Developmental wettability changes of soybean (*Glycine max* L) leaves and their impact on agrochemical behaviour

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Declaration of authorship

I, Diana Westfalia Morán Puente declare that this thesis and the work presented in it under the title: **Developmental wettability changes of soybean (*Glycine max* L) leaves and their impact on agrochemical behaviour**, are my own and has been generated by me as the result of my own original research. I confirm that this work has not been previously submitted for a degree or any other qualification at this university or any other institution and that when I have consulted the published work of others, this is always clearly attributed.

Essen, 4th May, 2011

Diana Westfalia Morán Puente

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LIST OF ABBREVIATIONS

γc	=	Critical surface tension
θ	=	Static contact angle
a.i.	=	Active ingredient
cos θ	=	Cosine of the contact angle
BBCH	=	Phenological scale (Biologische Bundesanstalt, Bundessortenamt und chemische Industrie)
CPA	=	Crop protection agent
EC	=	Emulsifiable concentrate formulation
EWC	=	Epicuticular wax crystals
EW	=	Epicuticular waxes
EWF	=	Epicuticular wax film
GS	=	Growth stage
GS/LP	=	Leaf position in the plant
IW	=	Intracuticular waxes
m	=	Slope of the linear phase in a Zisman plot
P	=	Probability
R^2	=	Coefficient of determination
SC	=	Suspension concentrate formulation
SEM	=	Scanning electron microscope
SNK	=	Newman Keuls test

Developmental wettability changes of soybean (*Glycine max* L) leaves and their impact on agrochemical behavior

SUMMARY

The macroscopic manifestation of the interaction of water or agrochemical spray droplets with plant surfaces is referred to as wetting. Besides large differences among species, for the same plant the leaf surface characteristics vary during plant development influencing leaf wettability, and as a consequence, the behaviour of agrochemicals on the target plant. Field observations indicate that wettability of soybean leaves changes as the plant develops. Soybean is one of the most important crops for agricultural production and food consumption; therefore much work to optimize the application of crop protection agents (CPA) has been done, in order to ensure healthy crops with better productivity and less impact on the environment. Spray liquid adherence is a relevant process in the delivery of active ingredients and so far no information is available about the properties of soybean leaf surfaces with respect to wettability by agrochemical sprays. The objective of this study was to characterize the wettability of soybean leaves developed at different growth stages to water and selected agrochemicals. In addition, penetration of active ingredients as a factor of the position of the leaves in the canopy was also investigated. Since azole fungicides are a significant part of soybean pest management, tebuconazole has been chosen as model CPA for this investigation.

The critical surface tension and Zisman plot slopes were validated as parameters to differentiate leaf surfaces varying in wetting properties. A good differentiation among leaf surfaces of diverse plant species was possible, especially with the slopes of the Zisman plot. Therefore the soybean leaf characterization included Zisman plots as well as the conventional contact angles of water, an illustrative wetting profile done with solutions with intermediate surface tensions, percentage of leaf coverage by water and penetration of radiolabelled tebuconazole.

The results indicate that wettability is a characteristic feature of each growth stage of soybean. More than 20-fold differences were found in the percentage of coverage by water of leaves located in the upper canopy as compared with leaves located at the base of the plant. Soybean plants at early stages of development have difficult-to-wet leaves because their leaf surfaces are covered with epicuticular wax crystals. Plants just before the reproductive stage have wettable leaves located in the upper canopy and on the lateral shoots because their leaf surfaces are covered by an epicuticular wax film only. The presented wetting profile indicates that wettability does not only depend on growth stage or position of the leaf in the plant, but can vary between discrete areas on a leaf, described herein as a patch. Wettable leaf patches appear mainly in close proximity of leaf veins in early developed leaves, but they are spread all over the leaf in leaves formed just prior to the beginning of the reproductive stage. Penetration of tebuconazole was 1.5 to 2-fold higher in wettable leaves located in the top of the plant. This is probably caused by higher transpiration rate of the upper canopy leaves which allows a greater translocation of the systemic active. For lower canopy leaves with lower transpiration rate, the systemicity of the active can be limited by a built-up concentration after penetration. Although, the positional differences in the plant, no differences were found in penetration between wettable and non wettable leaf patches within the same leaf, in spite of the differences in epicuticular wax deposition. This indicates that the penetration of tebuconazole is not affected by the presence of crystalline or amorphous epicuticular waxes.

The development of soybean plants causes changes in leaf surface fine structure which affect the coverage and wetting of water and agrochemicals. In contrast to monocots, like corn and sorghum which show a relatively abrupt transition from non-wettable to wettable, the situation is more complex with soybean. With respect to penetration, application of droplets with optimized wetting to leaf patches differing in epicuticular wax deposition results in similar rates of penetration. The information provided by this research is of importance when defining the timing for CPA application and the addition of adjuvants, factors which are influential on pest management. This characterization is also relevant for disease epidemiology studies and for a better understanding of plant development and adaptation.

Keywords: epicuticular wax crystals, epicuticular wax film, wetting, slope of the Zisman Plot, foliar penetration.

Entwicklungsabhängige Benetzbarkeit der Sojabohne (*Glycine max* L) und die Auswirkungen auf das Verhalten von Agrochemikalien

ZUSAMMENFASSUNG

Die makroskopische Beobachtung der Interaktion von Wasser oder Pflanzenschutzmitteltröpchen mit der Blattoberfläche wird als Benetzung bezeichnet. Bei einigen Pflanzenarten ändern sich die Eigenschaften der Blattoberflächen während der Pflanzenentwicklung. Solche Änderungen beeinflussen die Benetzbarkeit von Blättern und demzufolge das Verhalten von Pflanzenschutzmitteln auf der Zielpflanze. Feldbeobachtungen zeigen an, dass sich bei der Sojapflanze die Benetzbarkeit der Blattoberflächen entwicklungsabhängig ändert. Soja ist eine der wichtigsten Kulturpflanzen für die landwirtschaftliche Produktion und Ernährung. Es gibt zahlreiche Bemühungen, welche die Anwendung von Pflanzenschutzmitteln optimieren, um gesunde Sojapflanzen für eine bessere Produktivität bei weniger Einfluss auf die Umwelt zu gewährleisten. Der Bedeckungsgrad mit Flüssigkeiten ist ein relevanter Prozess in der Übergabe von Wirkstoffen. Es gibt zurzeit keine verfügbare Information über die Eigenschaften von der Blattoberfläche der Sojapflanze in Bezug auf die Benetzbarkeit durch Pflanzenschutzmittel. Ziel dieser Arbeit ist die Charakterisierung der Benetzbarkeit der Blattoberfläche mit Wasser und Pflanzenschutzmitteln bei Sojablättern in verschiedenen Wachstumsstadien. Darüber hinaus wurde die Aufnahme des Wirkstoffes in Abhängigkeit von der Positionierung der Blätter an der Pflanze bewertet. Da Azol-Fungizide eine wichtige Rolle bei Soja-Pflanzenschutz-maßnahmen haben, wurde Tebuconazol als Modellsubstanz für dieses Forschungsprojekt gewählt.

Die kritische Oberflächespannung und die Steigung im Zisman-Graph wurden als Parameter validiert, um Pflanzenoberflächen mit verschiedenen Benetzungseigenschaften zu differenzieren. Diese Charakterisierung umfasst, neben den Zisman-Graph, den herkömmlichen Kontaktwinkel von Wasser, den Bedeckungsgrad mit Wasser, ein illustratives Benetzungsprofil (Kontaktwinkel von Lösungen mit mittlerer Oberflächenspannung) und die Aufnahme von radioktiv markiertem Tebuconazol.

Die Ergebnisse zeigen, dass die Benetzbarkeit ein Merkmal ist, das jedes Wachstumsstadium der Sojapflanze charakterisiert. Der Bedeckungsgrad mit Wasser, der am oberen Teil der Pflanze befindlichen Blätter, ist 20-fach höher im Vergleich zu den am unteren Teil der

Pflanze befindlichen Blättern. Sojapflanzen im frühen Entwicklungsstadium sind unbenetzbar aufgrund einer mit kristallinen Epikutikularwachsen bedeckten Blattoberfläche. In der reproduktiven Phase sind die Blätter am oberen Teil der Pflanze und am Pflanzenspross sehr gut benetzbar. Solche Blattoberflächen sind mit einem amorphen epikutikularen Wachsfilm bedeckt. Die Benetzungsprofile zeigen, dass die Benetzbarkeit nicht nur vom Wachstumsstadium abhängt, sondern auch von der Position auf dem Blatt. Benetzbare Blattbereiche gibt es nur an der Blattader der in einem frühen Wachstumsstadium entwickelten Blättern. Diese Blattbereiche sind allerdings überall bei Sojablättern, die sich gerade vor der reproduktiven Phase befinden. Die Aufnahme von Tebuconazol war 1.5 - 2-fach höher in benetzbaren Blättern, die sich am oberen Teil der Pflanze befinden. Das ist wahrscheinlich aufgrund höherer Transpirationsraten, die eine höhere Translokation des sytemischen Wirkstoffes erlauben. Für die im unteren Teil der Pflanze befindlichen Blätter mit niedrigeren Transpirationsraten, kann die Translokation des Wirkstoffes durch eine aufgebaute hohe Konzentration beschränkt werden. Dies wird dadurch unterstützt, dass bei grundsätzlich unterschiedlicher Epikutikularwachsbedeckung keine signifikanten Unterschiede in der Wirkstoffaufnahme zwischen benetzbaren und nicht benetzbaren Blattbereichen innerhalb des gleichen Blattes gefunden wurden. Die gleichwertige Stoffaufnahme von Tebuconazol zeigt, dass die Epikutikularwachsbedeckung keine Rolle für die Barrierefunktionen der Blattoberflächen spielen.

Die Entwicklung von Sojapflanzen führt zu Unterschieden in der Feinstruktur der Blattoberfläche, die den Bedeckungsgrad und die Benetzung von Wasser und Agrochemikalien beeinflussen. Im Gegensatz zu Monokotylen, wie Mais und Sorghumhirse, die einen relativ scharfen Übergang von nicht-benetzbaren bis benetzbaren Blattoberflächen zeigen, ist die Situation bei Soja komplizierter. Bei Lösungen mit optimaler Anlagerung und Benetzung, im Bezug zur Wirkstoffaufnahme, erhält man bei unterschiedlicher Epikutikularwachsbedeckung ähnliche Tebuconazol-Penetrationswerte. Die Ergebnisse dieser Arbeit helfen bei der Bestimmung des richtigen Zeitpunktes der Anwendung von Agrochemikalien und den Zusatz von Hilfsmittel. Diese Charakterisierung ist relevant für epidemiologische Studien sowie für ein besseres Verständnis der Entwicklung und Adaptation von Pflanzen.

Schlüsselwörter: kristallinen Epikutikularwachsen, amorphen epikutikularen Wachsfilm, Benetzung, steigung im Zisman Plot, Aufnahme.

Cambios en la mojabilidad de las hojas de soya (Glycine max L) a lo largo del desarrollo de la planta y su efecto en el comportamiento de agroquímicos

RESUMEN

La observación macroscópica de la interacción de las gotas de agua o agroquímicos con la superficie de la hoja es conocida como mojado. Además de notables diferencias entre especies, en la misma planta las características de la superficie de las hojas pueden variar durante su desarrollo, afectando su mojabilidad y como consecuencia el comportamiento de agroquímicos. Observaciones de campo sugieren que la mojabilidad de las hojas de soya cambia durante su desarrollo. Debido a que el cultivo de soya es de los más importantes productos agrícolas y fuente nutricional, se han hecho muchos esfuerzos por optimizar el tratamiento con productos fitosanitarios. El propósito es tener cultivos saludables que aseguren una mejor productividad con menos impacto al ambiente. La adhesión de líquidos asperjados es un proceso de mucha importancia en la aplicación de agroquímicos, sin embargo actualmente se desconoce la influencia de las propiedades superficiales de la hoja de soya, y en general de plantas leguminosas, en la capacidad de mojado de estos. Con esta investigación se ha caracterizado la mojabilidad a agua y agroquímicos de hojas desarrolladas a diferentes estadios de crecimiento. Además se cuantificó la penetración de ingredientes activos en relación con la posición de la hoja en la planta. Se ha escogido tebuconazol como el compuesto modelo en la investigación, debido a que los fungicidas azoles son una parte muy importante del manejo de plagas en soya.

Se validó la llamada tensión superficial crítica y la pendiente de los gráficos de Zisman como parámetros para diferenciar hojas que varían en mojabilidad. Se obtuvo una buena discriminación con la pendiente de los gráficos de Zisman, y por eso la caracterización incluyo dichos gráficos así como también los convencionalmente utilizados ángulos de contacto de gotas de agua, un perfil muy ilustrativo de mojabilidad de la hoja hecho con líquidos con valores intermedios de tensión superficial, porcentaje de cobertura de las superficies de hoja con agua y penetración del fungicida tebuconazol.

Los resultados indican que la mojabilidad es definitivamente una característica muy típica de cada estadio de crecimiento de la planta de soya. El porcentaje de cobertura de la hoja con agua fue 20 veces más alto en hojas localizadas en la parte alta de la planta en comparación con las que crecen en la parte inferior. Plantas de soya que están en un temprano estadio de crecimiento poseen hojas que son difíciles de mojar como consecuencia de estar cubiertas con ceras epicuticulares cristalinas. Las plantas que están a punto de entrar al periodo de reproducción poseen hojas mojables, localizadas en la parte alta de la planta así como también en los brotes laterales. Dichas hojas están cubiertas por una capa amorfa de ceras epicuticulares. Los resultados indican que la mojabilidad no es solamente dependiente del estadio de crecimiento de la planta sino también de la sección de la hoja que es evaluada. Secciones mojables se encuentran solamente alrededor de las venas en hojas desarrolladas en estadios tempranos; sin embargo hojas desarrolladas antes que la planta entre en periodo reproductivo presentan estas secciones mojables por toda la hoja. El valor de penetración de tebuconazol fue 1.5 a 2 veces más alto en hojas mojables localizadas en la parte alta de la planta. Esto es probablemente debido a una mayor transpiración de estas hojas lo que permite mayor translocación del ingrediente activo. Por el contrario, en hojas con baja transpiración, como las ubicadas en la parte baja de la planta, la sistematicidad del ingrediente activo es limitada por la alta cantidad que se acumula después de la penetración. Esta hipótesis se origina en el hecho que secciones definidas como fácil y difícil de mojar dentro de una misma hoja resultaron en valores similares de penetración; a pesar que la deposición de ceras epicuticulares en estas secciones es muy diferente. Concluyendo así que la cobertura con ceras epicuticulares no tiene ningún efecto como barrera en la penetración de tebuconazol.

El desarrollo de la planta de soya provoca cambios en la estructura de la superficie de la hoja que afectan el grado de cobertura y mojado de agua y agroquímicos. En contraste con plantas monocotiledóneas como maíz y sorgo en las cuales la transición de no mojable a mojable es abrupta, la situación con soya es mucho mas compleja. Con respecto a penetración, la aplicación de una solución con optimo mojado resulta en valores similares de penetración de tebuconazol, incluso si se aplica en secciones de la hoja con diferente deposición de ceras epicuticulares. Esta investigación es de mucha importancia para la planeación del momento optimo de asperjado de productos fitosanitarios y la adición de adyuvantes, ambos factores muy decisivos en el control de plagas. Además es relevante para estudios de epidemiología y en general para un mejor entendimiento de los procesos de desarrollo y adaptación de plantas.

Palabras claves: ceras epicuticulares cristalinas, capa amorfa de ceras epicuticulares, mojado, pendiente del grafico de Zisman, penetración foliar.

GLOBAL INTRODUCTION

DEFINITION OF THE SCOPE AND OBJECTIVE OF THE PROJECT

The leaf epidermis is the outermost exposed layer of cells acting as an interface to the environment at the adaxial and abaxial side of leaves. The significance of the leaf epidermis as interface is recognized by its specialization and its particular features during plant evolution and development. Epidermal cells of leaves and all primary aerial organs synthesize constituents that form a continuous extracellular membrane of soluble and polymerized lipids called cuticle. It acts as permeability barrier against uncontrolled water loss, limits the movement of compounds such as ions and polar organic solutes from inside the plant and protects from abiotic and biotic impacts (Jeffree 2006). The plant cuticle varies in thickness between 0.1–10 µm and is linked to the underlying cell wall by the polysaccharides cellulose, hemicellulose and pectin (Holloway 1982). The biopolymer cutin constitutes 40-80% of the cuticle weight (Heredia 2003). Cutin is a high molecular weight polyester (Benitez *et al* 2004), consisting mainly of esterified hydroxylated and epoxy hydroxylated fatty acids with chain lengths of 16 and/or 18 atoms of carbon (Walton 1990). The ester bond in cutin can be hydrolysed under alkaline conditions to give the key monomers of cutin, namely the omega-hydroxy acids: 16-hydroxy palmitic acid and 18-hydroxy stearic acid (Benitez *et al* 2004). As described by Reina-Pinto & Yephremov (2009), cutin may also contain alpha omega dicarboxylic acids which are characteristic components of suberin, a secondary polyester in plants. It is widely accepted that the function of the cutin (and suberin) in plants is to provide a mechanically stable matrix while the waxes embedded (if present) in any of the mentioned biopolymers are responsible for water barrier properties (Schreiber 2010). In some plant species, the cuticle contains also cutan, which is a highly cross-linked biopolymer constituted by the same monomers as cutin but held together by non-ester bonds (i.e. polyether). For those plant species containing cutan, its ratio as related to the amount of cutin, varies according to the developmental stage of the cuticle (Heredia 2003). In the cutin matrix, soluble cuticular lipids are present, from which two wax fractions can be distinguished: the intracuticular waxes (IW) which are embedded within the cutin (Holloway 1982) and the epicuticular waxes (EW) which cover the outer side of the cuticle (Baker 1982). As reviewed by Koch and Ensikat (2008), cuticular waxes are complex monomer mixtures of homologue series of long-chain aliphatic with 20 to 40 carbons in the chain length mixed with wax esters having 60 or more atoms. Functionally, IW are part of the so called transport limiting skin which is located below the morphological outer surface of cuticles and often makes up about

10-20% of the total thickness of the cuticle. It is widely accepted that the mobility and solubility of xenobiotics is determined in this cuticular substructure and not in the sorption compartment underneath (Schönherr and Baur 1996). Differently, EW develop obviously crystalline protrusions called epicuticular wax crystals (EWC) or may appear as an amorphous layer called epicuticular wax film (EWF) (Jetter *et al* 2000). They determine the wetting properties of the leaf surface, e.g. whether a droplet sticks to or bounces off from a leaf. Therefore, both types of cuticular waxes are very important for studies on xenobiotic behavior. While agrochemical retention at the leaf surface is a phenomenon partially dominated by the physico-chemical properties of EW (see next section), penetration is a process restricted by the IW of the limiting skin (also called cuticle proper).

The barrier properties of each cuticle component or of the lipid membrane as a whole system are vital for the evolution and survival of plants. Therefore the cuticle has been widely studied as barrier to transpiration, for its role against biotic factors (viruses, bacteria, fungi and predators), as habitat for microorganisms and as bio-film where xenobiotics accumulate. The present study focuses on an aspect often investigated: the optimization of crop protection agents (CPA) efficacy.

Each component of the cuticle influences the way in which active ingredients move from the deposit to the target tissue. The involved partial processes are the adhesion, retention and spreading of CPA sprays as well as the penetration and translocation of the active ingredients into the plant. These processes are further complicated because the properties of the underlying biological structures vary with the developmental stage of the plants. The understanding of plant characteristics as affected by plant development and interaction with the environment is needed for optimizing performance and mode of action of CPA. For soybean, changes on leaf surface characteristics were suggested on the basis of field observations and appear not to be studied appropriately. There is hardly any information available, while for some monocots as maize enough evidence exists indicating changes in leaf wettability caused by plant development. Therefore the current research evaluates the changes of the leaf surface fine structure (especially EW deposition) of soybean caused by plant development. The investigation quantifies the influence of the developmental changes on three partial processes defining the behavior of agrochemicals: coverage, microscopic contact quality and penetration of active ingredients. The used model CPA is the systemic tebuconazole fungicide, an active that belongs to the triazoles, i.e. the most effective members of the fungicide portfolio against soybean rust.

In the next section a synopsis of the three publications is presented in order to identify the specific objectives of each publication. Methodology and results are comprised in the publication abstracts, located at the beginning of each chapter (publication). The publications are presented in chronological order and details about their publication or submission are given in the cover page. After the publications, a global discussion is presented where the key components of this research project are related to their appearance in the enclosed publications. In this section conclusive remarks as well as topics for future research are stated. It is followed by the reference section corresponding to the global introduction and discussion.

SYNOPSIS OF PUBLICATIONS

Publication 1: Critical surface tension measurement of plant surfaces: methodology and species dependence

The first publication focuses on the establishment of a method to discriminate plant surface wettability and to complement the information given by the widely used contact angle of water. While *contact angles* provide the threshold value for a droplet to run-off from a surface, the *critical surface tension* provides information about the spreading of a droplet on the surface. The critical surface tension is the surface tension of a liquid just low enough to cause spreading of the liquid on the particular surface i.e. the contact angle (θ) is becoming 0. Any liquid having a surface tension below the critical surface tension of a solid will spread on that surface. For low-energy surfaces like those of aerial plant organs, a linear relationship often exists between $\cos \theta$ and surface tension if the criterion of wettability is reached ($\theta = 90°$). By extrapolating the linear function to $\cos \theta = 1$ in a Zisman plot, the critical surface tension is estimated and the slope of the linear regression could be calculated. In this study the suitability of homologous liquids (dilution series of acetone, surfactant and fungicide) and non homologous liquid (selected tank-mix adjuvants) is compared to determine the critical surface tension of several plant species with different surface properties. There were only slight differences among the homologous systems, while the critical surface tension could not be calculated with the tank-mix adjuvants. While the critical surface tension for most plant species was around 30 mN/m, the Zisman plot slopes showed a clear dependence on species/surface characteristics. For wettable leaf surfaces like olive, cotton and kumquat, the

Zisman plot slope varied from -0.02 to -0.04; while for non-wettable leaves like soybean and maize, the slope varied from -0.11 to -0.13. These differences are in agreement with the leaf surface properties as observed by SEM.

Publication 2: Wettability of soybean (*Glycine max* L.) leaves by foliar sprays with respect to developmental changes

Having found a parameter that discriminates wettability of non-wettable leaf surfaces, allows characterizing the variable wettability and leaf fine structure of soybean. It has been observed under field conditions that the wettability of water on soybean plants varies among leaves at different positions in the canopy. It was obvious that such distinct developmental/positional changes would also affect the behaviour of agrochemicals. Therefore, the second part of the research characterizes soybean leaf surface wettability by water and agrochemical sprays at different plant development stages. The characterization was done by measuring contact angles of pure water and liquids with intermediate surface tension in different leaf patches, by quantifying the leaf area covered by water after spray application and creating Zisman plots for leaves belonging to different growth stages. These quantitative results were complemented by scanning electron microscope evaluation. When classifying soybean leaf surface wettability, a practical focus was given by providing recommendations to applicators on the optimization of active ingredient delivery by means of formulations or tank mix adjuvants. Diverse wettability measurements demonstrated that wetting is indeed a feature characteristic of each developmental stage of soybean leaves. Basically, adaxial surfaces of leaves located at the top of the canopy and growing on the lateral shoots were wettable, while leaves located at the base of the plant were difficult to wet. As suggested by coverage quantification of aqueous solutions, leaves located at the base (GS/LP 11-13) of greenhouse grown plants maintain their water repellency, even when the reproductive cycle begins. Abaxial leaf surfaces were in most of the cases difficult to wet, but a small increase of wettability was already perceived by the Zisman plot slope for leaves located in the upper canopy. By measuring contact angles of a solution with an intermediate surface tension on different leaf patches, an illustrative wetting profile could be drawn showing to what degree wetting varies (from >120° to <20°) as function of the leaf patch and leaf position on the plant. While the critical surface tension of leaf surfaces at different growth stages did not correlate with the observed changes, the Zisman plot slope accurately varied according the wettability changes. The slope of the Zisman plot is not representing the changes of leaf roughness (i.e. epicuticular wax deposition), but provides an insight into surface characteristics at the droplet-leaf interface.

Publication 3: Developmental and positional differences of surface wettability of soybean leaves and their impact on foliar penetration

Positional wettability differences of leaves in the plant and within the same leaf could potentially influence performance, selectivity and plant compatibility of agrochemicals. The reason for this is that active ingredients must be retained on the leaf surface before they penetrate through the transport limiting skin. This relationship of leaf wettability and penetration of active ingredients was the objective of the third publication. Penetration was measured on the adaxial surface of soybean leaves located at different positions in the plant. The penetration of tebuconazole was estimated from a tank-mix adjuvant system and a commercial EC formulation. For both spray solutions, the penetration of tebuconazole was 1.5 to 2 times higher for leaves positioned in the middle-top of the plant than for the first true leaves positioned at the base of the plant. While these striking differences were significant and practically meaningful, no significant differences in penetration were found within the same leaf between areas differing in wettability, in spite distinct deposition of epicuticular waxes. Because of this latter finding, it was concluded that positional penetration differences in the plant are not due to the macroscopically visible differential leaf-surface wax coverage. The implications of these findings for the timing and the adjuvants required during agrochemical application were discussed.

Publication 1

Critical surface tension measurement of plant surfaces: methodology and species dependence

Published and presented during the 8th international symposium on adjuvants for agrochemicals, USA (2007)

CRITICAL SURFACE TENSION MEASUREMENT OF PLANT SURFACES: METHODOLOGY AND SPECIES DEPENDENCE

ABSTRACT

Under equilibrium conditions the spreading properties of liquids for a given solid surface depend on their surface tension. The critical surface tension (γc) is the surface tension of a liquid just low enough to cause spreading of the liquid on the particular surface, i.e. contact angle $\theta = 0$. Any liquid having a surface tension below γc of a solid will spread on that surface. For low-energy surfaces, like those of aerial plant organs, a linear relationship often exists between cos θ and surface tension above the critical surface tension. This allows to estimate γc by plotting cos θ against surface tension and extrapolating to $\cos \theta = 1$ in a Zisman plot. In this study we compared the suitability of various liquid systems, differing in surface tension, for the determination of γc for several species with widely differing surface properties. The liquids used included homologous liquids with a solvent system, a surfactant, a fungicide formulation and a non-homologous system with selected tank-mix adjuvants. There was a surprisingly good rectilinear relationship ($R^2 > 0.95$) in the Zisman plots, particularly for the homologous liquid system with γc values in the range between 24 and 35 mN/m. The γc determined by the various methods did not differ more than γc values found in the literature for a given synthetic polymer like polyethylene. While the γc for most species was around 30 mN/m, there was a clear dependence on species or surface properties, demonstrated by widely differing slopes in the plot.

Key words: critical surface tension, plant surfaces, spreading properties, wettability, Zisman plot.

CRITICAL SURFACE TENSION MEASUREMENT OF PLANT SURFACES: METHODOLOGY AND SPECIES DEPENDENCE

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SUMMARY

The critical surface tension for spreading (γc) was determined in seven plant species with variable leaf surface properties by plotting the surface tensions of test liquids against the cosine of their contact angle (θ) and extrapolating to cos θ = 1 (θ = 0) in a Zisman plot. Homologous systems (solvent/water blend; anionic surfactant, commercial fungicide) and chemically distinct commercial tank-mix adjuvants were tested for its estimation. A linear relationship was obtained for lower surface tensions when the criterion for wettability was given. As a consequence, leaves with rough surfaces, due to crystalline waxes, could be used as well as those with smooth surfaces, enabling the evaluation of all plant species. There were only minor differences for γc among the homologous systems, while the use of different tank-mix adjuvants for its calculation was not possible. The Zisman plot showed that wetting of leaves followed the same rules with all species. The intercept and slope for the linear phase were correlated (R^2 = 0.94). While contact angles for pure water differed widely, the critical surface tension did not differ much among species. Differences in γc were rather low (extremes 24 and 35 mN/m), similar to the values found among chemically related polymers. The implications are discussed.

INTRODUCTION

The spray properties of foliar applied agrochemicals are often modified by adjuvants or by means of formulation to achieve an effective delivery to the respective plant surface under the given application parameters and environmental conditions (McWhorther 1982). Often agrochemical sprays are adapted to the specific crop or target plants to enable good wetting of the treated plant surface. Spray retention and coverage is a function of the physicochemical interaction at the interface of the droplets to the plant surface. The macroscopic manifestation of this interaction is referred to as wetting (Berg 1993). Leaf surface characteristics and their influence on its wettability have been widely investigated by scanning electron microscopy (e.g. Hall & Burke 1974; Holloway & Baker 1974; Jeffree *et al.* 1975; Wagner *et al.* 2003). Quantitatively, the wetting of leaves by spray liquids has been determined, e.g. by the leaf immersion method and estimation of the specific adhesional forces (Watanabe & Yamaguchi 1991), contact angle measurements of sessile, advancing or receding droplets (Watanabe & Yamaguchi 1992) and spread diameters of macroscopic or real spray droplets after evaporation on leaf surfaces (Abbott *et al*. 1990; Baur 2006). The contact angle has been proposed as a measure of wettability under two different approaches. One is the contact angle measurement of pure water droplets on the leaf surface (Hall &

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Burke 1974; Boyce & Berlyn 1988) which allows a rough classification of leaf surfaces (non, moderately, and easily wettable). The alternative is the usage of solutions with intermediate surface tension to account particularly for differences among leaf surfaces of difficult-to-wet-species (Forster & Zabkiewicz 2001), where pure water contact angles are inadequate because they do not discriminate (Forster *et al*. 2001). In that regard, contact angles of water-acetone solutions on foliar surfaces have been measured (Gaskin *et al.* 2005) and the resistance of leaf surface against wetting with water-methanol mixtures has been tried as well (Wagner *et al.* 2003).

When evaluating the interaction of an agrochemical spray with the leaf surface, it is a common observation that liquids of lower surface tension (~ energy) will spread over materials of higher surface tension and this is driven by the reduction of the total free energy of the system (Hansen & Pierce 1974). This real spreading ($\theta = 0$) occurs if the surface tension of the liquid is lower than that of the surface, and for artificial surfaces this relationship was applied by Zisman (Shafrin & Zisman 1960) to classify surfaces by the value of the so called critical surface tension for spreading (γc). For its calculation, the contact angles of a series of liquids with decreasing surface tension on a particular surface are determined under equilibrium conditions. Their cosines are then plotted against the surface tension of the liquids. The linear or quasi-linear relationship is extrapolated to $\cos\theta = 1$ ($\theta = 0$), i.e. the situation of real liquid spreading on a solid surface. Since most of the leaves possess non-polar low energy surfaces, the concept of Zisman can be used to assign to different plant species a second characteristic parameter for describing its wettability: the critical surface tension or energy.

Further to the known classification by the water contact angle, which gives the minimum wettability, the γc gives the threshold for optimum wettability. Our interest was to see to what extent the γc varied among species and how this related to surface tensions of agricultural sprays. The present approach compared the suitability of various liquid systems with decreasing surface tension for the determination of γc in plant species differing in leaf surface properties. We used a solvent/water blend system, an anionic surfactant and a fungicidal adjuvanted EC formulation. This enabled us to see whether the values obtained by the above systems agreed with the behaviour of a real product. Finally, a non homologous system with selected tank-mix products from different chemical classes and with diverse droplet spreading behaviour (Baur 2006) was tested for the estimation of γc. This is a system that relates more to the most often measured contact angles at certain concentrations and it was also used to estimate γc (Chambers *et al*. 1992). Differences among the liquid systems for the estimation of γc and its dependence on plant species are discussed.

MATERIALS AND METHODS

Plants

Pot grown plants of soybean (*Glycine max* L.), corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) were kept in the greenhouse at 26°C during the day and 22°C during the night and at 65% r.h. Lemon (*Citrus limon* L.) plant was grown in a pot on a window bench in the laboratory at 23°C and at approximately 30% r.h. One month prior to the evaluation, the plant was transferred to a growth chamber at the constant temperature of 29°C and 75% r.h. Olive (*Olea europaea* L.), apple (*Malus domestica* Borkh.) and

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kumquat (*Fortunella crassifolia* Swingle) were grown in pots in a growth chamber at the constant temperature of 29°C and 75% r.h. The third fully developed leaf of soybean, corn and wheat was used for the estimation of the contact angle. Top leaves of the three years old lemon plant were used. Olive plants were six months and apple and kumquat plants were one year old at the evaluation time.

Test Liquids for Wettability Measurement

For all liquid systems, tap water with the following characteristics was utilised: pH and electrical conductivity at 25°C: 7.3 and 622 µS/cm, respectively and hardness 2.83 mmol/L. For the solvent system, a series of 10, 20, 30, 40, 50, 60, 70 % (v/v) acetone in tap water was freshly prepared before measurements to avoid volatilisation of acetone. This resulted in surface tensions between 53.0 and 32.7 mN/m. For the surfactant system, Aerosol OT-B (sodium dioctyl sulfosuccinate, Cytec) was diluted in tap water at 0.01, 0.03, 0.05, 0.1 and 0.2 g/L (w/v) resulting in a surface tension from 42.0 to 23.1 mN/m. For the third system, a commercial EC formulation (a.i. tebuconazole) was prepared at 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.15 and 0.2 g ai/L (w/v) in tap water with corresponding surface tensions from 49.2 to 30.4 mN/m. For the last system, the following tank-mix adjuvants were used at 1.5 and 3.0 g/L (w/v) in tap water: Tween 80 (sorbitan monooleate ethoxylate; Uniqema), Frigate (fatty acid amine ethoxylate; ISK Biosciences) Mero (methyl esters of rape oil; BayerCropScience), Agridex (crop oil concentrate; Helena), Agral 90 (nonylphenol-ethoxylate; Syngenta), Geronol (Phosphate esters; Rhodia) and Trend 90 (isodecyl alcohol ethoxylate; Du Pont).

Contact Angle Measurement

Leaf strips of approximately 0.5 x 5.0 cm were placed on a sample holder. The strips were cut from the intercostal adaxial leaf surface since it is the most representative part covered by sprays (Abbott *et al.* 1990) and it has less topological structures beyond the cell level. Droplets (5 µl) of each solution were applied over the strips and the static contact angle under equilibrium (arithmetic mean of five values taken every 5 seconds from 90 -115 seconds after application, or else values taken after the equilibrium was reached) was measured by a goniometer DSA10 (Krüss GmbH, Hamburg 2002). Three repetitions were considered for calculating the average contact angle of each dilution in each plant species. The measurement was carried out at 23°C and at 30% r.h. Contact angles usually had coefficients of variability of about 6% within a whole range of 2-15%.

Surface Tension

The surface tension of the dilution series of each system was measured by the Wilhelmy plate method in a Tensiometer K100 (Krüss GmbH, Hamburg 2001). The given value represents the average of five values taken every five seconds from 475 - 495 seconds after the starting point. The measurement was carried out at 23°C and at 30% r.h.

Critical Surface Tension

The cosine of the average contact angles of each dilution series for each leaf surface was assigned to the ordinate and plotted against the corresponding surface tension. The resulting curve was extrapolated to $\cos\theta = 1$ (or $\theta = 0$) to obtain γc. The coefficient of determination (R^2), its probability, the confidence interval and the slope was calculated for each curve.

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Scanning Electron Microscopy (SEM)

Intercostal adaxial leaf sections were mounted on a sample holder and observed by SEM (Jeol JSM-5600LV, Japan) at an accelerating voltage of 10 – 15 kV and at a cooling sample temperature of -50°C.

RESULTS AND DISCUSSION

For the acetone/water blend, the anionic surfactant and the commercial fungicide systems, non linear functions were obtained for soybean when the cos θ was plotted against the surface tension for all solutions, including water (Fig. 1). However, towards lower contact angles a linear relationship was obtained for each test liquid and plant species, respectively, and the γc could be estimated under the same conditions as stated by Zisman for polymers (Shafrin & Zisman 1960). Thus, the linearity started at contact angles of 90° or below, meaning that this linearity was given exactly under conditions of wettability (Dörfler 2001). This classification of surfaces as being wettable relates to the wetting tension that becomes positive if the real contact angle for liquids on non-rough solid surfaces is below 90°. As will be shown below, transition from non-linearity to linearity in the Zisman plot is related to the measurement of real contact angles rather than apparent contact angles.

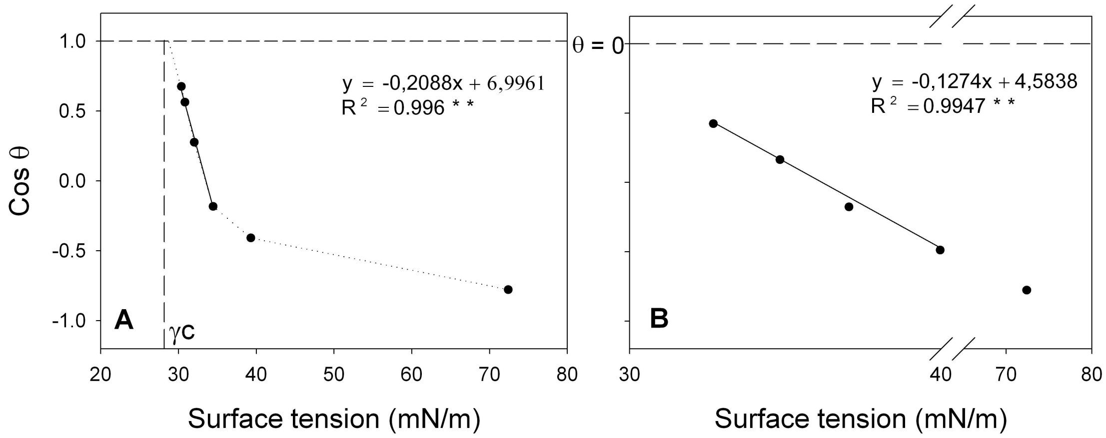

FIG. 1: Zisman plots for soybean leaf surfaces based on (A) a commercial fungicide system or (B) acetone/water blend system. The regression equation and determination coefficient (R^2) correspond to the solid line. Dotted line at Cos =1 (θ = 0) i.e. real spreading. The extrapolation of the linear part of the plots intersects the dotted line to the below critical surface tension (γc) value. ** = significant at P < 0.01

The contact angle related to spreading and thus to γc equals the advancing contact angle. This relates to a dry, clean and previously unwetted surface. These values can be obtained, 1) by rapid measurement immediately after droplet application or, 2) while applying further liquid to the surface or, 3) under conditions such that the drop contacts a new surface, or 4) by recording the contact angle of a sessile droplet on surface that is tilted until the droplets rolls off. In this study we preferred to measure the contact angles of sessile droplets in a dry environment (r.h. <30%), after leaving some minutes in order to avoid non-equilibrium conditions for the surfactant-induced surface tension

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reduction. This was clearly relevant as contact angle equilibrium was sometimes obtained only after 2-3 min and the angle changed significantly (>20°) during that time. This means that we had optimum conditions for a good estimate of γc but that there might be some deviations in the prediction of spreading behaviour of impacting droplets from the γc estimated from the Zisman plot (Fig. 5).

As shown in Fig. 1, the linear phase was considered for the calculation of γc. With one exception, all determination coefficients (R^2) for the acetone, surfactant and fungicide system were > 0.95 (Table 1) and statistically significant.

For soybean and corn plant, the γc values measured with the three systems were practically identical in each case (Table 1). In addition, the average γc was also identical with 28.3 ± 0.4 and 28.3 ± 0.3 for soybean and corn, respectively, indicating similar wetting properties for both species at this age. Soybean and corn both had a dense crystalline wax layer, while the topology of the epidermis was quite different (Fig. 2). Thus the physicochemistry of the surface waxes dominated in the determination of contact angle and critical surface tension.

TABLE 1: Critical surface tension (γc) of leaf surface from different species determined with the acetone, surfactant and fungicide system. Regression slopes (m) and determination coefficient (R^2) are given

Leaf surface	System	m	R^2	γc (mN/m)
Soybean	Acetone	- 0.13	0.995**	28.13
	Surfactant	- 0.14	0.960**	28.11
	Fungicide	- 0.21	0.996**	28.72
Corn	Acetone	- 0.11	0.993***	28.22
	Surfactant	- 0.14	0.863^{+}	28.09
	Fungicide	- 0.16	0.956**	28.59
Lemon	Acetone	- 0.04	0.974*	30.57
	Surfactant	- 0.04	0.997**	23.57
	Fungicide	- 0.04	0.994***	24.57
Wheat	Acetone	- 0.12	0.977*	27.51
	Surfactant	- 0.12	0.934*	25.17
Olive	Acetone	- 0.02	0.976*	33.01
	Surfactant	- 0.02	0.946*	32.31
Kumquat	Acetone	- 0.02	0.999*	35.19
Apple	Surfactant	- 0.12	1.000***	29.24

+, *, **, *** = significant at P < 0.1, 0.05, 0.01, and 0.001, respectively.

The similarity in the results for the estimate of γc with the three homologous systems is shown in Fig. 3. The solvent system was based on mixtures of water with acetone, an aprotic solvent with low surface tension which does not interact with leaf surfaces, e.g. does not dissolve waxes (Martin & Jupiter 1970; Holloway & Silcox 1985). The surfactant system used an anionic surfactant, sodium dioctyl sulfosuccinate, since it was found that anionic surfactants are not sorbed in the cuticle at normal pH values (Baur & Schönherr 1996) and at the concentrations used, no changes of the leaf surface properties were observed (by SEM evaluation). In contrast, the fungicide formulation

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contained substances which are able to enter the cuticle and surface active formulants which can change the leaf surface structure by altering the wax morphology.

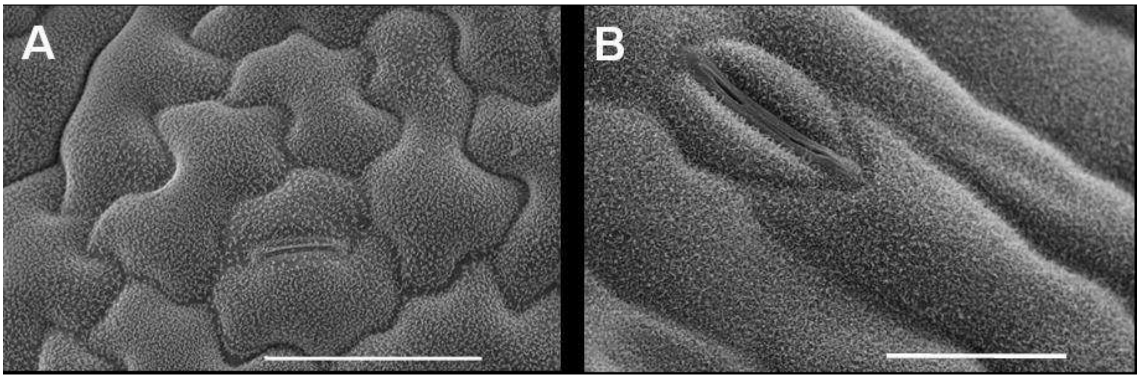

FIG. 2: Scanning Electron Microscopy showing the dense layer of crystalline waxes on the evaluated leaves of (A) soybean and (B) corn. Bars: 50 µm

When the spray drop was allowed to evaporate and the contact area was investigated by SEM, wax crystals appeared molten-like or collapsed (picture not shown). However, even though the test liquids were so different, the estimates of γc were comparable and the only difference was that the slopes differed slightly, which is quite common for different homologous liquids in a Zisman plot (Shafrin & Zisman 1960). Actually, the three systems intersect cos θ at exactly the same value, if the values with the highest surface tension (Fig. 3) are not used, which indicates a slight deviation from linearity. In spite of a low variability (<1.3%) found among liquid systems in the estimate γc of soybean and corn plants, a slight system slope dependency was observed for both species (Table 1, Fig. 3).

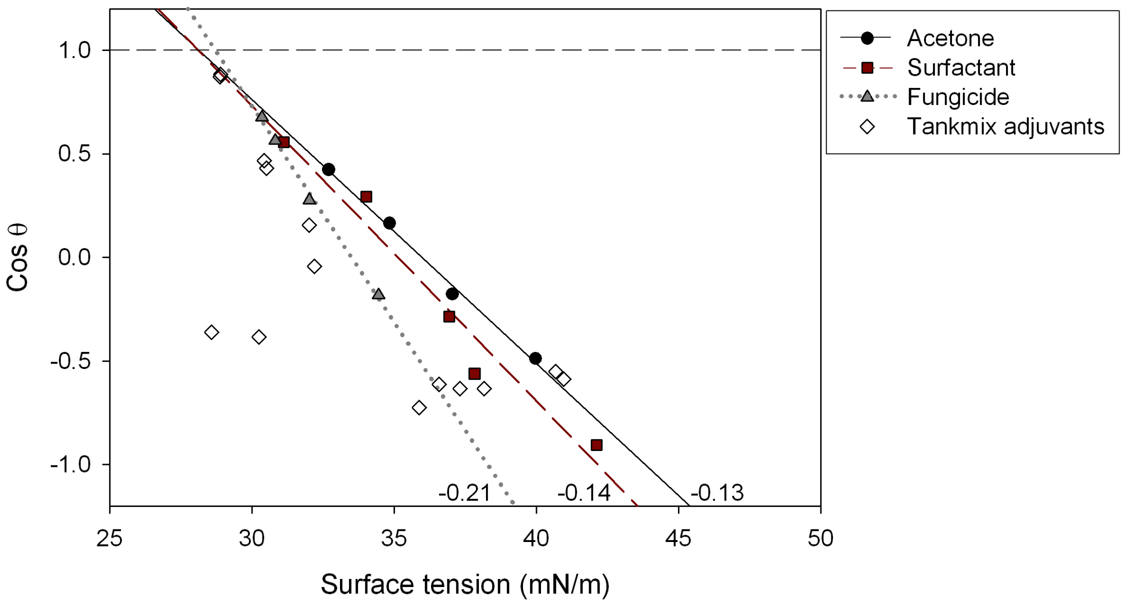

FIG. 3: Zisman plot for soybean leaf surface determined with acetone/water blends, anionic surfactant, commercial fungicide and different tankmix adjuvants. The slopes of the regression are given in the bottom part of the graph

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The fungicide formulation caused lower contact angles at the same surface tension. This was more pronounced at higher surface tension, i.e. lower surfactant concentrations. It is suggested that this was caused by rapid penetration of the surface active compound of the formulation, while the other systems did not penetrate and were generally static with respect to the measurement period.

It was not possible to estimate the γc of plant species with the fourth system. As is shown in Fig. 3, no relationship was found between the surface tension of the tank-mix adjuvants and the cos θ. Solutions made with different concentrations of a particular adjuvant resulted in the same surface tension, but different contact angles, on the leaf surface. It indicates that adjuvants caused changes on the leaf surfaces which modified their wetting characteristics. As observed by Abbot *et al*. (1990), we found the estimation of γc through contact angle and surface tension measurement using homologous liquid systems was not adequate to predict wettability of a complex mixture of commercial adjuvants on the leaf surface.

Fig. 4 shows the Zisman plots with all three systems for leaves from an easily-wettable lemon plant, and difficult-to-wet soybean and corn, respectively. The species dependence can be clearly distinguished with all systems. The coefficient of variation among the first three systems for the calculation of γc of lemon was high (14.3%) where a higher γc of 30.6 mN/m was found with acetone/water blends (Table 1). We reproduced these values and have no explanation as to whether the solvent system (acetone/water) deviated or whether the other systems changed the surface properties e.g. by interaction with oils from the lemon leaf. The slope of the regression equation was approximately 0.04 for the three systems for lemon. The low slope indicates that even a strong decrease in the surface tension of spray solutions will achieve only a small change in the wettability of the surface since the surface is already easy to wet. Similarly, low slopes were found for olive and kumquat plants and their γc of 32.7 and 35.2 mN/m respectively, coincided with the above mentioned γc of lemon found with the acetone system.

As indicated by Table 1, the most wettable plants had a γc that was higher by about 5 mN/m. Thus complete spreading can be realised at higher spray surface tensions. The γc of Table 1 are close to those found by McKay *et al*. (1987) for soybean, corn and wheat which were between 25 to 28 mN/m. It is not clear how accurate the determination of those γc values by McKay was as no data points or goodness of fit were shown. The critical surface tensions of Table 1 were in the range of magnitude of organic polymers. The calculated average γc of soybean leaves using the acetone and fungicide system in four different soybean plants (data not shown) was 28.0 mN/m. Minimum and maximum values varied between 25.5 – 29.6 mN/m with a coefficient of variation of 4.6%. These values do not differ more than the γc found in the literature for a given synthetic polymer like polyethylene.

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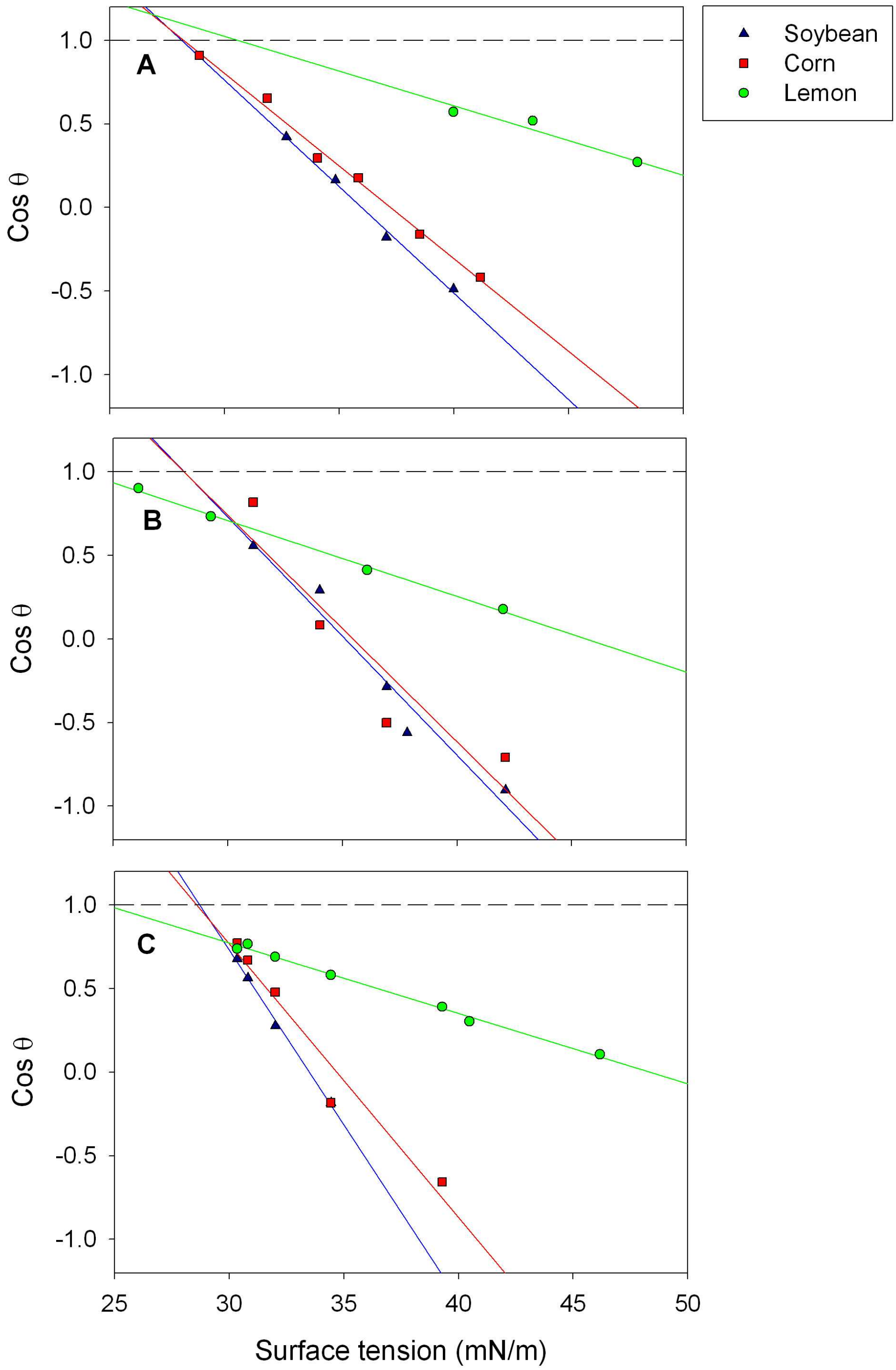

FIG. 4: Zisman plots for soybean, corn and lemon leaf surfaces determined with: (A) acetone system. (B) surfactant system. (C) fungicide system

Fig. 5 shows that all plant species evaluated followed a general trend. We have evaluated easily wettable dicot plants (olive and citrus) as well as difficult to wet dicots

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(soybean) and monocot plants (wheat). There was a strong relationship (R^2 = 0.94) between slope and intersection point and we expect that other species follow this law. One may wonder why this holds for plants that differ that much in the water contact angle. The cereals and soybean plants can have water contact angles much higher than 140° due to the crystalline surface waxes and this apparent contact angle results from the fact that water droplets rest essentially on the air between the wax crystals. The reason for the relationship shown in Fig. 5 is that with solutions of lower surface tension the air layer is substituted by the liquid and thus it is no longer a rough composite surface. Under the conditions of estimating the γc, the criterion for wettability is met. This is also an explanation for the applicability of the Zisman plot for rough surfaces that has been questioned by several authors, and also for artificial surfaces including powders (Hansen & Pierce 1974).

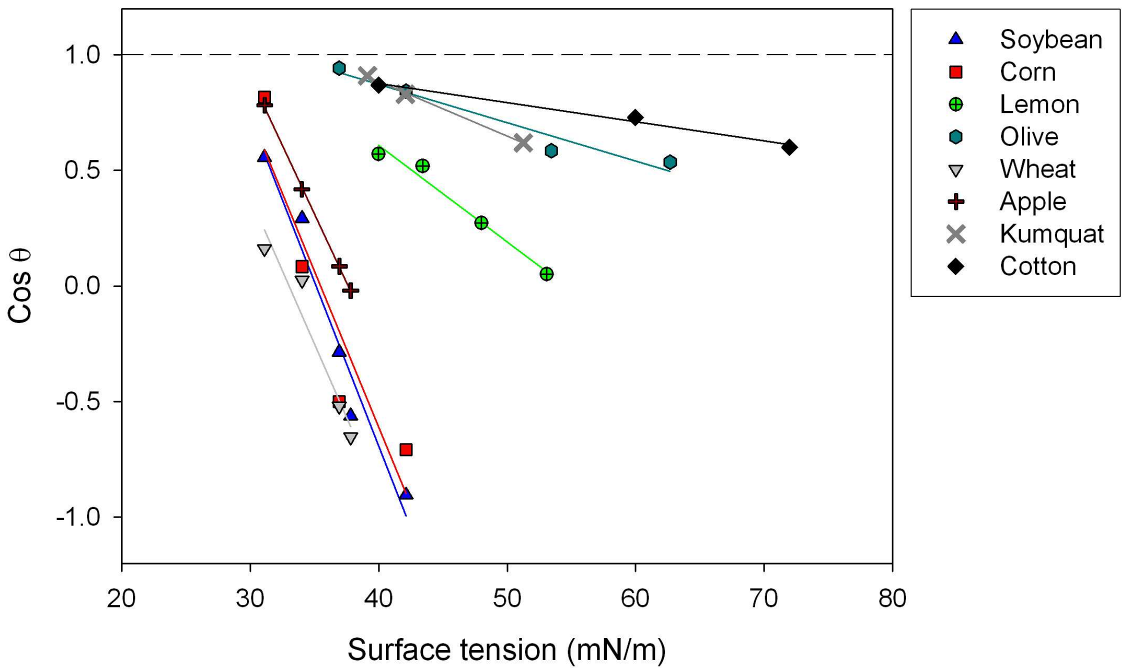

FIG. 5: Zisman plot for different plant species. Data points for soybean, corn, olive, wheat and apple were determined with the surfactant system while lemon and kumquat with acetone system (values for cotton surface from Baur 2005, unpublished)

The present study evaluated γc as a second parameter besides the often used water contact angle. In contrast to the very different water contact angles, the difference of γc among species was low and the practical implication of considering these threshold values is just to avoid run-off or enable real spreading, respectively. However, our results showed a species slope dependency and the regression slope is an interesting parameter to evaluate the wettability of different plant surfaces. The slope might provide an even better discrimination than the contact angle of 20% acetone-water mixtures which has been proposed for use instead of water contact angles (Boyce & Berlyn 1988; Forster & Zabkiewicz 2001).

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Publication 2

Wettability of soybean (*Glycine max L.*) leaves by foliar sprays with respect to developmental changes

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Wettability of soybean (*Glycine max* L.) leaves by foliar sprays with respect to developmental changes

Diana W Moran Puente[a,b*] and Peter Baur[a,b]

Abstract

BACKGROUND: Leaf wettability considerably defines the degree of retention of water and agrochemical sprays on crop and non-target plant surfaces. Plant surface structure varies with development therefore the goal was to characterise the wettability of soybean leaf surfaces as a function of growth stage (GS).

RESULTS: Adaxial surfaces of leaves developed at GS 16 (BBCH) were 10 times more wettable with water than leaves at the lower canopy (GS 13). By measuring contact angles of a liquid having an intermediate surface tension on different leaf patches, an illustrative wetting profile was elucidated, showing to what degree wetting varies (from >120° to <20°) depending on leaf patch and GS. While the critical surface tension of leaf surfaces at different GSs did not correlate with the observed changes, the slope of the Zisman plot accurately represented the increase in wettability of leaves at the upper canopy and lateral shoots (GSs 17 to 19, 21 and 24). The discrimination given by the slopes was even better than that by water contact angles. SEM observations revealed that the low wettability observed at early GSs is mainly due to a dense layer of epicuticular wax crystals. The Zisman plot slope does not represent the changes in leaf roughness (i.e. epicuticular wax deposition), but provides an insight into chemical and compositional surface characteristics at the droplet–leaf interface.

CONCLUSIONS: The results with different wettability measurement methods demonstrated that wetting is a feature that characterises each developmental stage of soybean leaves. Positional wettability differences among leaves at the same plant and within the same leaf are relevant for performance, selectivity and plant compatibility of agrochemicals. Implications are discussed.

Keywords: wetting; Zisman plot; critical surface tension; soybean (*Glycine max* L.); plant surfaces

1 INTRODUCTION

The quantity of active ingredient delivered to the plant surface and made available to reach its target predominantly determines the bioefficacy of many crop protection agents (CPAs). Besides drop size and spray properties, the degree of retention of agrochemical spray solutions is mainly a function of the intrinsic leaf surface wettability.[1] The strong interspecies variation in leaf surface wettability by rain water and agrochemical sprays is of high significance for CPAs, particularly for herbicide efficacy and selectivity.

Many factors are involved in the wetting phenomenon which is defined as the macroscopic manifestation of submicron physicochemical interactions at the droplet–leaf interface.[2] The influence of factors that are not under the control of applicators, such as environment and plant characteristics, must be known for optimising the delivery of an active ingredient. Some factors well understood are considered in models bringing together the controlled and non-controlled factors in order to predict several spray processes such as adhesion,[3] retention,[4] spreading[5] and subsequent uptake and translocation of xenobiotics into plants.[6,7] In these partial processes, the relevant timescales differ from milliseconds for spray retention to seconds for spreading of droplets, from minutes to hours for evaporation and from hours to days for deposit formation and (re)hydration.[8] The role of the leaf surface for these processes has been studied before and after spray evaporation. Spray liquid behaviour and surface properties can be characterised by measurement of spray retention and coverage of leaves or whole plants,[9–11] measurement of static contact angle with water[12,13] or solutions with intermediate surface tension,[14] hysteresis of advancing and receding contact angles of sessile droplets[4] and critical surface tension.[15,16] After evaporation of the droplet, spread diameters of macroscopic or real spray droplets have been used as indicators of wettability[17,18] with respect to leaf surface characteristics and concentration changes during evaporation.[8]

While there is generally a good agreement between the predicted and observed values of the above parameters, differences caused by leaf surface wettability during plant growth and de-

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velopment have largely been neglected. Such differences can result from changes in leaf size, shape, topology and trichome density, or from alteration of epicuticular wax composition and deposition. Rates of epicuticular wax production and synthesis of wax fractions change during expansion of leaves, and they also differ between adaxial and abaxial leaf surfaces.[19] It has been demonstrated that, during the development of maize plants, wax biosynthesis is modified with a subsequent variation in wax morphology.[20] For the tested varieties, this produces an increase in wettability at GSs 14 and 15 of the BBCH (Biologische Bundesanstalt, Bundessortenamt und chemische Industrie) code.[21] Up to the aforementioned growth stages, maize leaves were densely covered with crystalline waxes and water retention was found to be practically zero. In contrast, at GS 18 of the BBCH code, maize leaves were covered with an amorphous wax film only, and a retention value of 85% was recorded. In agreement with this, it has been reported that up to the fifth–sixth leaf of the seedling, maize leaves are covered by crystalline waxes which confer water repellency to the leaf surface.[22] The low wettability, in comparison with the upcoming stages, was attributed to changes in wax chemical composition.[23] Such a low initial wettability has to be considered for the performance of insecticides and fungicides and for the selectivity of maize herbicides. Changes in wax composition with age have been also reported in *Sorghum*.[24] The differential deposition of crystalline waxes and variation in wax chemistry, which influence the wettability of the leaf surface, have been demonstrated mainly by water contact angle measurements.[25]

It has been previously observed in the field that soybean leaves at different leaf positions on the plant are differently wetted by agrochemical spray solutions (Baur P, private communication, 2006). No information seems to be available on the differential wetting properties of soybean leaves across the plant at the age when most CPAs are applied. The objective of the present study was to characterise soybean leaf wettability as a function of growth stage, with particular reference to the practical implications for spray application of agrochemicals. For that, spray coverage and contact angles of water and solutions with intermediate surface tension were measured. Measurements were also made of the critical surface tension[26] and the slope of the linear phase of the Zisman plot as a third parameter to measure leaf wettability, discriminating well, for example, among plant species.[16] Quantitative results are supported by scanning electron microscope (SEM) micrographs.

2 MATERIALS AND METHODS

2.1 Plant material

Soybean (*Glycine max* L. cv. BRS133) seeds were sown in growth medium Standard Soil Typ ED73 (Einheits, Germany) and transplanted 7 days later to 4 L pots containing sandy soil. The plants were kept in a greenhouse under controlled environmental conditions: 29/25 °C day/night temperature, 80% RH and a 12 h photoperiod. Water was supplied as required. A fertiliser (%: 8 N, 8 P_2O_5, 8 K_2O, 0.01 B, 0.007 Cu, 0.013 Mn, 0.001 Mo, 0.005 Zn; Universaldünger; Bayer CropScience, Germany) was given every 2 weeks by diluting it at 20 mL L^{-1} water and supplying it by fertirrigation at 0.5 L plant^{-1}. Iron was provided separately. Pest management was done by soil application once or twice in the plant cycle of 0.5 mg plant^{-1} imidacloprid (Confidor SC350; Bayer CropScience) and 23 mg plant^{-1} triadimenol (Bayfidan SC312; Bayer CropScience). The identification key for the phenological GSs used in this study is based on the BBCH scale for soybean.[27] Stage 11 identifies plants with the first pair of true leaves unfolded (unifoliate leaves on the first node); stage 12 identifies plants with the trifoliate unfolded leaf on the second node and so subsequently as the leaves from the main shoot appear node by node. As the side shoots are formed, stage 21 identifies plants in which the first side shoot is visible. From here on, growth stage numbers are mentioned.

2.2 Liquid systems for wettability measurements

Tap water with the following characteristics was utilised: pH and electrical conductivity at 25 °C: 7.3 and 622 μS cm^{-1} respectively, hardness 2.83 mmol L^{-1} and surface tension 72 mN m^{-1}. For wettability profiling according to discrete areas on a leaf, described herein as patches, a moderately low-surface-tension solution (38 mN m^{-1}) was used by diluting Tween 80 (Croda) at 2.5 g L^{-1}. A fungicide system described elsewhere[16] was utilised to build the Zisman plot. A commercial EC formulation (AI: tebuconazole) was prepared at 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.15 and 0.2 g AI L^{-1} in tap water with corresponding surface tensions ranging from 49.2 to 30.4 mN m^{-1}.

2.3 Contact angle measurement

Plants were evaluated as the leaves were fully developed. Leaf strips of approximately 0.5 × 5.0 cm were placed on a sample holder. The strips for the adaxial leaf characterisation were cut from the second leaf quarter (from the leaf apice to the leaf petiole), and for the abaxial assessment they were cut from the third leaf quarter. Droplets (5 μL) of each solution were applied over the intercostal strip leaf patch, and the contact angle under equilibrium (arithmetic mean of five values taken every 5 s from 155 to 175 s after application, or else values taken after the equilibrium was reached) was measured by a DSA10 goniometer (Krüss GmbH, Germany). Three repetitions were considered for calculating the average contact angle of each dilution. Each repetition corresponds to the central leaflet at the mentioned GS of a different plant. The measurement was carried out at 23 °C and at 45% RH.

2.4 Surface tension

The surface tensions of the dilution series of the fungicide system, the intermediate surface tension solution and water were measured by the Wilhelmy plate method in a K100 tensiometer (Krüss GmbH, Germany). The given value represents the average of five values taken every 5 s from 475 to 495 s after the starting point.

2.5 Critical surface tension (γ_c)

The cosine of the average contact angle of the fungicide dilution series for each leaf surface was assigned to the ordinate and plotted against the corresponding surface tension.[26] The resulting curve was extrapolated to $\cos\theta = 1$ (or $\theta = 0$) to obtain γ_c. The coefficient of determination (R^2), the confidence interval and the slope were calculated for each curve.

2.6 Leaf wettability profiling

A leaf profiling was done in order to differentiate the wettability across the fully developed leaves at different GSs. Four leaf strips from each leaf side were cut and placed in a sample holder. Tween 80 at 2.5 g L^{-1} was used for wettability characterisation. A 3 µL droplet was placed in each third section of the leaf strip, and its contact angle at equilibrium was measured. Only one repetition was used because of the reduced area to be characterised. Each value represents the wettability of the proximate patch to the leaf edge, the middle patch and the proximate patch to the leaf middle vein; each leaf strip gives the information of the proximate patch to the leaf apice, the middle leaf patches and the adjoining patch to the leaf petiole. Only the adaxial surface was characterised, as it is the most representative part covered by sprays.[17]

2.7 Coverage

Leaves on nodes 1 to 6 (GS/LP 11 to 16) were analysed at GS 16, and leaves from the side shoots at GS 25. The number given as GS/LP does not represent the growth stage at which the measurement was recorded but the position of the leaf appearing on a node at the mentioned GS. Leaves growing on the same node from three different plants at GS/LP 11 to 16, 23 and 24 were used for water coverage quantification. Water containing the fluorescent tracer Blankophor (Lanxess, Germany) at 0.5 g L^{-1} was sprayed in a custom-built lab spray cabin (Check Tec, Germany) using the following parameters: nozzle AI11003-VS, 225 L ha^{-1}. After the leaves were dried, pictures of the strong blue-green signal were taken under UV light (366 nm; Reprostar3; Camag, Switzerland). A colour phase analysis of the fluorescent leaf patches covered by water was done with the image processing software AnalySIS 3.2 (Soft Imaging System, Germany). The reported value represents the leaf surface covered by water from the total leaf area.

2.8 Scanning electron microscopy

Leaves at GS/LP 11 to 16 were analysed at GS 16, leaves at GS/LP 17 and 18 were assessed at GS 20 and leaves from the side shoots were assessed at GS 25. GS/LP is also mentioned here to specify the position of the leaf in the canopy. Adjacent leaf patches to the veins, as well as intercostal adaxial and abaxial leaf patches, were cut and mounted with the help of forceps on a sample holder with double-sided adhesive tape. The sample holder was plunged into liquid nitrogen to produce frozen hydrated samples. The holder was then placed into the cryostation of the SEM (Jeol JSM-5600LV; Japan) and observed at an accelerating voltage of 3 kV and at a cooling sample temperature of $-50\,^{\circ}$C. No ice contamination was observed. Only the central patch not disturbed by the forceps was considered for examination.

3 RESULTS AND DISCUSSION

Field observations suggested that fully developed leaves located at the base of soybean plants are more difficult to wet by rain water or simple sprays without wetting agents than leaves located near the top of the plant or on the lateral shoots (Baur P, private communication, 2006). To corroborate this statement, the coverage of water, which is the result of both spray retention

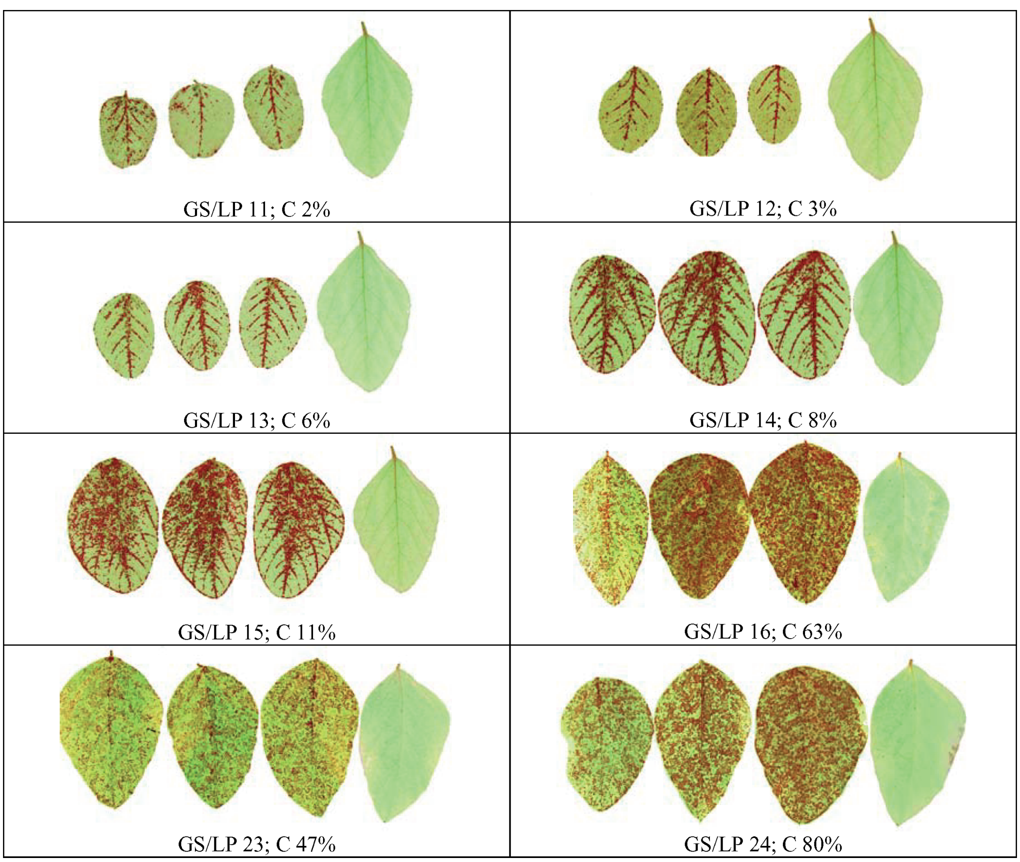

Figure 1. Characterisation of wettability by coverage with tap water (nozzle AI11003, 225 L ha^{-1}) on soybean (cv. BRS133) leaves. Red-coloured areas represent the leaf surface covered by water, which is denoted as % (C). The control leaf is placed to the right. GS/LP = leaf position in the canopy. GS/LP 23 and 24 grow on the same node as GS/LP 15 and 16 respectively, but they are found on the side shoots.

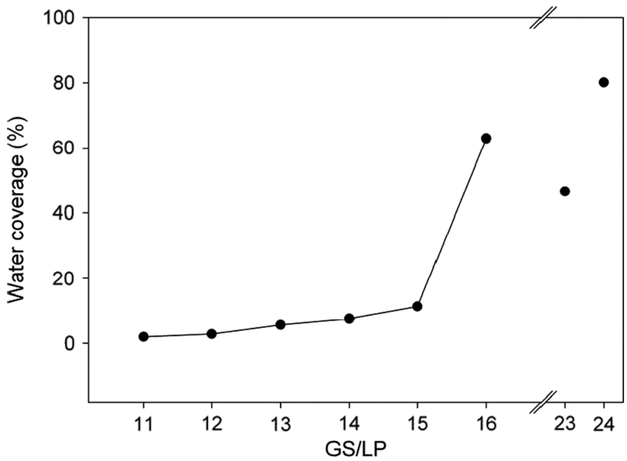

Figure 2. Dependence of coverage of aqueous sprays (nozzle AI11003, 225 L ha^{-1}) on leaf position of soybean plants (cv. BRS133). Leaves at GS/LP 23 and 24 grow on the same node as GS/LP 15 and 16 respectively, but they are found on the side shoots.

and droplet spreading,[8] was quantified on leaves at different plant positions (GS/LP), as shown in Fig. 1. The value of water coverage increased constantly from the base to the top of the plant (Fig. 2). Leaves at GS/LP 16 showed 30 times higher values for water coverage than leaves at GS/LP 11, and 10 times higher values than at GS/LP 13. An increase was also observed when comparing leaves on the same node but developed on the main stem against leaves developed on the side shoot (GS/LP 15 versus GS/LP 23 and GS/LP 16 versus GS/LP 24), demonstrating that leaves on side shoots were generally better wetted by water. Figure 2 shows the dependence of coverage by water on leaf position. GS/LP 24, a side shoot originating on the same node as GS/LP 16, was 15% better covered by aqueous solutions than GS/LP 16.

The red-coloured pattern in Fig. 1 shows that the leaf patch along the leaf veins retained more water, and how this area expanded when leaves from the base of the plant were compared with those on top. Actually, the results of this figure suggest the origin of the different wettability as a function of leaf position. Wetting started along the mid-vein. The side veins were then also increasingly wetted, followed by a distinctly wetted leaf margin. The intercostal areas were then wetted as well, initially spotwise (GS/LP 15), before full wettability was obtained. At GS/LP 16, the leaf veins were even no longer visible as distinct structures according to the wetting by water. As the plants were maintained in the greenhouse, this is a developmentally induced change and not due to erosion of waxes by rain.

In order to better understand this phenomenon, adaxial and abaxial leaf surfaces were studied by SEM. A dense layer of epicuticular wax crystals (EWCs) was observed uniformly on the adaxial and abaxial leaf surfaces from GS/LP 11 (Fig. 3) to GS/LP 14. This was also observed on the abaxial leaf surfaces from GS/LP 15 to 17, but not completely on their respective adaxial surfaces, which were found to be only partially covered by EWCs (GS/LP15 in Fig. 3; GS/LP17 in Fig. 4). Here, leaf patches around veins, previously found to be easily wetted by water, were not covered by EWCs. Such non-crystalline wax areas, sometimes beneath the crystals, are present on practically all leaf surfaces, and their ubiquitous existence as an amorphous wax layer was shown by Jeffree[28] and termed variously as a *non-waxy* area by Neinhuis and Barthlott[29] and as *epicuticular wax film* (EWF) by Jetter *et al*.[30]

The influence of EWCs on water retention is well understood.[25,31,8] EWCs have a (sub)micron dimension that causes a difficult-to-wet leaf surface essentially by reduction of the interfacial contact area between the water droplet and the plant surface. Pure water droplets cannot enlarge the contact area during impact and are unable to transfer the kinetic energy to the solid. Because of this, and the high surface tension of water, the drop might deform elastically, bouncing off, or drop shatter might occur, depending on droplet diameter and flight velocity.[32] By using a mutant genotype of the barley variety Ingrid,[33] which differs from Ingrid essentially by a ~80% reduction in epicuticular wax

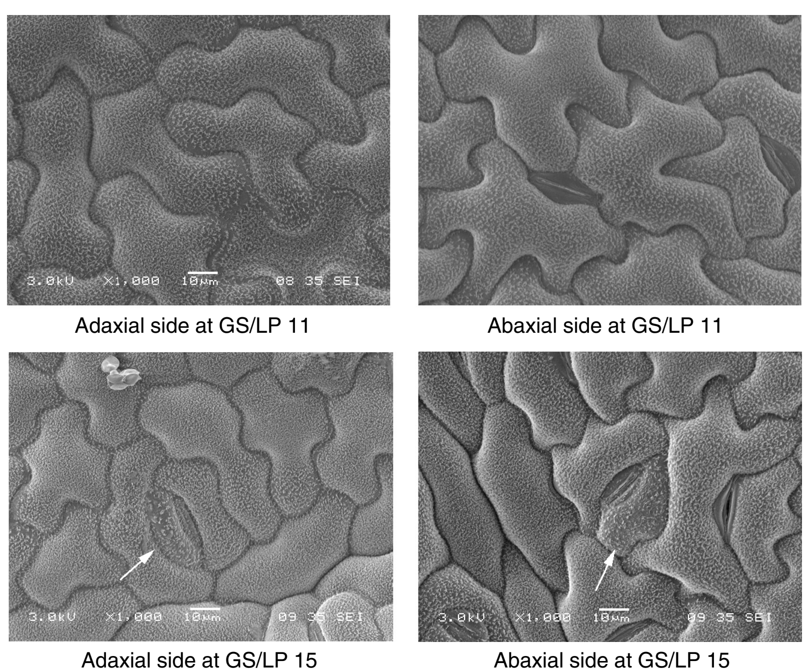

Figure 3. SEM micrographs (1000×) of soybean (cv. BRS133) leaf surfaces at different GS/LP.

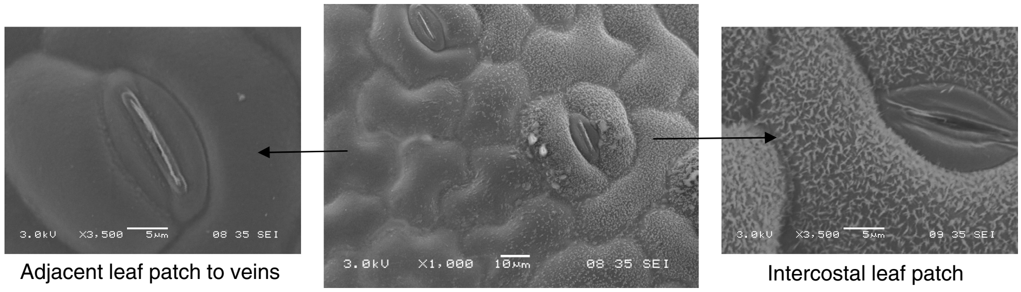

Figure 4. SEM micrographs (1000 and 3500×) of soybean (cv. BRS133) leaf adaxial surfaces at GS/LP 17.

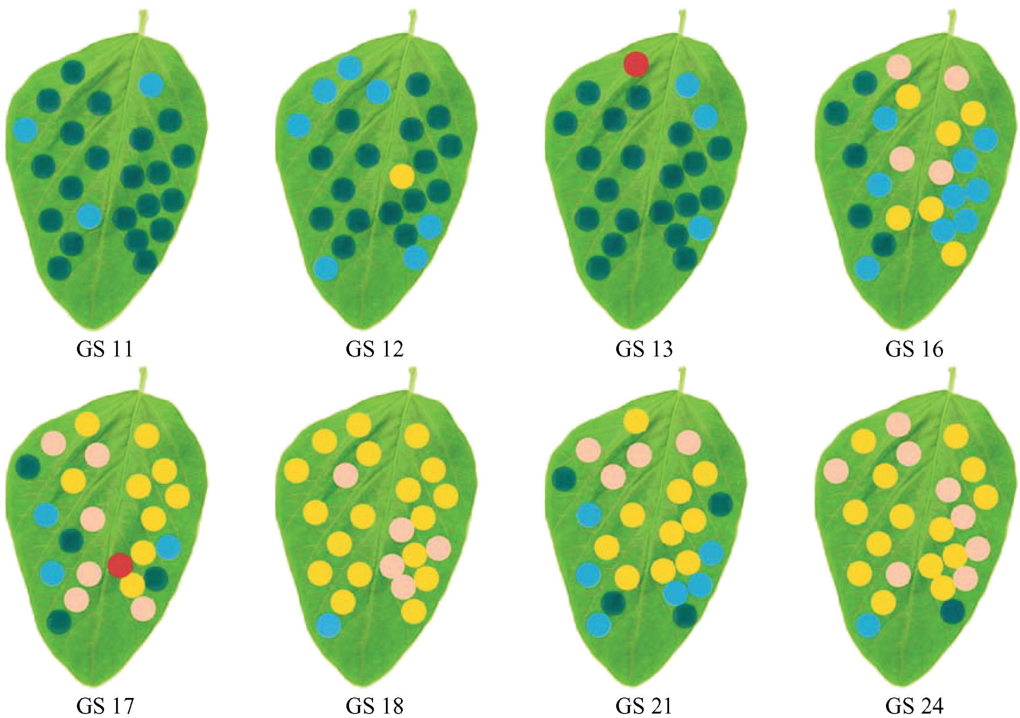

Figure 5. Influence of plant developmental stage on soybean leaf surface wettability. GS = BBCH growth stage. GSs 21 and 24 grow on the same node as GSs 13 and 16 respectively, but they are found on the side shoots. The coloured area represents the leaf patch where the contact angle of a 3 μL drop of Tween 80 (2.5 g L^{-1}) was measured. The colours define the value of the contact angle: dark blue $>120°$; light blue 90–120°; yellow 60–90°; pink 20–60°; red $<20°$.

amount and crystal density, a drastic increase in water retention (more than 30-fold) was reported.[8] This is of the same order of magnitude as the ratio of coverage at GS/LP 16 over GS/LP 11 (Figs 1 and 2).

The practical implication of these findings is that spray application of CPAs will be better retained by leaves located near the top of the canopy and on side shoots. Because these exposed leaves capture most droplets during spray application, reflection of droplets and deeper canopy penetration are reduced. Even if these lower-canopy leaves are met by simple sprays, only their leaf veins are wetted. For systemic active ingredients, this may still not affect performance if penetration and translocation can start from the leaf veins. Good penetration also requires good contact of the spray droplets to the leaf surface. On microscale contact, the stomatal area from both leaf sides at GS/LP 15 to 17 will be easily wetted by applied CPAs. As demonstrated by arrows in Fig. 3, leaf surface patches surrounding the stomata showed a less dense EWC coverage. This fact has great importance for agrochemical behaviour because the low density of EWCs in the stomatal area allows spray droplets to contact preferentially the stoma. For solutions with low surface tension, this could facilitate stomata infiltration, which is translated into higher CPA penetration as compared with the situation in which only cuticular penetration is considered.

Contact angles of a solution with an intermediate surface tension measured on the adaxial leaf surfaces of soybean plants were found to vary widely as well, depending not only on the growth stage chosen for the measurement but also on the selected leaf patch. As illustrated by colours in Fig. 5, fully developed leaves at GSs 11, 12 and 13 were dominated by contact angles that denote difficult-to-wet surfaces (i.e. contact angles higher than 90°). According to the lower contact angles dominating the majority of the leaf area, wettable leaves were encountered at GSs 17 and 18 and at GSs 21 and 24 growing on lateral shoots. The transition was identified at GS 16, at the latest, and this coincided with the observations by SEM and water coverage (Fig. 2). A comparison of

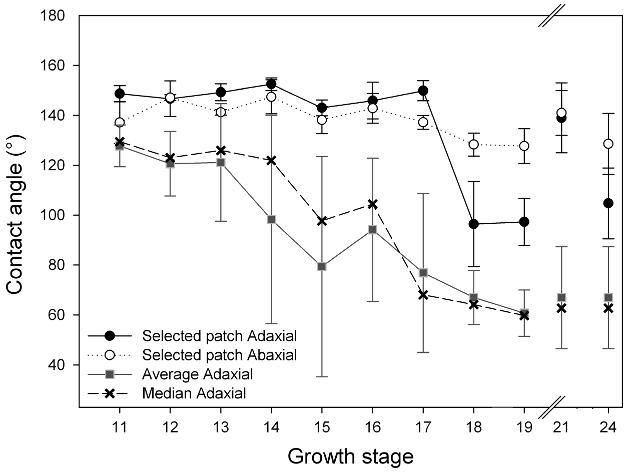

Figure 6. Contact angles of liquids measured on leaf surfaces at different growth stages in soybean (cv. BRS133). The black and white dots represent the contact angle of water ($n = 3$) on a selected leaf patch (intercostal patch at second leaf quarter for adaxial surface; third quarter for abaxial). The black squares represent the contact angle of Tween 80 (2.5 g L^{-1}) averaged from 24 leaf patches, while the bold crosses represent the median contact angle. The bars denote the standard deviation. GSs 21 and 24 represent leaves growing on the side shoots on the same node as GSs 13 and 16 respectively.

the results for coverage (Fig. 1) with those for contact angles in Fig. 5 may suggest a disagreement for GS 16. While coverage at GS 16 is already suggesting almost complete wettability by water, the contact angles in Fig. 5 suggest that the edge of the leaf is dominated by high contact angles, even though the solution used for the wetting profile had a lower surface tension than water. Each leaf taken for quantifying coverage came from a different plant, and Fig. 1 clearly shows that at GS 16 one of the repetitions (left side) had lower water coverage. The contact angles in Fig. 5 seem to match better the result for coverage of this left-side repetition. For a transition stage, this biological variability is to be expected. These findings underline the significance of leaf positioning and the information on the exact position on the plant where a parameter relating to plant surface wettability has been determined. For example, Fig. 6 shows contact angles of water for each growth stage measured within a selected leaf patch, as is typically done for wettability studies. Figure 6 also illustrates the average and median contact angles of a solution having intermediate surface tension, calculated from values presented in Fig. 5. As shown, GS 16 still shows a high contact angle for water ($>140°$), probably because the leaf patch taken was still covered by EWCs. If, instead of selecting a leaf patch for the measurement, the contact angles of a solution with an intermediate surface tension across the leaf are measured for calculating their average and median, values were obtained that better represent the changes in wettability among growth stages (■ and × in Fig. 6). As might be expected from Fig. 5, the standard deviation of the average contact angle (Fig. 6) is high for GSs 13 to 17. It is concluded that the leaf patch taken for measuring contact angles has to be carefully selected to be representative of the whole leaf and the growth stage of interest.

In some plant species a decrease in water repellency as a consequence of wax erosion after leaf expansion has been reported.[29] In the present case, the described growth-stage-dependent wettability seems to be constant even some weeks after leaf expansion. As suggested by coverage quantification of aqueous solutions (data not shown), leaves located at the base (GS/LP 11 to 13) of greenhouse-grown plants maintain their water repellency, even when the reproductive cycle begins. Some differences are to be expected under field conditions, because EWC deposition might be affected by leaf exposition to environmental factors (i.e. light, wind, drought periods, contaminants, CPA treatments).[19]

The observed differences in wettability during plant development are based on physicochemical changes in leaf epicuticular waxes. Water contact angles and wetting profiles, such as those shown in Table 1 and Figs 5 and 6 for adaxial surfaces, indicate changes in leaf surface roughness. These changes with growth stage could be due to a difference in wax yield[19] resulting in a less

Table 1. Contact angle of water ($n = 3$) and critical surface tension (γ_c) of soybean (var. BRS133) determined with the fungicide system[16] on the adaxial and abaxial leaf surface at different GSs. The GS order in the table relates to the node position in the canopy; thus, GSs 21 and 24 belong to secondary growth (lateral shoots) but appear on the node where GSs 13 and 16 originate, respectively. Regression slopes (m) and determination coefficients (R^2) are given

	Contact angle (deg) ± SD		m		R^{2a}		γ_c (mN m^{-1})	
	Leaf side		Leaf side		Leaf side		Leaf side	
GS	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
11	149 ± 3	137 ± 8	−0.15	−0.14	0.999*	0.969^n.s.	28.39	28.22
12	147 ± 7	147 ± 1	−0.21	−0.23	0.964^n.s.	0.972^+	29.10	29.87
13	149 ± 3	141 ± 1	−0.19	−0.21	1.000**	0.989^+	29.23	29.89
21	139 ± 14	141 ± 9	−0.10	−0.16	0.886^n.s	0.918^n.s	28.15	28.61
14	152 ± 2	147 ± 7	−0.26	−0.25	0.998*	0.989^+	30.14	30.25
15	143 ± 3	138 ± 5	−0.23	−0.19	0.986^+	0.995*	30.10	30.09
16	146 ± 7	143 ± 6	−0.18	−0.21	0.968^n.s	0.987^+	29.94	30.03
24	105 ± 14	129 ± 12	−0.06	−0.16	1.000**	0.999*	26.27	28.75
17	150 ± 4	137 ± 3	−0.11	−0.23	0.997*	0.998*	29.68	31.12
18	96 ± 17	128 ± 5	−0.04	−0.11	0.940^n.s	0.925^n.s	26.57	29.00
19	97 ± 10	128 ± 7	−0.04	−0.12	0.937^n.s	0.989^+	26.81	30.07

[a] +, *, **, *** = significant at $P \leq 0.1$, 0.05, 0.01 and 0.001 respectively; n.s. = not significant at $P = 0.1$.

dense crystalline wax coverage with higher wettability[8] and/or in leaf areas lacking wax crystals accompanied by a chemically distinguished surface. A considerable increase in wettability for the mutant emr1 of the back-cross barley cultivar Ingrid was reported[8] that was predominantly related to the above-mentioned decrease in wax crystal density with the EWC-deficient mutant, which was a consequence of reduced wax load.[33] In spite of chemical changes in surface wax chemistry being reported during development of individual leaves and for leaves at different positions on the plant,[34] this study does not consider the chemical composition of epicuticular waxes. It is well documented that the chemical wax composition correlates with the crystal morphology of many epicuticular waxes,[35–37] and such morphological changes are accurately represented by water contact angle measurements.[25] According to the presented SEM evaluation, no changes have been visually detected in the shape of EWCs among growth stages, which could provide evidence of a transitional change in chemical composition. The only change observed was in the transition area, where the structure of the crystals changes to a molten stage (Fig. 4). Independent of the evaluated growth stage, the contact angles of the solution with intermediate surface tension measured in patches where crystals were present were always high, as demonstrated by the blue-coloured areas in the leaf wetting profile (Fig. 5). Furthermore, low or no wetting with water has been observed on leaf surfaces covered by different wax crystalline structures (i.e. rodlets, threads, dendrites) having different wax composition, as long as the plant surface has been covered by a highly dense layer of them. The key for the hydrophobicity of surfaces then seems to be the morphology on the micron (unitary structure) and nanometer (binary structure) length scales rather than differences in composition and consequent surface energy.[38,39]

The measurement of water contact angles for the different growth stages was performed always in the same leaf patch (intercostal patch at second leaf quarter) without considering the presence or absence of crystals. The values shown in Table 1 for adaxial surfaces went from 140° at GS 11 to 97° at GS 19. Besides a change in roughness, this could also indicate a possible change in the chemical groups to which the water droplet was exposed, as the contact angle of water measured on single homologue constituents of EW has been demonstrated to vary.[25] At this point, it has to be considered that, starting at GS 16, the water droplet was not only resting on the EWCs but also on the EWF. Different wettability could be expected if the leaf surface were incompletely covered by wax, allowing water droplets to contact more hydrophilic cuticle components,[25] or if the chemical composition of this EWF were different. In this context, it has been reported that cuticular wax fractions of *Prunus laurocerasus* are arranged in layers with specific homologues exclusively in the EWF or in the intracuticular wax.[30] A gradual deposition with different homologues dominating in different leaf expansion periods has also been observed.[34] Thus, for a plant surface, different wax chemistry could be expected in the crystalline and amorphous EW. Here, the similar chemistry of the EWCs at different growth stages is not discussed; instead, it is indicated that the water droplets over EWCs were exposed to a different chemistry (i.e. surface energy) compared with the situation when the water droplets rested directly over the EWF.

In the case of the soybean cultivar BRS133, SEM micrographs revealed differences in EWC density only on the stomatal area (GS 15 in Fig. 3) of middle-upper-canopy leaves, while a drastic difference in EW deposition was observed on most of the evaluated surfaces of upper-canopy leaves (Fig. 4). These radical differences in roughness resulted in the measurement of apparent contact angles on those leaf patches covered by EWCs and real contact angles on the EWF. Quantification of coverage with water, contact angle measurement and the growth stage–Zisman plot slope dependence (Fig. 7) demonstrate chemical differences between EWCs and EWF starting at the latest at GS 16. In a previous work[16] it was indicated that linearity in the Zisman plot, which is used to calculate the critical surface tension, is given when the criterion of wetting is reached. By definition, the critical surface tension value characterises a solid surface by giving the surface tension of a liquid that completely spread over this specific surface. This linear phase represents the situation when a droplet of a low-surface-tension solution is not resting on the crystalline structures but infiltrates the crystals and rests over the EWF. Thus, real contact angles are measured and roughness is not taken into consideration any more. The critical surface tensions varied between 24 and 35 mN m^{-1} among plant species, being in the range of magnitude of organic polymers. Moran *et al*.[16] explained the low variation in critical surface tension among different plant surfaces with diverse wetting profiles, as a consequence of not considering roughness differences. However, it has been remarked that the slope of the Zisman plot differed considerably among plant species, suggesting that the slope represents differences in surface chemistry and by this means discriminates wetting dissimilarities. In the present study, EWC-EWF deposition characterises markedly the wetting of each growth stage, and the slope of the Zisman plot reflects it accurately (Fig. 7).

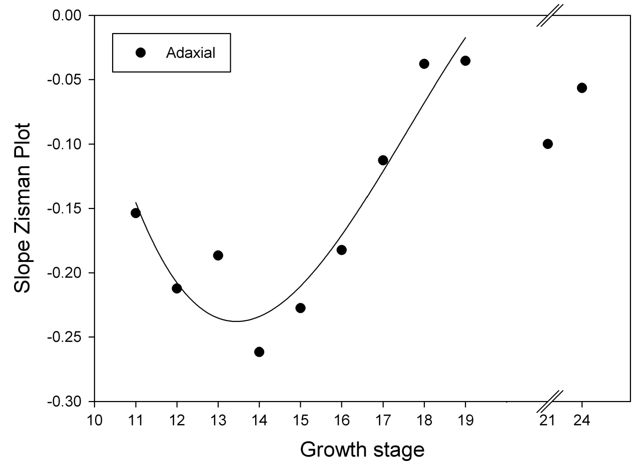

Figure 7. Plot of Zisman slopes against growth stage for the adaxial leaf surface of soybean (cv. BRS133). The solid line corresponds to leaves growing on the main shoot (GSs 11 to 19), and individual scatters represent the slope corresponding to leaves growing on lateral shoots (GSs 21 and 24). The contact angles were measured on the second leaf quarter (from the leaf apice to the leaf petiole).

The slopes of the Zisman plot for the adaxial leaf surface of GSs 11 to 16 ranges from −0.15 to −0.26, indicating that small changes in surface tension of solutions achieve big changes in contact angle, typical of difficult-to-wet surfaces.[11] Interestingly, the slope of the unifoliate leaves at GS 11 is less steep than the rest of the difficult-to-wet growth stages. When observing the corresponding SEM micrographs, it is clear that the leaf surface is covered by crystalline waxes, and no contact of the water droplet with the EWF is expected. There is no explanation for this, but the slope could be an indicator of a unique chemical feature of the unifoliate leaves. The slopes for adaxial leaf surfaces of GSs 17 to 19 and the GSs corresponding to leaves growing on lateral shoots (GSs 21 and 24) rank from −0.04 to −0.11. This indicates that even a high decrease in the surface tension of solutions will achieve a small change in the contact angle, as these surfaces are already wettable. Similar values were found for the wettable leaf surfaces of lemon, kumquat, cotton and olive.[16] The slopes for the abaxial surfaces suggested difficult-to-wet surfaces for the majority of GSs, but a small change was already perceived at GSs 18 and 19 and the secondary growth stages 21 and 24 (Table 1). For comparison, the water contact angle indicated a change in wettability on the adaxial leaf surfaces of GSs 18, 19 and 24, while all abaxial leaf surfaces were characterised by high contact angles classifying them as non-wettable surfaces (Fig. 6). Even though contact angles discriminate wetting differences, the slopes detected better the changes observed by the quantification of water coverage and by SEM evaluation of leaf microstructure.

As explained before, the critical surface tension does not consider the changes in roughness among surfaces, and this explains why in the current study the values of critical surface tension of adaxial and abaxial surfaces of all growth stages were similar (Table 1). Accordingly, in the Zisman plots the regression lines approximate at the extrapolation point (cos $\theta = 1 =$ real spreading). These low differences resemble the transition from a surface comprising CH_3 groups (21 mN m^{-1}) to one comprising CH_2 (31 mN m^{-1}), as measured for hydrocarbon surfaces.[26] In contrast, the slope of the Zisman plot not only reflects well the changes in wettability among different plant species[16] but also distinguishes changes in the same plant across plant development.

In another study, the impact of the surface wettability of soybean leaves on tebuconazole penetration was evaluated.[40] The result indicate that leaves developed at early growth stages have low wettability and also the lowest active ingredient penetration. This was demonstrated even with spray solutions in which the addition of wetting agents overcame the low wettability. These findings are relevant for rust control because its severity and sporulation is greater exactly on these leaves positioned at the base of the plant. While most fungicide applications against rust are done during the reproductive stage, protectant spraying is sometimes recommended at the advanced vegetative stage if high disease risk is given.[41] For these relatively early treatments, wetting agents and penetration enhancers are very useful. Fungicides against rust are often tank mixed with insecticides against aphids. The reason for this is that disease severity and pest incidence likely reach the threshold for application at the late vegetative to early reproductive stage. Thus, adjuvant recommendations based on leaf surface characteristics are similar for both CPAs. In addition, this information might be relevant for plant compatibility of fungicides, which can be critical under extreme environmental conditions. For example, lower-canopy leaves could be more affected because retained spray droplets do not spread. For droplets having the same volume, the concentration of agrochemical per leaf area differs with droplet spread diameter. Therefore, for the difficult-to-wet leaves in the lower plant canopy, the limited spread droplet diameter could result in agrochemical plant incompatibility. Post-emergent herbicides are applied earlier, and, as unifoliate (GS 11) and the two first trifoliate leaves (GS 12 and 13) are difficult to wet, some selectivity can be obtained. However, the EWC-based morphological selectivity of soybean decreases if wetting agents or other CPAs with built-in wetting agents are used.

4 CONCLUSION

There is clear evidence demonstrating that leaf wettability of soybean varies with development, and this potentially influences the performance, selectivity and compatibility of foliar-applied agrochemicals. Adjuvanted formulations or tank-mix adjuvants can overcome the low wettability of leaves developed at early growth stages (GSs 11 to 15). The addition of adjuvants should be considered during the vegetative and reproductive periods because these leaf surfaces maintain their hydrophobicity even when soybean inflorescences appear. After GS 15, adaxial surfaces of leaves growing on the main stem and on the lateral shoots are increasingly wettable. The information is useful for developing a spraying strategy based on suitable timing of active ingredient and adjuvant application. The results might also be relevant for plant disease epidemiology because pathogen incidence often relates to leaf wetness. Similar wetting profiles are expected for different cultivars but should be confirmed considering also different climate conditions. Likewise, the developmental changes in wettability will probably apply also to other important legumes such as *Pisum sativum* and *Arachis hypogaea*.

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Publication 3

Developmental and positional differences of surface wettability of soybean leaves and their impact on foliar penetration

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Errata in Figure 1:

- n.d. should be n.s. and means "no significance differences"
- In the second leaf, the green leaf patch should be colored in gray (60-90°)

DEVELOPMENTAL AND POSITIONAL DIFFERENCES OF SURFACE WETTABILITY OF SOYBEAN LEAVES AND THEIR IMPACT ON FOLIAR PENETRATION

ABSTRACT

Plant surfaces represent a challenge to formulators and applicators as they are the first interface for contact of impacting agrochemical spray droplets. Because of their active role in the adaptation of plants to the environment, they are biological systems constantly changing. The understanding of such systems leads to an efficient delivery of active ingredients. Knowledge of leaf surfaces characteristics at the time of a foliar application provides practical information for optimizing the timing and technique for application as well as the components of the spray solution. All factors together potentially enhance the biological performance of active ingredients, might reduce the amount to be utilized and the risk of negatively impacting the environment. This research deals with the changes produced by plant development in the wettability of soybean leaf surfaces and relates them with the penetration of tebuconazole.

Plant development causes changes in soybean leaf surface wettability among leaves formed at different leaf positions and patches within the same leaf. Leaves at the bottom of the plant are covered by epicuticular crystalline waxes and are consequently difficult to wet. Upper plant leaves and those located at secondary branches are instead covered by an epicuticular wax film, a characteristic which confers them higher wettability. This unevenness of soybean leaf wettability was so far not related to penetration of agrochemicals. The spray solutions tested included tebuconazole as combined with tank-mix adjuvants or prepared from an EC formulation. For both spray solutions, the penetration of tebuconazole was 1.5 - 2 times higher at GS/LP 16 (positioned at the middle-top of the plant) than at GS/LP 12 (first true leaves positioned at the bottom of the plant). Approximately 25% more tebuconazole is taken up by leaves located at GS/LP 17 in comparison with leaves developed only four nodes downward in the canopy. While those striking differences were observed among leaves positioned at different heights in the canopy, no significant differences in penetration were found

within the same leaf between areas differing in wettability, even though they differed in deposition of epicuticular waxes. Because of this last finding, it is concluded that for spray solutions in contact with the surface of soybean leaves, positional penetration differences in the plant are not due the macroscopically visible differential leaf surface wax coverage. The implication of these findings for the timing and the adjuvants required during agrochemical application are discussed.

Keywords: Soybean (*Glycine max* L), tebuconazole penetration, wetting, leaf surface, developmental changes

DEVELOPMENTAL AND POSITIONAL DIFFERENCES OF SURFACE WETTABILITY OF SOYBEAN LEAVES AND THEIR IMPACT ON FOLIAR PENETRATION

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SUMMARY

Plant developmental stage and position of leaves in the canopy influence significantly the wettability of soybean leaf surfaces. Leaves at the bottom of the canopy are difficult to wet as a result of a dense layer of epicuticular crystalline waxes. Middle and upper canopy leaves as well as those growing at secondary branches are better wetted by both water and agrochemical solutions. The transition in wettability is gradual and differences are also observed even within the same leaf. This study relates the developmental changes in wettability to the penetration of tebuconazole when combined with tank-mix adjuvants or used as an EC formulation. Penetration significantly increased progressively from the lower to upper canopy leaves. Tebuconazole penetration differed two fold between leaves separated in the canopy by only four nodes. However, no significant differences in penetration were recorded among patches differing in wettability within the same leaf. It is concluded that penetration of tebuconazole is affected by the leaf position but not by the leaf wetting patchiness due to differential deposition of epicuticular waxes. The implications for application timing and components of spray solutions are discussed.

Keywords: Soybean (*Glycine max* L), tebuconazole penetration, wetting, leaf surface, developmental changes.

INTRODUCTION

In order to optimize the uptake of agrochemicals, formulators and applicators deal not only with the chemical and physical properties of the active ingredients and carriers but also with variable and changing crop and weed surfaces. For foliar applied active ingredients, the outer part of the cuticle, which is referred to as limiting skin, represents the major resistance to the diffusion of pesticides (Schönherr & Baur 1994, Buchholz 2006). However, before active ingredients are in contact with it, they must be retained on the leaf surface. Field observations suggest that retention of water and spray solutions in soybean is influenced by the leaf position on the plant. These observations indicate that wettability progressively increases from the bottom to the top of the canopy and it is related to a differential leaf surface fine structure that is macroscopically visible (Moran & Baur 2007; pers. observation) and distinguished by SEM as changes in epicuticular crystalline wax (ECW) deposition (Moran *et al*. 2007).

The presence of a dense layer of ECW affects the performance of agrochemical solutions because of poor contact of the droplet to the leaf surface (Baur & Pontzen 2007). But this is not the case for low surface tension spray solutions (Gaskin *et al.*

2005) which are able to infiltrate ECW by displacing the air and coming into contact with the epicuticular wax film (EWF) (Moran *et al*. 2007). This good contact, and subsequent sorption of the active, is the first step for penetration and translocation, processes which are often measured on intact plants with radio-labelled compounds (Price & Anderson 1985; Baker & Chamel 1990; Schreiber & Schönherr 1992, Westwood *et al*. 1997). Thus, the influence of leaf surface fine structure in agrochemical spray related processes has been studied in different plants and it is still a controversial subject. This study, aims to better understand the role of surface fine structure for both wetting of soybean leaves and penetration of active ingredients as dependent on leaf position and plant developmental stage. We used tebuconazole, which is widely used in soybean particularly since it belongs to the most effective triazoles, i.e. preferred fungicide family for control of soybean rust (Miles *et al*. 2007).

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* L. cv. BRS133) plants were grown in propagation medium Einheits Erde Typ ED73 (Einheits; Germany) and transplanted later to pots containing sandy loam soil. The plants grew in a greenhouse under controlled conditions: 29/25°C day/night temperature, 80% RH and 12 hours photoperiod. Water was supplied as required. Fertilizers and crop protection agents were provided by basal application in water. The identification key for the phenological growth stages used in this investigation is based on the BBCH scale for soybean (Lancashire *et al*. 1991). From here on, growth stage (GS) or leaf position (GS/LP) numbers are mentioned. GS indicates the growth stage at which the plant was at the time the measurement was recorded; GS/LP numbers represent the position of petiole insertion at the node formed at the mentioned growth stage. For instance, GS 11 identifies plants with the first pair of true leaves unfolded (unifoliolate leaves on the first node); GS 12 identifies plants with the trifoliolate unfolded leaf on the second node and so subsequently as the plant develop and leaves appear node by node. Differently, GS/LP 12 identify leaves positioned at the second node but it does not imply that the measurement was done as plants were at GS 12. Instead penetration measurement was done for this position later at GS17 and 18 for the formulation and tank-mix system, respectively.

Leaf Wettability Profiling

Soybean leaf wettability profile was made by contact angle determination. The measurements were done by applying a 3µl droplet of a low surface tension solution (38 mN/m) obtained by diluting Tween 80 at 2.5 g/L. 4 leaf strips from each leaf side (left and right side to the main vein) were cut and placed in a sample holder. The droplet was applied in each third segment of the leaf strip and its contact angle at equilibrium was measured by a goniometer DSA10 (Krüss GmbH; Germany). Contact angles values are not reported hereby but can be found elsewhere (Moran & Baur, 2010 submitted). Here we present only the classification, i.e. the wetting leaf patchiness resulting from them.

Spray Solution Systems for Penetration Assessment

^{14}C labelled Tebuconazole ((RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol; Bayer Wuppertal) was used as tracer in a mixture of Folicur SC 570 (a.i. 0.5 g/L), methyl ester of rapeseed oil (1 g/L) and an emulsifying agent (0.2 g/L). The second spray solution system mixed with the radio-labelled tracer was an emulsion resulting from diluting tebuconazole to 0.5 g/L from a Folicur EC formulation. In both cases, before application spray solutions containing the ^{14}C tracer were left shaking 18 hours. The final radioactivity was ≈ 160 Bq in a 5µl droplet.

Penetration of tebuconazole into Soybean Leaves

Plants at GS17 were used for the measurement of penetration with the EC formulation system, while plants at GS18 were taken for the tank mix system. In order to differentiate between penetration from the wettable and non-wettable adaxial leaf areas defined by difference in EW deposition, a droplet from each spray system as described above was applied 1 cm right to the leaf middle vein and a second droplet was applied 1 cm left to the leaf edge. One day after application, the ^{14}C tebuconazole residue on the leaf surface was removed by cellulose acetate (5% w/v cellulose acetate powder in acetone) stripping technique (Silcox & Holloway 1986). For a complete recovery of the non penetrated active ingredient, application areas were stripped twice. The cellulose acetate strips were dissolved in acetone and their radioactivity was determined by means of a Liquid Scintillation Analyzer (Packard Instrument; USA) after addition of Lumasafe Plus scintillation cocktail (Lumac; The Netherland). Penetration was defined as the fraction of applied ^{14}C tebuconazole no recovered by cellulose acetate stripping.

RESULTS AND DISCUSSION

Tebuconazole is a systemic and curative fungicide which penetrates the plant cuticle and is acropetally translocated in the plant. Beside plant characteristic, application technique and environmental factors; particularly the degree of retention, contact quality to the leaf/plant surface and cuticular penetration of active ingredients vary according to the formulation type or the adjuvants used in the tank mix (Schönherr & Baur 1994, Baur & Pontzen 2007). Two spray systems have been used in the present study: a tank-mix simulating spray solution system in which the radio-labelled a.i. is mainly dispersed and partly emulsified and a spray solution prepared from an EC formulation in which the radio-labelled tracer and the a.i. of the spray solution are emulsified. Additionally to the described differences, adjuvants contained in the spray solutions are expected to influence the bioavailability of the tracer. Therefore, spray solutions systems can not be compared. The intention was instead, to investigate, for each case, the influence of the developmental stage dependent leaf wettability on tebuconazole penetration. Figure 1 shows a wetting profile for fully developed leaves formed at growth stages 11, 12, 16 and 17 with the spots having different levels of grey indicating the degree of wettability. Black leaf areas represent difficult to wet leaf surfaces covered by ECW which are a typical feature of GS 11-13; while gray and white coloured areas correspond to wettable leaf patches covered by just EWF which characterize leaves at GS 17-19. Such an epicuticular wax load disparity within the same leaf has been recorded also for leek (Rhee *et al*. 1998) and for different growth stages of corn (Hennig-Gizewski & Wirth 2000) and sorghum (Atkin & Hamilton 1982). For soybean, the differential deposition of EW is macroscopically visible by a change of the green darkness, which allows to distinguish droplets resting on ECW or EWF. As a result, the penetration of tebuconazole can be exactly related to the changes in wettability produced by the absence or presence of ECW and is indicated by dots in Figure 1. Starting at GS/LP 14, penetration was measured in areas differing in wettability and EW deposition. While wettability differed largely no significant differences in penetration were found between leaf patches. This indicates that once an agrochemical spray having low surface tension established good contact to the leaf surface, the presence of ECW does not influence foliar penetration of actives. Similar observations have been made for cereals by Giessler *et al*. (2010) where no differences in penetration of three active ingredients differing in physico-chemical properties were found between the barley back cross cultivar Ingrid and its mutant genotype emr1, genotypes differing entirely in EW load (Jansen 2007).

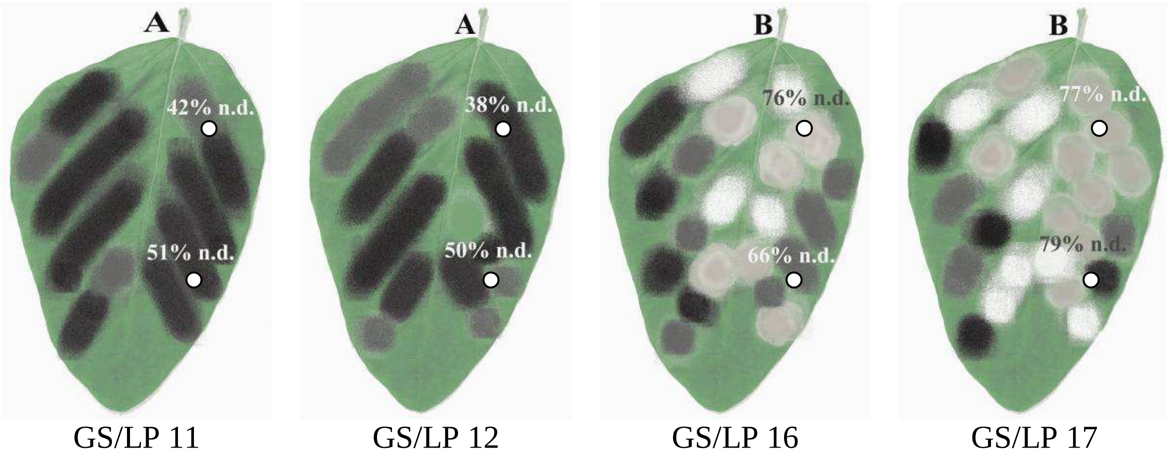

FIGURE 1: Leaf wettability profiles at diverse leaf canopy position (GS/LP). Levels of grey of leaf area represent leaf wetting patchiness as measured by contact angle: black >120°; dark gray 90-120°; gray 60-90°; white <60°. The numbers indicate the percentage of tebuconazole penetration (tank mix system) and the white dots the application area. Different capital letters near the leaf petiole denote significant differences in tebuconazole penetration among GS/LP. "n.s." means no significant differences on penetration between wetting patches ($p < 0.05$, SNK).

Independently from leaf patches assessed, penetration of tebuconazole increased as plant ages (Figure 2). Because of the lack of differences on tebuconazole penetration between the two application areas within the same leaf, percentage of penetration can be analyzed as a unique value representing the leaf position. The penetration of leaves positioned at GS/LP 16 was significantly higher by about 2 and 1.5 fold for formulation and tank mix systems, respectively than leaves at GS/LP 12. For instance, for the tank mix system around 45% of the applied tebuconazole was taken up by leaves at GS/LP 12 while about 70% penetration was found for leaves positioned only four nodes upwards. An important practical implication of tebuconazole penetration results lies in the relationship of rust incidence to leaf position. Changes of susceptibility to soybean rust associated with plant age and leaf position have been recently reported (Srivastava *et al*. 2009). A tendency was found indicating that leaf positioned at GS/LP 12 showed greater disease severity and sporulation than leaves located at GS/LP 17. According to our findings, GS/LP 12 leaves show not only low wettability but also the lowest tebuconazole penetration. Therefore, formulation or tank mixes which contain wetting agents and penetration enhancers are more useful for spraying fungicides at very early stages. Triazoles, the most important members of the fungicide portfolio against soybean rust, are usually applied at the beginning of flowering or pod formation but also as preventive treatment. The presented soybean wettability profiles are particularly relevant for the preventive applications.

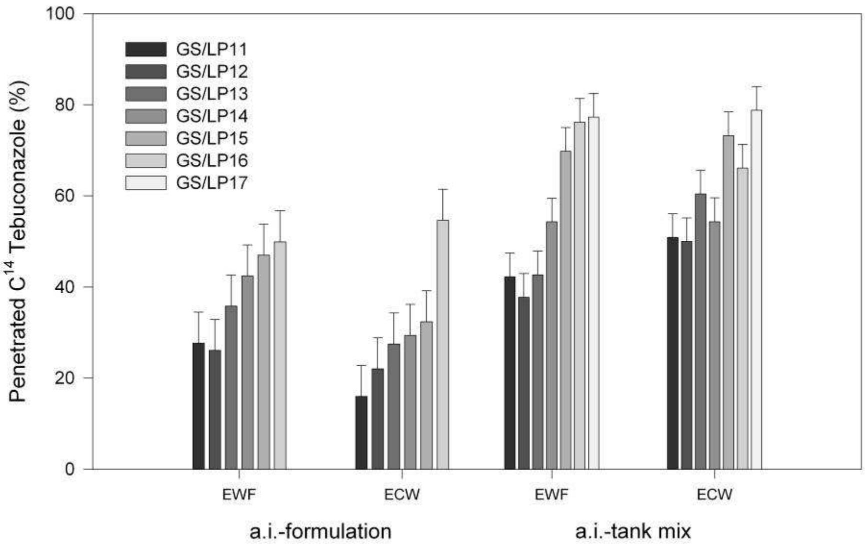

FIGURE 2: Percentage of penetration of tebuconazole after 24h from EC formulation and tank mix spray solution as function of plant leaf position (GS/LP) and epicuticular wax deposition. ECW = epicuticular crystalline waxes; EWF = epicuticular wax film.

As penetration of the a.i. between leaf patches differing in wettability was similar, it can be concluded that the increase in penetration among leaves at different positions in the plant is not related to the changes on leaf surface fine structure (EW deposition) found according to plant development stage. Other developmental factors which can not be explained with the data taken in the present study are involved. Since tebuconazole moves along the xylem and translocation is expected to be affected by changes on transpiration, it would be reasonable that lower stomata resistance found in the upper canopy leaves as compared to lower leaves (Teare & Kanemasu 1972) is responsible for the higher penetration of tebuconazole at GS/LP 15 and progressing leaves upward. Counting of stomata per leaf area in the cultivar BRS133 indicates that the abaxial leaf surface has approximately 2.5 times more stomata than the adaxial side and their density increase with the height of the canopy. Similarly, trichome density is 1.5 times higher at abaxial than at adaxial leaf surface. For abaxial surfaces, trichome density increased two fold as leaves at GS/LP 17 are compared to GS/LP 13 (Moran 2007, non published data). In addition, the barrier properties of the leaf cuticle can differ with plant development and often the permeability is higher for the leaf surface with higher number of stomata (Norris & Bukovac 1968). All factors together, lower stomata resistance, higher stomata density and higher cuticular permeability can be responsible for the increased penetration and translocation of tebuconazole at upper plant leaves. A generally higher transpiration and lower barrier properties for foliar penetration at this later stage is probable since the soybean plants have developed a strong root system and with normal water supply shortage is then not a problem.

The above mentioned increase of penetration with leaf position occurred for both spray system used for the evaluation. It is worth to mention that in spite of the continuously increase on penetration from one leaf position to the next coming, it is statistically not significant. Instead, significance ($p<0.05$) allocates the leaves growing at different plant positions into two and three penetration groups for the tank mix adjuvant and formulation system, respectively (Figure 3). For the tank mix system, leaves at GS/LP 11-14 showed significantly lower penetration of tebuconazole than leaves at GS/LP 15 and 16; whereas for the formulation system, penetration was gradually increasing from the bottom (GS/LP 11-13) to the top (GS/LP16) of the plant.

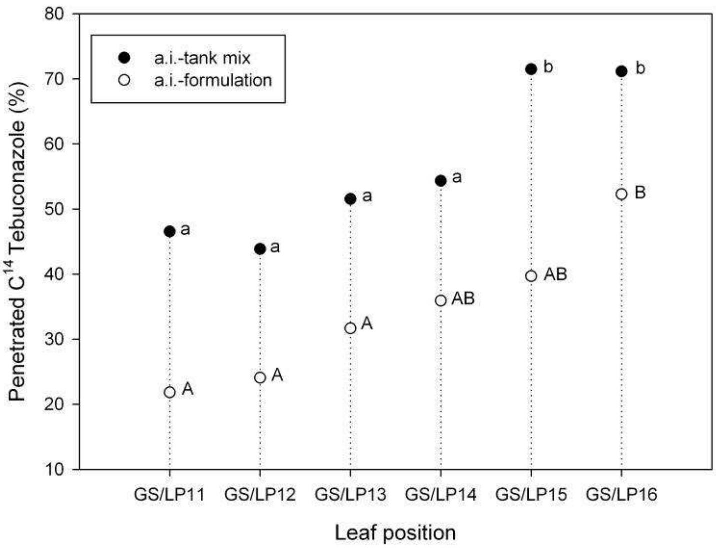

FIGURE 3: Differential penetration of tebuconazole (%) after 24h from leaves located at middle-top of the plant (GS/LP 14-16) and at the bottom (GS/LP 11-13). Different letters denotes significant differences ($p < 0.05$, SNK). Spray systems are not comparable (see text) and statistic analysis was done separately.

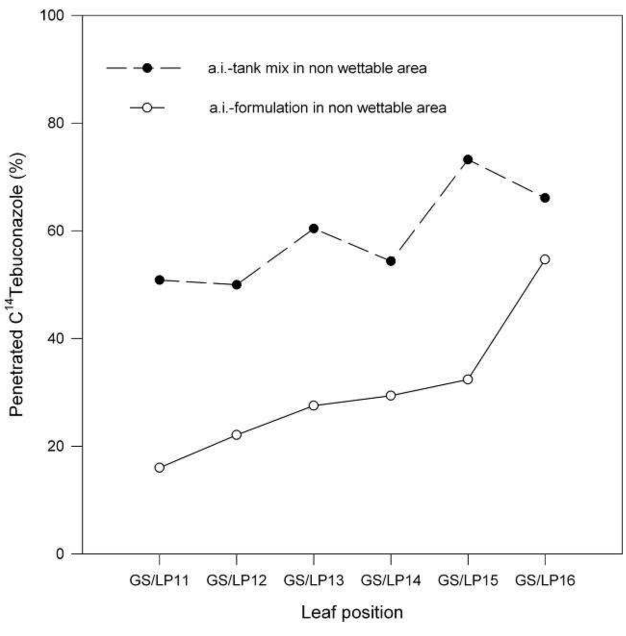

FIGURE 4: Impact of leaf position on tebuconazole penetration after 24h from EC formulation and tank-mix solution systems in leaf patches covered with ECW.

Another remarkable observation was a trend observed for the formulation spray system to decrease the difference of tebuconazole penetration in the non-wettable patches among GS/LP as compared to the tank mix system. As shown in Figure 4 an almost linear increase was found for the formulation. One of the components of the EC formulation is known to modify epicuticular wax fine structure. According to the result here presented, this would not make a difference in penetration. However, the adjuvant is also known to swell the cuticle barrier of plants increasing transpiration. As proposed above, hypothetically the differences of tebuconazole penetration among GS/LP might be a result of increasing transpiration from the lower to the upper canopy leaves. A swelling adjuvant which increases cuticular transpiration would then tend to equalize the penetration of a.i. among GS/LP. A detailed study on a.i. penetration though isolated cuticles belonging to different GS/LP would answer this question.

The results hereby and previously presented demonstrate that plant developmental leaf patchiness of wettability is not related to the speed or extent of penetration of tebuconazole. On the other side, developmental changes (differing from wettability) occurring among leaves at different position in the canopy affect the penetration of tebuconazole indicating higher foliar penetration up to GS/LP 14. The outcome of our wetting and penetration characterization according growth stage and leaf position highlight the importance of GS/LP 14-16 for the behaviour of agrochemicals, application timing and adjuvant selection for soybean crop protection programs.

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GLOBAL DISCUSSION

RESEARCH TOPICS AND RELATIONSHIP TO ENCLOSED PUBLICATIONS

Epicuticular waxes

As stated by Jeffree (2006), EWC confer water repellence; keep the leaf surface clean and dry; protect from short wave radiation, damage by acid rain as well as from air pollution and climbing by insects; discourage attachment of microorganisms and play a vital role in host-pathogen recognition. The protection given by EWC indicates their relevance in the responsive adaptation of plants to the environment at which they are exposed in each developmental stage.

The EW which form a waxy bloom on the surfaces of many plants are hydrophobic, soluble in organic solvents and solid at room temperature. They might form crystalline three dimensional structures (EWC) varying in morphology and being even visible as a white or bluish coloration on some fruits like grapes or plums or on the leaves of cabbage. But they could also be present as a thin film (EWF) which can be only seen by high resolution microscopy.

The chemistry of the waxes has been widely investigated in the past (reviewed by Koch and Ensikat 2008); however, many of those studies do not differentiate between the intra- and epicuticular waxes and the extensive data published so far does not allow discrimination. Even though the chemical composition of plant waxes is highly variable among plant species and organ ontogeny, the main component classes described in the literature are: primary and secondary alcohols, ketones, ß-diketones, fatty acids and aldehydes. Other components have been reported as well, such as triterpenoids, polymerised aldehydes, oligomeric hydroxy fatty acids and flavonoids (Table 1).

Table 1. Major constituents of plant leaf waxes (http://lipidlibrary.aocs.org/Lipids/comp_plant/index.htm)

Compound	Structure	
n-Alkanes	$CH_3(CH_2)xCH_3$	21 to 35C
Alkyl esters	$CH_3(CH_2)xCOO(CH_2)yCH_3$	34 to 62C
Fatty acids	$CH_3(CH_2)xCOOH$	16 to 32C
Fatty alcohols (primary)	$CH_3(CH_2)yCH_2OH$	22 to 32C
Fatty aldehydes	$CH_3(CH_2)yCHO$	22 to 32C
Ketones	$CH_3(CH_2)xCO\ (CH_2)yCH_3$	23 to 33C
Fatty alcohols (secondary)	$CH_3(CH_2)xCHOH\ (CH_2)yCH_3$	23 to 33C
β-Diketones	$CH_3(CH_2)xCOCH_2CO\ (CH_2)yCH_3$	27 to 33C
Triterpenols	Sterols, *alpha*-amyrin, *beta*-amyrin, uvaol, lupeol, erythrodiol	
Triterpenoid acids	Ursolic acid, oleanolic acid, etc	

Elmore *et al* (1998) reported the composition of epicuticular waxes for soybean leaves differing in the level of pubescence. Since they used chloroform for wax extraction, the obtained fraction can not be limited to EW, as they proposed, but might also contain IW. They found the following chemical composition:

- alkanes ranging from C20 – C32
- even and odd primary alcohols from C18 – C34
- long chain, monobasic even and odd numbered carboxylic acids, ranging from C14 to C34
- saturated and unsaturated pentacyclic triterpenes. The unsaturated pentacyclic triterpenes were members of the a-amyrin (precursor of ursolic acid) and b-amyrin (precursor of Boswellic acids).
- the cinnamic acid derivative: r-hydroxy cinnamic acid
- hexacosanoic acid methyl esters

As cited in the second publication, Jetter and Schäffer (2001) reported that triterpenoids, specifically ursolic and oleanolic acid, are found and dominated the composition of IW in *Prunus laurocerasus*. According to Holloway (1970), the contact angle of water on a surface composed by the single homologue ursolic acid is 89° (without considering surface roughness). Such contact angles were only found in soybean leaf patches which were not covered by EWC. It could be then interpreted that the pentacyclic triterpenes found by Elmore *et al* (1998) might not be presented or exposed in EWC. However this can not be confirmed with the research work.

Environmental factors may change the chemical wax composition, however it has been demonstrated that they mainly influence the total wax load rather than the wax composition (Koch and Ensikat 2008). Wettability of soybean fully developed leaves at the base of the plant was low, not only after fully leaf expansion, but also later when the reproductive cycle begins. That means that greenhouse plants maintain the observed low wettability over time. As discussed in the second publication, this is not expected in the field because environmental factors could lead to a decrease of total wax load (Koch and Ensikat 2008) modifying consequently the wettability of leaf surfaces.

First theories in wax biosynthesis were recapitulated by Bianchi *et al* (1985) who indicate that long chain fatty acids are generated by adding C2 units to precursor molecules until a specific chain length is obtained. The fatty acyl chains obtained enter different reaction pathways: a) decarboxylation to alkanes, b) reduction to aldehydes and primary alcohols, c) release as free acids, d) esterification to yield esters. Recent genetic studies in *Arabidopsis* have improved the understanding of the involved mechanisms in elongation of fatty acids and of the subsequent modification of the elongated products into primary alcohols, wax esters, secondary alcohols, and ketones (Samuels *et al* 2008). The use of forward and reverse genetic approaches allows creating mutant *Arabidopsis* plants which differ from the wild type in their stem EW deposition. While the wild type is covered by EWC, the mutants are covered by EWF only. Those studies have led to the identification of the enzymes involved in fatty acid elongation and biosynthesis of some wax components (alkanes synthesis has not yet been elucidated), as well as speculative transporters required to deliver lipids to the cuticle. In the epidermal cells, lipids must move from the place of generation, proposed to be the endoplasmic reticulum, to the plasma membrane and finally across the cell wall to the cuticle. The transference from the endoplasmic reticulum to the plasma membrane is unknown and it is speculated that ABC transporters and lipid transfer proteins are involved in the exportation of lipid from the cell (Kunst and Samuels 2009).

As it has to be expected, studies using *Arabidopsis* wild and mutant lines do not distinguish between EW and IW, but focus on the total wax. Initial fractioning steps were done at the beginning of this decade by using glycerol as cryo-adhesive followed by solvent extraction that allowed wax fraction separation (Jetter *et al* 2000). As aforementioned, it was evidenced with *Prunus laurocerasus* that components such as triterpenoids, which are reported in the chloroform extracted wax composition of many species, are exclusively located in the IW. SEM observations of the surface of a *Quercus pubescens* leaf, from which the EWC were mechanically stripped, showed that these crystalline structures do not arise from the cuticular proper but from a continuous covering layer of apparently amorphous wax (Jeffree 1996). This means that EWC originate from a chemically different EWF. For this study, this means that in those leaves with different EW deposition, leaf patches lacking EWC are covered by a layer of EWF. At this point it is worth to mention that the distinct Zisman plot slopes of wettable and unwettable soybean leaves evidences that covered leaf patches with EWC differ chemically from patches covered by EWF only. As it is explained there, the Zisman plot slope discriminates in very easy and practical manner leaf surface characteristics (i.e. surface energy) independently on their roughness.

An interesting fact to discuss from the first publication is that the values of critical surface tension found for different plant species vary within the range of a hydrocarbon surface comprised of CH_3 groups (21 mN/m) to the one of CH_2 (31 mN/m). Referring to leaf components containing CH_2, Baur *et al* (1997), Baur (1998) and later on Buchholz and Schönher (2000) indicate that the lipophilic pathway across cuticles of cyclic and aliphatic organic non-electrolytes is constituted by methylene groups (CH_2) of cutin and amorphous waxes. They reported that in spite of the differential IW load and composition among species, diffusion seems to take place in a similar chemical environment given by the methylene groups of cutin and IW. Now, referring to leaf components containing CH_3, as stated by Holloway (1970), in EWC the aliphatic chains are arranged in several monomolecular layers with chains placed perpendicularly to certain planes in the crystalline structure. As a result, the ends of the chains exposed to the surface are methyl groups (CH_3). This is also expected for plants like soybean because its dominating EW homologues are alkanes, having CH_3 exposed in both sides of the chain. All this information together led to the conclusion that for plant surfaces might be the situation of a water droplet exposed to the methyl groups of the EWC or to the methylene groups of the amorphous waxes of IW accidentally exposed by imperfections at the leaf surface (and probably of the EWF). Because of this, it could be hypothesized that the critical surface tensions of diverse plant species represent the change methylene/methyl group of cuticular waxes. Some data supporting this hypothesis are the critical surface tensions of EWC covered leaf surfaces like soybean and corn, which are ≈ 28 mN/m. In contrast, a non-EWC covered leaf surface like kumquat has a value of 35 mN/m, probably because the droplet is in contact with the methylene groups of the amorphous EWF. However, the data corresponding to diverse growth stages of soybean (second publication) reject this hypothesis. The critical surface tension did not represent the change of EW deposition. Actually, EWF covered leaves showed a slightly lower critical surface tension.

Initial research in EW characterization reported a distinct coherence between chemical composition and morphology. However, recent work has challenged this theory indicating that under varying crystallization conditions a same homologue could also crystallize in more than one type of three dimensional structures (Koch and Ensikat 2008). In contrast, the same wax morphology can represent different chemical compositions as is the case for wax type platelets of *Allium porrum* which are dominated by ketones, platelets of *Triticum sp.* and *Eucalyptus sp.* dominated by primary alcohols and for *Saccharum officinarum* which are mainly composed by aldehydes. Similarly, wax tubules occur in four chemical sub-types as reported by Jetter and Riederer (1999). For future research, it would be interesting to determine the Zisman plot slopes in leaves covered by the same crystallite type and density, which results in surfaces with similar roughness and wetting (as measured by contact angles), but differing in surface energy.

Influence of leaf surface fine structure on wetting

It has been proposed that in nature, hydrophobic surfaces are a product of a micro- and nanostructure or of coverage with low surface energy material which confers water repellency to the surface. The necessity of having the mentioned two scale structures in order to present high apparent contact angles (< 150°C) has been questioned (Ma and Hill 2006; Kijlstra *et al* 2002). However, recent studies indicate the need of a hierarchical structure produced by the rough surface of the epidermal cells (and trichomes) in the micro scale with the submicrometer-sized asperities given by the three-dimensional EW. Once that both characteristics have been reached, hydrophobic leaves will show stable high static contact angles and low contact angle hysteresis (< 10°) (Bhushan *et al* 2009). In such surfaces, contaminants are carried away by water droplets converting them in what is called *self cleaning surfaces*, a phenomenon described under the name of *Lotus effect* (Neinhuis and Barthlott 1997). The lotus leaves (*Nelumbo nucifera*) have protruding nubs in the micro scale and are covered in the sub-micro scale with EWC. Important crop plants, such as rape, soybean and maize, have also hydrophobic surfaces in specific developmental stages. Typical observations on these leaf surfaces are contact angles higher than 130° for water and low retention/coverage of water and agrochemical sprays. For leaf surfaces, spray retention describes the fraction of droplets which remain on or stick to the applied surface after impact (Baur and Pontzen 2007), while coverage is the result of retention and spreading. In the case of foliage applied agrochemicals, retention is governed by the above explained characteristics of the surface of the target organ, the physico-chemical properties of the spray solution, application techniques and environmental factors. For water and agrochemical retention, differences are to be expected among plant species. Hunsche *et al* (2006) characterized retention of the fungicide Mancozed for the adaxial leaf surface of apple, bean and kohlrabi. Apple and bean surfaces are covered by EWF and showed lower retention values than kohlrabi having EWC mainly composed of alkanes. The values of retention correlated negatively to roughness (as measured by contact angle of water + acetone droplets), wax load, total mass of alkanes and especially to the amount of C_{29} alkane.

Differences of retention of water have been also found for the same specie among cultivars. Mutants of maize and barley having low load or no EWC have been studied focusing on the implication of EW deposition for water retention. Publication 2 and 3 show the case of a mutant (emr1) of the back cross barley cultivar Ingrid evaluated by Baur and Pontzen (2007). In the same context, Beattie and Marcell (2002) used 11 wax mutant cultivars of maize to identify relations between leaf surface hydrophobicity and leaf surface properties. Some of the properties studied with the so-called glossy mutants were: wax (no discrimination of EW and IW) load, crystal morphology and quantification of surface area covered by EWC. For the mutant gl26, the contact angle was 35° lower than in the wild type (147°); however, both contact angles are, by definition, still representative for difficult to wet surfaces. On the mutant gl26, the planar leaf surface area covered with EWC and the wax load was reduced as compared to the wild type. The mutant had almost 8-fold lowered EWC coverage. In spite of the dramatic decrease of surface area covered by EWC, high contact angles (112°) were still found. As comparison, the barley mutant emr1 (Baur and Pontzen 2007), mentioned in the second and third publication, showed only 4-fold lower EWC coverage and this partially accounts for the reduction of the water contact angle from ≈ 145° to 85° (Moran 2010, unpublished data).

The SEM pictures of the maize cultivars evidenced the great reduction of EWC. In surfaces densely covered with EWC, it is said that the roughness produced by the morphology on micron and nanometer scale is the most important factor for hydrophobicity (Ma and Hill, 2006). For gl26 (maize) and for emr1 (barley) which are not densely covered by EWC, the following argument indicates that the surface energy of the dominating EW homologue is the decisive factor to produce such high/low contact angle, respectively. As reported by Beattie and Marcell (2002), the waxes of the mutants are mainly constituted by esters (≈ 40%), while the mutant barley emr1 is composed mainly by alcohols (≈ 54%) and in a lower proportion by esters (≈ 20%) (Jansen 2007). Considering the contact angles of water on surfaces constituted by single homologues (Holloway 1970), it is not surprising that a surface, mainly composes by esters (i.e. the mutant maize), exhibits higher contact angles than the barley leaf surface mainly composed by alcohols. This shows again the importance of the chemical group to which the droplet is exposed. In such study (Beattie and Marcell 2002), it would be worthy to measure the Zisman plot slopes to account for surface characteristics at the droplet-leaf interface, besides the normal wetting characterization made with contact angle of water. In order to account for roughness differences, it would be also meaningful to measure contact angles of solutions with intermediate surface tension. Creation of Zisman plots could be also useful because of the important information about roughness provided by the non-linear phase of the Zisman plot (explanation below). This research area will be covered by us in the future by using cuticular-wax mutant cultivars.

In the following example the Zisman plot slope helped to distinguish changes of surface characteristics (Baur and Moran 2008; unpublished data). The methodology was used to evaluate the effect of an herbicide formulation on leaf surface properties of two rape cultivars having similar EWC deposition, as observed by SEM. The application of the herbicide resulted in a drastic decrease of EWC in the cultivar 1, which was represented by a decrease of 14° in the contact angle of water. For cultivar 2, there was a reduction of the density of ECW and a change of the shape of the crystalline structures as observed by SEM. The contact angle of water was 11° lower in the treated surface. The critical surface tension was measured for both cultivars and no differences were found between treated and non-treated surfaces, in spite of the huge differences in EW deposition. Interestingly, the slopes of the Zisman Plot account for the observed differences and it was the parameter giving the best discrimination. For both cultivars the slope of the Zisman Plot was reduced by approximately 0.1. In the cultivar 1 with drastic reduction of EW, the slope is representing the situation of a droplet in contact with the ECW and then with the EWF on the treated surface. That is similar to the discrimination given by the slope with soybean leaves at difference growth stage (publication

2). In the cultivar 2, the slope is accounting for the quality of EW deposition. With the information of cultivar 1, one could think that the slope indicates differences of roughness. But with cultivar 2 was clear that the discrimination was related to chemical changes. As conclusion, the slope accounts for chemical and compositional characteristics of the leaf surface. This is also supported by publication 1, where this magnitude of difference (≈ 0.1) was found for leaf surfaces with distinct chemical and compositional surface characteristics, such as soybean and lemon.

Different from the mutants of maize and barley, soybean leaves do not show a decrease of EW load, but leaf patchiness with spots covered by either EWC or EWF. In the second paper is explained how these EWF covered leaf patches appear and become wider as leaves at different positions in the canopy are compared. As result, leaves developed at different growth stages or growing at different canopy positions are dominated either by high or low contact angles. If a leaf patch is selected for a measurement, it is likely to find extreme contact angles and conclude that the change of wettability is actually abrupt. However, if wettable and non-wettable leaf patches across the leaf are considered and compared to the upcoming leaf in the canopy, a gradual increase of wetting is observed. This is exactly meant in the second publication when soybean wettability is described as gradually changing.

The gradual increase of wettability is clearly demonstrated by the leaf patchiness seen in the wetting profile (second publication). If this patchiness is not considered but a selected leaf patch is taken for wettability measurements, then an abrupt change in wettability is quantified among growth stages. At this respect, Zisman plot helped to understand the changes. Interestingly, not only the linear phase of the Zisman plot provides relevant information of the surface but also its non-linear phase. The following figure and statements were not included in the publications but proposed as a concept in the first publication. For low energy surfaces, linear relationships are found for solutions which surface tension produces contact angles lower than 90° on a specific surface. Figure 1 shows clearly that the slope of the linear phase discriminates the wettability at diverse plant growth stages (discussed in details in the second publication). This linear relationship is calculated when the droplet of the liquid reaches the criterion of wetting. Therefore, as aforementioned the slope provides insights of the surface energy.

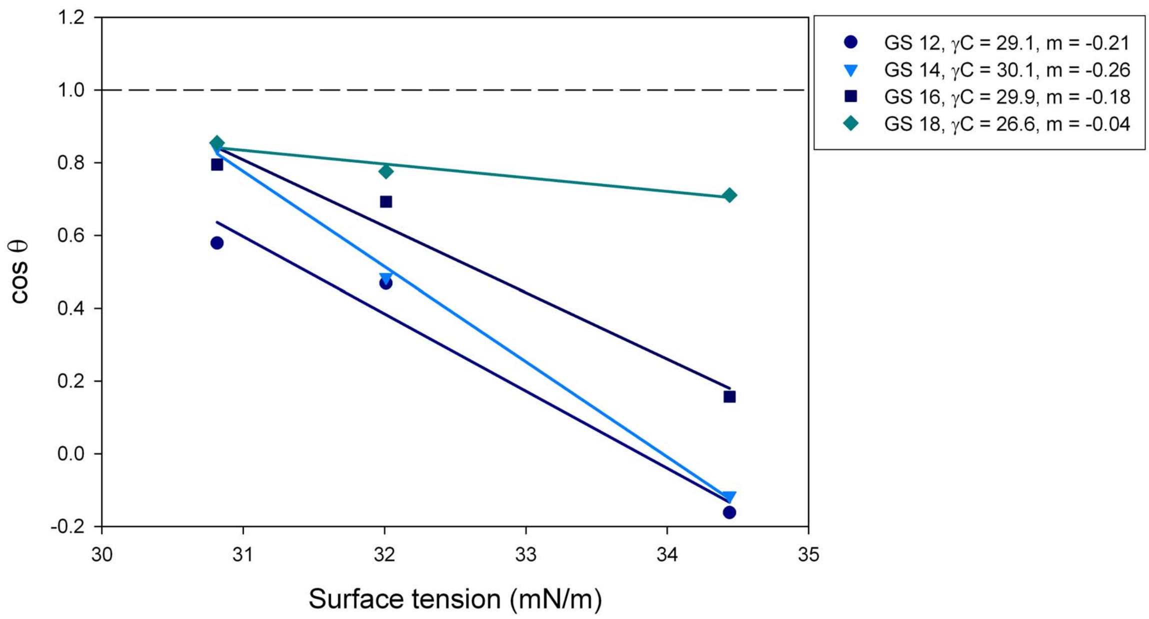

Fig. 1. Zisman plot for soybean (cv. BRS133) leaf surfaces corresponding to growth stages (GS) 12, 14, 16 and 18. The solid lines symbolize the linear function. Dashed line at cos θ = 1 denotes real spreading (θ = 0). The extrapolation of the function intersects the dashed line and critical surface tension (γc) is obtained. The slope (m) of the line is also shown in the legend.

Zisman plots were also drawn for solutions with higher surface tension (40 to 70 mN/m) (no shown). For non-wettable GSs the obtained relationship was not linear but better fit to a degree 3 polynomial function. A line could be drawn at cos θ = 0 (θ = 90°) indicating the theoretical division between easy and difficult to wet leaf surfaces. Interestingly, the GSs having most of their "xy pairs" above this threshold line correspond to wettable leaves covered by EWF. In these leaves, roughness is produced by a.o. cell topology and trichomes. Below the line are the curves which belong to difficult to wet surfaces covered by EWC. For GS 11, the second publication describes an inconsistency with respect to the Zisman plot slope. The adaxial leaf surface was covered by EWC (likely by alkanes with exposed methyl groups) exactly as it was for GS 12 to 16; however the slope of the function for GS 11 differed from the other EWC covered leaves. An explanation for this could not be given. Thus, it was hypothesized that this could be an indicator of an unique characteristic of the also unique soybean unifoliate leaf. The non-linear phase of the Zisman plot of GS 11 shows a similar curve to the other rough leaf surfaces (GS 12-16). If differences are present for GS 11, they are likely to be related to surface energy. The Zisman plot in its linear and non linear phase gave valuable information which fit to the results of the leaf wettability characterization.

As theoretical exercise, it could be of interest to use the extrapolation concept of the linear phase in the Zisman plot for the non linear phase. In practice, 140° is the maximum contact angle which could be properly measured in the laboratory. If cubic functions fit and the value of “y” is extrapolated to the respective cosine of 140°, a surface tension could be estimated, which provides two key information points.

First, this would be the surface tension required for a solution to account for roughness differences for a specific surface. Historically, the contact angle of water is being replaced by contact angles of solutions with low/intermediate surface tension (≈ 80% water + 20% acetone) to account for roughness differences of difficult to wet leaves (reviewed by Forster *et al* 2010). Those values are used for modeling and predicting agrochemical behavior, even though they present practical and theoretical difficulties. The practical problem of such solutions is that acetone is under constant evaporation with a subsequent change in surface tension and contact angle. The theoretical problem is that the proportion water-acetone is not specific to the surface of interest. While it might be optimal for soybean, it might not be for a slightly less wettable surface. This has been stated in details recently by Forster *et al* (2010). As a solution for these difficulties, the extrapolated surface tension of the cubic functions could provide more leaf surface specific information. Spray solutions having the resulting tension value could be prepared and used for leaf roughness discrimination. Adjuvants which do not change the properties of the leaf surface are recommended (see wetting profile of the second publication and liquid systems of the first publication) instead of the constantly evaporating acetone solutions.

Secondly, the value obtained could represent a threshold value for repulsion from the surface. If applied with a pipette, a droplet of a solution having the extrapolated surface tension will produce a contact angle of 140°. In this situation, the droplet will have minimal contact with the leaf surface and consequently its kinetic energy will not be transferred to the surface (Baur and Pontzen 2007). Therefore the energy of cohesion of the molecules in the liquid remains higher than the potential energy of adhesion to the surface. As result, the droplet will not suffer deformation. Assuming the same droplet size utilized for the creation of Zisman plots, a gently applied droplet having higher surface tension than the extrapolated will likely be repulsed from the surface. For soybean adaxial surfaces, the described value was calculated (Table 2) and it will be called *critical surface tension for repulsion.* The methodology used for its calculation applies only for rough surfaces. The reason for this is simply that no contact angles higher than 90° were reported for non-rough surfaces (EWF covered leaves), even with liquids as water. Therefore, only for GS 11 to 16 the data fits a cubic function allowing extrapolation to 140°. For validation of this concept, experimental work should be done and contact angles of liquids with surface tensions below the value for water (50 – 70 mN/m) should be measured.

According to the first interpretation while water (i.e. ≈ 70 mN/m) will be a suitable liquid to discriminate roughness of leaves at GS 16, a solution having a surface tension of 53 mN/m will be required for GS 11-15 (Table 2). The extrapolated surface tension varies from 48.5 to 53.3 for GS 11- 15. For a unique solution with 53 mN/m, slightly different contact angles are expected which values would successfully discriminate roughness.

According to the second interpretation, the values of Table 2 provide specific information on the surface roughness. Further experimental work should be done in this respect for dynamic (i.e. real) systems because the size of the droplet and the impact velocity are decisive factors for a droplet to shatter or bounce off (Forster *et al* 2010). The values presented here have been calculated with static contact angles at equilibrium conditions and interpretations should be done carefully. Though, some empirical assumptions can be done for same size of droplets applied with a pipette. For GS 11, a liquid with surface tension higher than 49 mN/m would be repulsed from the hydrophobic surface with high probability. Here, in order to increase droplet adhesion, spray solutions should have a surface tension lower than 49 mN/m; while in order to have real spreading the surface tension of the liquid should be lower than 28 mN/m (publication 2). As leaves become more wettable, the critical surface tension for repulsion is higher. For GS 16, liquids with surface tension lower than water will not be repulsed. In this case, adjuvants providing better spreading are more important than adjuvants improving adhesion.

Table 2. Critical surface tension for repulsion of soybean (var. BRS133) adaxial leaf surface at different GS. The value characterizes the surface while indicating the required surface tension of a liquid for reaching a contact angle of 140°

GS	Critical surface tension for repulsion (mN/m)
11	48.8
12	48.5
13	50.7
14	51.1
15	53.3
16	70.5

The importance of retention and spreading for agrochemicals has been discussed in details in the publications. But so far, the role of these values for artificial surfaces has not been yet mentioned. Pesticides are not only applied to leaf surfaces but also to artificial porous and non-porous surfaces. For leaf surfaces, spreading is desirable, among others, to allow stomata infiltration of CPA. Instead, for porous surfaces while adhesion should be optimized, spreading should be avoided. The reason is that at contact angles close to liquid spreading, liquids tend to penetrate these porous surfaces, a process which decreases the amount of active ingredient available on the surface. Knowing the critical surface tension for spreading and for repulsion of liquids for a specific surface, pesticide application to non-plant surfaces could be optimized as well.

The utilization of the Zisman plot allowed identifying the developmental changes affecting wettability of soybean leaves. With the critical surface tension for spreading was possible to define the required surface tension of a solution in order to totally spread over the surface of a leaf belonging to a specific growth stage/leaf position. With the slope of the linear phase of the Zisman plot an insight to the surface energy independently of roughness could be gained. With the non-linear phase of the Zisman plot was possible to allocate leaves at different growth stages in groups depending on their coverage by EWC or EWF (roughness). Moreover with empirical calculations coming from the cubic phase of the Zisman Plot, the concept of critical surface tension for repulsion of gently applied solutions was introduced. With both critical surface tensions (spreading and repulsion) threshold values could be obtained to predict adhesion and spreading of gently applied liquids on biological and artificial surfaces.

This research has given enough information to characterize the wettability of soybean as related to plant development in a very practical way. The publications provide further implications for agrochemical behavior.

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