contribute to the inherent immunity of the plant (Reina-Pinto and Yephremov 2009).

4.4 Light reflection and temperature management

The fate of radiation incidents on plant cuticles is strongly influenced by the topography of the plant surface. The abundance of epicuticular wax crystals, for example in glaucous cultivars, is associated with a higher reflection of irradiation. The reduced absorbed irradiation leads to reduced tissue temperatures, which enable better tolerance to drought and heat (Mulroy 1979) but may be adverse for an optimal photosynthesis (Pfündel et al. 2006). While both wax crystals and trichomes contribute to the reflection of irradiation, wax crystals have been demonstrated to be especially effective reflectors of ultraviolet wavelengths and hence represent a protective shield against the detrimental effects of UV-B and UV-A radiation (Holmes and Keiller 2002). Ultraviolet radiation is also known to be absorbed by aromatic terpenoids present in the intracuticular waxes of many plant species (Krauss et al. 1997).

5 The cuticular waxes in relation to the foliar penetration of agrochemicals

The cuticular waxes are not only the first site that agrochemicals interact with; they are in fact the most important barrier to their penetration. A huge share of the solute is repelled by the hydrophobic epicuticular wax layer and runs off the leaf immediately. This barrier needs to be paced to reach the continuous wax film, the cuticle, and the underlying epidermal cells. Some solutes might enter into the leaves through the stomata if the surface tension of the liquids is very low ($< 30 \text{ mN m}^{-1}$) or in response to hydraulic activation of the stomata (Burkhardt et al. 2012).

The pathway of penetration into the cuticle is perceived to be locally distinct for lipophilic non-ionic and ionic solutes. One of many clues for this hypothesis is the fact that extraction of

cuticular wax is known to increase the penetration of lipophilic agrochemicals by factors from 10 to 1000 (Schönherr and Riederer 1989), but it has rarely any effect on ionic agrochemicals (Schönherr 2000). Preferential sites for the penetration of ionic solutes (e.g. glyphosate) are currently critically discussed, and it is hypothesized that they could be located at the base of and in the trichomes and in the vicinity of stomata (Fernández and Eichert 2009, Schreiber 2008). Lipophilic substances on the other hand enter via the lipophilic domains of the cuticle, whereas the wax-rich outer layer inheres the transport-limiting barrier (Riederer and Friedmann 2008). Interestingly, pure water seems to be able to penetrate through both pathways (Schreiber 2008).

The penetration of lipophilic substances is quite well understood and well described with a solubility-mobility model (Buchholz 2006). According to this model, the permeability P (m s^{-1}) of a molecule in cuticular wax depends on the thickness of the wax layer l_{wax} (m) and is proportional to the wax/water partition coefficient K_{ww} of the molecule and the diffusion coefficient D ($\text{m}^2 \text{s}^{-1}$):

$$P = \frac{DxK_{ww}}{l_{wax}}$$

The solubility of a molecule in cuticular wax is measured by K_{ww} , which is the quotient of the equilibrium concentrations in wax and in water. K_{ww} values are determined using reconstituted cuticular wax and water comprising the radiolabeled molecule (Schreiber and Schönherr 1992). The solubility in wax was observed to be 3-10 times lower than in the whole cuticle, which is explained by Schreiber and Schönherr (2009) with the high share of crystalline wax structures which are impermeable for solutes. Schreiber (2006) compared the K_{ww} of two species with different wax structures and demonstrated 10 times lower K_{ww} for the more crystalline species. In any case, the main factor influencing the solubility of a solute is its lipophilicity. The cuticle/water partition coefficient (K_{cw}) is considered to be directly proportional to K_{ww} (Schönherr and Baur 1994) and was shown to vary by a factor of 1000 due to the lipophilicity of the substance (Riederer 1995). Therefore, it is possible to estimate the solubility of a substance in the cuticular wax by consulting its octanol/water partition coefficient (K_{ow}), which is a measure for its lipophilicity (Riederer and Friedmann 2008).

The mobility of a molecule in wax is described by the diffusion coefficient. For the determination of D , reconstituted waxes are loaded with radiolabelled compounds and the diffusion of the radiolabel into a desorption medium is measured (Schreiber and Schönherr 1993). The desorption medium must be free of the diffusing substance at all times to ensure independence of the diffusion from the concentration gradient and thus to separate between solubility and mobility. Diffusion data of cuticles reflect the data of reconstituted waxes, and the waxy coating is subsequently seen as the rate limiting factor of diffusion (Kirsch et al. 1997). The strong influence of the cuticular waxes on the mobility shows no dependence on the wax amount or thickness, but to the species-specific variation of wax compositions (Baur et al. 1999). The chemical composition largely determines the architecture of the waxes, which itself establishes the pathway for diffusing solutes. Diffusion is the process of jumping between voids which occur exclusively in the amorphous regions of the cuticular wax. The crystalline regions on the other side represent obstacles for passing compounds and increase the tortuosity of the diffusion path (Schreiber 2006). Diffusion is hence highest in species with a high ratio of amorphous to crystalline cuticular waxes. Increasing temperature may lead to enhanced mobility since both the volume of amorphous regions increases (depending on the thermodynamics of the wax constituents) as well as the occurrence of voids within the amorphous regions (Baur, Buchholz, et al. 1997). Because of the limited volume of the amorphous regions, it is reasonable to consider the cuticular wax a size-selective barrier (Schreiber and Schönherr 2009c), but the size selectivity of the overall cuticle is not affected by wax extraction, and size selectivity is hence considered to be limited by the cutin matrix (Baur, Marzouk, et al. 1996).

6 Overcoming the waxy barrier: usage of adjuvants in agrochemical spray application

The efficiency of pesticide application is largely restricted by an inadequate interaction with the leaf, including rebound, run-off, crystallization, and degradation. This problem is best approached by improving the formulation of products with adjuvants, while the adjuvants may be selected according to the physicochemical characteristics of the active ingredient and the

purpose of the application. Adjuvants are capable of mediating between the specific active ingredient and the penetration-limiting barrier of the target plant. Adjuvants may be classified according to their mechanism as a) humectants, which prevent drying of the deposited spray liquid by reducing the deliquescence point; b) surfactants, which mediate the contact of the spray liquid with the cuticle surface; and c) penetration accelerators, which operate in the cuticle interior. However, an adjuvant may combine properties of all three classes or display different properties, such as the prevention of crystal formation. Surfactants and accelerators are considered to interact with the cuticular waxes, whereas the humectant effect is restricted to interactions with the spray solution and it is thus not further explained in this thesis.

6.1 Sticking- and wetting-improving adjuvants

Surfactants are amphiphilic substances with a hydrophilic head and a lipophilic tail which is mostly constituted of hydrocarbons. They are classified according to their head group as non-ionic, anionic, cationic, or amphoteric surfactants. While different purposes may require different surfactant classes, non-ionic ones are the most used in agriculture (Castro et al. 2014). Non-ionic surfactants often contain ethoxylated sequences which increase their hydrophilic character, also expressed as the hydrophilic-lipophilic balance (HLB) (Hess and Foy 2000). Surfactants adsorb at interfaces and lower the surface tension between them. They are hence able to improve spreading and sticking of the aqueous spray solution on the waxy leaf surface and enable a solute flow within otherwise impregnable voids between epicuticular wax crystals and inside the stomata. This is an important, in hydrophobic species even crucial, feature of surfactants for improving the penetration of a solute. It is noteworthy that extensive spreading may lead to coalescence of spray droplets and a subsequent run-off, a phenomenon which requires consideration especially for spray formulation addressing non-hydrophobic species (Zabkiewicz 2007). The surface tension of a solution is reduced by increasing ethoxylated units (Stevens and Bukovac 1987) and surfactant concentration up to the critical micelle concentration (cmc). At the cmc, the previously single surfactant molecules start to aggregate to micelles by connecting their lipophilic tails and cease to adsorb at interfaces.

6.2 Penetration-accelerating adjuvants

In the last two decades, surfactants were found to not only influence the interaction with the leaf surface, but to furthermore accelerate the diffusion through the cuticle by factors of several magnitudes. Determination of the accelerating effect is done *in vitro* in an accelerating chamber comprising either cuticles or reconstituted waxes, where both set-ups have been repeatedly shown to bring comparable results (Kirsch et al. 1997, Schreiber and Schönherr 1993). Both methods have the advantage of excluding solubility processes as well as surface effects and measuring only diffusion. Fundamental insights were gained into the acceleration mechanism from these experiments. It was found that accelerators affect only the lipophilic pathway of diffusion, whereas the diffusion of hydrophilic substances is not impacted (Perkins et al. 2008, Shi et al. 2005). Accelerators affect the diffusion stronger in species with a higher share of crystalline waxes (Šimáňová et al. 2005) and for substances with lower initial diffusion coefficient (Schreiber and Schönherr 2009a). The acceleration effect within a class of accelerators is unspecific and depends solely on their concentration in the wax (Burghardt et al. 1998, Schreiber 1995, Schreiber et al. 1996, Šimáňová et al. 2005). The K_{ww} or the lipophilicity of an accelerator is hence the property which determines its acceleration potential. The acceleration potential differs between adjuvant classes by about several orders of magnitude. Besides surfactants, accelerating properties are also ascribed to n-alkyl-esters (Schönherr et al. 2001, Šimáňová et al. 2005), aliphatic alcohols (Schönherr 1993) and even to pesticides such as chlorfenvinphos (Baur, Grayson, et al. 1996) and permethrin (Baur, Grayson, et al. 1997). However, the reason behind the varying acceleration potential between substance classes remains yet unresolved.

7 Objective

Waxes, being the direct barrier to the environment of most plant tissues, exhibit a multitude of functionalities crucial for the survival of the plant. The barrier of the waxes to agrochemical spray delivery can be bypassed by adjuvants; however, the consequences of this action on other functionalities of the cuticular waxes and the integrity of the plant organism have hardly been studied. The present work aims to contribute to this understanding with an emphasis on shielding against uncontrolled water loss and the subsequent consequences for mechanisms of drought stress resistance.

The objective of the first chapter is to thoroughly review the historical development of research and theories on the possible changes that adjuvants may cause to cuticular waxes, and to summarize the literature on the consequences of these alterations for the barrier functionality of the waxes. Where no literature can be found, hypotheses concerning potential effects based on the known relationships between cuticular wax structure and composition and wax functions are made. Uncertainties in the scientific literature caused by either research gaps, methodological problems or contradictory studies are identified.

Out of this review arose the hypothesis that plants may lose more water through their cuticles after application of permeability-increasing adjuvants. Plasticizing adjuvants enhance the permeability of the cuticular waxes for incoming agrochemicals, and prior research has also showed a consequential increase in water diffusion *in situ*. Considering the extent of the conundrum of the establishment of the transpiration barrier in a complex leaf organism, it is important to study these effects *in vivo*. Thus, in the second part of the present work, the effect of adjuvants on cuticular transpiration is investigated in a controlled experiment on *Brassica oleracea* and *Malus domestica*. *B. oleracea* and *M. domestica* exhibit very different epicuticular wax structures, respectively amorphous and crystalline, and it is hypothesized that the species would thus react differently to the adjuvants. To better understand the relationship between possible effects and adjuvant properties, adjuvants with and without surface activity and with differing solubilities (estimated by their K_{ow}) are applied. Their effects on minimum conductance, total conductance, leaf wettability, epicuticular structures, photosynthesis and phytotoxicity is then monitored over a period of 14 days to further investigate the reversibility of possible changes. The adjuvants are diluted to 0.1 % and sprayed until the leaves are dripping wet in order to imitate application in agricultural practice.

An increase in cuticular water loss does not necessarily mean that the plant experiences more drought stress. Drought resistance of plants is established through a complex interplay between different mechanisms. On a physiological level, plants may effectively maintain turgidity and hence functionality even at high water loss by adjusting osmotic potential or elasticity of the cell walls. Thus, to fully understand the effect of adjuvants on physiological drought resistance, cuticular transpiration must be evaluated in conjunction with turgor-related parameters. The pressure-volume curve is widely accepted to be the most thorough method for understanding the dynamics of water potential, but a reliable analysis of pressure volume

curves is difficult due to a lack of objective and reproducible analysis techniques. In the third chapter of this thesis, an R-package ('pvcurveanalysis') is developed to address the need for a standardized determination of turgor loss point, osmotic potential at full turgidity, apoplastic fraction and modulus of elasticity. The goal of the package is to eliminate the need for subjective assessments of curve characteristics and to improve the reliability of the analysis when curves consist of few data points or contain random noise. The package was developed using data derived from apple and tested on data from an experiment on *B. oleracea* showing the plasticity of pressure volume curve parameters in response to drought.

Chapter B

ALTERATIONS OF CUTICULAR WAXES AND THEIR FUNCTIONALITY THROUGH ADJUVANTS

submitted to Crop Protection in 2022

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1 Introduction

All interactions of plants with their environment are regulated by the plants' surfaces. The outer layer of the cuticular surface, the cuticular waxes, establish an effective barrier that also prevents agrochemical sprays from penetrating plants. Sticking and spreading of agrochemical spray droplets is strongly impaired in plants exhibiting water-repellent ('superhydrophobic') leaf surfaces (Taylor 2011). Superhydrophobic epicuticular wax layers are exhibited by many plants, including many important agricultural crop plants such as wheat, rice, barley, cabbage and soy (Neinhuis and Barthlott 1997). Even after successful deposition on the surface, the penetration of solutes through the cuticle into deeper tissues is further impeded by the cuticular waxes (Buchholz 2006). To overcome this barrier, agrochemical solutes require specifically designed adjuvants, which change the cuticular waxes in a way that facilitates penetration. Such adjuvants are thus commonly used to enhance the effectiveness of agricultural chemicals, including herbicides, fungicides, insecticides, and foliar fertilizers. Embedding the active ingredient into a well-designed formulation enables efficacy at low quantities and thus decreases costs and pollution (Appah et al. 2020). Adjuvants thereby substantially help meet the demanding requirements of regulators and consumers aiming to achieve sustainable use of agrochemicals.

Cuticular waxes do not only shield against penetrating agrochemicals, but also against a multitude of other environmental influences. One of the most important functions of the cuticular

waxes is the sealing against uncontrolled water loss. The waxes that are embedded in the cuticle effectively restrict transpiration to the stomata (Schreiber and Schönherr 2009d). Many further functions are attributed to the cuticular waxes, including protection against abiotic and biotic stresses or shielding against UV radiation and pollutants, as well as mechanical stabilization of the cuticle (Shepherd and Griffiths 2006). Adjuvant-induced alterations of the waxy barrier might thus not only improve agrochemical penetration, but they may also affect the interaction of the cuticle with its environment. Here, we hypothesize that changes of the waxy barrier through adjuvants influence relevant processes such as cuticular transpiration or susceptibility to pathogens and pests. To test this hypothesis, we review the existing literature on adjuvant-induced alterations of cuticular waxes and their consequences for the functionality of the waxes. We aim to provide an integrated, synthesized overview of the current state of knowledge. We also aim to highlight knowledge gaps and indicate relevant scientific fields for future research.

2 Methodology

The interdisciplinary Google Scholar literature search engine was used to identify appropriate literature. To find research on adjuvant-induced modifications of cuticular waxes, we used the following search term:

```
surfactant OR adjuvant
AND cuticular OR intracuticular OR epicuticular
AND wax
AND alteration OR erosion OR modification OR transformation
OR plasticization OR solubilization OR adsorption
```

Literature on the impact of adjuvants on selected aspects of the functionality of the cuticular waxes was searched with the following query:

```
surfactant OR adjuvant
AND cuticular OR intracuticular OR epicuticular
AND wax
AND superhydrophobicity OR hydrophobicity OR
contamination OR pathogen OR infection OR fungi OR
transpiration OR conductance
```

The Google Scholar search results are ranked by relevance; therefore, hits were screened until fifty consecutive hits were irrelevant for the review topic. The cited as well as the citing references of the selected literature were furthermore screened to ensure that all relevant literature was identified. Due to the limited research on the chosen topic, all sources that were identified were analyzed, regardless of their quality. In cases where quality concerns arose, these are discussed and potential explanations for contradictory research results are presented.

3 Modification of cuticular waxes by adjuvants

The most widely used adjuvant types for the formulation of agricultural sprays are surfactants. The amphiphilic properties of surfactants enable them to modulate the interaction of the spray liquid with the plant surface (Appah et al. 2020). The amphiphilic character of surfactants also enables them to aggregate to micelles in a liquid environment at concentrations above their specific critical micelle concentration. Lipophilic substances such as waxes may segregate into the lipophilic core of adjuvant micelles, a process which is known as solubilization (Tamura et al. 2001). Thus, ever since the first hint towards adjuvant-induced alterations of the leaf surface was provided by Wortmann in 1965 up until today (Hagedorn et al. 2017), surfactants are hypothesized to decrease the thickness of the wax barrier by solubilizing cuticular waxes, thereby enabling the penetration of agrochemicals. However, in later investigations surfactants were found to solubilize only minor amounts of waxes. Moreover, adjuvants lacking amphiphilic properties were soon found to improve penetration of agrochemicals as well, rendering the solubilization theory an unlikely explanation for the majority of penetration enhancements caused by adjuvants (see section 3.2 for more details). Nowadays, the improved penetration is thought to originate mainly from accelerated diffusion through an increase in permeability of the waxes caused by plasticization.

3.1 Changes of structural, rheological and thermotropic properties through wax plasticization

Adjuvants can accelerate the diffusion of agrochemicals by several orders of magnitude (Schreiber and Schönherr 1993). The target site of accelerating adjuvants has been proposed

to be the cuticular waxes, motivated by the observation that the effect was equally strong on leaves as on reconstituted waxes (Kirsch et al. 1997; Schreiber and Schönherr 1993). Further, the accelerating effect was found to be comparable if the accelerating adjuvant was applied combined with or separate from the agricultural active ingredient, and thus the effect was proposed to be related to persistent modifications of the cuticular waxes (Kirsch et al. 1997; Schreiber and Schönherr 1993). Already in 1995, it was hypothesized that acceleration might be caused by plasticization of waxes (Schreiber 1995). Plasticizers are commonly used in the chemical industry to improve the elasticity of materials such as plastics. According to the free-volume theory, plasticizers wedge between amorphous polymers where they connect by loose dipole-dipole interactions (Marcilla and Beltrán 2004). Thereby they increase the free volume, lowering the transition temperature and increasing the fluidity of waxes. Waxes softened by plasticizers allow accelerated penetration, since the energy needed for diffusion is reduced (Marcilla and Beltrán 2004).

The plasticizer theory has emerged as the leading paradigm of the acceleration action of adjuvants. Plasticizer-associated changes through adjuvants were observed for the first time on reconstituted *Hordeum vulgare* wax with alcohol ethoxylates via electron spin resonance (ESR) (Schreiber et al. 1996) and soon afterwards also via nuclear magnetic resonance (NMR) (Schreiber et al. 1997). The increase in fluidity was similar to a temperature increase of about 20-30 °C. Evidence for plasticizing of the cuticular waxes through adjuvants was repeatedly demonstrated afterwards using a multitude of techniques including Dynamic Mechanical Thermal Analysis (DMTA) (Auweter et al. 2010), Differential Scanning Calorimetry (DSC) (Schreiber et al. 1997; Perkins et al. 2005; Fagerström et al. 2014), Atomic Force Microscopy (AFM) (Grant et al. 2008) and Small Angle x-ray Scattering (SAXS) (Webster et al 2018).

The plasticization theory was established through studies on the behavior of artificial wax systems, and plasticization was later also found to occur in reconstituted waxes, cuticles and leaves (Table 1). In the first *in vivo* study, leaves of *Prunus laurocerasus* were investigated through thermal microscopy (SThM) (Perkins et al. 2005). SThM enables localizing changes in the transition temperature of melting on the leaf. The results revealed that the transition temperature of melting was depressed after the application of adjuvant droplets in a pattern that reflects the typical distribution of active ingredients in the outer annulus of the droplet after

the evaporation of the liquids (Basi et al. 2012). The depression was strongest in the deposition annulus, while in the inner region of the droplet a network of depressed areas and unaffected areas was detected. These *in vivo* studies provide indications on how adjuvant-induced plasticization might influence agricultural crops or weeds growing in the field. However, to date no studies have investigated plasticization *in situ*. Thus, the practical relevance of this observation needs to be explored in future research programs in order to understand how the spraying of plasticizing agents affects field crops.

In most plasticization studies, alcohol ethoxylates and alkyl esters have been used; a recent study by Webster et al. (2018) expanded the horizon to mineral oils and lecithin. An environmentally friendly series of polysorbate adjuvants also used in agriculture has been shown to plasticize industrially processed carnauba wax in a polymer science study (Zhang et al. 2016). Due to the importance of sustainability in plant cultivation, a great task for future studies would be to see if this finding can be transferred to undisturbed plant wax systems.

The degree of plasticization depends on the adjuvant concentration in the cuticular waxes and the distinct properties of the substance class. Generally, the available evidence indicates that the more soluble an adjuvant is in waxes, the higher will be the increase in fluidity (Schreiber et al. 1996, Fagerström et al. 2014) and the stronger the changes in phase transition temperatures of the waxes (Perkins et al. 2005, Webster et al 2018).

Table 1: Studies on the effect of adjuvants on the rheological and thermotropic properties of plant cuticles and reconstituted plant waxes.

| Adjuvant | Probe | Effect and method used | Source |
|--|--|--|---------------------|
| 0.2 % alcohol ethoxylate C12E5 | <i>Beta vulgaris</i> wax | AFM relative stiffness: Elastic modulus ratio [E2/E1], E2 treated surface, E1 untreated surface = 0,77 | Grant et al. 2008 |
| 0.12 % (SThM), 0.2, 2, 20 % (DSC) alcohol ethoxylate C12-C15E7 (Synperonic A7) | <i>Prunus laurocerasus</i> leaf (SThM) wax (DSC) | SThM: Reduction of melting phase transition temperature from 69 °C to 43 - 37 °C. DSC: Reduction of melting phase transition temperature from 62 °C to 54 °C for 0.2 and 2 mg, no effect for 0.02 mg | Perkins et al. 2005 |

| | | | |
|--|--|---|---------------------|
| 0.12 % (SThM), 0.2 – 20 % (DSC) alcohol ethoxylate C12-C15E20 (Synperonic A20) | <i>Prunus laurocerasus</i> leaf (SThM) and wax (DSC) | No effect on melting phase transition temperature | Perkins et al. 2005 |
| 0,1 % Oxalcohol ethoxylate C10 | <i>Prunus laurocerasus</i> leaf cuticle | DMTA: Reduction of glass transition temperature from 30°C to -40 °C | Auweter et al. 2010 |

Plasticizers have been considered to act by modifying exclusively the amorphous waxes ever since an NMR study by Schreiber et al. (1997) provided evidence on this effect. In the mentioned study, an amorphous (octadecanoic acid) and a crystalline (dotriacontane) wax were labeled for NMR analyses and included into barley wax during the recrystallization process. The labeled waxes were expected to associate with their homologues in the barley wax. The study revealed that the amorphous region increased substantially in fluidity after adjuvant application, but the crystalline region remained unchanged. A later study, however, challenged this view. Fagerström et al. (2014) used a biphasic crystalline model wax to investigate the capability of accelerators to influence crystalline regions. They found an increase in softness after adjuvant application in the purely crystalline wax using the Quartz Crystal Microbalance with Dissipation (QCM-D) technique. The different changes in fluidity of dotriacontane-containing wax systems measured by Fagerström et al. (2014) using QCM-D and by Schreiber et al. (1997) using NMR may seem surprising, yet we propose they may be explained by differences in the measurement techniques.

NMR only observes the fluidity of the direct environment of the labeled molecules, whereas QCM-D measures the fluidity of the overall wax. According to the widely accepted model of the spatial arrangement of cuticular waxes, the single dotriacontane molecule is integrated into a tightly packed crystalline network (Reynhardt and Riederer 1991), where it is essentially immobile. Thus, the fluidity of the environment of the single wax molecule as measured by NMR is not changed. However, in crystalline wax mixtures a volume fraction consisting of

chain-ends and associated functional groups forms an amorphous space between two adjacent crystalline regions (Reynhardt 1997). Fagerström et al. (2014) found no change in crystalline structures using X-ray diffraction and thus suggested that adjuvants may not change the crystalline structures directly, but get absorbed into the spaces between the adjacent crystalline wax domains. The adjuvants wedge between the crystalline wax domains and adsorb to the waxes, increasing the space within crystalline regions and thereby increasing the fluidity of the overall wax (Figure 1).

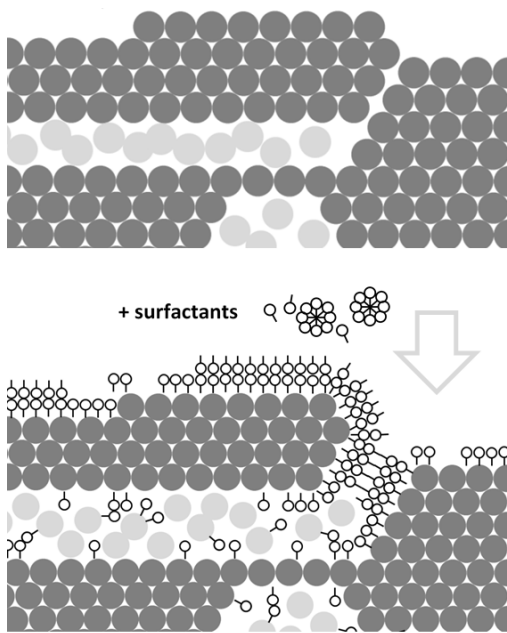


Figure 1: Schematic overview of surfactant adsorption and absorption in a wax system consisting of crystalline (dark) and amorphous (pale) waxes. The free volume within the amorphous domains may be increased by adjuvants. Furthermore, according to Fagerström et al. (2014), crevices between individual crystalline wax domains may get enlarged by adsorbed adjuvants. Surfactants may further form admicelles on the epicuticular wax surface and within the wax.

Widening of the crevices between crystalline phases might contribute significantly to enhanced penetration of substances. Using a time-of-flight secondary ion mass spectrometer (ToF-SIMS) to estimate the penetration path of alcohol ethoxylates through the wax layer of a *Prunus laurocerasus* leaf, Perkins et al. (2008) proposed that accelerators might form new penetration paths, especially in leaves with a high share of crystalline waxes, such as those of *P. laurocerasus*. An alcohol ethoxylate surfactant with a high EO content lacking plasticization ability was strongly retained by the wax layer. A low EO alcohol ethoxylate with plasticizing ability, on the other hand, penetrated quickly and in a more direct path. Since the epicuticular

wax of *P. laurocerasus* is known to be composed of > 99 % of crystalline long-chain aliphatics (Jetter et al. 2000), plasticization of only the amorphous parts should not result in such a direct penetration path. The finding that the acceleration effect is more pronounced in species with higher shares of crystalline waxes (Šimáňová et al. 2005) is additional evidence of a previous underestimation of the effect on crystalline phases. However, the individual contribution of plasticization of the amorphous vs. the crystalline phase to the overall enhanced permeability after adjuvant application is yet to be determined. This knowledge would not only contribute to answering a central question concerning the mechanism of adjuvant-induced plasticization of cuticular waxes, but also allow better prediction of the degree of plasticization in plants whose chemical or physical wax composition - and thus their share of amorphous to crystalline waxes - is known. As a result, the degree of penetration acceleration through adjuvants could be partially forecasted by the wax composition of the plant.

3.2 Modification of epicuticular waxes

The first hint on adjuvant-induced changes of the epicuticular waxes was provided in 1965 by Wortmann using transmission electron microscopy (TEM). This was followed by observations with the more precise scanning electron microscopy (SEM), which revealed species and substance class dependent alterations of epicuticular waxes by adjuvants. Strong alterations were found after application of alcohol ethoxylates in species with delicate epicuticular wax crystals like *Brassica oleracea* var. *gemmifera* (Noga et al. 1991), *Brassica oleracea* var. *gongylodes* (Wolter et al. 1988; Noga et al. 1987; Noga et al. 1991; Neinhuis et al. 1992), *Brassica oleracea* var. *botrytis* (Tamura et al. 2001; Räscher et al. 2018) (Figure 2) or *Brassica oleracea* var. *italica* (Song et al. 2021). *Brassica* species are a rewarding sample to study alterations of epicuticular waxes due to the complex three-dimensional crystal network of the waxes. The most delicate filamentous parts of the wax vanish after application of some adjuvants (Figure 2c, d), whereas the thicker rodlets may appear broken off (Figure 2d). Alterations were also apparent in the rosette-like clusters of platelets of *Glycine max* (Damato 2017; Hagedorn et al. 2017) and in the platelet-like epicuticular waxes of *Triticum aestivum* (Noga et al. 1991) and *Hordeum vulgare* (Baales et al. 2022). No such alterations were visible in species with smooth epicuticular waxes, such as *Beta vulgaris* (Noga et al. 1987), *Zea maize* (Stevens and Bukovac 1987), *Lyc-*

persicon esculentum (Tamura et al. 2001), *Ipomoea purpurea*, *Spirodela polyrhiza* and *Portulaca oleracea* (Falk et al. 1994), and in *Malus domestica* (Räsch et al 2018). Strongly hydrophilic surfactants may cause little erosion of epicuticular waxes (Räsch et al 2018) (Figure 2).

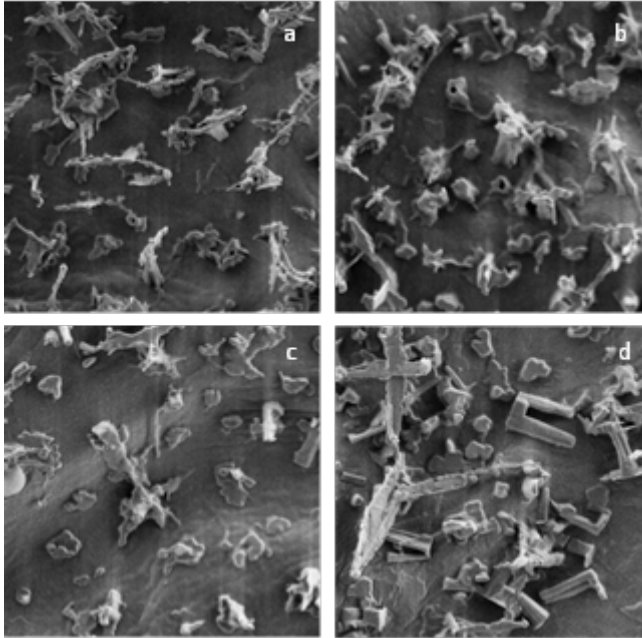


Figure 2 Scanning electron microscope (SEM) micrographs showing changes in the leaf surface microstructures on *Brassica oleracea* var. *gongylodes* two days after the spraying of adjuvants diluted to 0.1 % (Räsch et al. 2018). The finer structures deteriorated slightly after treatment with the hydrophilic surfactant alkylpolyglycoside (b) and more strongly after treatment with the more lipophilic surfactant polyoxyethylated tallow amine (c) in comparison to treatment with pure water (a). Spraying of an adjuvant lacking surface activity, rape seed methyl ester (d), led to the strongest alteration. The characteristic tubes of *B. oleracea* appeared folded and laid flat on the surface instead of protruding upwards.

The exact mechanism of adjuvant-induced alterations of epicuticular waxes is still not fully understood. The early idea of solubilization of waxes by surfactants persists (Hagedorn et al 2017), although the belief in adjuvant solubilization has weakened following experiments that found only minor solubilized quantities of bulk cuticular waxes by common adjuvants after shaking waxes and adjuvant solutions in vials for several days; non-ionic octylphenol adjuvants at concentrations above the critical micelle concentration were found to solubilize only very small amounts of the alkane pentacosane (Stevens and Bukovac 1987) and the triterpenoid cholesterol (Tamura et al. 2002) and none of the alkane dotriacontane (Tamura et al. 2002). Solubilized wax quantities were also very low in reconstituted *Solanum lycopersicum* fruit and *Brassica oleracea* var. *italica* leaf wax (Tamura et al. 2001). All experiments showed that solu-

bilization increased with increasing concentration of the adjuvant and with decreasing hydrophilic-lipophilic balance (HLB) or ethylene oxide (EO) content. However, the wax quantities that were solubilized by adjuvants were negligible, especially considering the aggressive extraction methods used. A more recent study reopened the debate of surfactant-induced wax solubilization (Hu et al., 2020), but the authors revoked their finding shortly afterwards due to methodological problems. Riederer and Schönherr (1990) analyzed the wax amount and composition of isolated cuticles of *Citrus aurantium* and *Pyrus communis* L. var. *Bartlett* after the application of a range of adjuvants of different classes, finding no significant changes. However, all studies researching solubilization focused on quantifying solubilized wax amounts. Whereas cuticular waxes are likely not solubilized in large amounts by surfactants, the alterations of only the fragile epicuticular waxes with their high surface-area-to-volume ratio may still be caused by solubilization.

Many studies have investigated alterations of epicuticular waxes by alcohol ethoxylate surfactants (Wolter et al. 1988; Noga et al. 1987; Noga et al. 1991; Neinhuis et al. 1992; Tamura et al. 2001; Hagedorn et al. 2017; Baales et al. 2022). *Brassica oleracea* var. *gongyloides* waxes were altered even more strongly by rape seed methyl ester, an adjuvant lacking surface activity (Räsch et al 2018) (Figure 2). Consequently, plasticization offers an alternative explanation for the alterations of epicuticular waxes. Adjuvant-induced plasticization might leave the delicate wax structures more susceptible to cracking and erosion. However, systematic studies providing a comprehensive picture on the mechanism behind adjuvant-induced changes of epicuticular waxes are lacking.

3.3 Adjuvant adsorption

Despite considerable efforts in studies investigating alterations of epicuticular waxes to remove surfactant depositions by washing (Noga et al. 1991; Hagedorn et al 2017; Baales et al. 2022), shaking (Noga et al. 1987) or desorption with distilled water (Tamura et al. 2001), it is possible that residual adsorbed surfactant molecules remained and covered the waxes. Surfactants may aggregate to monolayers on hydrophobic surfaces (Tiberg 1996) such as waxes. The adsorbed surfactant molecules may cover the crystal network or fill the gaps between the crystals. This needs to be considered as an alternative explanation of the appearance of smaller crystals following adjuvant application in case of surfactants as also postulated by

Burkhardt et al. (2018). Further research is required to improve discrimination between eroded waxes and a mere camouflaging of the waxes by adsorbed adjuvants. Chemical imaging microscopy may possibly assist here. Stimulated Raman scattering (SRS) microscopy is an emerging tool for the chemical imaging of cuticular waxes (Littlejohn et al. 2015; Farber et al. 2019) and could potentially be used to discriminate between waxes and adsorbed monolayers of surfactants.

Theoretically, surfactants that are adsorbed to the cuticle may change its physical properties, with potential impact on the penetration of substances. Adsorbed surfactants may change the wettability of the leaf surface. Further, surfactants may aggregate to sub-monolayers or admicelles, which may allow water molecules to partition into their inner hydrophilic regions (Tiberg 1996; Claesson and Kjellin 1999) (Figure 1). Adsolubilized water inside the cuticle may form continuous water pores. Such aqueous pores influence the movement of water in the cuticle (Tredenick et al. 2021).

3.4 Regeneration of waxes

Reversibility of plasticization has not been demonstrated directly but may be estimated from studies showing the reversibility of the accelerating effect. The accelerating effect of a range of alcohol ethoxylates in both isolated cuticles and in reconstituted wax has been shown to decrease with decreasing concentration in the wax and to have stopped after full desorption of the adjuvants (Schreiber and Schönherr 2009a). Diffusion into the wax was much quicker than desorption, and plasticization was thus considered by the authors to establish much more quickly than it is reversed. Desorption is not favored in field-grown plants by a desorption medium unlike in the *in vitro* reversibility studies. Thus, the affinity for the adjuvants to diffuse out of the lipophilic cuticle might be very low under natural conditions and plasticization might persist for a comparatively longer time.

The reversibility of the superficial alterations of epicuticular waxes by adjuvants was first demonstrated by Wortmann (1965) within a week after adjuvant application on *Brassica napus* L. using transmission electron microscopy (TEM). About two decades later, Wolter et al. (1988) used scanning electron microscopy (SEM) to show that the filamentous waxes of *Brassica oleracea* var. *gongylodes* L., which had vanished after application of droplets containing

an alcohol ethoxylate (EO 9.5), did not recover in young or mature leaves in a timeframe of seven days. In a comparative experimental setup, the filamentous crystals of *Brassica oleracea* var. *gongylodes* L. regenerated to a large extent within a week (Neinhuis et al. 1992). However, the wax structures at the droplet periphery, where surfactants are known to accumulate, were damaged irreversibly. In a recent study by Baales et al. (2022), wax platelets were visible again within hours after a surfactant treatment.

No studies have investigated how long adjuvants will remain adsorbed to the leaf surface.

4 Adjuvant-induced alterations: consequences for the functionality of the waxy barrier

4.1 Protection against water loss

Being the direct barrier to the atmosphere, cuticular waxes have developed a multitude of functions which are critical to terrestrial life. Their most important function is the restriction of uncontrolled water loss (Schreiber and Schönherr 2009d). Transpiration through the cuticle is mainly restricted by the impregnating waxes (Isaacson et al. 2009; Leide et al. 2007), but it is still unclear how exactly the transpiration barrier is established (Riederer and Schreiber 2001; Fernández et al. 2017). The thickness of the wax layer has repeatedly been found to not correlate with cuticular transpiration and chemical composition. The structural characteristics of the waxes appear to provide a better explanation of the capability of the cuticle to restrict water movement (Jetter and Riederer 2016; Zeisler-Diehl et al. 2018). It appears unlikely that cuticular transpiration can be explained solely by investigating the waxes. In the ecophysiology literature, the idea of hydrophilic pathways through which water may pass the cuticle including the wax layer prevails (Schreiber and Schönherr 2009; Fernández and Eichert 2009). Accordingly, a tortuous pathway for traversing aqueous molecules is established by connected hydrophilic domains. These hydrophilic domains may be composed of trichomes, polysaccharides and other polar moieties of the cuticle, which protrude into the hydrophobic wax layer and offer water molecules a site for adsorption (Fich et al. 2020). Water clusters, either originating from the plant tissue or from the environment, may thereby establish a continuous route through the wax layer (Fernández et al. 2017). This route has been termed ‘polar pore’,

'aqueous pore' and recently 'dynamic aqueous continuum' (Fernández et al. 2017), and its establishment through the cuticular waxes is probably closely linked to the cuticle structure (Tredenick et al. 2017).

Considering how much of a conundrum it is to understand the principles of the cuticular transpiration barrier, it is still surprising how little is known on the impact of adjuvants on cuticular transpiration. Cuticular transpiration was first discovered to rise in response to a range of adjuvants in isolated, astomatous leaf cuticles of *Citrus aurantium* and *Pyrus communis* (Riederer and Schönherr 1990). All investigated polyoxyethylene surfactants increased cuticular transpiration, except for the highly hydrophilic sodium dodecyl sulphate (SDS). *C. aurantium* reacted most strongly to Brij30 with an increase by a factor of 15 and *P. communis* to a polyoxyethylene tallow amine with an increase by a factor of 152. A recent study by Zeisler-Diehl et al. (2022) observed six different species but only found a significant increase in cuticular transpiration if the investigated alcohol ethoxylate surfactants were applied at very high concentrations. The study by Räscher et al. (2018) provided the first evidence of an *in vivo* increase in cuticular water loss after adjuvant spraying on *Brassica oleracea* leaves, but not on *Malus domestica* leaves. Rape seed methyl ester, an adjuvant lacking amphiphilic properties but with the highest wax solubility among the adjuvants studied, increased cuticular and residual stomatal (= minimum) transpiration of *B. oleracea* the most, by a factor of 10. The effect reverted within days. The very hydrophilic alkyl polyglycoside adjuvant had no effect on minimum transpiration, similarly to the hydrophilic SDS in the study by Riederer and Schönherr (1990). Baales et al. (2022) found no significant increase in residual transpiration *in vivo* on barley leaves after spraying of two hydrophilic alcohol ethoxylates at non-toxic concentrations.

Hydrophilic adjuvants could potentially contribute to the hydrophilic pathway in the cuticle (Fernández and Eichert 2009) and thus potentially also enhance the mobility of transpiring water in the cuticle. However, the available evidence suggests that very hydrophilic adjuvants are less potent than their more hydrophobic counterparts in increasing cuticular transpiration (Riederer and Schönherr 1990; Räscher et al. 2018). Some hydrophilic adjuvants do not increase cuticular transpiration at all (e.g. Baales et al. 2022). Instead, the increases in cuticular water loss might be better explained by the plasticization ability of an adjuvant, e.g. its K_{ow} (Räscher et al. 2018). Plasticization leads to increased permeability of the waxes for lipophilic substances

(Schreiber 2006). Water permeabilities and diffusion coefficients for many lipophilic substances were found to be correlated by several studies, which indicates that water can penetrate both via the hydrophilic path as well as the waxy pathway (Schreiber and Schönherr 2009). Plasticization of the waxes may thus in turn increase water permeability. However, the available studies are too sparse to postulate an understanding of the mechanism. Further in-depth investigations of the adjuvant properties that lead to increases in cuticular transpiration are required.

Cuticular transpiration has a negligible impact on the overall transpiration when plants are grown with appropriate water supply. However, it might become important if crops are exposed to water deficit situations. Under conditions of severe drought or frost, the survival of the leaf depends on effectively restricting non-photosynthetically-productive water loss through the cuticle and the closed stomata (Tranquillini 1982; Nguyen et al. 1997). Evidence suggests that certain adjuvants might strongly increase cuticular transpiration (Riederer and Schönherr 1990; Räscher et al. 2018). Application of such adjuvants before or during drought or frost might therefore be detrimental to the water balance of the leaf and could cause irreversible drought damage. In contrast, what appears as a disadvantage for cultivated crops might be an advantage for weed control. For example, herbicide application in the cool spring and autumn months is a widespread strategy to reduce initial weed pressure. Formulating the herbicide with an adjuvant capable of increasing cuticular transpiration might enhance the efficiency of this measure under water-limiting conditions. So far, however, there is no scientific publication on the effect of enhanced cuticular transpiration by adjuvants on the overall water balance of crops. It is thus unknown to what extent the effects observed on isolated cuticles and on detached leaves can also be observed on plants growing in an agricultural ecosystem.

4.2 Water repellency and self-cleaning

Leaf surfaces are structured hierarchically by trichomes, cuticular folds and epicuticular waxes. Increasing degrees of structuring generally result in increasing hydrophobicity, which renders the leaves difficult to wet and establishes an efficient self-cleaning mechanism (Barthlott et al. 2016). Water droplets on hydrophobic surfaces move already at low tilt angles and collect deposited substances on their way while rolling off the leaf. This prevents the accumulation of contaminants like dust, pollutants and microbial spores (Barthlott and Wollenweber

1981). Another beneficial effect of hydrophobicity is that it decreases leaf wetness duration and hence reduces the germination rate of most fungal spores (Butler 1996).

The natural hydrophobic barrier limits the efficiency of agrochemical sprays and requires the addition of adjuvants. Surfactants lower the surface tension of the spray solution and thereby improve sticking and spreading. This quality makes them the most used adjuvant types for improving foliar applications (Castro et al. 2014). However, adjuvants do not only improve the wetting behavior of the spray solution they are added to; they have also been found to enhance the wettability of leaf surfaces. Neinhuis et al. (1992) demonstrated that water repellency and self-cleaning of *Brassica oleracea* var. *gongylodes* L. leaves was strongly inhibited after application of a diluted EO 9.5 alcohol ethoxylate. Adherence of dirt particles was strong on the treated side. Simulated rain washed off the depositions, if the whole leaf was treated with the adjuvant in a manner that was equivalent to complete wetting in the field. If single droplets were applied, as might happen especially in sheltered parts of the canopy, the simulated raindrops including the collected dirt particles were accumulated preferentially at the treated spots. Hagedorn et al. (2017) observed improved wettability of *Glycine max* leaves after application of the ethoxylated triglycerols Agnique® SBO10 and XP ED 75 and following rinsing of the leaf with water. No such effect was observed in response to four ethoxylated diglycerols. Variations in HLB could not explain the differences in effects. Simultaneously, alterations of the epicuticular wax crystals were observed and used to explain the improved wettability. Similarly, in studies by Räscher et al. (2018) and Baales et al. (2022), the wettability of *Brassica oleracea* or *Hordeum vulgare*, respectively, were enhanced after spraying adjuvants for weeks or days, respectively, and associated in both cases with altered leaf surfaces. In fact, leaves with smoother epicuticular wax layers appear to have a lower innate hydrophobicity (Wolter et al. 1988; Neinhuis et al. 1992). However, residually adsorbed surfactants that cover the epicuticular waxes and thereby make them appear eroded could in turn attract water particles. An adsorbed surfactant layer needs to be considered as an alternative explanation for the reduced hydrophobicity, as highlighted in chapter 3.3.

Many consequences may arise from facilitated contamination with dust, pollutants, and microbial spores through impeded self-cleaning after adjuvant application, yet causal studies on this topic are entirely lacking.

4.3 Pathogen infection

The impeded self-cleaning and water repellency may have consequences for the susceptibility to pathogen infections. Lowered hydrophobicity not only changes the capability of the pathogen to attach to the leaf surface, but it might also influence the leaf wetness duration, an important driver of spore germination (Duetting et al. 2003; Eigenbrode 2004; Bullock and Federle 2011). In theory, the interaction with pathogens might be influenced by alterations of the cuticular waxes after adjuvant application through several other mechanisms. For example, specific chemical or physical characteristics of cuticular waxes may serve as cues for fungal infestation or may be used by insect pathogens to recognize potential hosts (Müller and Riederer 2005; Hansjakob et al. 2011; Yeats and Rose 2013). They may also trigger defense responses (Reina-Pinto and Yephremov 2009). Plasticizing adjuvants could interfere with plant susceptibility to pathogens in additional ways. A more permeable cuticle may either render the plant easier to penetrate or allow for faster transmission of defense signals (Ziv et al. 2018).

Three studies have observed enhanced infection susceptibility after adjuvant application. Noga et al. (1990) applied alcohol ethoxylates to *Phaseolus vulgaris* L. and *Brassica oleracea* var. *gongylodes* L. leaf discs and inoculated them with *Botrytis cinerea*. The adjuvant treatments led to up to three times larger lesions on *B. cinerea*, whereas the abundance of lesions on *P. vulgaris* was only slightly affected. The leaves of *B. oleracea* exhibit superhydrophobicity, a feature that may be of importance for their pathogen defense, whereas the smooth, fluid-like epicuticular wax of *P. vulgaris* might not be central to the plant's defense strategy. Similarly, Rogiers et al. (2005) found a relationship between adjuvant application and *Botrytis* infection in *Vitis vinifera*. Incidence and severity of infection increased from 25% after treatment with a pure fungicide to 40 - 100% for a combination of the fungicide with the evaluated adjuvants. However, specific adjuvants may result in direct phytotoxicity in specific crops - depending on tissue age, application setup and adjuvant concentration - ultimately leading to necroses (Furmidge 1959; Knoche and Noga 1991; Hunsche and Noga, 2011; Basi et al. 2012). Since *Botrytis* penetrates injured areas more easily, phytotoxicity may be an alternative explanation for the increased infection susceptibility.

A recent study by Song et al. (2021) showed increased attachment of *Salmonella typhimurium* after application of a surfactant to the non-glossy version of a near-isogenic line of *B. oleracea* var. *italica*. After surfactant application, attachment of the pathogen to the glossy *B. oleracea* var. *italica* was similar to the attachment on the non-glossy equivalent. The surfactant application was associated with a reduced contact angle of the leaf and a smoother appearance of the leaf surface. As described in chapter 3.3, surfactant adsorption may explain this phenomenon, yet the authors concluded that enhanced pathogen attachment was caused by erosion of the waxes.

The available studies provide some hints towards a relationship between adjuvant application and susceptibility to pathogen infections. However, research is sparse and studies under field conditions are entirely lacking. There is a large need for more systematic studies to clarify the relationship between adjuvant-induced alterations and pathogen infection or disease severity.

5 Conclusion and outlook

Adjuvants alter the diffusion-limiting cuticular waxes in a way that penetration of agrochemicals is greatly improved. Many adjuvants act as plasticizers on the cuticular waxes, thereby increasing fluidity and reducing phase transition temperatures. Plasticized waxes exhibit enhanced permeability, and this in turn probably facilitates water loss through the waxy barrier. A range of further impacts on the functionality of the waxy barrier through increased permeability is possible, including changes in the susceptibility to pathogen infection. Furthermore, the hydrophobicity of the leaf surface may be affected by adjuvant adsorption or by erosion of fine crystals.

Further research is required to enhance knowledge on how to improve both security and efficiency of adjuvant-containing sprays. Ideally, scientific developments will fill the gap of research connecting the heterogeneous adjuvants with the diversity of wax properties (Zabkiewicz 2007; Castro et al. 2014). Most studies on adjuvant effects on cuticular waxes have concentrated on the adjuvant class of alcohol ethoxylates, whereas other chemical categories are

underrepresented. Toxicological concerns are to date restricting the use of alcohol ethoxylates. Many alternative adjuvants with improved ecotoxicological profiles are available (Müller et al. 2002; Green and Beestman 2007; Castro et al. 2014), but their influence on the waxy barrier has barely been investigated.

Finally, scientific research would benefit from better alignment with the way sprays are applied on farms. Adjuvants are added to agrochemical formulations for many purposes, including improved manufacturing processes, prolonged shelf-life, and finally to improve efficacy. These adjuvants are mostly surfactants, and their effect on cuticular waxes is largely unknown. Furthermore, in agronomic field practice, and in particular in commercial formulations, mixtures of adjuvants are ubiquitous. Farmers regularly combine several products in one spray tank, and sometimes tank-mix adjuvants are added to the already existing in-can adjuvants of the products. Addressing knowledge gaps related to the implications of such practices would help establish a target-oriented approach to choosing or developing adjuvants that improve spray efficiency while reducing negative effects on crops.

Chapter C

AGRICULTURAL ADJUVANTS MAY IMPAIR LEAF TRANSPIRATION AND PHOTOSYNTHETIC ACTIVITY

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1 Introduction

Foliar application is a common practice in crop production systems to supply nutrients or to deliver agrochemicals for disease and pest control. Thus, adjuvants are usually incorporated into agrochemical formulations to improve the biological efficiency of foliar sprays. Permeability-increasing adjuvants are known to alter the physicochemistry of plant surfaces (Damato et al. 2017, Noga et al. 1986, Rogiers et al. 2005, Schreiber, 2006). The cuticle is the outer layer of the leaf surface and thereby determines the interaction with surface deposited liquids or solids. The cuticle does not only limit agrochemical sprays from penetration, but its most important function is the protection against water loss (Bartlett et al. 2012, Zeisler and Schreiber, 2016). Waxes are the main cuticular component restricting cuticular transpiration (Leide et al. 2007) and are considered a physical limitation for the diffusion of water molecules (Shepherd and Griffiths, 2006). Hence, a low cuticular water permeability is an ideal trait for effective water use and is commonly targeted in breeding for drought tolerance (Gutierrez et al. 2010, James et al. 2008). A more permeable cuticle increases transpiration without an associated advantage in CO₂ assimilation (Blum, 2009). A lowered cuticular conductance hence prolongs

survival during impending desiccation (Gutierrez et al. 2010, James et al. 2008, Nguyen et al. 1997).

Most commonly, surfactants are used to decrease the surface tension of the spray liquid, thereby improving the retention and spreading on the target surfaces (Hunsche et al. 2006, Kraemer et al. 2009). The critically debated polyoxyethylated tallow amine (POEA) is a ubiquitously used surfactant in herbicides. Newer generations of surfactants, such as alkyl polyglycosides (APG) are considered to be more environmentally friendly and have become the most important surfactants based on natural raw materials (Balzer, 1996). Surfactants as well as other adjuvants might also act as penetration improving agents. It is yet unknown which chemical characteristics determine the penetration accelerating potential of adjuvants. However, it is known that the penetration accelerating potential within a class of adjuvants is determined by its solubility in the cuticular waxes (Burghardt et al. 1998). POEA acts as a penetration improving agent, as well as rapeseed methyl ester (RME). Both are employed in agrochemical formulations as in-can and tank mix adjuvants, also due to their capability to act as a solvents for lipophilic active ingredients (Baur et al. 1999, Brausch and Smith, 2007, Charlemagne, 1999). Penetration accelerating adjuvants are known to alter the permeability of the cuticles, particularly of the cuticular waxes (Schreiber, 2006) and may thereby influence the barrier properties of the cuticle.

The disruption adjuvants add to the cuticular barrier might have consequences for the plant. Furthermore, adjuvant-induced changes of the plant surfaces or in the tissue metabolism may lead to phytotoxicity (Jursik et al. 2013, Knoche et al. 1992). Thus, the trade-off between the use of adjuvants in agricultural production systems and the influence of those adjuvants on plant health itself and the environment should be optimized. Until now, very little emphasis has been given to the adjuvant's impacts on crop water balance or drought tolerance. Hence, we hypothesized that adjuvants can alter cuticular water conductance (g_{\min}) and affect wax morphology and photosynthetic activity in dependence on the adjuvant's properties. We tested this hypothesis using adjuvants of largely differing chemistry and solubility in waxes on the leaves of apple and kohlrabi. The surfaces of kohlrabi leaves exhibit multi-dimensional epicuticular waxes and have a moderate surface wettability. In contrast, leaves of apple have a smooth surface and a high wettability.

2 Materials and Methods

2.1 Plant material and growth conditions

Kohlrabi (*Brassica oleracea* cultivar *gongylodes*) and apple (*Malus domestica* cultivar *Bittenfelder*) plants were germinated and then pre-grown in a greenhouse during late summer 2017 with supplemental light (SON-K 400, DH Licht, Germany) reaching a photoactive radiation (PAR) of about $190 \mu\text{mol m}^{-2} \text{s}^{-1}$ at plant canopy level. The seedlings were grown in ED 73 standard substrate until three (kohlrabi) or four (apple) leaves were fully developed. Plants were then transferred into a controlled environmental chamber with $\text{PAR} = 115 \mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level, 12 h day/night, radiation source: white fluorescence lamps (MasterPL-L4P, Philips, The Netherlands). The average day/night temperature was $25 \text{ }^\circ\text{C} / 21 \text{ }^\circ\text{C}$ and the relative humidity was 75 % / 86 %, respectively. Plants were irrigated automatically via capillary matting. Plants with homogenous growth were selected for the conduction of the experiment. On the selected plants, the two youngest, fully developed leaves (3-8 days old leaves for both kohlrabi and apple) were labeled 1 day prior to the adjuvants application. At the last day of the experiment, the marked leaves used for measurements were 17-22 days old. The first three fully developed youngest leaves of each selected plant were labeled for g_{min} measurement and the second three fully developed youngest leaves were labeled for g_{tot} measurement prior to the start of the experiment. Similarly, scanning electron microscope (SEM) observation and contact angle (CA) measurement were performed on a treated, fully developed, youngest leaf from each treatments. Leaf mass per area (LMA) and leaf dry matter content (LDMC) were determined from the same leaves used for g_{min} measurement.

2.2 Leaf traits and scanning electron micrographs

Leaf mass per area ($\text{LMA} = [\text{mg cm}^{-2}]$) and leaf dry matter content ($\text{LDMC} [\text{mg g}^{-1}]$) were determined at the starting day of the experiment from the water-saturated-leaf fresh weight (FW_s), the dry weight (DW) and the leaf area (A). FW_s was determined by cutting off the petioles with a razor blade and saturating the leaves with the cut petioles immersed in water for 1 h. Leaf petioles were carefully blotted dry with a soft tissue and immediately weighted. DW was determined by drying of the leaf at $50 \text{ }^\circ\text{C}$ until constant weight.

Leaf surface micrographs were taken on the adaxial leaves 2 days after adjuvant treatment (DAT). The leaves were mounted on the sample stubs and air-dried in a desiccator with silica gel. Pictures were recorded without conductive coating by a scanning electron microscope (SEM) (LEO 1530, Carl Zeiss AG, Oberkochen, Germany) at 1 kV.

2.3 Adjuvants and foliar application

The adjuvants were selected based on their contrasting mode of action (table 1). The treatment solutions were diluted (0.1 % (w/v)) one hour before application in demineralized water. The emulsion containing RME was in a homogenous state through constant stirring until application. The other adjuvants, APG and POEA were hand-shaken before application. Leaves were treated on both sides (adaxial + abaxial) by dipping the leaves into a beaker with demineralized water (as control treatment) or into the surfactant solution or emulsion, respectively. This treatment procedure was almost similar to the application pattern of spraying the leaves until dripping wet in practice. Adjuvant deposition was calculated by dividing the weight loss of the beaker by the leaf area of the dipped leaves.

Table 1: List of selected adjuvants and their properties derived from the product data sheet or from chemical data bases (logKow for POEA and RME).

| Adjuvant | CAS number | Molecular structure | LogKow |
|--------------------------------------|-------------|---|--------|
| Alkyl polyglycoside (APG) | 157707-87-4 | not applicable | 3.7 |
| Polyoxyethylated tallow amine (POEA) | 61791-26-2 | C ₂₈ H ₅₇ N ₁ O ₅ | 5.9 |
| Rapeseed methyl ester (RME) | 112-62-9 | C ₁₉ H ₃₆ O ₂ | 7.4 |

2.4 Contact angle

Contact angle was determined in order to analyze the persistence of adjuvant-induced alterations of the physicochemical characteristics of the leaf surfaces after adjuvant treatment.

Therefore, 5 μL pure water droplets ($n = 10$) were applied to the surfaces as indicated in previous reports (Matos and Rosado, 2016, Pariyar et al. 2017). In contrast to smooth surfaces, where 2 μL droplets are commonly measured, the varying surface energy of rough contact surfaces (leaf surfaces) is best examined by a droplet volume of 5 μL . Determinations were done 0, 2, 7 and 14 (only kohlrabi) DAT. Zero DAT measurements were carried out 1 h after treatments, when treatment solutions had dried on the leaf surfaces. For this purpose, leaves were fixed either adaxial or abaxial onto glass slides with double-sided adhesive tape (Tesa SE, Hamburg, Germany) and the contact angle was recorded using a droplet shape analyzer (DSA30, Krüss GmbH, Hamburg, Germany). Since left and right angles of the droplet were not significantly different, means were calculated from the pooled data from both left and right angle.

2.5 Minimum epidermal water conductance (g_{min})

Minimum epidermal water conductance was determined by the gravimetric measurement of water loss of detached leaves as described in previous reports (Burkhardt and Pariyar, 2014, Kerstiens, 2006, Sack and Scoffoni, 2011). Measurements were conducted 0, 2, 7 and 14 DAT, whereas 0 DAT measurements were carried out 1 hour after treatments. The leaves ($n = 6$) were detached including 2 cm of the petiole using a razor blade, put in water and allowed to saturate for 1 h. The petioles were then sealed with melted paraffin wax (melting point 44 $^{\circ}\text{C}$) to prevent water loss. The sealed leaves were placed in an empty Eppendorf-tube (3 ml) covered with Parafilm to ensure erect stand and put on a sample holder in a shaded area of the growth chamber in a controlled environment. The temperature and relative humidity data were recorded (Tinytag, Gemini Data Loggers, Chickester, UK). During the experiment, mean temperatures were (22.6 \pm 0.36) $^{\circ}\text{C}$, (22.7 \pm 0.19) $^{\circ}\text{C}$, (22.6 \pm 0.32) $^{\circ}\text{C}$ and (22.5 \pm 0.33) $^{\circ}\text{C}$, mean relative humidity were (80.6 \pm 3.8) %, (81.9 \pm 2.32) %, (82.2 \pm 2.21) % and (81.1 \pm 2.52) % on 0, 2, 7 and 14 DAT, respectively. The leaf was weighed on a digital balance (4 digits) every 10 min for the first 90 minutes, every 20 min for the subsequent 120 minutes and every 30 min for the last 210 minutes (420 minutes total). After a critical value of the relative water deficit was reached, the leaf weight decreased linearly with time leading to constant water loss. This turnover point indicates the minimum threshold for stomatal water loss. g_{min} ($\text{mmol m}^{-2} \text{s}^{-1}$) was determined from the measurements of the last 120 min, where the water loss

curve achieved linearity for all adjuvant treatments (figure 2). The leaf area was determined by photographing the leaf after the measurement horizontally from a fixed height and angle together with a 2 x 2 cm² red size reference and processing of the image with the image processing software Easy Leaf Area (O'Neal et al. 2002).

2.6 Total epidermal water conductance (g_{tot})

Total water conductance was determined by the gravimetric measurement of water loss of detached leaves at constant water saturation. Total conductance was measured 0, 2, 7 and 14 DAT. Zero DAT measurements were carried out 1 hour after treatments. Leaves ($n = 6$) were detached including 2 cm of the petioles using a razor-blade and immediately re-cut under water to prevent embolism. The leaves were then put in a 3.5 ml cuvette containing distilled water, which was sealed with parafilm to prevent water loss from evaporation and to ensure erect positioning. The leaves in the tubes were placed in a holder and put at block-randomization in the controlled environment growth chamber under white fluorescence lamps (MasterPL-L4P, Philips, The Netherlands), which emitted 115 $\mu\text{mol m}^{-1} \text{s}^{-1}$ photoactive irradiation at leaf level. The detached leaves were kept under constant ventilation of a fan to ensure a constant driving force for transpiration. Weighing was done 2 h after putting the petioles in water to allow water saturation and adaptation of the cut leaves to the measurement environment. The weights were determined subsequently every 30 min over a 2 h period. The leaf area and the total water conductance were determined by the same methods used for g_{min} (section 2.5).

2.7 Maximum fluorescence and photosynthetic efficiency

To evaluate whether different adjuvants influence the photosystem reactions of kohlrabi and apple leaves, chlorophyll fluorescence measurements on leaves were recorded by pulse-amplitude modulated (PAM) chlorophyll fluorometry combined with saturating pulse analysis of fluorescence quenching. This measurement provides quantitative information on the quantum yield of photosynthetic energy conversion and potential phytotoxic effects of agrochemicals. The induction curves were recorded for kohlrabi (3rd leaf from base, 17-21 days old) and apple (4th leaf from base, 17-21 days old) at laboratory conditions (ambient CO₂ concentra-

tion) using an imaging PAM fluorometer (ImagingPAM, Heinz-Walz GmbH, Effeltrich, Germany). The fluorometer is equipped with 96 blue light-emitting diodes (peak at 470 nm) used for fluorescence excitation, actinic illumination and saturation pulses. Fluorescence images (640×400 pixel) were taken on the fully expanded leaves with one measurement per plant. Plants were dark-adapted (30 min) prior to the measurements. For each measurement, one representative (circular for droplet application or rectangular) area of interest (AOI) was selected. The recorded images were analyzed by ImagingWinv2.46i (Heinz-Walz GmbH, Effeltrich, Germany). Ground fluorescence (F_o) was recorded after leaf illumination by the blue light-emitting diodes ($0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$), the maximum fluorescence (F_m) was determined after a blue light saturation pulse of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The yield of variable chlorophyll fluorescence (F_v) was calculated as $F_m - F_o$, while the maximum photochemical efficiency of the photosystem II (PSII) was calculated as F_v/F_m . After the first saturation pulse, actinic light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) was switched on and saturation pulses ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) were applied at 20s interval within a period of 4 minutes.

2.8 Statistics

All measurements were conducted on individual leaves of different biological replications. Data analysis and statistics were performed using the R statistical program (R Development Core Team 2017) or in SPSS (v.25). Graphs were made either with SPSS or with SigmaPlot (v.13.0). Data was tested for normal distribution (Sapiro-Wilk test) and homogeneity (Levene -test). Cuticular conductance data was transformed logarithmically for the statistical analysis based on Baur (1997) since conductance data follows a log-normal distribution. Statistical analysis was performed by one-way analysis of variance (1-way ANOVA) and the Tukey's (honestly significant difference or HSD) pairwise comparison or Duncan multiple range test ($p \leq 0.05$) was used to determine the significant differences among the treatment groups.

3 Results

3.1 Leaf traits

The leaf mass per area (LMA) of kohlrabi and apple was $(3.4 \pm 0.5) \text{ mg cm}^{-2}$ and $(3.0 \pm 0.5) \text{ mg cm}^{-2}$, respectively. An almost 3 times higher leaf dry matter content (LDMC, mg g^{-1} fresh mass) was observed for apple leaves ($232.7 \pm 30.9 \text{ mg g}^{-1}$) compared to kohlrabi ($80.7 \pm 9.9 \text{ mg g}^{-1}$).

3.2 Epicuticular wax microstructure

The pronounced epicuticular wax microstructure of kohlrabi leaves was altered by adjuvant application as observed in SEM micrographs (figure 1b-d). However, this effect was not observed in the smooth epicuticular wax layer of apple (micrographs not shown). Kohlrabi leaf surfaces are characterized by filamentous epicuticular wax crystals, which are composed of tubes and very fine filaments (figure 1a). Adjuvant application led to a change in the wax microstructure, and the fine filaments branching from the tubes vanished on the treated leaf surfaces (figure 1b-d). RME-treated leaf surfaces appeared furthermore affected as the tubes folded and laid flat on the surface instead of protruding upwards (figure 1d). In addition, flat and thin structures appeared on the leaf surfaces.

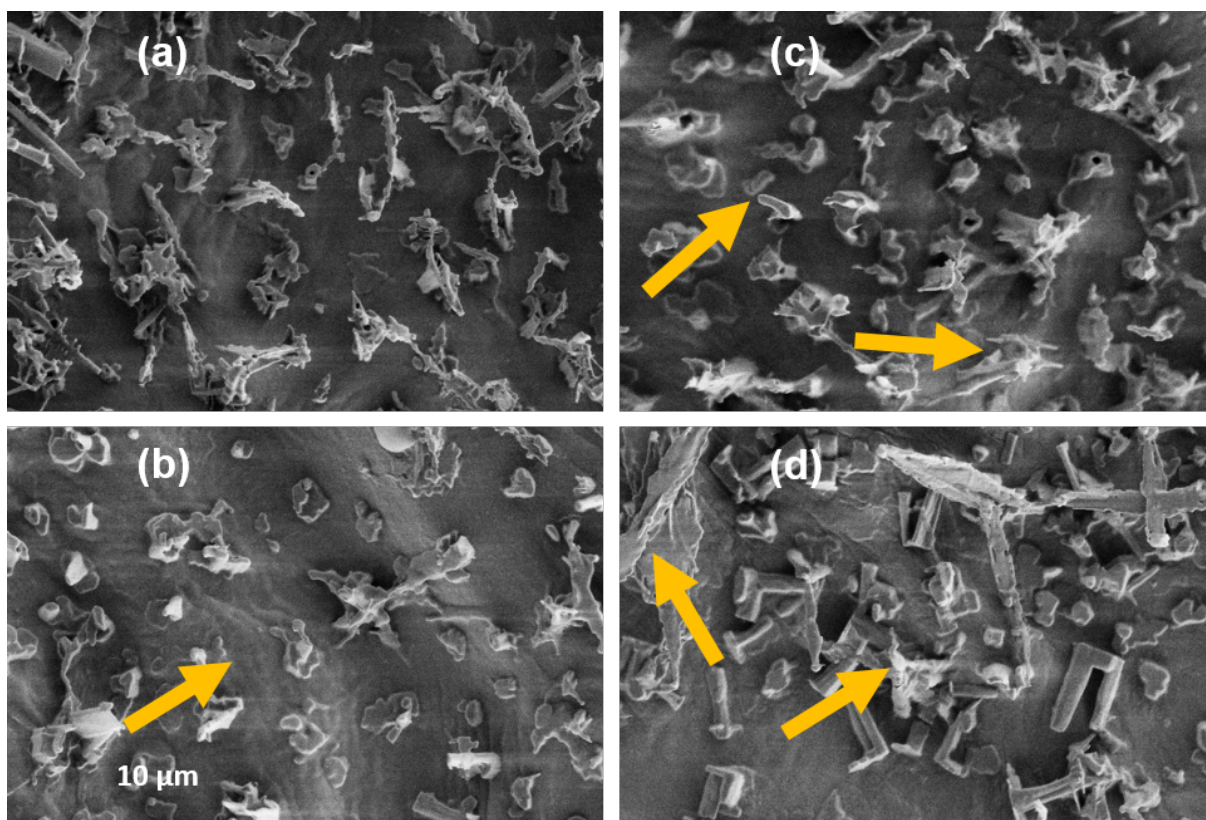


Figure 1: Scanning electron microscope (SEM) micrographs showing the changes in the leaf surface microstructures (shown by arrows) on *B. oleracea* after adjuvant treatment. SEM pictures were captured 2 days after the treatments either with pure water (a) or with 0.1 % (w/v) alkyl polyglycoside (APG) (b), polyoxyethylated tallow amine (POEA) (c) or rapeseed methyl ester (RME) (d).

3.3 Contact angle

Contact angle (CA) of the sessile droplets of pure water on kohlrabi ($130^{\circ} - 135^{\circ}$) was higher than on apple ($96^{\circ} - 98^{\circ}$) leaves. Moreover, CA on kohlrabi adaxial leaves was $135 \pm 2.5^{\circ}$ (mean \pm SE) and on kohlrabi abaxial leaves was $130 \pm 1.4^{\circ}$. In contrast, CA on apple adaxial leaves was $96 \pm 1.1^{\circ}$ and on apple abaxial leaves was $98 \pm 0.4^{\circ}$.

Contact angle of kohlrabi leaves was significantly lower after APG or RME treatment on 0 and 2 DAT as compared to the treatment with demineralized water on both adaxial and abaxial surfaces (figure 2a, table 2). A strong decrease of CA after RME treatment was observed on both sides of kohlrabi leaves. However, CA after RME treatment increased and became similar to CA of the water-treated control at 7 DAT on adaxial and 14 DAT on abaxial leaf sides (table 2). CA of APG-treated adaxial and abaxial leaves was depressed but adjusted to the control at

7 DAT (adaxial) and 2 DAT (abaxial) (table 2). POEA-treated leaves showed significantly decrease in CA on adaxial leaves for all measurement days (0-14 DAT), however, it did not differ at 0 and 2 DAT, but decreased significantly at 7 and 14 DAT on abaxial leaves (figure 2a, table 2) as compared to water treatment.

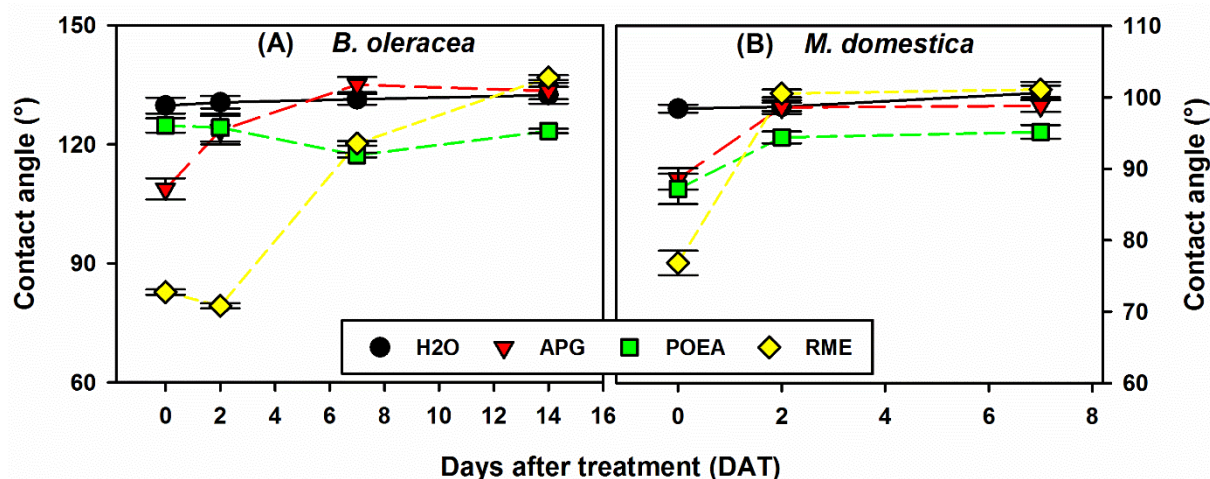


Figure 2: Effects of adjuvants on contact angle of water droplets (5 μ L) on kohlrabi (*B. oleracea*, (A) and apple (*M. domestica*, (B) abaxial leaves 0, 2, 7 and 14 (only kohlrabi) days after adjuvant treatment (DAT). The measurements at 0 DAT were done 1 h after application. The treatments were either water or 0.1 % (w/v) alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME). Values: mean \pm SE (n = 10, except n = 9 for RME application on kohlrabi adaxial leaves). Significant differences are shown in table 2 below.

The contact angle of water on apple leaves (adaxial, abaxial) was significantly reduced by adjuvants at 0 DAT on both adaxial and abaxial leaves (figure 2b, table 2). CA of APG and RME-treated leaves (i.e. both adaxial and abaxial) increased and became similar to water-treated control already at 2 DAT (table 2). POEA-treated leaves showed significant decrease in CA on adaxial leaves 0 and 2 DAT, but it increased and became similar to water-treated leaves on 7 DAT (table 2). However, CA of POEA on abaxial leaves significantly decreased on all measured days (0, 2 and 7 DAT) as compared to water treatment (figure 2b, table 2).

Table 2: Contact angle data and statistical comparisons between the treatments measured on the leaves treated either with water or with 0.1 % (w/v) alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME). The measurements were done subsequently on 0, 2, 7 and 14 days (only kohlrabi) after treatment application. The measurements at 0 DAT were done 1 h after application. Values: mean \pm SE (n = 10, except n = 9 for RME application on kohlrabi adaxial leaves). Significant differences were at $P < 0.05$ (Tukey-HSD/Duncan test) and denoted by different letters (a, b, c, d), where (a) > (b) > (c) > (d). The same letters (a, a, a, a) in all treatment groups for each measurement days (0, 2, 7, 14) denotes that the values are non-significance ($P > 0.05$).

| Crop | Leaf side | DAT | Treatments | | | | Statistical Test |
|----------------------------------|-----------|-----|----------------|----------------|---------------|---------------|------------------|
| | | | H2O | APG | POEA | RME | |
| <i>B. oleracea</i> (Kohlrabi) | Adaxial | 0 | 135 ± 4 (a) | 99 ± 1 (b) | 111 ± 8 (b) | 75 ± 1 (c) | Duncan-test |
| | | 2 | 138 ± 2 (a) | 126 ± 1 (b) | 120 ± 3 (c) | 81 ± 1 (d) | Duncan-test |
| | | 7 | 130 ± 4 (a) | 128 ± 1 (a) | 101 ± 5 (b) | 128 ± 1 (a) | Duncan-test |
| | | 14 | 130 ± 1 (a) | 131 ± 1 (a) | 106 ± 1 (b) | 128 ± 1 (a) | Duncan-test |
| | Abaxial | 0 | 130 ± 2 (a) | 109 ± 3 (b) | 125 ± 2 (a) | 83 ± 1 (c) | Tukey-HSD |
| | | 2 | 131 ± 2 (a) | 124 ± 4 (a) | 124 ± 4 (a) | 79 ± 1 (b) | Tukey-HSD |
| | | 7 | 131 ± 1 (a) | 135 ± 2 (a) | 117 ± 1 (b) | 120 ± 1 (b) | Tukey-HSD |
| | | 14 | 132 ± 2 (a) | 134 ± 2 (a) | 123 ± 1 (b) | 137 ± 1 (a) | Tukey-HSD |
| <i>M. domestica</i> (Apple) | Adaxial | 0 | 96 ± 2 (a) | 88 ± 4 (b) | 86 ± 2 (b) | 75 ± 1 (c) | Duncan-test |
| | | 2 | 100 ± 1 (a) | 101 ± 1 (a) | 97 ± 1 (b) | 101 ± 1 (a) | Tukey-HSD |
| | | 7 | 100 ± 1 (a) | 98 ± 2 (a) | 100 ± 1 (a) | 100 ± 1 (a) | Tukey-HSD |
| | Abaxial | 0 | 98 ± 1 (a) | 89 ± 2 (b) | 87 ± 2 (b) | 77 ± 2 (c) | Tukey-HSD |
| | | 2 | 99 ± 1 (a) | 99 ± 1 (a) | 94 ± 1 (b) | 101 ± 1 (a) | Tukey-HSD |
| | | 7 | 101 ± 1 (a) | 99 ± 1 (ab) | 95 ± 1 (b) | 101 ± 1 (a) | Tukey-HSD |

3.4 Water loss

Leaf water loss 1 h after adjuvant treatment from the excised saturated and sealed (petiole) kohlrabi leaves at room temperature differed between experimental treatments. In general, the curves were characterized by high initial water loss due to high leaf water content and open stomata. This tendency declined strongly afterwards, indicating the proceeding closure of stomata and conversion into a phase of constant water loss (minimum transpiration). At this stage, it can be assumed that stomata are closed, but residual transpiration through the partially closed stomata could be possible (Figure 3, shown with horizontal dash-lines and arrows). The APG-treated leaves showed a water loss behavior similar to the control leaves (Figure 3a, b). The onset of stomatal closure of POEA-treated leaves happened after an average of 154 min at a constant water loss of $13.4 \pm 4.43 \text{ mg min}^{-1}$ (Figure 3c) and thereby took longer than that of APG and control treatments, which leveled after 131 and 129 min to a constant water loss of $8.5 \pm 1.88 \text{ mg min}^{-1}$ and $6.5 \pm 2.21 \text{ mg min}^{-1}$, respectively) (Figure 3a, b). The RME-treated leaves were characterized initially by a less steep decline of water loss and a delay of stomatal closure leading to a remarkably prolonged time until the curve leveled after 192 min to constant water loss ($31.5 \pm 11.90 \text{ mg min}^{-1}$) (Figure 3d).

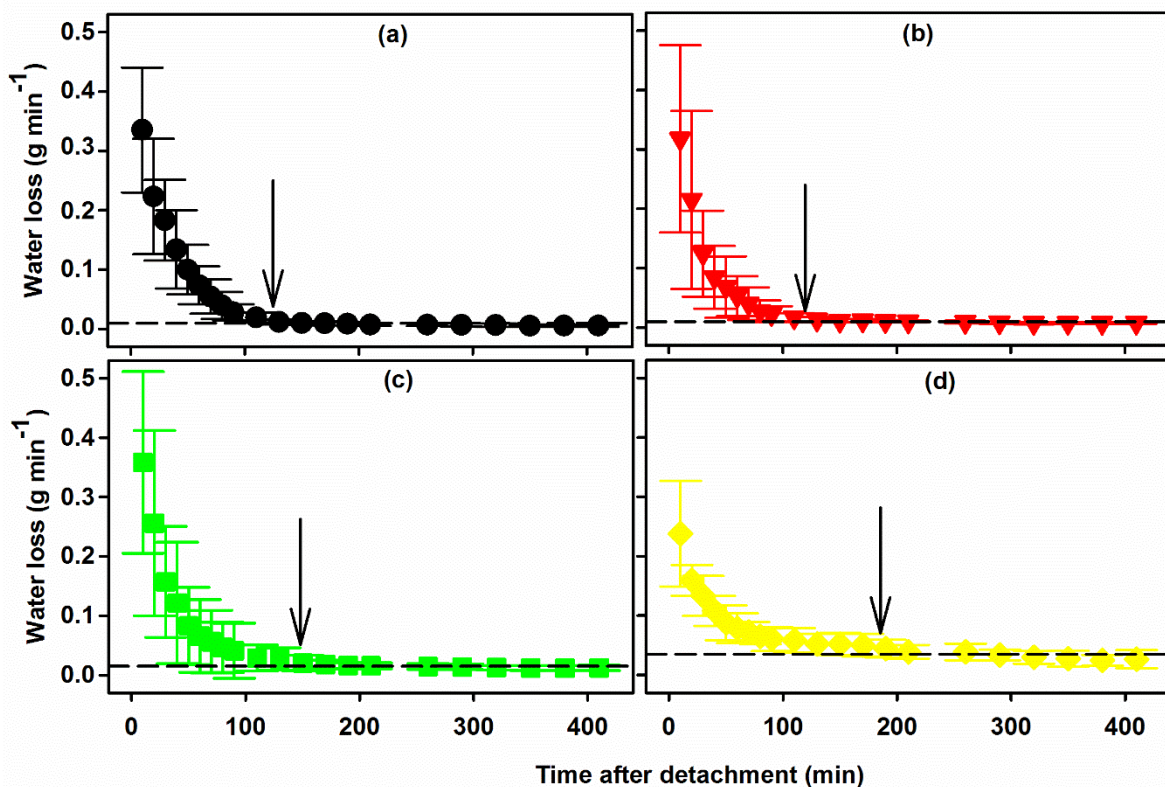


Figure 3: Comparison of temporal water loss trend of detached kohlrabi (*B. oleracea*) leaves as affected by water as control treatment (a) or by adjuvant treatments (b-d). Leaves were detached one hour after treatments either

with water or with 0.1 % (w/v) alkyl polyglycoside (APG) (b), polyoxyethylated tallow amine (POEA) (c), rapeseed methyl ester (RME) (d). Water loss values of a single leaf at a given time were linearly interpolated from the water loss curves measured successively within the time range at room temperature, since several weight measurements could not be made at the same time. Horizontal dash-lines and arrows represent the turnover point just before the constant water loss from excised leaves and indicate the minimum threshold for stomatal water loss. The turn over points were: water (at 129 min and constant water loss of $6.5 \pm 2.21 \text{ mg min}^{-1}$), APG (at 131 min and constant water loss of $8.5 \pm 1.88 \text{ mg min}^{-1}$), POEA (at 154 min and constant water loss of $13.4 \pm 4.43 \text{ mg min}^{-1}$), RME (at 192 min and constant water loss of $31.5 \pm 11.90 \text{ mg min}^{-1}$). Values: mean \pm SE (n = 6).

3.5 Minimum (g_{\min}) and total (g_{tot}) epidermal water conductance

Minimum epidermal water conductance (g_{\min}) of kohlrabi leaves was significantly increased by RME treatment on 0 and 2 DAT, which was an about 10.4-fold increase as compared to the water-treated control leaves (figure 4a). However, RME-treated leaves adjusted to the level of the untreated leaves 7 DAT. APG treatment did not have a significant effect on g_{\min} . In contrast, POEA treatment doubled minimum conductance, and no reversibility of the effect was observed during the investigated time span (14 DAT). Minimum epidermal conductance of apple leaves increased significantly with RME treatment only at 7 DAT as compared to the untreated control leaves (figure 4b, supplementary table 2). In contrast to kohlrabi, the share of g_{\min} on g_{tot} did not increase in apple leaves treated with RME due to the associated increase of g_{tot} (supplementary table 1).

No significant effect of the adjuvant treatments on g_{tot} was observed for both kohlrabi and apple (figure 4c, d), although total conductance increased by 50 - 100 % with RME on 0 and 2 DAT as compared to water-treated control leaves (figure 4c, d).

Minimum and total epidermal conductance of apple were concordantly non-significantly increased by the adjuvants in a way that the share of minimum on total water conductance was left unaltered (supplementary table 1). This was not the case for kohlrabi. Notwithstanding the increases in g_{\min} , g_{tot} was left unaltered by the adjuvants. Consequently, the contribution of g_{\min} to g_{tot} increased after application of RME or POEA for kohlrabi. The share of minimum on total conductance of untreated control kohlrabi leaves was 3.5 %, whereas RME treatment increased the share to 41.7 % on 0 DAT (supplementary table 1).

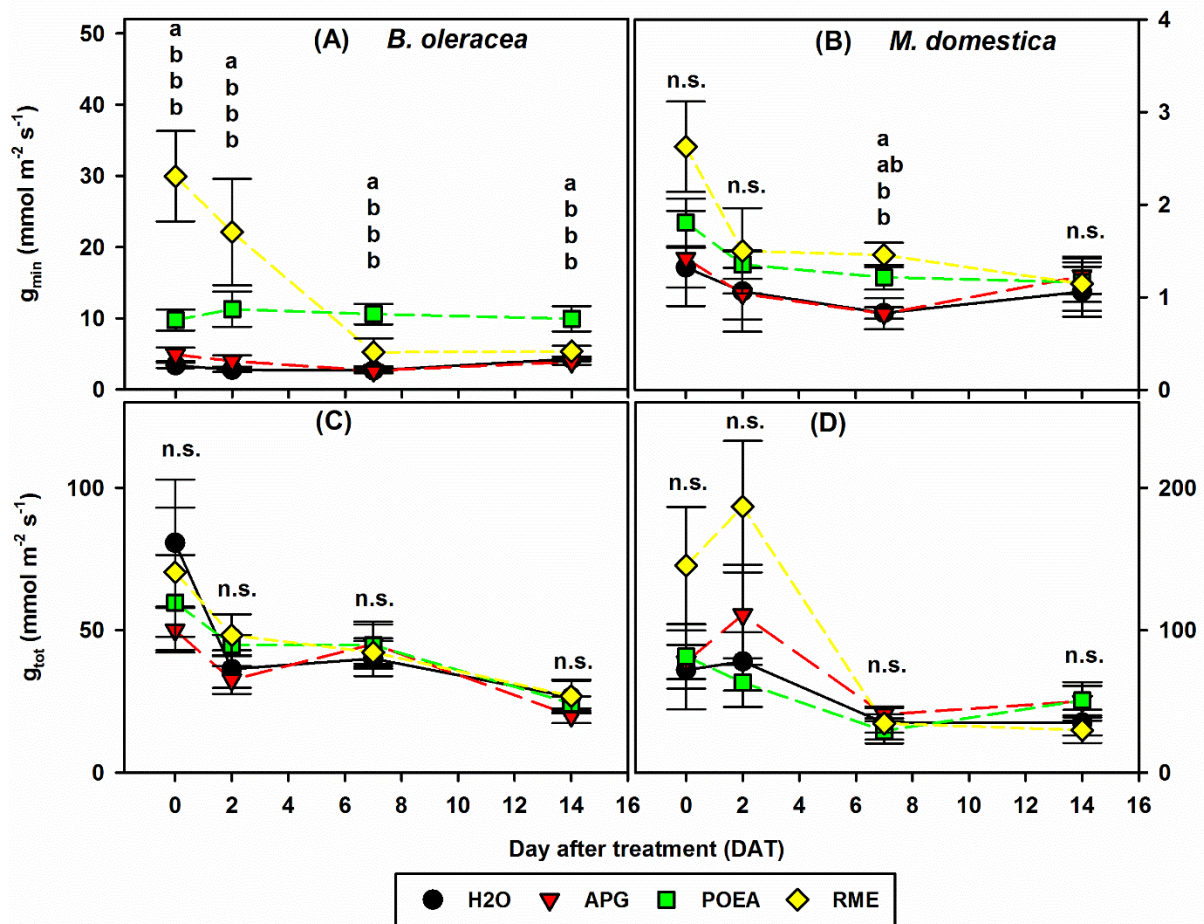


Figure 4: Effects of adjuvants (0.1% w/v) on minimum epidermal conductance (g_{min}) and total epidermal conductance (g_{tot}) on kohlrabi (*B. oleracea*) (A, C) and apple (*M. domestica*) (B, D) leaves, respectively. Adjuvants were alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME), and water as control treatment. Measurements were done 0, 2, 7 and 14 days after adjuvant treatment (DAT). Zero DAT measurements were performed 1 h after treatment applications. Values: mean \pm SE ($n = 6$). Significant differences were at $P < 0.05$ (Tukey-HSD/Duncan test) and denoted by different letters (a, b, c, d), where (a) > (b) > (c) > (d). n.s. = not significant ($P > 0.05$).

3.6 Fluorescence yield and photosynthetic efficiency

Maximum fluorescence and photochemical efficiency (F_v/F_m) varied between plant species for the tested adjuvants. POEA had an effect on photosystem reactions on kohlrabi but not on apple leaves. In kohlrabi, maximum fluorescence was affected in both the dark (F_m) and light (F_m') phase of the photosystem kinetics (figure 5a), but F_v/F_m was affected only in the light phase (figure 5c). In apple, neither F_m and F_m' nor F_v/F_m was affected by adjuvant application (figure 5b, d).

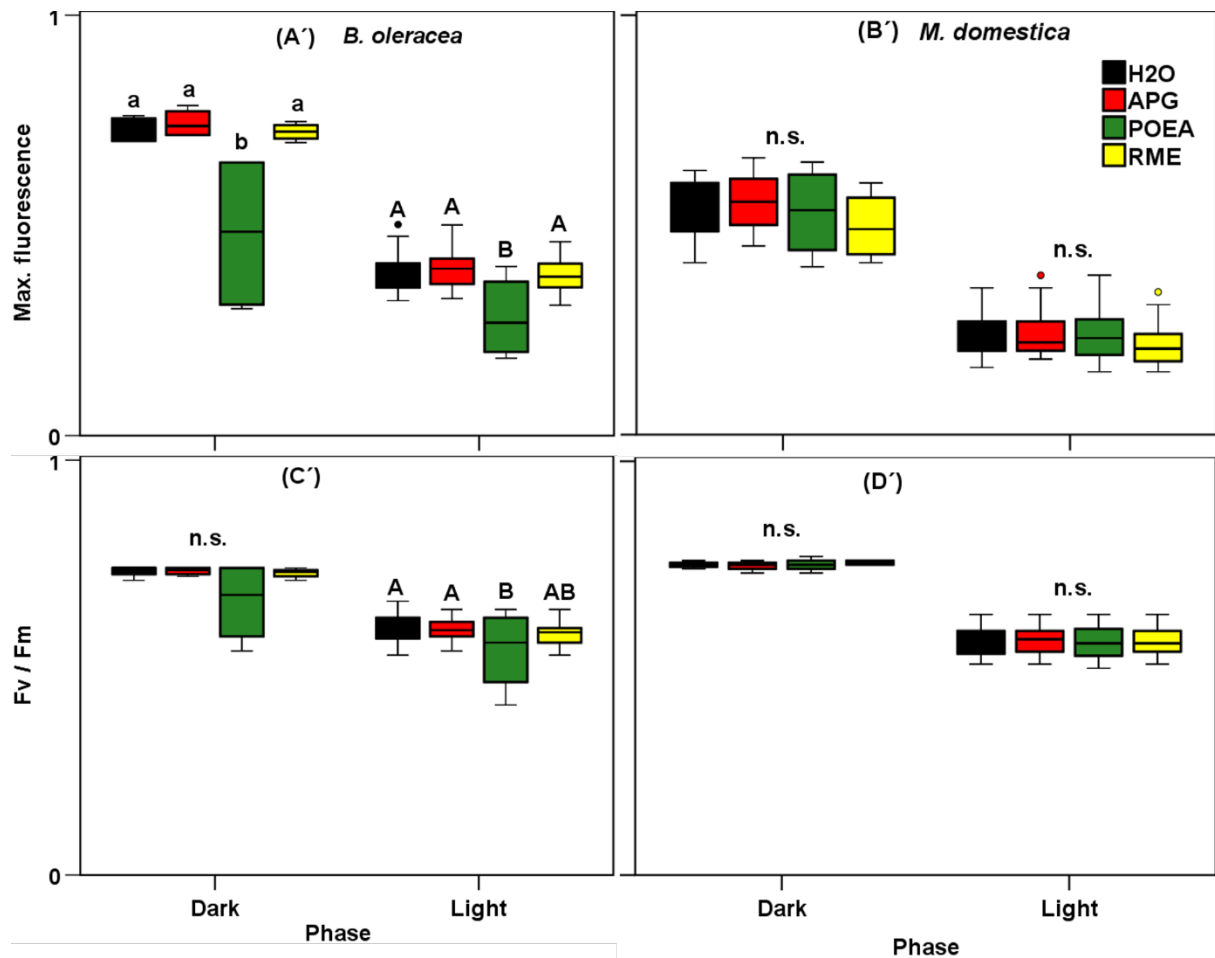


Figure 5: Comparison of maximum fluorescence yield and photochemical efficiency (F_v/F_m) in PSII as affected by the application of adjuvants (0.1 % w/v) on kohlrabi (*B. oleracea*) (A', C') / apple (*M. domestica*) (B', D') adaxial leaves. A single droplet (5 μ L) was applied for each treatment (control: water, alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME)) on the same leaves with 4 biological replications. Photosystem kinetics measurements were performed 1 day after treatments. Before measurements, plants were dark adapted for 30 minutes. Bars: mean \pm SE ($n = 4$, dark phase, $n = 20$, light phase). Significant differences were at $P < 0.05$ (Tukey-HSD test) and denoted by different letters (a, b or A, B for dark or light phase). n.s.: not significant ($P > 0.05$).

4 Discussion

Kohlrabi (*B. oleracea*) and apple (*M. domestica*) differed largely in their susceptibility to adjuvant-induced alterations on surface properties. Some of the main reasons for the different responses of the two species might be the varying leaf traits and their wax barriers. Kohlrabi had a lower LDMC than apple, but their LMA was similar. LDMC as a functional leaf trait reflects physiological functions, such as transpiration, and is a valid predictor for the parameters of stomatal regulation (Krober et al. 2015). A higher LDMC enables a plant to keep the integrity of the leaf even at low moisture and might be linked to the different transpiration responses

of the crop species to the adjuvant application. Besides that, surface characteristics of apple and kohlrabi are varying largely. The hypostomatic kohlrabi leaves were characterized by filamentous wax structures and a higher abaxial CA (130°) than the amphistomatic abaxial apple leaves (98°) with their flat epicuticular surfaces. Such differences in leaf surface traits and wax microstructures contributed to the physical-chemical interaction between adjuvant and leaf surface, and might have determined their varying responses to the applied adjuvants.

In this study, the effect of adjuvant was closely linked to an alteration of the epicuticular wax morphology. Similar alterations on epicuticular waxes by ionic and non-ionic surfactants have been reported previously (Damato et al. 2017, Knoche et al. 1992). The decreased contact angle after adjuvant application indicates a decrease of the roughness of the leaf surface as observed in SEM micrographs (Figure 1b-d). In addition, minor residues of the adjuvants on the surfaces might also have long-term effect on the contact angle. This alteration of epicuticular waxes implies an associated alteration of the intracuticular waxes, which are discussed to constitute the transpiration barrier in leaves (Jetter and Riederer, 2016, Zeisler and Schreiber, 2016, Zeisler et al. 2018) and might explain the associated increase of g_{\min} . Similar changes on wax film alterations by surfactants were also reported by Pambou et al. (2018). Such phenomena may be due to the plasticizing effects of accelerators (Schreiber, 2006) or due to a solubilization of wax filaments (Knoche et al. 1992, Noga et al. 1986). Adjuvants interact with leaves in different ways in order to improve the efficiency of the spray liquid. Surfactants lower the surface tension of the liquid and thereby enhance e.g. the wetting. Furthermore, surfactants might improve penetration of the active ingredients via lowering the surface tension to such an extent that the spray liquid might enter crevices within the cuticular waxes. However, it is generally assumed that an accelerated penetration is established by plasticizing of the cuticular waxes. As a consequence, the plasticized waxes will allow an accelerated penetration since the energy needed for diffusion through the waxes is lowered (Fagerström et al. 2014, Schreiber, 2006). It is yet unknown which properties determine the penetration accelerating effect of adjuvants, however, it is known that the acceleration effect is strongly linked to the adjuvant's solubility in the cuticular waxes (Burghardt et al. 1998, Schreiber 1995, Šimáňová et al. 2005). In theory, a higher permeability for active ingredients should result in higher water permeability and vice versa. In fact, a Kow-dependent increase of cuticular water

conductance was already proven using a range of polydisperse surfactants on isolated, stomatous cuticles (Baur et al. 1999, Riederer and Schreiber, 2001). This study provides the first *in vivo* proof of an increase of the water permeability of leaves by adjuvants. Concordantly to the theory, $\log K_{ow}$ might be closely linked to the potential of the adjuvant to increase minimum conductance. RME with the highest $\log K_{ow}$ of 7.4 increased g_{min} 1 h after application 10-fold, POEA with a $\log K_{ow}$ doubled g_{min} and APG with a $\log K_{ow}$ of 3.7 did not alter g_{min} .

Furthermore, the adjuvant effect was found to be reversible on living plants, as has by now also only been shown on isolated cuticles (Schreiber, 2006). Exceptionally, POEA-treated kohlrabi adaxial leaves did not recover the effects of POEA on cuticular water permeability even at 14 DAT. This might be due to the potential phytotoxic effect of POEA on kohlrabi leaves (figure 5a, c, supplementary figure 2), since adjuvant molecules accumulation in confined areas are related to the phytotoxic side effects (Hunsche and Noga, 2012). The decrease in photosynthetic activity by POEA (figure 5c) might support the potential phytotoxic effect on kohlrabi leaves. A similar phytotoxic effect was also reported in sunflower (Jursik et al. 2013).

Table 3: Comparison of the contribution of minimum epidermal conductance (g_{min}) to total conductance (g_{tot}) measured on leaves treated with 0.1 % (w/v) adjuvants - alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME). g_{min} and g_{tot} were sampled 1 h after treatment applications. Values: mean (n = 6) and calculated as $g_{min} / g_{tot} * 100$.

| Treatments | g_{min} / g_{tot} ratio (%) (<i>B. oleracea</i>) | g_{min} / g_{tot} ratio (%) (<i>M. domestica</i>) |
|------------------|--|---|
| H ₂ O | 3.5 | 1.1 |
| APG | 8.0 | 1.2 |
| POEA | 9.5 | 1.4 |
| RME | 41.7 | 1.3 |

Minimum epidermal transpiration (g_{min}) was relatively low and contributed only a small share of 3-6 % (kohlrabi) and 1-2 % (apple) to the total transpiration (g_{tot}) under the transpiration-favoring conditions of the study. Adjuvant application increased minimum transpiration in

case of kohlrabi up to 10-fold, so it accounted for 42 % of the total transpiration (table 3). However, the response of apple leaves to adjuvant application was less pronounced compared to kohlrabi. The fact that kohlrabi has stomata on both upper and lower leaf side might have also influenced transpiration. In addition, a stronger increase of g_{\min} following RME application on stomatous side of apple leaves was observed (supplementary figure 1), suggesting that stomatous leaves might be more affected by adjuvant application, similar to the findings of a previous study (Burkhardt et al. 2012). Moreover, a relatively higher increase in g_{\min} following adjuvant application in association with respective g_{tot} was observed for kohlrabi, however, such a relationship was not observed for apple. Leaves might have down-regulated stomatal conductance after adjuvant application, therefore the effects of adjuvants were negligible for g_{tot} . The down-regulation of stomatal conductance implies a reduction of photosynthetic water use, and the effective use of water might be severely impaired (Blum, 2009). Kohlrabi (both sides stomatous) and apple (abaxial stomatous) showed different strategies for g_{tot} , which is similar to the leaf economic spectrum of reflecting physiological functions (Krober et al. 2015). However, further studies on plant level are necessary to estimate whether an adjuvant-induced increased cuticular water conductance impairs the crop water balance to a significant extent.

5 Conclusion

In our study, we showed that agricultural adjuvants can increase minimum epidermal water conductance. Thereby drought tolerance of horticultural crops might be decreased, especially at repeated application. Moreover, we found that the increase of g_{\min} depends on the crop and the adjuvant used. Effects of adjuvants were more pronounced in kohlrabi (*B. oleracea*) in terms of g_{\min} , wax morphogenesis and photosynthetic activity than in apple (*M. domestica*). Additionally, the solubility of the adjuvant in cuticular waxes (expressed as $\log K_{\text{ow}}$) may be linked to its ability to increase g_{\min} . Among selected adjuvants, alkyl polyglycoside ($\log K_{\text{ow}}$ of 3.7) had no effect on plant water loss and plant photosynthetic performance. POEA and RME ($\log K_{\text{ow}}$ of 5.7 and 7.4, respectively) increased minimum epidermal conductance 10- and 2-fold, respectively. An increased non-photosynthetic water loss might affect water use effi-

ciency of crop plants. Furthermore, at impending desiccation, the survival of the plants depend on their capability to keep the transpirational water loss low. Application of some adjuvants before or during drought or frost might therefore affect the water balance of crop plants negatively.

Chapter D

“PVCURVEANALYSIS” – A NEW R PACKAGE FOR EFFICIENT GENERATION AND ANALYSIS OF PRESSURE VOLUME CURVES

resubmitted to Physiologia Plantarum (2022) after a rejection with an invitation to resubmit

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1 Introduction

The availability of water within and across biomes is expected to change as a consequence of global warming (Mankin et al. 2019). This highlights the need to gain detailed knowledge about the drought responses of plants. Evolutionary adaptations have led to a number of coping strategies, ranging from drought avoidance by water-storing organs to drought tolerance through physiological features. The diversity of properties related to efficient water use has necessitated the use of a variety of indices to predict a plant's performance under drought conditions and to screen for plant traits that convey drought tolerance (Lenz et al. 2006).

For decades, water potential at turgor loss has been one of the most widely used determinants of physiological drought tolerance (Bartlett et al. 2014). The turgor loss point is often recognized as a 'higher-level' drought tolerance trait, since it describes the range of water potentials at which the leaf maintains its turgidity and thereby its functionality (Lenz et al. 2006). Across species, it is related to other important determinants of drought tolerance, such as the soil water potential at which wilting is permanent, hydraulic safety margins and also water availability within and across biomes (Bartlett et al. 2012, Bartlett et al. 2016, Zhu et al. 2018). Water potential at turgor loss is classically derived from a plot of water potential as a function of relative water content, known as a pressure volume curve (Andersen et al. 1991). Four additional parameters describing cellular and structural properties related to drought performance can be derived from pressure volume curves: (1) the apoplastic water fraction, (2) the relative water content at the turgor loss point, (3) the osmotic potential at full turgidity, which

is highly responsive to drought (Bartlett et al. 2014) and (4) the modulus of elasticity, which may be linked to photosynthetic capacity, sclerophylly and drought tolerance (Nadal et al. 2018, Salleo and Nardini 2000).

The methodology for analyzing pressure volume curves has been introduced in the 70ies, and has been refined on several occasions (Tyree and Hammel 1972, Tu 2008, Sack et al. 2011, 2011b, Bartlett et al. 2012). However, common approaches are based on a poorly reproducible inspection of the measurement data, aiming to visually discriminate between the strongly decreasing and the linear parts of the curves. The limited reproducibility of results produced with these methods has been criticized since the 1980s (Schulte and Hinckley 1985). In addition, visual estimations may not produce accurate predictions of the turgor loss point (supplementary table 3). Visually determining the turning points may produce greatly different estimations (supplementary figure 3). This large discrepancy among the results of visual curve analyses stresses the importance of a standardized and reproducible analysis protocol.

The R-package 'pvcurveanalysis' presented here is designed to address the need for both standardized and quick trait determination. It provides a toolbox for generating, analyzing and displaying pressure volume curves based on experimental datasets. The package builds on well-established procedures (Sack et al. 2011, Bartlett et al. 2012), but it eliminates the need for subjective assessments of curve characteristics. The package also improves the reliability of the analysis, when curves consist of relatively few data points or contain random noise. The functionality of the package is demonstrated using data derived from a controlled experiment on *B. oleracea* plants showing the interrelation of pressure volume parameters as well as their plasticity in response to drought stress

2 Biological principles of pressure volume curves

Pressure volume curves are derived experimentally by repeated measurements on detached leaves as described in detail, for instance, by Lenz et al. (2006). In brief, water potential is measured on leaves that are exposed to desiccation after previous saturation to full turgidity.

Pressure volume curves are obtained by plotting the obtained bulk leaf water potential as a function of relative water deficit. Bulk leaf water potential (ψ_{\square}) is composed of pressure potential (ψ_p) (turgor), osmotic potential (ψ_o) and matrix potential (Shackel 1987). Since the influence of the matrix potential is negligible, the relationship can be approximated as:

$$\psi = \psi_o + \psi_p \quad (1)$$

Turgor pressure is the main driver of water potential in a hydrated leaf. With advancing dehydration, turgor is lost at a rate determined by the cell wall stiffness, i.e. the modulus of elasticity (\mathcal{E}), leading to a strong decline of leaf water potential. With increasing turgor loss, the contribution of pressure potential to the overall leaf water potential decreases, and the influence of the osmotic potential becomes increasingly apparent. After the turgor loss point (π_{tlp} , water potential at turgor loss point or RWD_{tlp} , relative water deficit at turgor loss point), the pressure potential has no further influence, with the osmotic potential becoming the main driver of leaf water potential. The osmotic potential reflects the influence of cell solutes on the bulk leaf water potential. Osmotic potential is commonly expected to decline linearly with declining bulk leaf water potential. In a fully turgid leaf, the osmotic potential (π_o) is associated with the solute concentration in the cells. When turgor pressure is zero and osmotic potential is infinite, the remaining water in the leaf is found in extracellular compartments of the leaf, i.e. in the apoplastic fraction (a_f).

3 Analysis with the *pvcurveanalysis* package

3.1 Input data

The data analysis routine is implemented in the programming language R (R Core Team 2021) and available as a package ('pvcurveanalysis') on R's server network CRAN (<https://CRAN.R-project.org/package=pvcurveanalysis>). The functions of the package require data to be formatted as a 'data frame' with columns of equal length, with records for individual samples provided in chronological order. The names of the columns containing the required data may be specified as function arguments. For computational efficiency, all functions are applied to entire data frames and evaluated individually for each sample. Data are generally required to

be numeric, the sample ID must be an integer and the time of measurement must be specified using R's date and time format POSIXct. Before the data frame is processed, it is checked for correct order and data types. The code also implements plausibility checks to detect inconsistencies arising from erroneous unit assignments or shifted decimal separators.

3.2 Analysis routine for pressure volume curves

3.2.1 Initial Plateaus

Before applying any package functions, the user is required to remove initial plateaus in the ψ_{\square} versus RWD plots. Plateaus are data points at the beginning of the curve with a stronger initial decrease in water potential compared to the following points. Such plateaus, which may happen due to excessive apoplastic water uptake during rehydration, may otherwise lead to bias in the curve parameters, and they also interfere with the fitting procedure (Kubiske and Abrams 1990).

3.2.2 Saturated Fresh Mass

Measuring saturated leaf mass accurately is a prerequisite for obtaining reliable pressure volume curve parameters. Yet the leaf mass after saturation often deviates from the turgid mass, i.e. water potential after saturation is not zero, as also seen in the example data provided in the package (Arndt et al. 2015). In such cases, estimation of saturated fresh mass by the function 'FMSaturated' helps to improve the quality of the pressure volume parameters. Above the turgor loss point, a linear relationship between water content and water potential exists (Sack et al. 2011). Based on this premise, the function fits fresh mass values above an approximated turgor loss point linearly to water potential. The point where water potential of the linear regression line is zero equals the saturated water content.

3.2.3 Relative Water Deficit

Relative water deficit (RWD, %) can be calculated by the function 'RelativeWaterDeficit' from fresh mass (FM, g), dry mass (DM, g) and saturated fresh mass (FM_s, g) of the leaf as:

$$RWD = \frac{11 - (FM - DM)}{FM_s - DM} * 100 \quad (2)$$

3.2.4 Turgor Loss Point

For pressure volume curves, the negative inverse of bulk leaf water potential ($-\psi^{-1}$, measured in MPa^{-1}) is plotted as a function of relative water deficit (%). The function 'TurgorLossPoint' can be applied to pressure volume data to determine the coordinates at the turgor loss point. The Gauss-Newton algorithm of the R base function 'nls' is used to fit the following equation, which combines an exponential and a linear function, to the data:

$$-1\psi^{-1} = a * e^{b*RWD} + c * RWD + d \quad (3)$$

The linear part of the equation models the contribution of the osmotic potential on the inversed water potential. There is no uniform opinion in the literature on the best way to model pressure potential. For example, Schulte and Hinckley (1985) describe it as an exponential or a power function, in the pressure volume curve fitting excel tool from landflux.org however, a quadratic equation is used (landflux.org/resources/PV_Curve_Fitting_5.6.xls). We have decided here to use an exponential function, since results were best with it. The function 'nls' requires the estimation of starting values. Since parameters of pressure volume curves can vary substantially across species, starting values are estimated individually for each curve (table 1).

Table 1: Formulas for the estimation of starting values for the combined fit using the Gauss-Newton algorithm of the R core base function 'nls'. y = negative inverse of water potential in case of pressure volume curve data or conductance in case of leaf drying curve data, x = (RWD). last5 = last five values of the curve data.

| Parameters | Formula for starting value |
|------------|---|
| a | $\max(y)$ |
| b | -0.5 |
| c | $\text{slope}_{\text{last5}}$ |
| d | $\min(y) - \text{slope}_{\text{last5}} * \max(x)$ |

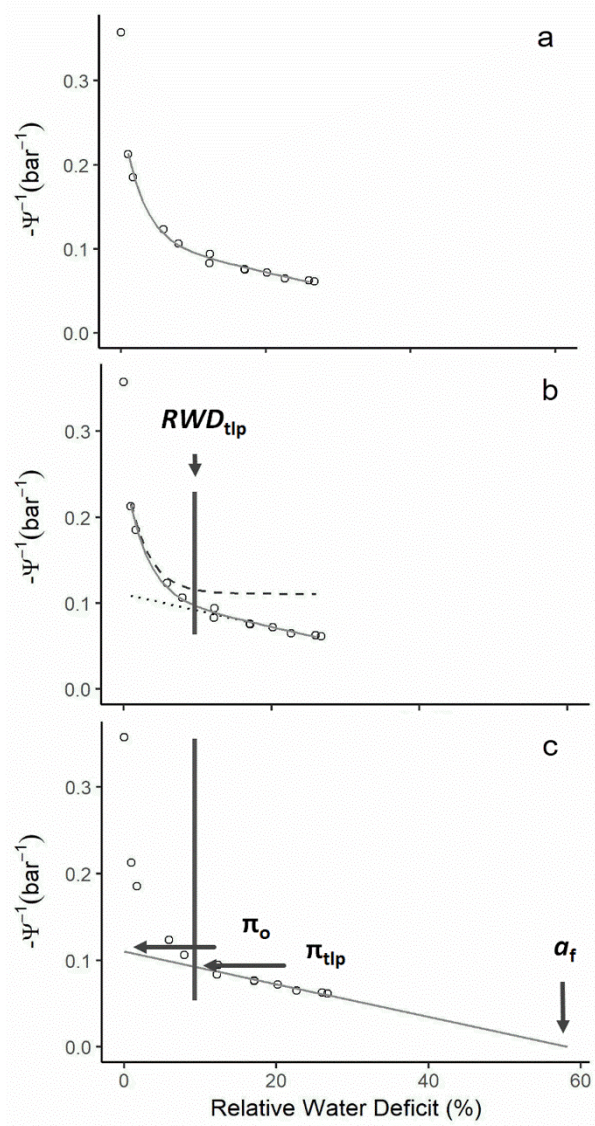


Figure 1: The pressure volume curve fitting routine of the functions ‘TurgorLossPoint’ (a, b) and ‘OsmoticPot’ (c). (a) Original data, with water potential ($-\psi^{-1}$) plotted against relative water deficit (RWD) with the fit. (b) The fit is separated into its exponential (dashed line) and linear (dotted line) parts and their point of minimum distance is relative water deficit at turgor loss point (RWD_{tlp}). (c) In the function ‘OsmoticPot’ the water potential data following the turgor loss point is linearly fitted and the interception points of the linear fit with RWD_{tlp} and with the y and the x axis are determined. These intercepts are water potential at turgor loss point ($-1/\pi_{tlp}$), osmotic potential at full saturation ($-1/\pi_o$) and apoplastic fraction ($100-a_f$), respectively.

After fitting (figure 1a), the linear and exponential parts of the combined fit are extrapolated across the range of relative water deficit measurements. The *RWD at turgor loss point* ($RWD_{tlp, \%}$) is then determined using the coefficient from the fit, as the point where the distance between the linear and the exponential curve reaches its minimum (figure 1b):

$$RWD_{tlp} = \frac{\log_e \frac{c}{a*b}}{b} \quad (4)$$

3.2.5 Osmotic potential and apoplastic fraction

The function 'OsmoticPot' allows separation of the osmotic potential ($-\psi_o^{-1}$, measured in MPa⁻¹) from the overall water potential. It returns the related parameters *osmotic potential at full hydration* (π_o , MPa) and *apoplastic fraction* (a_f , %). First, the function 'TurgorLossPoint' is employed to determine RWD_{tip} (%). By applying a linear fit to the water potential measurements following the turgor loss point, the influence of osmotic potential is identified.

The RWD and ψ coordinates at which the osmotic regression line is zero represent *osmotic potential at full hydration* and *apoplastic fraction*, respectively (figure 1c). *Water potential at turgor loss point* (π_{tip} , MPa) is determined by intersecting RWD_{tip} with the linear regression line (figure 1c).

3.2.6 Pressure potential and modulus of elasticity

The function 'ModElasticity' first calculates the turgor loss point and osmotic potential by using 'TurgorLossPoint' and 'OsmoticPot' (figure 2a). Pressure potential (ψ_p , MPa) is calculated as the difference between water potential (ψ , MPa) and osmotic potential (ψ_o , MPa) (figure 2b). The pressure potential values before the turgor loss point are fitted linearly, RWD (%) is transferred to RWC and then the *modulus of elasticity* (\mathcal{E} , MPa) is calculated as the slope of the fit (figure 2b):

$$\mathcal{E} = \frac{\Delta\psi_p}{\Delta(RWC)} \quad (5)$$

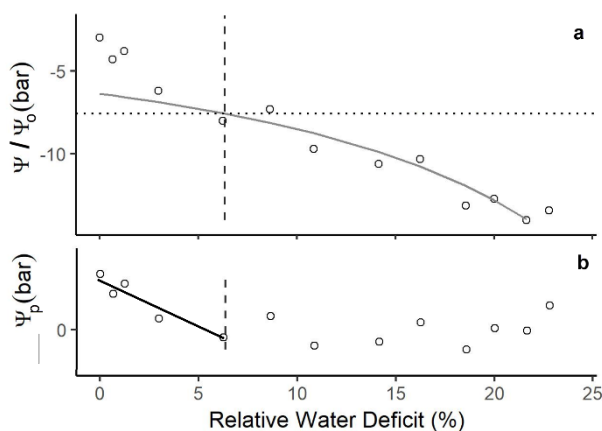


Figure 2: Pressure volume curve fitting routine of the function 'ModElasticity' to obtain pressure potential and modulus of elasticity (ϵ). (a) Water potential data (ψ_{\square} , points) with the coordinates of the turgor loss point (dashed lines) and the osmotic potential fit ($\psi_{\square o}$, solid line) (calculated with 'TurgorLossPoint' and re-converted from $-\psi_{\square}^{-1}$ or $-\psi_{\square o}^{-1}$, respectively). (b) Pressure potential data ($\psi_{\square p}$) are the residuals of the osmotic potential and leaf water potential data, derived by subtraction. The pressure potential data are then fitted linearly (solid line) after using all data points with relative water deficits lower than or equal to the deficit at turgor loss. After transferring the fit from RWD (%) to RWC, modulus of elasticity (ϵ) equals the slope of the fitted line.

3.3 Test of functions with experimental data

The package functions were developed and refined using data from an experiment conducted on apples. They were then tested with data from a trial on kohlrabi, which produced the data that are stored in the 'pvcurveanalysis' package under the name 'pressure_volume_data'. The data were analyzed as explained in the vignette of the 'pvcurveanalysis' package.

3.4 Experimental set-up

The kohlrabi plants (*Brassica oleracea* var. *gongylodes*) were cultivated in a growth chamber with a photoperiod of 12 hours for 5 weeks. Soil moisture in the pots was maintained at ~40-60% with a drip irrigation system. Half of the plants were exposed to a reduced soil moisture regime of ~10-30% (drought-stressed) for 7-8 days before the measurements.

In preparation for measurements, the youngest fully developed leaves were detached from the plants the previous night, weighed (to determine "fresh mass at harvest"), and immersed in tap water for 12 hours. Saturated fresh mass was determined immediately before the first mass measurement. During acquisition of the pressure volume and leaf drying data, the average temperature was 24.7°C (absolute minimum of 20.1°C and absolute maximum of 26.8°C) and the average relative air humidity was 47.2% (absolute minimum of 35.1%, absolute maximum of 62.6%). The leaves were then exposed to desiccation. Leaves were weighed with an analytical digital scale (precision: 1 mg) and leaf water potential was determined using a Scholander pressure chamber. The measurements were continuously repeated for 12 hours. Dry mass was measured after 48 hours of drying in a drying oven at 60°C.

3.5 Data analysis

The 'pvcurveanalysis' package was used for the analysis of the experimental data. A few of the pressure volume curve samples could not be analyzed, because measured data were too sparse. For two of the 16 samples, insufficient observations around the exponential or the transition from the exponential to the linear sections of the curves prohibited the use of the 'TurgorLossPoint' function (see sample 1 and 6 in supplementary figure 3). To inform the users, the function returns an error message for such samples, stating that the data is insufficient for the function to be applied. As a result, the function 'OsmoticPot', which relies on the output of the 'TurgorLossPoint' function, could not be applied to these two samples either. In addition, 'OsmoticPot' estimated the apoplastic water fraction (a_f) to be negative in some cases, which is conceptually impossible. These results indicate that the data following the turgor loss point were not of sufficient quality for a linear regression in all samples and hence both a_f and osmotic potential at full saturation (π_o) need to be interpreted with care. The function 'ModElasticity' produced no results in one further case, because too few data points had been measured before the turgor loss point. Sufficient data points before the turgor loss point are also a prerequisite for the 'FMSaturated' function, which was therefore not used as an estimator for saturated fresh mass.

3.6 Pressure volume curve analysis

Table 2: Relative water content (RWC) at harvest, maximum water potential measured ($\psi_{\square\max}$) and the pressure volume curve parameters of 5-week-old kohlrabi plants in response to normal watering or 7 days of severe drought stress (40–60% or 10-30% soil moisture) as determined by the functions 'TurgorLossPoint', 'OsmoticPot' and 'ModElasticity' of the 'pvcurveanalysis' package for R. The data for the pressure volume curves were measured after overnight saturation (> 12 h) of the leaves. Values are means \pm two standard errors (2 SE), $n_{10-30\%} = 8$, $n_{40-60\%} = 6$ except for modulus of elasticity, where $n_{10-30\%} = 7$ and $n_{40-60\%} = 6$. Pressure volume curve parameters are: RWD_{tip} = relative water deficit at turgor loss point, π_{tip} = water potential at turgor loss point, a_f = apoplastic water fraction, π_o = osmotic potential at full saturation, ϵ = modulus of elasticity.

| Treatment | RWC at harvest (%) | $\psi_{\square\max}$ (-MPa) | RWD_{tip} (%) | π_{tip} (-MPa) | a_f (%) | π_o (-MPa) | ϵ (-MPa) |
|-----------|--------------------|-----------------------------|------------------------|---------------------------|------------------|------------------|-------------------|
| 10-30% | 63.8 \pm 2.32 | 0.40 \pm 0.148 | 9.3 \pm 2.59 | 0.98 \pm 0.111 | 19.2 \pm 24.87 | 0.87 \pm 0.103 | 7.2 \pm 1.07 |
| 40-60% | 96.9 \pm 1.92 | 0.30 \pm 0.052 | 14.7 \pm 5.68 | 1.03 \pm 0.167 | 19.9 \pm 14.49 | 0.83 \pm 0.129 | 6.7 \pm 1.96 |

After exposure to 7-8 days of drought stress, the RWC at harvest had dropped far below RWD at the turgor loss point ($RWD_{t_{lp}}$) (table 2). This implies that the plants had become so strongly dehydrated that their water potential was permanently below $\pi_{t_{lp}}$. Correspondingly, the leaves of the water-stressed plants appeared flaccid during the last 4 days of the drought phase. Overnight saturation of the harvested leaves successfully aligned the maximum water potential of watered and drought-stressed leaves (table 2). Except for a lower $RWD_{t_{lp}}$, no further differences in the pressure volume curve parameters between the two watering regimes were apparent (table 2), but $\pi_{t_{lp}}$ and π_o were found to be strongly correlated ($r = 0.92$, $p < 0.001$, figure 3).

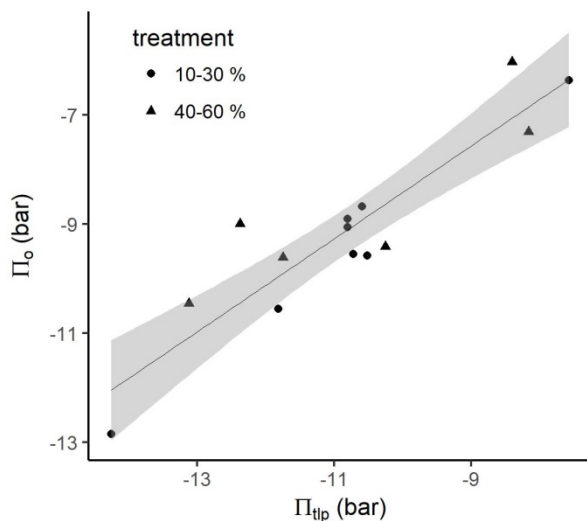


Figure 3: Relationship between turgor loss point ($\psi_{t_{lp}}$) and osmotic potential at full saturation (ψ_o) in the kohlrabi plants watered either to 10-30% (circles) or to 40-60% (triangles) soil moisture. The grey area indicates the 95% confidence interval of the linear fit, $r = 0.92$, $p < 0.001$. Results are shown for all analyzable samples from the pressure_volume_data dataset.

4 Discussion

4.1 Implications of the trial

Pressure volume curve parameters respond to drought in multiple ways (Bartlett et al. 2012). Very common is a lowering of turgor loss point (π_{tlp}), associated with an accumulation of solutes (decreasing osmotic potential at full saturation (π_o)). Plants can also improve their drought tolerance by relocating water outside the cell walls, which would be reflected by increasing a_f , or they can change the flexibility of their cell walls (ϵ) (Kozlowski and Pallardy 2002). In our results only RWD_{tlp} responded to the drought treatment (table 2). A reduction of RWD_{tlp} in response to drought is hard to interpret, but it is likely unfavorable for survival under drought (Bartlett et al. 2012). However, π_{tlp} and π_o were strongly correlated, which is consistent with previous demonstrations (Bartlett et al. 2012) (figure 3).

4.2 Required data quantity and quality

The decrease of relative water content in detached leaves depends on many factors, such as the (micro) climate and anatomical or physiological characteristics of the leaves. The drying rate of detached leaves is thus difficult to predict, making generation of homogeneous pressure volume curves challenging. In the example dataset, data points were sparse, in some cases before, in others after and also in the direct vicinity of the turning points. Sparse data in the region of the turning points was not limiting for the application of any function, since the turning behavior of the curves can be predicted well based on a few data points only. However, the results of functions employing linear regressions ('FMSaturated', 'OsmoticPot' and 'ModElasticity') are greatly affected by sparse data. For this reason, in cases where fewer than three data points are available for linear regression, the functions return warning messages instead of numeric results. Also for small datasets with more than three data points, apoplastic fraction, osmotic potential at full saturation and modulus of elasticity need to be interpreted with care. This was observed in our analysis for the apoplastic fraction, a value which must conceptually be positive but became negative in some cases. Nevertheless, data quality was high enough to produce the expected correlation between water potential at turgor loss point and osmotic potential at full saturation (figure 3) (Bartlett et al. 2012).

4.3 Separation of the linear and the exponential components of the curve

Pressure volume curves are characterized by an initial strong decline, which continues until turgor has been lost. After the turgor loss point, the curves reveal the underlying linear response to water deficit of the osmotic potential values. To our knowledge, the separation of the two phases, which is of decisive importance for the calculation of all curve parameters, has so far been done either visually or by defining a threshold value for the slope of the curves. Applying such thresholds to the raw data provides an objective measure, but it suffers from the problem that the possible options for the turning point are restricted to the set of data points that were actually measured. However, measurements may not be very dense and subject to random variation, especially around the transition point of the curve. Applying thresholds to the raw curve data might then lead to ambiguous or erroneous results. Both the visual appraisal and the threshold procedure may introduce errors into the analysis that may compromise its accuracy. The procedures also limit reproducibility, as demonstrated here (supplementary table 3) and discussed in earlier assessments (e.g. Schulte and Hinckley 1985, Andersen et al. 1991). The functions implemented in the 'pvcurveanalysis' package address this problem by making use of the biological foundation of the curve. They apply a combined exponential and linear model to the curve data. The turgor loss point is derived by calculating the point of the lowest distance between the linear and the exponential components of the curve. The point of turgor loss is then derived from the fitted curve, which ensures that the point's location is relatively independent of the data density and quality around the turning point.

5 Conclusion

The R-package presented here provides open source software for convenient analysis of experimentally determined pressure volume curve data. It follows analysis procedures described in detail in the literature (Tyree and Hammel 1972, Schulte and Hinckley 1985, Bartlett et al. 2012). Parameters derived from these curves are widely used to characterize a plant's performance under regimes of water restriction and to explain the underlying physiological mechanisms (Bartlett et al. 2012). In times of intensifying drought stress events, there is a growing interest in understanding drought responses and in methods to quickly determine reliable parameters for screening plants for adaptation to drought stress. This package addresses this

need by providing functions for reproducible, objective and quick generation of pressure volume and leaf drying curves, thereby eliminating the need for subjective estimation by visual interpretation of curve shapes. The algorithms introduced here and implemented in the 'pvcurveanalysis' package performed reliably to generate meaningful results, as demonstrated for the experimental data on the drought stress response of kohlrabi plants.

Discussion

The present work contributes to the scientific community by bringing a better understanding of a topic which had been first studied decades ago but has not received much attention since then. Scientific progress on the topic of adjuvant-induced alterations of cuticular waxes focused on understanding the penetration of pesticides, but the effects on the functionality of the cuticular waxes has hardly been investigated. This doctoral thesis brings these two topics together, and thereby creates awareness for an overlooked effect which could help explain unexpected behavior of plants after application of agricultural sprays, especially in response to the defense of plants towards environmental stresses, such as drought.

The progress generated by this thesis is, in the first instance, the filling of the current gap of an in-depth review of modifications of cuticular waxes by adjuvants. Adjuvants alter the diffusion-limiting cuticular waxes such that penetration of agrochemicals is tremendously improved. Many adjuvants act as plasticizers on the cuticular waxes, thereby increasing fluidity and reducing phase transition temperatures. Plasticizing adjuvants were long assumed to exclusively influence amorphous waxes, but more recent research calls for a reassessment of this assumption. Thus, the present work proposes a more comprehensive explanation of the permeability-increasing mechanism of adjuvants which expands the explanatory approach to influences on the crystalline phases of waxes as well as to the permeability-enhancing effect that may result from surfactant-admicelles adsorbed into wax crevices. This expanded explanation might also help to understand the effects of adjuvants on pesticide penetration. Pesticides may penetrate into the plant over various pathways, the waxes being one of them. Alterations of the permeability properties of the leaf waxes will alter penetration depending on the properties of the applied molecules.

In addition to increases in permeability, adjuvants were also found to decrease hydrophobicity, i.e. increase wettability, and to alter the appearance of epicuticular waxes in micrographs. In the past, these observations were attributed to the solubilization action of surfactants. Here, alternative reasons for the apparent erosions are proposed. First, the adsorption of adjuvant molecules onto the wax surface could cause a smoother appearance in micrographs, especially if adjuvants were not washed off after application. Second, plasticizing adjuvants

could cause a loss of the integrity of the fine epicuticular wax structures, which would make them more susceptible to erosion.

Based on the known relationships between properties of cuticular waxes and their functionality, hypotheses on possible adjuvant-induced changes of the cuticular wax functions were derived. Many influences are conceivable, yet there is very little and inconclusive research on this subject. In particular, an increase in permeability of the waxes could lead to a higher cuticular transpiration. A facilitated penetration of pathogens is also likely, since the leaf waxes determine the interaction of pathogens in multiple ways. An increase in wettability or hydrophilicity may ease the attachment of many pathogens or could prolong leaf wetness duration and hence improve germination of fungi. Further, alterations in the hierarchical structuring of the leaf surface could hamper the reflection of irradiation, thereby increasing leaf temperature and inducing photochemical damage.

In a second step, and after consideration of the possibilities and the restraints of the infrastructure available, the focus of the next chapter of the present thesis was chosen to be the investigation of the possible effects that adjuvants might have on cuticular transpiration *in vivo*. This research question has not been addressed so far and this published study provides the first available evidence. *Brassica oleracea* and *Malus domestica* were chosen as model organisms due to their very different epicuticular wax characteristics. *B. oleracea* leaf surfaces exhibit highly-structured crystalline waxes, whereas *M. domestica* leaf surfaces are covered by an amorphous wax layer. Amorphous waxes are generally thought to be less effective shields against water loss and to be more affected by plasticizers. However, the minimum water loss of *M. domestica* was hardly influenced by the adjuvants. The effect on *B. oleracea*, on the other hand, was highly significant, but reverted within 14 days. This finding supports the hypothesis made about the location of action of adjuvants in the first chapter of the thesis. The former conviction of the amorphous waxes being most affected by plasticizers was challenged theoretically and also through empirical evidence in this study.

Particularly insightful was the finding that the potential of the adjuvant to increase minimum transpiration was not determined by the presence of surface activity, but related to the plasticization capability of the adjuvant, as estimated by its $\log K_{ow}$. Theoretically, adjuvants with surface activity (=surfactants) could establish “water-filled pores” traversing the cuticle,

thereby also leading to an increased mobility of exiting water molecules. However, in this study, no increase in minimum transpiration was observed with the surfactant with the highest surface activity; rather, the increase was highest for the adjuvant lacking surface activity but having the highest $\log K_{ow}$. Furthermore, the highest alterations in the structuring of the leaf surfaces were also observed in response to the adjuvant with the highest $\log K_{ow}$ and not to the surfactants. This finding validates the hypothesis of plasticization being an alternative explanation of eroded epicuticular waxes after adjuvant application, which was proposed in the first chapter of this thesis.

The most urgent research question which arose from this study is whether the increase in cuticular transpiration comes with consequences for drought stress resistance. A low cuticular transpiration is an important mechanism for avoiding drought stress, but plants are complex organisms, and increasing one risk factor for experiencing drought stress might be compensated by adjusting another. On a physiological level, the most important drought resistance mechanisms are related to keeping transpiration low and maintaining turgor.

Despite the importance of turgor-related parameters and the well-established method for their measurement with pressure volume curves, the analysis of pressure volume curves currently suffers from a lack of alternatives to subjective assessment. We found that the currently widely-employed method of visually detecting the turgor loss point leads to different results for each observer. For the third chapter of this thesis, an R tool was coded and made available for the scientific community which meets the need for standardization by detecting the turgor loss point via a modeling approach. In the pressure-volume curve, continuous leaf desiccation leads to a decline in water potential due to a loss of turgor and osmotic potential. Osmotic potential declines in response to desiccation continuously in a linear fashion until water in the symplast is depleted. Turgor declines exponentially until it is fully lost (= turgor loss point). This is when the underlying linearly-declining osmotic potential is revealed. Fitting the curve to a combined exponential and linear model and identifying the point of the lowest distance between the two models allowed for the determination of the turgor loss point.

Reliable detection of the turgor loss point determines the quality of all other pressure volume parameters because the turgor loss point is used to separate between the influence of osmotic

and pressure potential on the overall water potential. Osmotic potential at full turgidity, modulus of elasticity and apoplastic fraction were then derived following established procedures, and the results were outputted as both data lists and figures. The package's functionality and the plasticity of pressure volume curve parameters in response to drought stress were demonstrated using data derived from a controlled drought stress experiment on *B. oleracea* plants.

From the research presented in this thesis, it becomes clear that adjuvants influence cuticular waxes and their functionalities in many ways, including the alteration of the permeability of cuticular waxes. This leads to profound increases in cuticular water loss and thus possibly to an increased susceptibility of the entire plant to drought. From here, a subsequent experiment on *B. oleracea* in order to analyze the effect of plasticizing adjuvants on transpiration in conjunction with turgor-related traits, and ideally also first-level drought indicators, would be helpful to understand whether adjuvants indeed increase drought susceptibility of the entire plant. Furthermore, many more effects of adjuvants on the functionalities of cuticular waxes are conceivable, especially a facilitated penetration of pathogens, since the leaf waxes determine the interaction of pathogens in multiple ways. An increase in wettability or hydrophilicity may ease the attachment of many pathogens or could prolong leaf wetness duration and hence facilitate germination of fungi. Furthermore, alterations in the hierarchical structuring of the leaf surface could hamper the reflection of irradiation, thereby increasing leaf temperature and inducing photochemical damage. These hypotheses could provide interesting subjects for further research. Further research would also benefit from a proximity to the application praxis in agriculture. Although application of adjuvants was done in this study in a manner that imitated application in the field, consecutive applications and application of adjuvant mixtures on farms are standard. Crop plants could thus be exposed to far higher adjuvant loads. Additionally, application might not be carried out on well-watered plants but on already drought-stressed plants, where stomata are fully closed and an increase in cuticular transpiration might be critical for the survival of the plant.

Abbreviations

| | |
|------------------|-------------------------------------|
| AB | abaxial |
| AD | adaxial |
| APG | alkyl polyglycoside |
| a_f | apoplastic fraction |
| AFM | atomic force microscopy |
| ψ_{\square} | bulk leaf water potential |
| cm | centimeter |
| CA | contact angle |
| cmc | critical micelle concentration |
| DAT | days after adjuvant treatment |
| °C | degree Celsius |
| DSC | differential scanning calorimetry |
| DM | dry mass |
| DMTA | dynamic mechanical thermal analysis |
| ESR | electron spin resonance |
| EO | ethylene oxide |
| e.g. | exempli gratia, for example |
| figure | figure |
| FM | fresh mass |
| g | gram |
| i.e. | id est, that is |

| | |
|----------------------|--|
| last5 | last five values of the curve data |
| LDMC | leaf dry matter content |
| LMA | leaf mass per area |
| log | logarithm |
| F_m' | maximum fluorescence yield |
| MPa | megapascal |
| μg | microgram |
| mg | milligram |
| mmol | millimole |
| g_{min} | minimum epidermal conductance |
| \mathcal{E} | modulus of elasticity |
| NMR | nuclear magnetic resonance |
| no. | number |
| n | number of replications |
| n.s. | not significant |
| $\log K_{\text{ow}}$ | octanol-water partition coefficient |
| ψ_o | osmotic potential |
| π_o | osmotic potential at full hydration |
| r | Pearson's correlation coefficient |
| % | percentage |
| F_v/F_m | photochemical efficiency of photosystem II |

| | |
|--------------------|--|
| PSII | photosystem II |
| PAR | photosynthetic active radiation |
| POEA | polyoxyethylated tallow amine |
| ψ_p | pressure potential |
| p | probability of error |
| PAM | pulse amplitude modulated |
| QCM-D | quartz crystal microbalance with dissipation |
| RME | rapeseed methyl ester |
| RWC | relative water content |
| RWD | relative water deficit |
| RWD _{tip} | relative water deficit at turgor loss point |
| FMs | saturated fresh mass |
| SEM | scanning electron microscopy |
| s | second |
| SAXS | small angle x-ray scattering |
| SE | standard error |
| SRS | stimulated raman scattering |
| SThM | thermal microscopy |
| ToF-SIMS | time-of-flight secondary ion mass spectroscopy |
| g _{tot} | total epidermal conductance |
| TEM | transmission electron microscopy |
| var. | variety |

π_{tlp} water potential at turgor loss point

w/v weight per volume

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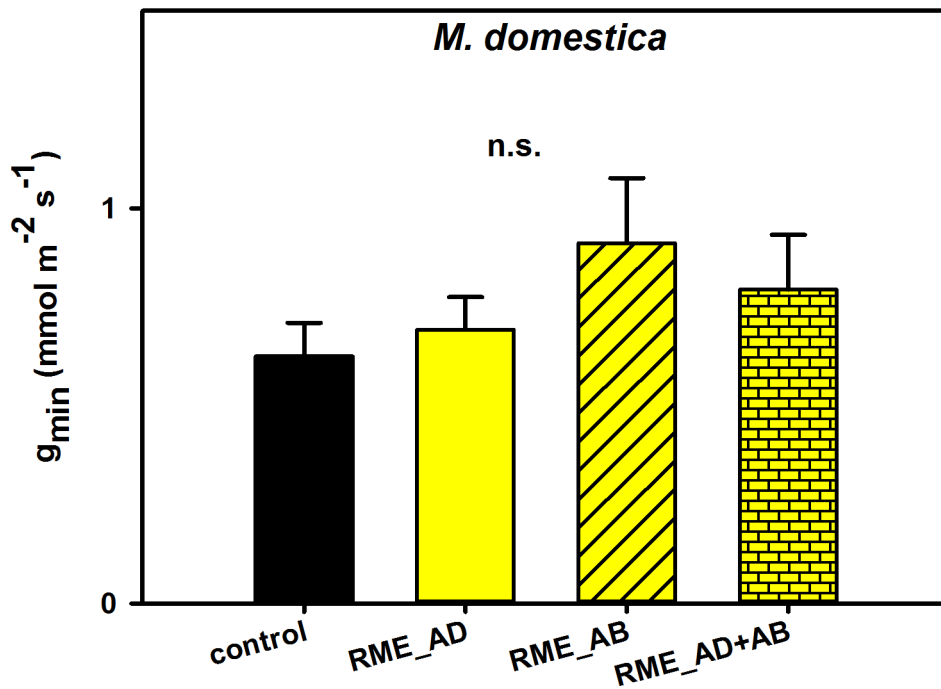
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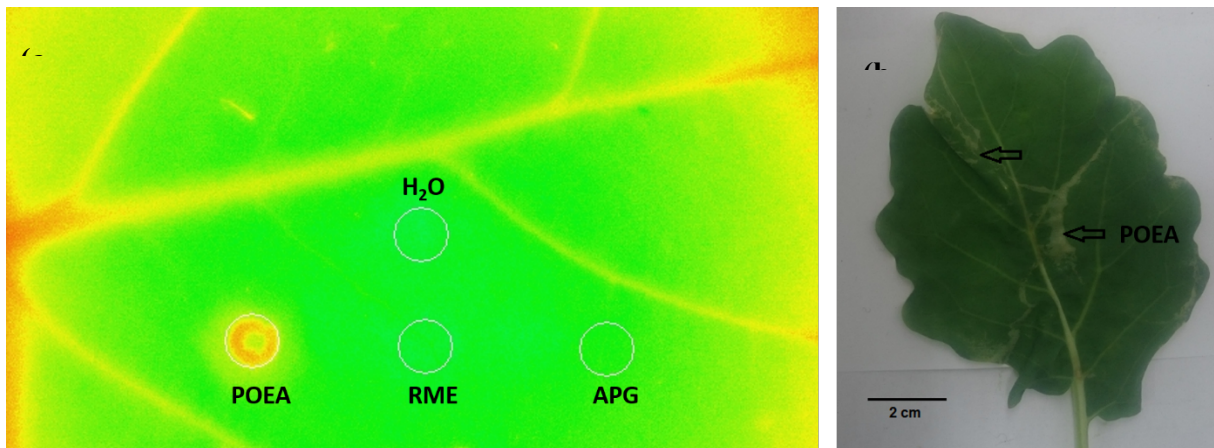
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Supplementary Material



Supplementary figure 1: Comparison of minimum epidermal conductance (gain) after application of rapeseed methyl ester (RME, 0.1 % (w/v)) adjuvant on apple (*M. domestica*) leaves either adaxial (AD, astomatous) or abaxial (AB, stomatous) or both sides (AD + AB), respectively. Measurements were done 1 days after adjuvant treatment. Bars: mean \pm SE (n = 6). n.s.: not significant (P > 0.05, Tukey-HSD test).



Supplementary figure 2: (a) Comparison of the effects of adjuvants (0.1% w/v) applied on kohlrabi (*B. oleracea*) adaxial leaf compared to water as control treatments based on the fluorescence yield. Maximum fluorescence picture was assessed 2 min after illumination on the leaf, one day after treatment. A 5 μ L droplet was applied for each treatment (control: water, alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME) on the same leaf. The circular marks indicate the area where droplets were applied. (b) Effect of POEA (0.1% w/v) on kohlrabi leaf where leaf tissue damage is shown by arrows. A kohlrabi leaf was sprayed until the leaf got saturated with the spray solution. The picture was taken 2 days after adjuvant treatment.

Supplementary table 1: Comparison of the contribution of minimum epidermal conductance (g_{min}) to total conductance (g_{tot}) measured on leaves treated with 0.1 % (w/v) adjuvants - alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME). g_{min} and g_{tot} were sampled 1 h after treatment applications. Values: mean (n = 6) and calculated as $g_{min} / g_{tot} * 100$.

| Treatments | g_{min} / g_{tot} ratio (%) (<i>B. oleracea</i>) | g_{min} / g_{tot} ratio (%) (<i>M. domestica</i>) |
|------------------|--|---|
| H ₂ O | 3.5 | 1.1 |
| APG | 8.0 | 1.2 |
| POEA | 9.5 | 1.4 |
| RME | 41.7 | 1.3 |

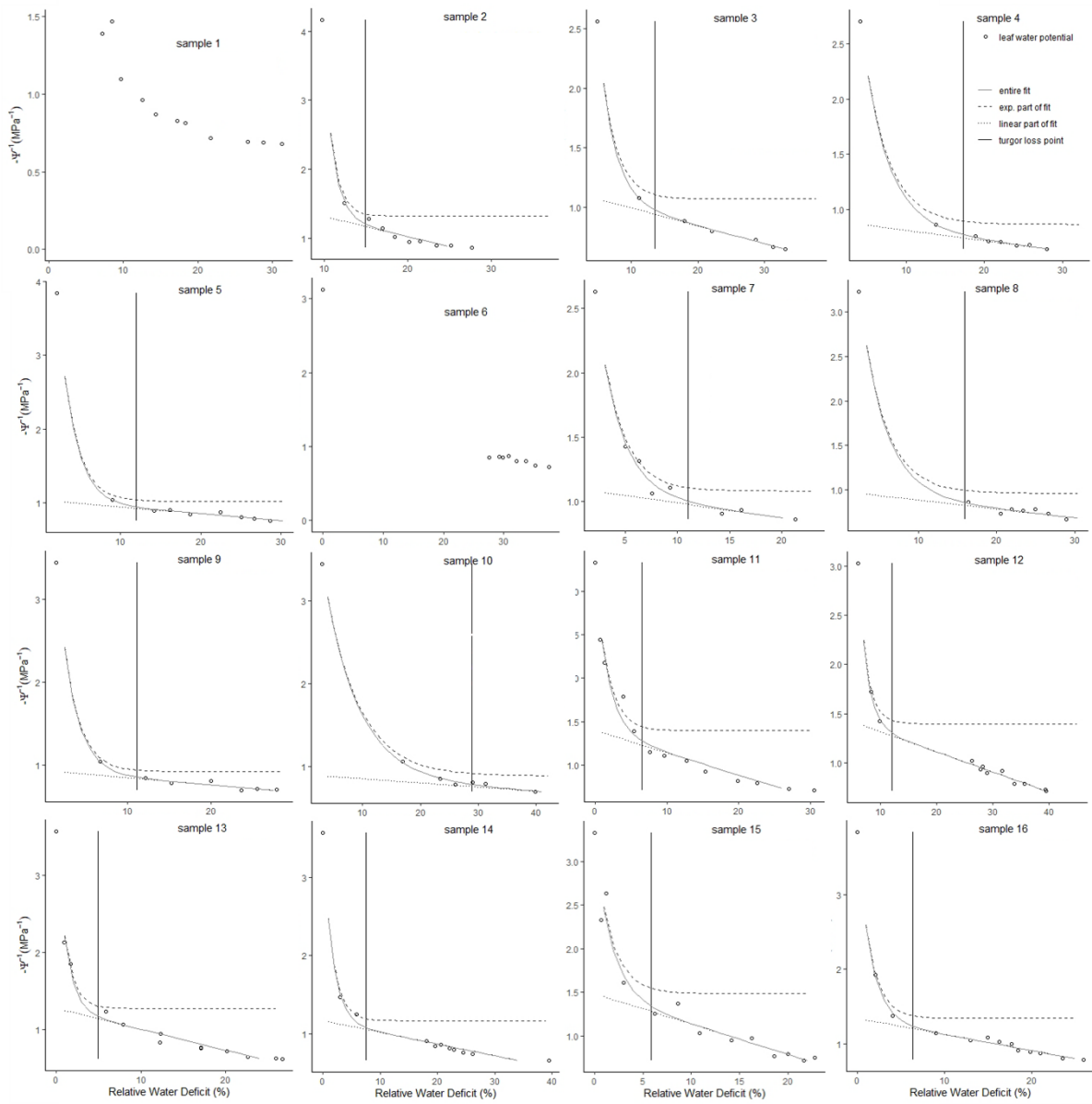
Supplementary table 2: Minimum epidermal conductance (g_{min}) or total epidermal conductance (g_{tot}) data and the statistical comparisons between the treatments measured on the leaves treated either with water or with 0.1 % (w/v) alkyl polyglycoside (APG), polyoxyethylated tallow amine (POEA) and rapeseed methyl ester (RME). The measurements were done subsequently on 0, 2, 7 and 14 days after treatment application. The measurements at 0 DAT were done 1 h after application. Values: mean \pm SE (n = 5-6). Significant differences were at $P < 0.05$ (Tukey-HSD/Duncan test) and denoted by different letters (a, b, c, d), where (a) > (b) > (c) > (d). The same letters (a, a, a, a) in all treatment groups for each measurement days (0, 2, 7, 14) denotes that the values are non-significance ($P > 0.05$).

| Crop | g_{min} or g_{tot} | DAT | Treatments | | | |
|----------------------------------|------------------------|-----|--------------------------|-------------------------|--------------------------|--------------------------|
| | | | H ₂ O | APG | POEA | RME |
| <i>B. oleracea</i> (Kohlrabi) | g_{min} | 0 | 3.40 \pm 0.37 (b) | 4.92 \pm 0.94 (b) | 9.76 \pm 1.47 (b) | 29.96 \pm 6.35 (a) |
| | | 2 | 2.80 \pm 0.28 (b) | 4.05 \pm 0.80 (b) | 11.29 \pm 2.48 (b) | 22.11 \pm 7.47 (a) |
| | | 7 | 2.80 \pm 0.22 (b) | 2.67 \pm 0.38 (b) | 10.59 \pm 1.45 (a) | 5.23 \pm 1.96 (b) |
| | | 14 | 4.30 \pm 0.33 (b) | 3.89 \pm 0.41 (b) | 9.94 \pm 1.78 (a) | 5.36 \pm 0.77 (b) |
| | Gott | 0 | 80.67 \pm 22.31 (a) | 50.05 \pm 7.89 (a) | 59.70 \pm 16.69 (a) | 70.34 \pm 22.67 (a) |
| | | 2 | 36.40 \pm 6.61 (a) | 32.56 \pm 4.89 (a) | 44.83 \pm 3.56 (a) | 48.26 \pm 7.33 (a) |

| | | | | | | |
|--------------------------------|------------------|----|----------------------|-----------------------|----------------------|-----------------------|
| | | 7 | 40.05 ± 6.15 (a) | 45.15 ± 6.96 (a) | 44.80 ± 8.22 (a) | 42.27 ± 4.89 (a) |
| | | 14 | 23.53 ± 5.76 (a) | 20.00 ± 2.63 (a) | 24.29 ± 2.51 (a) | 26.87 ± 5.78 (a) |
| <i>M. domestica</i> (Apple) | g_{\min} | 0 | 1.32 ± 0.22 (a) | 1.42 ± 0.51 (a) | 1.81 ± 0.26 (a) | 2.63 ± 0.49 (a) |
| | | 2 | 1.06 ± 0.43 (a) | 1.04 ± 0.28 (a) | 1.35 ± 0.15 (a) | 1.50 ± 0.46 (a) |
| | | 7 | 0.83 ± 0.06 (b) | 0.82 ± 0.17 (b) | 1.22 ± 0.13 (ab) | 1.46 ± 0.13 (a) |
| | | 14 | 1.06 ± 0.27 (a) | 1.23 ± 0.19 (a) | 1.16 ± 0.21 (a) | 1.15 ± 0.29 (a) |
| | g_{tot} | 0 | 71.11 ± 27.67 (a) | 77.75 ± 11.99 (a) | 81.62 ± 22.57 (a) | 145.45 ± 41.11 (a) |
| | | 2 | 78.05 ± 20.47 (a) | 110.81 ± 35.07 (a) | 63.26 ± 17.05 (a) | 186.88 ± 46.29 (a) |
| | | 7 | 34.90 ± 11.70 (a) | 40.88 ± 4.72 (a) | 29.31 ± 8.76 (a) | 34.57 ± 6.55 (a) |
| | | 14 | 35.17 ± 8.97 (a) | 50.11 ± 13.46 (a) | 50.64 ± 10.30 (a) | 29.86 ± 9.05 (a) |

Supplementary table 3: Relative water deficit at turgor loss point (RWD_{tlp} , %) as estimated by trial participants (estimator no. 1-5), who visually interpreted pressure volume curves ($n = 16$) derived from experiments on kohlrabi plants exposed to either 10-30% or 40-60% soil moisture ('pressure_volume_data' dataset in the 'pvcurveanalysis' package). Values are the means ± two standard errors (2 SE).

| Treatment | Estimator no. | | | | |
|-----------|---------------|------------|------------|------------|-----------|
| | 1 | 2 | 3 | 4 | 5 |
| 10-30% | 10.0 ± 1.9 | 9.6 ± 3.1 | 12.5 ± 1.7 | 17.4 ± 2.4 | 8.1 ± 2.7 |
| 40-60% | 12.4 ± 4.4 | 11.2 ± 3.1 | 15.3 ± 2.9 | 20.0 ± 3.4 | 8.7 ± 2.9 |



Supplementary figure 3: Pressure volume curves from the pressure_volume_curve dataset as analyzed by the TurgorLossPoint function and the estimated RWDs at turgor loss point of the trial participants (still missing xx - will draw in as soon as I found the raw data – sorry!). Two curves could not be analyzed: sample 1 could not be analyzed due to the sparse data in the exponential part of the curve, and figure 6 could not be analyzed due to sparse data in the region of the transition from the exponential to the linear curve.