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Deposits of foliar-applied herbicides:

characterization and relevance for bio-efficacy as evaluated

in selected weed species

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M.Sc. Sabin Basi

aus

Kathmandu, Nepal

Referent: Prof. Dr. G. Noga

Korreferent 1: Prof. Dr. P. Schulze Lammers

Korreferent 2: PD Dr. J. Burkhardt

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Deposits of foliar-applied herbicides: characterization and relevance for bio-efficacy as evaluated in selected weed species

The main focus is to elucidate and characterize the effect of formulation on the deposition patterns and the consequences for the foliar uptake of herbicides, either through cuticular or stomatal pathways. Glyphosate (Gly) and diquat were chosen as model hydrophilic, systemic or contact foliar applied herbicides. The formulations were prepared either with surfactants or hygroscopic salts. The ethoxylated rapeseed oil (RSO) surfactants of different number of ethylene oxide (EO) units were added to affect the deposition behaviour of active ingredients on the leaves. Likewise, to understand the stomatal uptake process, hygroscopic salts were added to the formulation to induce hydraulic activation of stomata (HAS). The formulation droplets were applied on plant leaves with kinetic energy (from a defined height with a monodroplet generator), and/or without kinetic energy (with a micropipette in contact with the plant surface). Easy-to-wet species, *Stellaria media* and/or *Viola arvensis*, and difficult-to-wet species, *Setaria viridis* and/or *Chenopodium album*, were selected. The formulation residues were characterized using a scanning electron microscope integrated with/without energy dispersive x-ray microanalysis. Deposit residue, in terms of its pattern or hydraulic activation of stomata, were discussed in relation to foliar uptake of herbicides affecting the biomass or the chlorophyll fluorescence of the tissue. The major results can be summarized as follows-

1. A simple and robust pneumatic monodroplet generator was constructed for drop-on-demand application of aqueous pesticide solutions. Increasing the impulse width and orifice diameter led to an increase in the droplet diameter. In contrast, increased pressure and reduced surface tension of the liquid led to a decreased droplet diameter. With optimum adjustments, monodroplets with diameters ranging from 533 to 1819 μm were generated.

2. The kinetic energy of the droplet had no consistent effect on the deposit structure or the bio-efficacy of Gly formulations. On the other hand, RSO surfactants differing in the number of EO units, affected both the deposit structure and the bio-efficacy of the formulations, depending upon the plant species. In easy-to-wet species, the higher EO units of RSO surfactant failed to affect the deposit area of Gly and its bio-efficacy. But in difficult-to-wet species, higher EO units of RSO surfactant reduced the deposit area of Gly and enhanced its bio-efficacy.

3. The non-significance of RSO surfactant in affecting the area of diquat residue on the easy-to-wet leaves coincided also with its non-significant changes on the chlorophyll fluorescence parameters and the herbicide-induced desiccation potential of the leaves. In contrary, on difficult-to-wet leaves, decreased deposit area of diquat droplet was associated with stronger modifications of chlorophyll fluorescence parameters, and the increased desiccation potential of diquat.

4. Hygroscopic salts led to hydraulic activation of stomata (HAS). Kosmotropic salts (Na_2SO_4) slightly reduced the surface tension of Gly solution and formed amorphous globular structures of Gly-salt deposit. In contrast, chaotropic salts (NaNO_3 or NaClO_3) led to strong reduction of the solutions' surface tension and formed a colloidal mixture flowing through periclinal walls and even into the stomata. Kosmotropic Na_2SO_4 had no impact on the Gly bio-efficacy, but the chaotropic NaNO_3 enhanced the Gly bio-efficacy yielding in results equivalent or even better than that obtained with the organosilicone Break Thru S233.

Deposition von Blatt-applizierten Herbiziden: Charakterisierung und Bedeutung für die biologische Wirksamkeit, untersucht an ausgewählten Unkrautarten

Der Schwerpunkt dieser Studie liegt auf dem Einfluss von Formulierungshilfsmitteln auf die Eigenschaften der Depositionen und deren Auswirkung auf die kutikuläre oder auch stomatäre Aufnahme von Herbiziden. Dazu wurden Glyphosat (Gly) und Diquat als hydrophile, systemische oder bei Kontakt wirkende blatt-applizierte Herbizide als Modellsubstanzen ausgewählt. Die Formulierungen wurden entweder mit Tensiden oder hygroskopisch wirkenden Salzen zubereitet. Das ethoxylierte Rapssamenöl-Tensid (RSO) mit unterschiedlichen Ethylenoxid (EO) Anteilen wurde zugefügt, um die Depositionseigenschaften der Aktivsubstanzen zu beeinflussen. Zum besseren Verständnis der stomatären Aufnahme wurden der Formulierung hygroskopische Salze zugesetzt, um somit die hydraulische Aktivierung der Stomata (HAS) zu induzieren. Die Formulierungströpfchen wurden entweder mit kinetischer Energie (aus definierter Höhe mittels Einzeltropfengenerator) oder ohne kinetische Energie (mittels Mikropipette) appliziert. Als relevante Unkrautarten wurden die leicht zu benetzende *Stellaria media* und *Viola arvensis*, sowie die schwer zu benetzende *Setaria viridis* und *Chenopodium album* herangezogen. Die Depositionen wurden mittels Rasterelektronenmikroskopie in Kombination mit der Röntgenstrahlmikroanalyse untersucht. Die Größe der Ablagerungsrückstände sowie die induzierte hydraulische Aktivierung der Stomata wurden in Relation zur Aufnahme des Herbizids durch das Blatt evaluiert. Darüber hinaus wurde die Auswirkung der Ablagerungsrückstände auf die Biomasse und die Chlorophyll-Fluoreszenz des Gewebes diskutiert. Die wichtigsten Ergebnisse lassen sich wie folgt zusammenfassen:

1. Für die an dem tatsächlichen Bedarf orientierte, zielgerichtete Applikation von wässrigen Lösungen wurde ein robuster, pneumatischer Einzeltropfengenerator konstruiert. Eine Erhöhung der Impulsweite und des Düsendurchmessers hatte eine Vergrößerung des Tropfendurchmessers zur Folge. Im Gegensatz dazu führten erhöhter Druck und reduzierte Oberflächenspannung der Flüssigkeit zu einer Verringerung des Tröpfchen-Durchmessers. Bei optimaler Anpassung resultierten Einzeltropfen mit einem Durchmesser von 533 bis 1819 μm .
2. Die kinetische Energie der Tröpfchen hatte keinen gleichmäßigen Effekt auf die Ablagerungsstruktur oder die biologische Wirksamkeit der Gly-Formulierungen. RSO Tenside hingegen veränderten mit verschiedenen EO-Anteilen die Ablagerungsstruktur und die biologische Wirksamkeit der Formulierungen in Abhängigkeit von der Pflanzenart. Bei leicht zu benetzenden Unkrautarten hatte der EO-Anteil von RSO-Tensiden keinen Einfluss auf die Depositionsfläche von Gly und seine biologische Wirksamkeit. Bei schwer zu benetzenden Arten führte die Zunahme von EO-Anteilen in RSO-Tensiden zu einer Verringerung der Depositionsfläche von Gly und zu einer Steigerung der biologischen Wirksamkeit.
3. Der nicht signifikante Einfluss von RSO-Tensiden auf die Diquat-Depositionsfläche bei leicht zu benetzenden Blättern stimmte mit den nicht signifikanten Veränderungen der Chlorophyllfluoreszenzparameter und dem Herbizid-induziertem Austrocknungspotenzial der Blätter überein. Im Gegensatz dazu führte eine verringerte Depositionsfläche von Diquat-Tröpfchen zu stärkeren Veränderungen der Chlorophyllfluoreszenzparameter des Gewebes und höherem Austrocknungspotenzial von Diquat.
4. Hygroskopische Salze führten zur hydraulischen Aktivierung der Stomata (HAS). Kosmotropische Salze (Na_2SO_4) verringerten die Oberflächenspannung der Gly-Lösung geringfügig und resultierten in der Ausbildung von amorphen, kugelförmigen Strukturen von Gly-Salzablagerungen. Im Gegensatz dazu führten chaotropische Salze (NaNO_3 or NaClO_3) zu einer starken Reduzierung der Oberflächenspannung der Lösung und damit zur Ausbildung kolloidaler Mischungen, welche durch periklinische Zellwände und sogar bis in die Stomata strömten. Kosmotropisches Na_2SO_4 hatte keinen Einfluss auf die biologische Wirksamkeit von Gly, wohingegen das chaotropische NaNO_3 die biologische Wirksamkeit von Gly steigerte und zu einem äquivalenten, wenn nicht sogar besseren Ergebnis führte als bei Zusatz des organosilicone-basierten Adjuvant Break Thru S233.

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List of abbreviations

%	percentage	cm	centimeter
σ_{sl}	surface–liquid interfacial energy	<i>C. album</i>	<i>Chenopodium album</i> L.
C_{fr}	Concentration of formulation residue over cuticle	CaSO ₄	calcium sulfate
C_w	Concentration of formulation in cell wall	C_D	drag coefficient
D^o	diffusivity of molecule having zero molar volume	CGP	commercial glyphosate product
J_s	evaporation flux	Chl a	chlorophyll a
K_{cw}	Partition coefficient cuticle/water	ChlF	chlorophyll fluorescence
K_{ow}	Partition coefficient octanol/water	CMC	critical micelles concentration
K_{wxfr}	Partition coefficient wax/formulation residue	CPA	crop protection agent
M_o	amount desorbed from cuticle	DOD	drop-on-demand
T_c	surface temp at centre of droplet	EDX	energy dispersive x-ray microanalysis
T_e	surface temp at edge of droplet	E_{ERE}	excess rebound energy
d_d	droplet diameter	EO	ethylene oxide
d_j	jet diameter	Fig.	figure
d_m	maximum droplet spread diameter	F_m	maximum fluorescence
m_R	mass of solute	F_o	ground fluorescence
t_f	drying time	F_v/F_m	maximum efficiency of PSII
β'	free volume available for diffusion	g	acceleration due to gravity
μ_l	liquid viscosity	g	gram
ρ_a	air density	Gly	glyphosate
ρ_a	air viscosity	h	hour
ρ_l	liquid density	h	translocation rate
σ_l	liquid surface tension	ha	hectare
σ_{sv}	surface-vapour interfacial energy	HAS	hydraulic activation of stomata
ΔP_l	pressure change	HLB	hydrophilic lipophilic balance
Δx	path length across membrane	keV	kilo electro volt
μg	microgram	kg	kilogram
μl	microlitre	μm	micrometer
ANOVA	Analysis of variance	μmol	micromole
BT	Break Thru S233	a.i.	Active ingredient
l	litre	kPa	kilopascal
LSD	least significant difference	RH	relative humidity
m	meter	RSO	rapeseed oil
<i>M. domestica</i>	<i>Malus domestica</i> Borkh.	RWC	relative water content
m ²	square meter	s	second

Ma	Marangoni number	<i>S. media</i>	<i>Stellaria media</i> L.
mg	milligram	<i>S. viridis</i>	<i>Setaria viridis</i> L.
ml	millilitre	SE	standard error
mM	millimolar	SEM	standard error of means
mm	millimeter	Spp.	species
mm ²	square millimeter	ST	surface tension
mmol	millimole	t	time
mN	millinewton	USD	US dollar
ms	millisecond	V DC	volt direct current
N	Newton	<i>V. arvensis</i>	<i>Viola arvensis</i> L.
n	number	v/v	volume/volume
Na ₂ SO ₄	sodium sulfate	<i>We</i>	Weber number
NaCl	sodium chloride	<i>Y(NPQ)</i>	quantum yield of regulated energy dissipation
NaClO ₃	sodium chlorate	<i>Y(II)</i>	effective PSII quantum yield
NaNO ₃	sodium nitrate	θ	equilibrium contact angle
nm	nanometer	θ_A	ascending contact angle
°	degree	SMD	souter mean diameter
°C	degree centigrade	θ_R	receding contact angle
P	probability	<i>D</i>	diffusion coefficient
Pa	Pascal	<i>DRP_t</i>	drift reduction potential
PAR	photosynthetic active radiation	<i>J</i>	flux
PSI	photosystem I	<i>MV</i>	characteristic molecular volume
PSII	photosystem II	<i>Oh</i>	Ohnesorge number
<i>R</i>	droplet radius	<i>P^o</i>	permeance
<i>r</i>	radial coordinate in droplet	<i>u</i>	air velocity
<i>r</i>	Pearson's correlation coefficient	<i>v</i>	velocity
<i>R</i> ²	coefficient of determination	<i>z</i>	depth into the leaf
<i>Re</i>	Reynolds number	β	surface tension-temperature coefficient
km	kilometer	(NH ₄) ₂ SO ₄	ammonium sulfate

A. Introduction

1. Background

Use of foliar applied chemicals plays a crucial role in the modern agriculture. Despite of its worldwide criticism for environmental pollution, the global market of agrochemicals is ever increasing. It was worth USD 48.0 billion in the year 2011 and is expected to reach USD 71.3 billion by 2018 (Transparency Market Research, 2013). Since decades the herbicides form the largest category in the overall crop protection market, contributing about USD 19.9 billion in 2011. On the other hand, significant efforts have been made by public and private institutions to make a better and more rational use of pesticides in order to reduce their overall application.

Around 80% of the pesticide may be lost during spraying due to drift (15%), rebound (30%), run-off (20%), and other processes (15%) like evaporation and photolysis (Knowles, 2001). Hall et al. (1993) pointed out that overall efficiency of foliar applied chemicals use might be only 1% or less. Thus, continuous efforts have been devoted to minimization of such losses, either by improving the application technology or by adjusting the physico-chemical properties of the formulation. As identified, there are several efficiency losses whose proper management can drastically reduce the pesticide use. Such loopholes arise in each single step of the foliar application.

Foliar application is a complex process and may be affected by the physico-chemical properties of the spray solution, operating conditions of the application device, environmental factors and surface and plant factors, respectively (Bukovac, 1985). Equivalently, foliar application of herbicide is also a complex process and could be affected by above-mentioned factors. Despite of its complexity, the whole process is grouped into three simple steps: a) delivery, b) deposition and c) uptake/translocation (Fig. 1). The delivery of hydrophilic herbicide having pure water as carrier can be adjusted by the physico-chemical properties of the liquid, environment and application system. Here, major losses include drift (Sarker et al., 1997, Green, 2001), evaporation (Ramsey et al., 1992), interception and reflection (Reichard et al., 1998; Webb et al., 1999). Likewise, in the deposition stage, the interaction between the liquid, the plant surfaces, and the environment, determines the fate of spray residues on the foliage. Here, the most relevant losses comprise run-off (Furnidge, 1962), evaporation (Ramsey et al., 1992) and photolysis (Green and Hazen, 1998). Finally, in the uptake phase the close interaction between formulations, plant and environment is essential for the spatial distribution of a.i., reduced concentration gradient, adsorption in the cuticle (Schönherr and

Baur, 1994), compartmentalization, detoxification (Monaco et al., 2002) as key factors for the bio-efficacy. In the entire application process, the adjustment of the liquid properties is the more precise way to reduce the uncertainty in the bio-efficacy (“*Spray and pray*”). A schematic model, depicting the role of liquid properties of formulations affecting the efficiency of foliar applied hydrophilic herbicide, and the possible loophole for losses in the system is depicted in Fig. 1.

2. Adjuvant

The physico-chemical properties of the liquids might be adjusted by the proper combination of adjuvants. Adjuvants are substances without significant pesticide properties and might be added to agrochemicals or to pesticide spray mixtures to improve or modify their activity (Underwood et al., 2001). Several reviews on adjuvants explain and summarize their use and mode-of-action (Kirkwood, 1993; Hazen, 2000; Tu et al., 2001). The main chemical classes of adjuvants, in the order of commercial importance, comprise surfactants, oils, polymers, polymer-forming compounds and inorganic salts (Holloway, 1993).

Surfactants are probably the most important adjuvants. Miller and Westra (1998) divided surfactants into five major classes with respect to their chemical composition: non-ionic surfactants, crop oil concentrates, nitrogen-surfactant blends, esterified seed oils and organosilicone surfactants. Surfactant affects not only the surface active phenomena, but also improve the dispersing/emulsifying, sticking properties, and enhance absorption (Kirkwood, 1993; Miller and Westra, 1998). Surfactants are usually amphipathic organic compounds having both a hydrophobic tail and a hydrophilic head group. And occasionally, the head groups are esterified with ethylene oxide units (EO) or a propylene oxide chain to increase its hydrophilic nature (Wang and Liu, 2007).

Apart from benefits, there are also some risks associated with the use of surfactants. Surfactants might lead to formation of smaller droplets (Lefebvre, 1993), later leading to the losses due to drift, evaporation etc. The rapid uptake of a.i. due to surfactant can cause localized tissue death (Scherrick et al., 1986; Liu, et al, 1996; Kirkwood et al., 2000), consequently, reducing the a.i. movement out of this zone (Merritt, 1982; Geiger and Bestman, 1990; Zabkiewicz, 2003). Likewise the superspreader has its own disadvantage of faster drying, reduced uptake, and the higher runoff (Liu, 2003).

In the following chapters, the application process (Fig. 1) has been studied in its single steps, and the role of liquid properties as changed by adjuvants, affecting the overall efficacy of foliar applied systemic hydrophilic herbicide, is explained.

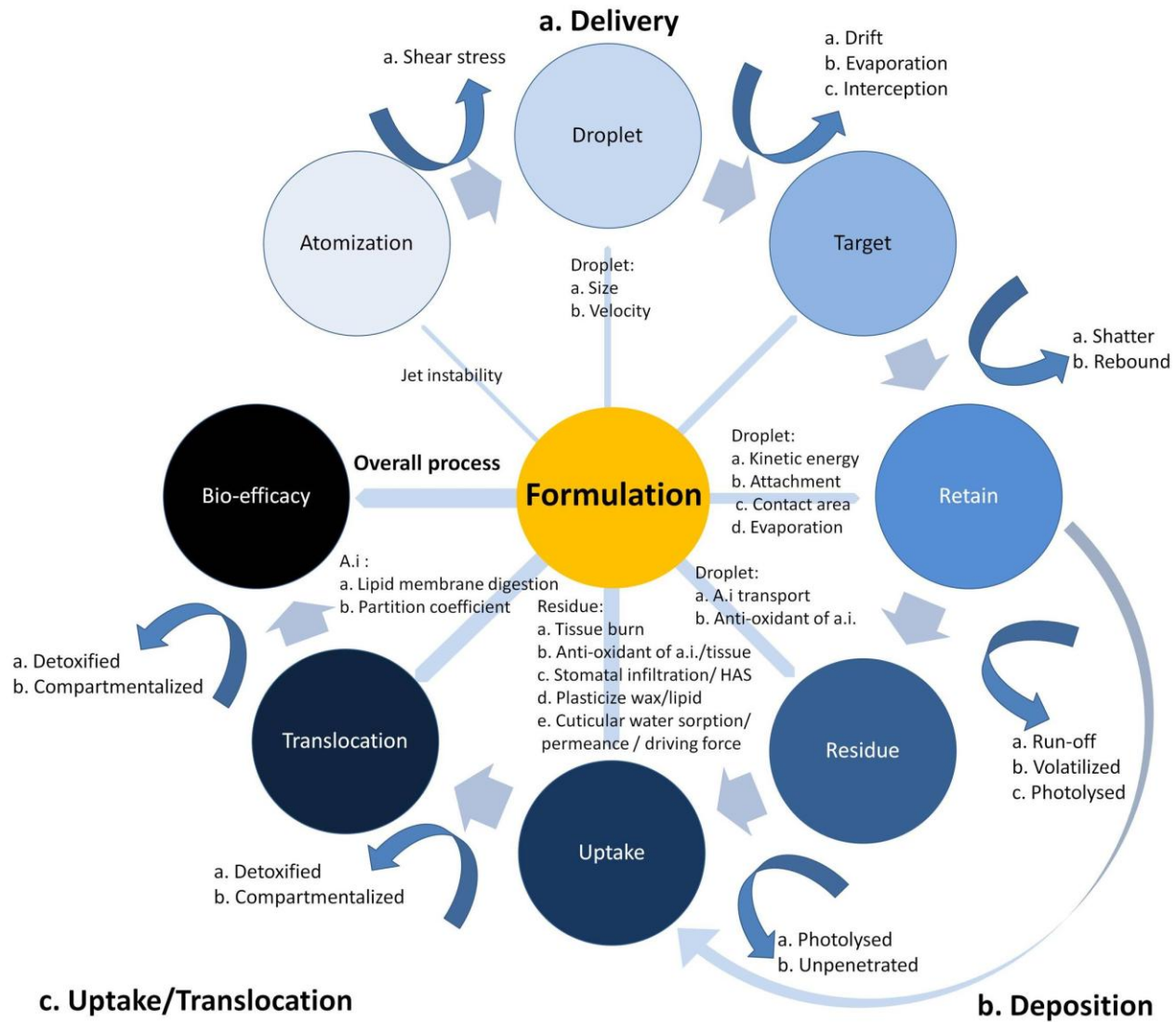


Fig.1. Proposed model showing the effect of formulations and the possible losses during the application of foliar applied systemic hydrophilic herbicides.

3. Delivery

The delivery of foliar sprays is usually achieved by sprays from the nozzle, by providing sufficient energy from hydraulic, gaseous, centrifugal, kinetic, thermal and electrostatic energy (Mathews, 2008). Apart from the design itself, liquid properties viz. surface tension and viscosity could affect the delivery of pesticide from the sprayers. Likewise, environmental conditions such as wind and humidity or temperature can support drift and evaporative volatilization of the spray, respectively. An overview about the most important effects of liquid properties of formulations affecting the delivery process is provided below.

3.1. Atomization

The physico-chemical properties of the liquid can directly affect the success of the atomization process. The basic principle of atomization is to disintegrate the liquid jet or sheet to individual droplets. Instability of the jet, caused either by the liquid itself or by other external means such as high-velocity air, mechanical energy applied externally through a rotating or vibrating device, leads to a breaking of the jet into individual droplets (Lefebvre, 1993).

As liquid is issued from a nozzle with sufficient pressure, it forms a jet after attaining minimum jet velocity (Sticker and Sofer, 1991). The perturbation provided either due to liquid surface tension (Rayleigh mechanism), or other mechanical stress cause droplet break-up from the jet. Linear stability analysis of Yim (1996), shows that the diameter (d_d) of such detached droplet due to capillary instability is given by,

$$d_d = d_j(1.5\pi\sqrt{2 + 3Oh})^{1/3} \quad (1)$$

where,

$$Oh = \frac{\mu_l}{\sqrt{\rho_l \sigma_l d_j}} \quad (2)$$

Accordingly, droplet diameter (d_d) increases with the increase in the jet diameter (equivalent to nozzle diameter) (d_j) and the Ohnesorge number (Oh) (eqn. 1). In this way, the liquid properties, influencing the Oh (eqn. 2) finally affect the droplet diameter (eqn. 1). In this context the viscosity (μ_l) has the positive effect, while the density (ρ_l), surface tension (σ_l) negative effect on droplet diameter.

The further increase in the jet velocity leads to the formation of turbulent jet and forms a sheet. Its break-up behaviour is more complex, resulting from the interaction with the surrounding air. Disintegration occurs when the wave amplitude reaches a critical value and fragments of sheet are torn off. Surface tension causes these fragments to contract into irregular ligaments, which then collapse into droplets of variable sizes according to the Rayleigh mechanism (Lefebvre, 2011). Empirical correlations are derived to express the relationship between the mean droplet size in a spray and the variables of liquid properties, gas properties, flow conditions, and nozzle dimensions (Lefebvre, 1993). The droplet size data is presented in terms of average diameters such as Sauter mean diameter (SMD), which is the diameter of the droplet whose ratio of volume to surface area is the same as that of the entire spray. For the hydraulic nozzle of plain-orifice, Elkoth (1982), provided the empirical equation,

$$SMD = 3.08 \mu_l^{0.385} (\sigma_l \rho_l) \rho_a^{0.06} \Delta P_l^{-0.54} \quad (3)$$

Thus, the Sauter mean droplet diameter (*SMD*) accounts not only for the operating condition, i.e. pressure change (ΔP_l), but also in the liquid properties (eqn. 3). The *SMD* increases with the increase in the viscosity (μ_l), surface tension (σ_l) and density (ρ_l) of the liquid and vice versa, in addition to changes in air density (ρ_a). Similar, other empirical equations, specific to different nozzles types, have been developed (Jasuja, 1974; Kayano and Kamiya, 1978; Snyder et al., 1989 etc.).

Droplet size has inverse relation to drift (Bird et al. 1996; Bouse et al., 1990) and, however, has a direct relation to the lifetime of evaporating droplet (Elliott and Wilson, 1983; Sirignano, 1999) as well as rebound and shatter losses. Thus, the optimal droplet size for highest efficacy has to be identified in each case. As an example, deposition of 0.1% solution of ortho X77 on citrus leaf was highest when droplet size was 240 to 340 μm (Salyani, 1988). Although, the efficiency of spray application depends also on velocity distribution, volume distribution pattern, entrained air characteristics, spray sheet structure and the structure of individual droplets (Miller and Butler Ellis, 2000), the droplet size, and the liquid property affecting the velocity, and its drift is illustrated below.

3.2. Droplet velocity

Droplet size influences the droplet velocity and all related aspects such as drift and the impaction. Neglecting buoyancy and Cunningham's correction, and assuming no air

displacement, forces equilibrium according to Newtons' second law gives the differential equation for the velocity of the droplet (adapted from Perry et al., 1984, c.f. Mercer et al., 2006),

$$\frac{dv}{dt} \rho_l d_d = gK\rho_l d_d - \left(\frac{3}{4} C_D \rho_a |v - u|(v - u)\right) \quad (4)$$

where, ρ_l , v , d_d is the liquid density, droplet velocity, and droplet diameter respectively; ρ_a , u is the dynamic viscosity and velocity of air respectively; K is the height of application; g is acceleration due to gravity and the C_D is the drag coefficient as given by:

$$C_D = \frac{24 (1 + 0.14Re^{0.7})}{Re} \quad (5)$$

And the Reynold number (Re) can be derived as,

$$Re = \frac{\rho_a d |v - u|}{\mu_a} \quad (6)$$

Thus, decreasing the droplet diameter (d_d) and the density of liquid (ρ_l), the droplet acceleration ($\frac{dv}{dt}$) could also be decreased (eqn. 4).

3.3. Drift

The linear relation, for total drift potential (DRP_t) is prepared by Nuyttens (2007). The drift reduction potential, DRP_t (%) in the spray having a proportion of total volume of droplets smaller than 200 μm in diameter (V_{200}) or 50 μm (V_{50}) is given as:

$$DRP_t(\%) = 100.7 - 4.24 V_{200} \quad (R^2 = 0.78) \quad (7)$$

$$DRP_t(\%) = 91.6 - 53.0 V_{75} \quad (R^2 = 0.76) \quad (8)$$

It is clear that the drift reduction potential (%) reduces with the decrease in the droplet size (eqn. 7 and 8) or in the increase in the proportion of droplet diameter 200 or 50 μm . In typical air-assisted deposition in fruit crops, distribution can reach 65% on foliage, 25% on the ground and 10% as drift (Zabkiewicz, 2003).

4. Deposition

The deposition of droplet and finally the a.i. on the plant leaves is the inter-relation between canopy characteristics, liquid characteristics, and the environmental conditions. Study on canopy characteristics, as done by Mercer (2006), is beyond the scope of this review. Thus, the emphasis is given in the deposition behaviour over the basic component of canopy, the leaves, in affecting the efficacy of sprays. The physico-chemical property of the liquid affects, not only on its behaviour over the leaf surface, whether it sticks or not, but also, to determine how the residues would be formed once the droplet dries. The details of the intermediates are elucidated in the following chapters.

4.1. Droplet impaction

When a droplet impacts on a leaf surface, three events may happen: 1) the droplet adheres to the leaf; 2) it bounces off the leaf; 3) it shatters into smaller droplets that may adhere to the surface nearby (Mercer et al., 2006). Leaf surface roughness and hydrophobicity is known to govern the adhesion/bouncing transition. The falling droplet contains kinetic energy, due to its vertical velocity, and potential energy, which is mainly due to its surface tension (Yarin, 2006; Bertola, 2008). As the droplet spreads out, the initial kinetic energy is partially converted into potential energy through increases in droplet surface area. The droplet reaches a point of maximum spread, from which it then recedes back towards a more spherical shape returning under the force due to the surface tension. During the expansion and recession phases the droplet loses energy by friction. If friction energy losses are large the droplet is retained, else it rebounds or shatters. Butler Ellis et al. (2004) examined the effect of liquid properties and application technology on spray retention in range of situations representative of practical pesticide application. As they demonstrate, retention on whole plants was strongly influenced by plant growth and canopy properties. Thereby, high leaf surface hydrophobicity reduced adhesion. As a decision support tool, several mathematical models are available for determination of retention of xenobiotics, such as AGDISP 8.24 (AGDISP, 2010) or modified AGDISP (Forster et al., 2012) for aerial based sprays or the ground based track sprayer (Grayson et al., 1991).

4.1.1. Rebound

Experiments on rigid flat surfaces have shown, after the deformation and flattening of a droplet upon impact, the excess surface energy enables the droplet to the fast retraction of the

drop after the maximum radius has been reached. The maximum spreading (d_m) of the droplet after impaction can be calculated from Mao et al. (1997) as follows,

$$\left[\frac{1}{4} (1 - \cos \theta) + 0.2 \frac{We^{0.83}}{Re^{0.33}} \right] \left(\frac{d_m}{d_d} \right)^3 - \left(\frac{We}{12} + 1 \right) \left(\frac{d_m}{d_d} \right) + \frac{2}{3} = 0 \quad (10)$$

where d_m = maximum droplet spread diameter, d_d = droplet diameter, θ = static contact angle, and, Weber number (We) and Reynolds number (Re) are calculated as:

$$We = \frac{\rho_l d_d v_d^2}{\sigma_l} \quad (11)$$

$$Re = \frac{\rho_l d_d v_d}{\mu_l} \quad (12)$$

where, ρ_l , μ_l , and σ_l denote liquid density, viscosity, and surface tension, respectively, and v_d is the droplet velocity.

The criteria for the droplet to rebound could be derived from the maximum spreading d_m of the droplet, on its excess rebound energy (E_{ERE}) (Mao et al., 1997) as follows:

$$E_{ERE} = \left[\frac{1}{4} \left(\frac{d_m}{d_d} \right)^2 (1 - \cos \theta) - 0.12 \left(\frac{d_m}{d_d} \right)^{2.3} (1 - \cos \theta)^{0.63} + \frac{2}{3} \left(\frac{d_d}{d_m} \right) - 1 \right] \quad (13)$$

If excess rebound energy (E_{ERE}) of the droplet is > 0 (eqn. 13), droplet rebounds, else wise it is retained on the surface. The later phase of retraction and rebound is adjusted by the surface property of the liquid (Mao et al., 1997). While the drop deformation occurs so rapidly, on the order of 5 ms, the surface tension at the deformed surface will not be the equilibrium surface tension, but the dynamic surface tension (Forster et al. 1998; Chaidron et al., 1999; Crooks and Boger, 2000). Thus, the faster surfactant molecule below CMC reduces the tendency of recoil, since there is less surface energy available (Crooks et al., 2001). Likewise, kinetic energy of the impacting droplet at the beginning of the relaxation phase is reduced by viscous dissipation suppressing rebound (Crooks et al., 2001). Air inclusion inside the droplet produced from air injection nozzle, results in more droplet adherence, and the retention is in comparison to the one of a finer spray (Faggion et al., 2006). Lake and

Marchant (1983) proposed a concept of the critical speed, for a droplet impacting upon a leaf surface, below which capture is taken to occur and above which the droplet rebounds.

4.1.2. Droplet shatter

At the higher energy state, excess to the bouncing of the droplet, it might shatter. Mundo et al. (1995) considered energy balances and derived a criteria for the shattering of droplets as,

$$We^2Re > K^4 \quad (14)$$

where, K is a constant equivalent to 57.7 over a wide range of leaf surface. Thus, the shattering of the droplet depends upon the liquid properties, apart from the kinetic energy of the falling droplet (eqn. 14).

4.1.3. Retention

At this stage, excess energy is completely dissipated and droplet posses a minimum energy that is equal to the static surface energy (Mao et al., 1997). The final spread area of the droplet is a weak function of the impact velocity for drops of liquids that are free of surfactant or contain a slowly transporting surfactant. By contrast, for a highly active surfactant, the final spreading radius increases considerably with increasing impact velocity (Zhang and Basaran, 1997). The effect of impact velocity on spreading diminishes with increasing viscosity (Werner et al., 2007). The retraction of droplet is reduced in the presence of surfactant; below CMC concentration, faster surfactants are more effective on this phenomenon (Aytouna et al., 2010). Furthermore, the leaf surface characteristics play a major role on this static stage as explained in the previous section.

4.2. Run-off

The amount of spray retained on the surface raises until the surface drops increase to a critical size, above which they begin to slide down the surface and drip off, a phenomenon defined as run-off. The relative retention for any liquid/surface combination may be derived from Furnidge (1962) as,

$$F = \theta_M \sqrt{\frac{\sigma_l(\cos\theta_R - \cos\theta_A)}{\rho_l}} \quad (15)$$

Accordingly, a larger retention factor (F), for particular combination of formulation (given by the surface tension (σ_l) and density (ρ_l)) and leaf type (given by contact angle

hysteresis as difference between ascending contact angle (θ_A) and receding contact angle (θ_R) and their arithmetic mean (θ_M) will retain more spray than another combination with a smaller F (eqn. 15). Thus, the higher the contact angle hysteresis, the higher the retention. And to increase the contact angle hysteresis, Chen et al. (1991) proposed (i) increase the freedom of the surface molecules to reorder, (ii) increase the load and time allowed for surfaces to remain in contact, and (iii) increase the rate of separation or retraction.

4.3. Droplet contact angle

The angle made by the droplet at its equilibrium with the contact surface is defined as contact angle. The angle thus formed is the resultant equilibrium state, resulting from the interaction between three energies over the droplet: surface-vapour σ_{sv} , the surface-liquid σ_{sl} , and the liquid-vapor interfacial energy (i.e. liquid surface tension) σ_l . The equilibrium contact angle (θ) is determined from these quantities by Young's Equation (Quééré, 2005),

$$\cos \theta = \frac{\sigma_{sv} - \sigma_{sl}}{\sigma_l} \quad (16)$$

Thus, lowering the surface tension of the liquid, the droplet contact angle with the surface is also decreased (eqn. 16). In addition to the surface tension of the liquid, the surface-liquid energy as influenced by the leaf surface properties is also equally important, in determining the droplet contact angle. Leaf surfaces are rough and heterogeneous varying among plants and environments, and also with their surface energies. Epicuticular waxes comprise the upper most layer of the leaf. It is composed mainly of non-polar constituents, and may be shaped as crystalline form (e.g. platelets, rodlets, scales, granules) or as an amorphous (smooth) layer (Barthlott et al., 1998). Wax chemical composition (Kolattukudy, 1980; Baker, 1982; Chachalis et al., 2001; Hunsche et al., 2006), orientation and physical structure of the wax platelets may all affect the contact angle and wettability of surface (Wanamarta and Penner, 1989; Falk, 1994). Leaf roughness is further contributed by the microstructure, i.e. distribution of trichomes (Levin, 1973) containing hydrophilic (Uphof, 1962; Choi et al., 2001) or lipophilic (Kelsey et al., 1984; Wollenweber, 1984; Li et al., 2002) compounds or leaf hairs, as well as by the presence of stomata. Ridged periclinal cell walls or veins etc. can also provide micro-roughness.

In rough surface, the penetration of liquid inside the rough surface is inhibited, and air is entrapped inside liquid-surface interface, and will maintain the highest contact angle,

which is also known as Cassie's mode (Nakajima, 2011 and ref. within). The decrease in the surface tension of the liquid or in its roughness will allow the liquid to flow inside the rough surface also known as Wenzel mode, and will have lower contact angle compared to that in Cassie's mode. Likewise, at such condition, droplet spread will be higher than on smooth leaf surface (Singh and Sharma, 2001). Thus, the reduced surface tension of a spray droplet, might lead the spread further from the point of deposition, approaching an infinitely thin liquid layer over the surface (Hartley and Graham-Bryce, 1980). Such droplets do not only result in larger spread area, but also dry faster (Liu and Zabkiewicz, 1997).

4.4. Deposit residue

Once the droplet is deposited on the leaf surface, it dries in a time-span of few minutes (Yu et al., 2009) to a few hours (Xu et. al, 2011). While drying, droplet physico-chemical properties, substrate structure characteristics, and environmental factors determine the properties of a.i. residue on the leaf surface (Stevens and Baker, 1987; Falk, 1994; Baur and Pontzen, 2007). Residue of a.i. may be distributed over the droplet footprint, as a number of individual masses, often associated with specific morphological trichomes, guard cells, or concentrated in an annulus at the periphery of the residue footprint (Bukovac et al., 1995). Leaf micro roughness affects the amount and quality of the interface between the deposit and the leaf (Falk, 1994; Green, 2001). The distribution of deposits is crucial for the penetration of a.i (Baur and Pontzen, 2007; Fears and Pontzen, 2008; Kraemer et al., 2012). For instance, it affects the amount of a.i. per surface unit, and the driving force for the diffusion mediated penetration process (Liu, 2004; Price, 1982). Faers and Pontzen (2008) proposed that low coverage with adjuvant, corresponds to a good uptake. Likewise, low surfactant concentration below the cmc favored particles to be deposited in an annulus and adjuvant separated in the centre, while its intermediate concentration produced annuli containing both particles and adjuvant, and the high surfactant concentrations favored the larger deposits (Fears and Pontzen, 2008). By understanding the different processes during droplet drying, the particles could be induced to be deposited in the desired location within the droplet periphery. As observed on smooth artificial surface, three forms of deposition pattern could be highlighted in the prospect for enhancing foliar application of chemicals.

4.4.1. Peripheral deposit

An outward flow of liquids in a drying drop is produced when the contact line is pinned, so that liquid that is removed by evaporation from the edge of the drop must be replenished by a

flow of liquid from the interior. In the evaporating droplet, the evaporation flux $J_s(r, t)$ is given by Deegan et al. (2000),

$$J_s(r, t) \approx (R - r)^{-\lambda} \quad (17)$$

where,

$$\lambda = \frac{\pi - 2\theta_c}{(2\pi - 2\theta_c)}$$

and, R is the droplet radius, r is the radial coordinate and θ_c is the contact angle.

Thus, at the periphery, where, r approaches R , the evaporation rate $J_s(r, t)$, is the highest. Due to this higher evaporative flux at the edge, and the pinned contact line, the changes in the volume of the drop must be replenished by a flow of liquid from the interior. This movement of liquid, deposits the mass of solute (m_R) in the ring, at a time (t) is given by Deegan et al. (2000).

$$m_R \approx [t]^{2/(1+\lambda)} \quad (18)$$

The solute transfer to the periphery of the droplet is the function of time, and ultimately of the evaporation flux. This flow is capable of transferring 100% of the solute to the contact line (eqn. 18) and thus accounts for the strong perimeter concentration of many strains (Deegan et al., 2000). This leads to a popularly known 'coffee-ring' effect (Deegan et al., 1997), and occurs irrespective of gravitational effect, electric fields, temperature gradient (Deegan et al., 2000), and even in a very high viscous (up to 900 mPa s^{-1}) Newtonian fluid (Faers et al., 2010). This peripheral deposit could be enhanced by addition of surfactant of low molecular weight (low EO unit) (Hunsche and Noga, 2011).

4.4.2. Centralized deposit

In the surfactant free system, the surface tension is greatest at the top center of the droplet due to lowest temperature at the liquid-air surface caused by the longer thermal conduction path (Hu and Larson, 2006). If the Marangoni flow, caused by surface tension gradients, is pronounced, this might lead the radial inward surface flow leading to the centralized deposition of the particles. Under conditions that particles don't affect fluid flow, thermal conductivity, and other physical-chemical properties of the solvent is very less, and the

number density of particles near the droplet free surface is too small, the Marangoni number is as follows,

$$Ma \equiv -\beta(T_e - T_c) - t_f/\mu_l R \quad (19)$$

where, β is the surface tension-temperature coefficient, μ_l viscosity, R radius of contact line, T_e surface temp at edge, T_c surface temp at centre, t_f is the drying time.

The higher the Ma number (eqn. 19), the higher is the velocity above the droplet vortex, and more pronounced is the central deposition (Hu and Larson, 2006). Thus, the surface tension-temperature coefficient β has a positive effect in the transfer of the solutes to the centre of the droplet. Likewise, equivalent to the surface tension-temperature coefficient, the surfactant molecules can also induce the Marangoni stress. Below cmc, there will be a higher concentration of surfactant in the air-water interface at the drop edge. This will in turn set up a surface tension gradient at this interface, increasing towards the drop centre and resulting in an inward Marangoni surface flow (Faers, 2007). However, above the cmc the phenomenon ceases and particles are deposited in the annulus. Additionally, if the droplet is unable to pin the contact line, as it is the case on Teflon surface, all the particles could be transported to the centre of the droplet forming a centralized deposit (Deegan et al, 2000; Hunsche and Noga, 2011).

4.4.3. Uniform deposition

In presence of centre-enhanced evaporation around the droplet periphery, the capillary force is weak thus resulting in uniform homogenous deposits (Deegan, et al, 2000). Likewise, the dimension of particle also accounts for the deposition pattern. The presence of ellipsoidal particles could halt the coffee-ring effect, and form the homogenous deposits (Yunker et al, 2011). The non-Newtonian shear thinning fluid can also form a homogenous a.i. deposit (Faers et al., 2010). Apart from these known factors, there might be several factors yet unexplored influencing deposition pattern. Since the deposition involves the transportation of particles, their mass also might influence the deposition pattern (Deegan, 2000). Particularly, it might be important when efflorescent crystalline salt, are to be deposited.

5. Uptake/translocation

The liquid physico-chemical properties affect both the uptake and translocation processes of active ingredients. Thereby, liquid properties influencing the residue formation could affect the uptake behaviour, stomatal infiltration of xenobiotics, and accelerate the cuticular penetration.

There are two possible pathways hypothesized for the penetration through foliage- a. Cuticular pathway and b. Stomatal pathway. The penetration of xenobiotics through cuticular pathway is a diffusion process (Price, 1982), while the stomatal pathway is a mass flow process (Gaskin, 1995). Cuticular pathway was detected by observing the uptake process through astomatous plant cuticle. However, detection of stomatal pathway couldn't be identified directly, due to the size limitation of stomata. Though, Eichert et al. (2008), indirectly proved the stomatal pathway, showing the uptake of large nanoparticles, that otherwise would be impossible through the cuticle. Additionally, xenobiotics uptake of low surface energy, within few minutes after application otherwise impossible through cuticular pathway (Stevens et al., 1991), supports the presence of stomatal uptake pathway. Before that, stomatal uptake, was supposed to be through the cuticle of guard cells, and was termed as peristomatal cuticular uptake (Sargent, 1961).

5.1. Cuticular uptake

Cuticular uptake is hypothesized as a concentration gradient mediated diffusion process (Price, 1982; Schönherr and Riederer, 1989). Thus, both the membrane and the liquid properties play a vital role on the uptake of xenobiotics through cuticle. Such properties affecting the cuticular uptake are given below.

5.1.1. Membrane properties and penetration paths

Leaf surfaces are covered by a thin, continuous cuticular membrane, which minimizes water loss from the plant and also acts a barrier for the penetration of foliar applied chemicals (Riederer and Schreiber, 2001; Schreiber, 2005). It is composed of a framework of polymeric cutins with embedded cuticular waxes, and soluble waxes deposited on the surface as epicuticular barrier (Kolattukudy, 1980). The epicuticular waxes are the first barrier for cuticular penetration, and its removal significantly increases permeability (Hall and Jones, 1961; Shafer and Bukovac, 1987). The cuticle is chemically and physically heterogeneous. There are three components of the cuticle, which could provide a medium in which the solute might diffuse: the cutin, embedded waxes and water. The polymer cutin constitutes the

matrix for embedded intra cuticular waxes and forming the basis for the deposition of epicuticular wax films and crystals. Cuticle thickness evaluated on a number of species ranged from 0.7 μm for *Prunus laurocerasus* to 13.5 μm for *Olea lancea* (Martin and Juniper, 1970). Two pathways were proposed to explain the cuticular transport of xenobiotics: the lipophilic and the hydrophilic pathway.

i. Lipophilic pathway

The pathway not only leads the uptake of lipophilic compounds but also the hydrophilic compounds. The hydrophilic compound permeates across the pathway, through cutin and wax domain (Burghardt and Riederer, 2006).

ii. Hydrophilic pathway

Although the basic cutin framework is composed of esterified hydroxy fatty acids, free hydroxyl groups confer some polar properties to the cutin (van Overbeek, 1956). It was suggested that water molecules are absorbed to polar moieties located in the cuticular membrane (Schönherr, 2000; Schreiber, 2005), such as unesterified carboxyl groups (Schönherr and Bukovac, 1973), ester and hydroxylic groups (Chamel *et al.*, 1991) in the cutin network and carboxylic groups of pectic cell wall material (Schönherr and Huber, 1977). According to this model, water molecules might lead to the formation of “polar pores” (Schönherr, 1976; Schreiber *et al.*, 2001) or “aqueous pores” (Schönherr, 2000; Schönherr and Schreiber, 2004) or “water-filled pores” (Schreiber, 2005). Thus, high humidity enhances the transport capacity of the penetration route of hydrophilic solutes by increasing the size or number of such polar pores (Schönherr, 2001). Likewise, carbohydrate fibers are extending from the underlying wall and middle lamella into the cuticle (Norris and Bukovac, 1968). Such fiber might also provide a possible hydrophilic pathway extending from the aqueous apoplast to close proximity to the external surface of the cuticle (Hoch, 1979). The mean radius of polar pore was determined to be in the ranges of estimated 0.46 nm (Schönherr, 1976) to 4.8 nm (Eichert and Goldbach, 2008).

5.1.2. Diffusion

The rate of movement of xenobiotics by diffusion process is described by Fick's first law, and modified for membranes by Nobel (1974 c.f. Price, 1982) as,

$$Uptake = \text{Effective area } (A) \times \text{Flux } (J) \quad (20)$$

where, A comprises 1. % of the contact area available for penetration and 2. area covered by the spray deposit, and (J) comprises the penetration force (Price, 1982). At a later stage, Schönherr and Baur (1994) disintegrated the Flux (J) into simple forms for the heterogeneous plant cuticle. The flux of hypothetical molecule having zero molar volume through the cuticle having permeance (P°) is given by,

$$J = P^\circ(K_{wxfr}C_{fr} - K_{cw}C_w) \quad (21)$$

where,

$$\text{Log } P^\circ = \log \frac{D^\circ}{\Delta x} - \beta' x MV \quad (22)$$

$$\frac{D^\circ}{\Delta x} = k_o^* (\text{m s}^{-1}) \quad (23)$$

k_o^* is the slope of first order linear equation between $-\ln\left(1 - \frac{M_t}{M_o}\right)$ and time (hr).

M_t is the amount of solute desorbed (gm) in the underlying liquid from the outer surface of CM at time t,

M_o is the amount desorbed (gm) plus those remaining in the CM after termination of desorption.

D° = Diffusivity of a hypothetical molecule having zero molar volume

Δx = Path length across membrane

MV = Characteristics molecular volume (Abraham and Gowan, 1987)

β' = Free vol. available for diffusion, Slope of straight line between $\log D$ vs. MV (Schreiber and Schönherr, 1993)

K_{wxfr} = Partition coefficient wax/formulation residue = Conc. of formulation in wax/ conc. of formulation in residue (Reiderer and Schönherr, 1984)

C_{fr} = Conc. of formulation residue over cuticle

K_{cw} = Partition coefficient cuticle/water = Conc. of solute in cuticle/ conc. of solute in water (mathematical model available in Sablijic et al., 1990). Instead of K_{cw} , Schönherr and Baur (1994) proposed to use K_{ow} , for having only a minor deviance.

K_{ow} = Partition coefficient octanol/water = Conc. of solute in octanol/ conc. of solute in water (which is < 1 (hydrophilic a.i.) and > 1 (lipophilic a.i.).

C_w = Conc. of formulation in the water of cell wall

Likewise, the permeance ' P ' of the 1 m thick membrane (p) as given by Crank (1975 c.f. Schönherr and Riederer, 1989),

$$p = DK_{cw} \quad (24)$$

where, $K_{cw} = K_{ow}$ (following Schönherr and Baur, 1994)

D = Diffusion coefficient

Thus, the uptake of xenobiotics is a function of liquid, environment and plant properties. By adjusting the liquid property - decreasing the molar volume (MV) (eqn. 22) (Kerler and Schönherr, 1988), and increasing the octanol-water partition coefficient (K_{ow}) (eqn. 24) - the 'Permeance (P)' could be increased, increasing the flux (J) (eqn. 21). Likewise, increasing the concentration of solute (C_{fr}), the 'driving force ($K_{wxfr}C_{fr} - K_{cw}C_w$)' could be increased (eqn. 21). However, to increase the 'driving force' by maximizing ' $K_{wxfr}C_{fr}$ ', ' $K_{cw}C_w$ ' should be turned as low as possible (eqn. 21). This is possible either by, i. rapid uptake into cells, followed by translocation and/or metabolism, ii. continuous decrease in the volume of the formulation residue on the leaf surface or iii. dissociation of weak electrolytes as soon as they appear in the cell wall.

Increasing the concentration of a.i. (C_{fr}) in the droplet led to its higher penetration (Knoche, 1994; Liu, 2003). Using the mathematical model, Mercer (2006) concluded that, the cuticular penetration rate of a.i. ($\frac{\partial C_{fr}}{\partial t}$) is inversely related to the radius (r) of the droplet,

$$\frac{\partial C_{fr}}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left(rD \frac{\partial C_{fr}}{\partial r} \right) + \frac{\partial}{\partial z} \left(D \frac{\partial C_{fr}}{\partial z} \right) - hc \quad (25)$$

where, t is time, r is radius of the droplet, z is depth into the leaf and h is rate of translocation of a.i., and D is the diffusion coefficient.

The uptake process continues even up to several days (Gaskin and Holloway, 1992; Liu and Zabkiewicz 1997), even though the droplet is already dried to its residues. And, Krämer et al. (2009), showed that a.i. is not always deposited homogenously in the droplet footprint. Realistically, the a.i. might be deposited in the periphery of the droplet, or centrally deposited, apart from its homogenous distribution over the droplet footprint (Deegan et al,

2000; Hu and Larson, 2006, Krämer et al., 2009, Hunsche and Noga, 2011). Thus, the diffusion of a.i. might be related more to its residue area rather than the droplet coverage area.

Accelerator adjuvants (of relatively small molecular weight) maintain maximum driving force and at the same time assure maximum mobility of active ingredients in the limiting tissue, thus enhancing the penetration rate of a.i. (Holloway and Edgerton, 1992; Stock et al., 1993; Schönherr and Baur, 1994). Such adjuvants might function in multiple ways for enhancing the a.i. uptake, such as a) reduce the surface tension of the liquid, b) plasticize the epicuticular wax or lipid membrane, c) increase the a.i. concentration by its compaction in residue, d) increase the cuticular water sorption, e) increase the association between a.i. and surfactant. Low surface tension of solution extends its contact area even to the periclinal walls, which might not be possible at its higher energy. Likewise, it is in debate that some surfactants might plasticize the epicuticular waxes in its mixed micelles, while many agreed (Noga et al., 1991; Knoche et al., 1992; Manthey and Nalewaja, 1992; Schreiber, 1995), others couldn't find such interactions (Ruiter et al., 1988; Stevens and Bukovac, 1987; Falk et al., 1994). The plasticizing effect of the wax increases with the decrease in the EO unit of non ionic surfactant (Schönherr, 1993; Perkins et al., 2005).

Surfactant "might" disrupt the biological membrane too, by binding to the lipid molecule (at/above CMC) or the protein (below CMC), and solubilize it into its micelles or into smaller polypeptide chains, respectively (Helenius and Simons, 1975). The surfactants of higher EO units (15-20), having very poor spreading properties, compacts the deposit residue (Krämer et al, 2012) and enhances the uptake process. Likewise, water sorption of cuticle is increased with increasing EO unit in ionic surfactant, which coincided also with the highest glyphosate uptake (Coret and Chamel, 1993). Nevertheless, neither EO content nor spreading, have an influence on the efficiency of compounds of intermediate polarity (Stock et al., 1993). The number of EO units also affects the partitioning of a.i. and adjuvant in the droplet residue on the surface. Surfactants of larger EO unit led to higher association between the a.i. and surfactant (Hunsche and Noga, 2011). Partitioning of a.i. and adjuvant together in the dry residue of the droplet allowed a higher uptake of a.i. (Faers and Pontzen, 2008). Thus, it is likely that more than one phenomenon is causing this differential behavior of EO unit, and the accelerator adjuvant.

5.2. Stomatal pathway

Stomatal uptake of foliar applied chemicals constitutes the other pathway (Stevens et al. 1991, 1992; Gaskin, 1995). Reducing the surface tension of liquid below 30 mN cm^{-1} can lead to a stomatal uptake (Schönherr and Bukovac, 1972). Later, Burkhardt et al. (2012) showed that spatial expansion of dendritic salt crystals due to humidity fluctuations could hydraulically activate the stomata. Formation of such hydraulic links significantly enhanced the foliar uptake of applied chemicals. Additionally, hygroscopic salts of chaotropic nature as classified according to the Hofmeister series (Zhang and Cremer, 2006) could reduce the surface tension of the liquid drastically and thus lead to a very high infiltration of foliar applied chemicals inside the stomata.

5.3. Translocation

Once herbicide has penetrated the leaf or stem cuticle, there are still many barriers that can affect movement to its site of action. A number of selectivity mechanisms that differ between weeds and crops are the result of differential compartmentation (e.g. vacuole; DiTomaso et al., 1993) or its inactivation by metabolism (Hess, 1985). If the herbicide is not immobilized in some manner, it is available for long-distance movement in the plant through the xylem and/or phloem transport systems (Monaco et al., 2002). Environmental conditions favoring optimum photosynthesis (high light, adequate soil moisture, and moderately warm temperatures), thus will maximize herbicide movement, but rapid foliage kill will result in poor transport and poor root kill. Likewise, translocation agent may assist with movement in the appropriate vascular system and onward to sites permitting herbicidal activity (Field et al., 1995).

The nonlinear simulation model (ERMESSE) presents the complete picture of uptake and distribution of xenobiotics in the plant system (Satchivi et al, 2000a). The model predictability ratio was 1 ± 0.3 in 61% of comparison, while simulating it with published experimental results comprising of 18 herbicides from several chemical families in 25 species (Satchivi et al, 2000b). Using the model, phloem sap velocity, transpiration rate, relative humidity and air temperature had a positive effect on both uptake and translocation of xenobiotic on hypothetical near to plant parameters, while the molecular size (molar volume) and cuticle thickness had a negative effect. Likewise, Partition coefficient ($\log K_{ow}$) had the positive effect on absorption. However, on translocation, it increased with the increasing partition coefficient at the beginning, but at higher coefficient (2-5) translocation declined sharply, probably due to the increasing affinity with the lipophilic cuticle (Satchivi et al,

2001). Though, it has to be noted that, in the simulation the interaction effect is not considered. Sieve tube transfer, phloem unloading, and transfer of xenobiotics between phloem and xylem depend upon the phloem sap velocity, and the xylem transfer rate depends upon transpiration rate (Satchivi et al, 2000a).

6. Objectives of the experiments

As explained above, the liquid properties of formulations have a multiple effect on the efficiency of foliar applied agrochemicals. However, the main focus of the experiments was to study the formulations affecting the deposit residue, ultimately affecting the foliar uptake through cuticular or stomatal pathways. Surface tension of the formulations was affected by addition of ethoxylated rapeseed oil (RSO) or other synthetic surfactants. Ethoxylated rapeseed oil molecule consists of varying unit of ethylene oxide (EO) chain (in average, 5, 10, 30 and 60 units) inserted between the alcohols and the fatty acids in its triglycerides. Thus, different RSO surfactants, though contain variable hydrophilic EO units, but have the constant lipophilic fatty acid units. Likewise, the different hygroscopic salts were added to affect its surface tension and crystallization behaviour while the formulation droplet is drying. Isopropylamine glyphosate was used as model hydrophilic systemic foliar applied agrochemicals, while the diquat dibromide was used as model hydrophilic contact foliar applied agrochemical, for the foliar uptake studies. The formulations were treated with and/or without kinetic energy on the leaves. Deposit residue as affected by these factors was studied in relation to the uptake and bio-efficacy of the agrochemicals. The specific objectives of the experiments are explained below-

1. To understand the effect of liquid properties on the generation of monodroplets from the monodroplet generator.
2. To study the effect of kinetic energy of droplet and the surfactant in the formation of deposit residue on the leaf surface and its final effect on bio-efficacy of the systemic herbicide glyphosate.
3. To study the effect of deposit residue on uptake of the contact herbicide diquat dibromide with the aid of chlorophyll fluorescence.
4. To study the effect of different hygroscopic salts on stomatal uptake and bio-efficacy of glyphosate.

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B. Evaluation of a pneumatic drop-on-demand generator for application of agrochemical solutions

1. Introduction

Techniques for producing uniform monodrops of agrochemicals have an obvious advantage over other sprayers, not only for studying the behaviour of individual droplets on plant surfaces (Reichard et al., 1998) but also because of the efficient target oriented application that makes it possible to reduce losses and off-target environmental hazards. In precise weed control, the designs of patch sprayers (Paice et al., 1995), direct injection devices (Rockwell and Ayers, 1996; Walgenbach et al., 2011) and microsprayers for dosing the herbicide solution in an automated individual weed detection and control system (Soegaard and Lund, 2007) are well documented. However, considering that even a few droplets of herbicide solutions are sufficient to control weeds (Krämer et al., 2009), the use of micro-sprayers, as shown by Nieuwenhuizen et al. (2010) for high-viscosity gel-like formulations, offers the potential to further reduce the application rate and unnecessary environmental contamination of herbicides. Thus, the use of a monodroplet generator for targeted application of liquid agrochemical solutions as single droplets has significant potential.

Devices as simple as flickering needles (Merritt and Drinkwater, 1977) to a more sophisticated piezoelectric droplet generator (Reichard et al., 1998) have been used for generating droplets in studies of droplet behaviour on plants. However, none of these devices have been widely accepted. Although piezoelectric droplet generators have gained considerable interest, their dependence on fragile moving ceramic piezo-crystals reduces their robustness (Zabkiewicz et al., 2004). Difficulty in dismantling and cleaning these units between different formulations makes them even more inconvenient.

To overcome the aforementioned shortcomings, we constructed the pneumatic drop-on-demand (DOD) droplet generator, as described by Chandra and Jivraj (2002), with specific adjustments. The focus of our work was to evaluate the ability of the system to produce monodroplets of agrochemical solutions under laboratory conditions to reach predetermined volumes. To accomplish this goal, specific settings of the droplet generator that affect the droplet diameter and the adjustments for different herbicidal formulations (including the use of adjuvants) were exploited.

2. Materials and Methods

2.1. Design concept

The design of our droplet generator (Fig. 1) is a modified version of the equipment presented by Cheng and Chandra (2003); our design has a larger liquid chamber but no buffer tank, leading to equipment-specific operating parameters. Briefly, the main body consists of a cylinder fixed between stainless steel plates sealed with inert buna-nitrile rubber O-rings. The upper body had an inlet for a cross-junction fitting. One end of the fitting was used for letting in the pressurised nitrogen gas, and the other end is for exhausting the cylinder with a pin valve (150 divisions) (M-flow 25, NS 2.5, Vögtlin Instruments AG, Switzerland). The lower end of the cylinder was connected to a nozzle containing a sapphire orifice (Swiss Jewel Company, Philadelphia, PA, USA) through a tube (PVC, 0.3 m length). The gas flow inside the cylinder was regulated by a solenoid valve (model: LEO65A001V-24C16701, Asco Joucomatic, Germany) operated by the current impulses of an impulse generator (Institute of Agricultural Engineering, University of Bonn, Germany) operating at 12-V DC.

2.2. Equipment operation

In principle, droplets are separated from a liquid jet released through the nozzle due to jet breakup behaviour (Dimmock, 1950). For a short current impulse from the impulse generator, the solenoid valve opened, permitting a flow of nitrogen gas into the cylinder. A portion of the gas is used to eject a liquid jet through the orifice, and a droplet is detached from the jet. Venting of excess gas caused negative pressure inside the cylinder, causing the remaining portion of the jet to retract inside the cylinder. This inhibited the formation of further droplets; thus, generating a single drop-on-demand (DOD) was possible. The liquid level in the cylinder was maintained at 10 mm, and the nozzle was kept at the liquid level throughout the experiment.

2.3. Test solutions

Deionized water and glyphosate solutions were tested. The surface tension (ST) of the glyphosate solution (43 mM; unformulated isopropylamine; Monsanto Europe S.A., Belgium) was reduced by adding surfactants (Tween[®] 60 (Sigma-Aldrich Chemie GmbH, Germany), Break Thru[®] S233 (Evonik Goldschmidt GmbH, Germany) and Silwet[®] L-77 (Momentum Performance Materials, USA) (0.1% v:v)) from 68.42 to 37.26, 24.23 and 20.91

mN m^{-1} , respectively. The static surface tension of the solution was determined by the pendant drop method (DSA 30E, Krüss GmbH, Germany).

2.4. Impulse width and supply pressure

An impulse width between 3.75 and 27.75 ms was provided at the interval of 2.75 s. The pressure was regulated in the range between 35 and 185 kPa by a pressure regulator (0-20,000/ 1000 kPa, 0-28 l min^{-1} , Kayser GmbH, Germany) and one additional precision manometer (0-100 kPa; Manometer Preiss Empeo-KBM, Sankt Augustin, Germany).

2.5. Orifice diameter

Synthetic sapphire orifices with various diameters (508, 381, 254, 152 and 102 μm ; Swiss Jewel Co., Philadelphia, USA) were used.

2.6. Exhaust vent

A pin valve with a regulator knob (150 divisions) was used. Droplets were collected at three vent opening positions, i.e., the maximum, minimum, and average values (divisions) of exhaust vent size at which droplet generation was possible. In specific cases, droplets were collected at further one vent opening position, between average and its extreme range, leading to unequal number

of replications. Data for vent opening are presented as a percentage of the maximum vent opening, where 0% means fully closed, and 100% means fully open.

2.7. Droplet size determination

All liquid solutions contained fluorescein dye (1 mg l^{-1}) (fluorescein sodium salt, Sigma-Aldrich Chemie GmbH, Germany). The addition of fluorescein had only a negligible effect on the surface tension of the liquids (surface tension: water, 73.15 mN m^{-1} ; water + fluorescein, 74.89 mN m^{-1}). Ten droplets of water or 5 droplets (orifice diameter: 508, 381, 254 μm) or 10 droplets (orifice diameter: 152 and 102 μm) of glyphosate solutions were collected. After adding water to a constant volume of 10 ml in a volumetric flask, fluorescence was measured by a spectrofluorophotometer (RF-1501, Shimadzu Europe GmbH, Germany) at an excitation wavelength of 483 nm and an emission at 514 nm. The droplet volume was determined from a standard emission curve (calculated from 7 concentrations and 3 replications; $R^2 > 0.99$), and later, the droplet diameter was calculated by assuming that the droplet gets a spherical shape once it detaches from the nozzle. To

control possible error due to photo-degradation of fluorescein, the liquid chamber of the droplet generator was covered with dark paper. Droplets were collected in dark-colored vials, and the solutions used to establish the standard emission curve were collected from the droplet generator itself.

2.8. Data analysis

The main effect of the parameters on the droplet diameter was identified, and the ranges were given wherever needed. Pearson's correlation coefficient and its significance were determined with SPSS (Statistics 18.0, SPSS Inc., IL, USA). The regression analysis and graphs were built in Microsoft® Office Excel® 2007 (2006 Microsoft Corporation, Redmond, WA, USA) or OriginPro 8.0.63.988 (OriginLab Corporation, MA, USA).

3. Results and Discussion

A simple and robust pneumatic droplet generator (Fig. 1) with DOD and continuous mode was constructed based on the model proposed by Chandra and Jivraj (2002). Our driving force to construct this system was the possibility of using it to study droplet dynamics under laboratory conditions, with potential applications in precision weed control systems. Compared with the popular piezoelectric droplet generator, the system is robust and durable because it does not contain fragile moving piezoelectric crystals, is easy to handle, operates under larger ranges of working conditions (i.e., surface tension, viscosity, temperature etc.), and could easily generate not only small droplets but also larger droplets.

After building sufficient pressure inside the liquid chamber, the jet was released through the nozzle, a single droplet separated from the jet, and the remaining portion of the jet was retracted. Further increases of the impulse width or supply pressure induced the formation of satellite droplets, and subsequently, a stream of liquid. The production of a stream was not desired. However, by releasing the extra pressure that had built up in the chamber through the exhaust vent, monodroplets could be generated again (Table 1). In this system, the exhaust vent worked as an attenuator, and there is no direct relation between it and the impulse width. However, the opening of the exhaust vent required adjustments according to the orifice size and liquid properties (e.g., surface tension).

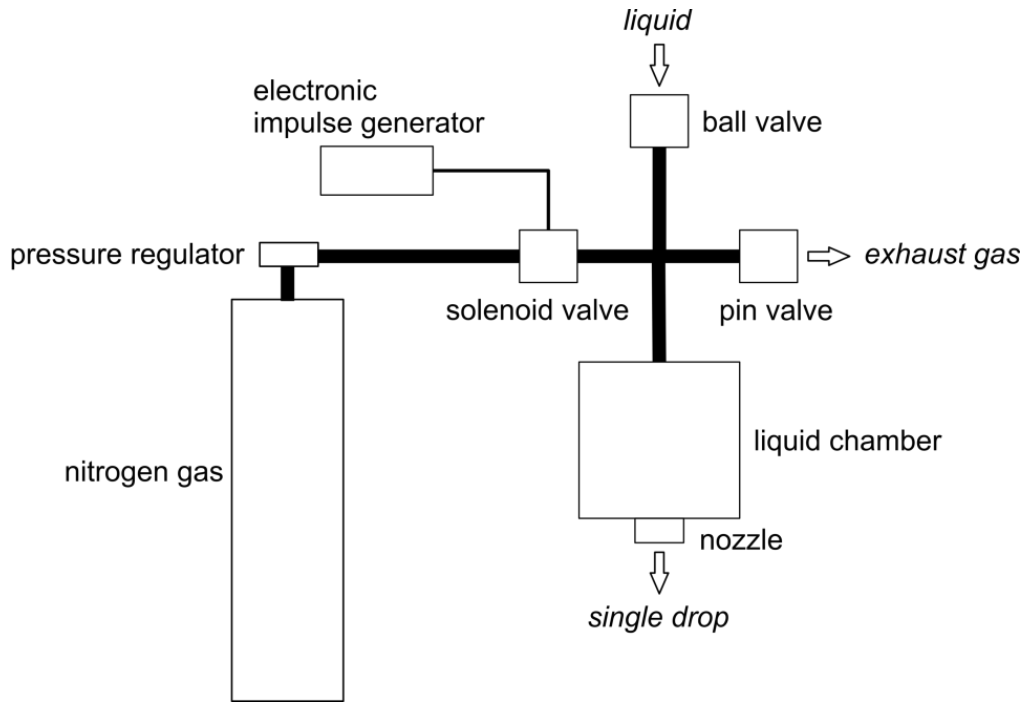


Fig. 1. Schematic representation of the droplet generator with its different components (not to scale).

Table 1. Effect of the impulse width (ms) on the diameter of the water droplet (μm) produced by various orifices with the following diameters a. 152 μm , b. 254 μm and c. 508 μm and variable vent opening (%) at which droplet generation is possible at a constant pressure of 35 kPa.

Impulse width (ms)	a. 152 μm		b. 254 μm		c. 508 μm	
	Vent opening (%)	Droplet diameter (μm)	Vent opening (%)	Droplet diameter (μm)	Vent opening (%)	Droplet diameter (μm)
3.75	0	N/A ¹	45.3- 49.8	961.5 - 1099.4	51.3 - 53.7	1622.7 - 1819.5
5.75	36.8 - 36.9	657.4 - 719.5	70.9- 75.8	985.2 - 1011.1	69.9 - 71.1	1552.2 - 1668.3
7.75	46.5 - 50.2	709.1 - 780.1	77.7- 83.9	890.5 - 917.8	78.9 - 83.1	1441.9 - 1672.2
9.75	51.4 - 54.5	791.9 - 826.1	83.3- 88.3	941.9 - 987.5	85.9 - 89.1	1453.0 - 1572.0
11.75	54.7 - 58.7	814.6 - 875.6	80.0- 89.1	955.7 - 997.7	88.8 - 90.4	1493.9 - 1596.8
15.75	58.0 - 60.0	840.6 - 899.1	93.1- 93.7	1000.4 - 1154.1	96.5 - 98.8	1573.3 - 1681.4
19.75	62.5 - 63.6	853.0 - 866.9	93.8- 95.4	1116.7 - 1238.7	99.6 - 99.9	1606.8 - 1691.0
23.75	65.7 - 66.9	915.8 - 920.2	94.6- 94.9	1142.5 - 1239.0	100	N/A
27.75	68.2 - 71.0	977.1	94.4 - 95.3	1179.6 - 1190.3	100	N/A

¹N/A = Droplets could not be generated

3.1. Effect of impulse width and orifice size on the diameter of water droplets

In general, increasing the impulse width increased the droplet diameter (Table 1). This effect is most obvious for the orifices with diameters of 152, 254 and 508 μm . However, for larger orifices (254 - 508 μm), this linear relationship was observed only at higher impulse widths (>7.75 ms). Significant ($P \leq 0.05^*$) or highly significant ($P \leq 0.01^{**}$) differences were observed between the impulse width (after 7.75 ms) and the droplet diameter generated from the orifices of diameters 152 μm ($r = 0.79^{**}$, $n = 24$), 254 μm ($r = 0.88^{**}$, $n = 29$), and 508 μm ($r = 0.59^*$, $n = 20$). The exception was 381 μm , which was not significant ($r = 0.54_{ns}$, $n = 9$). Increasing the orifice diameter increased the droplet diameter; a linear relationship ($R^2 > 0.98$) was identified between the mean droplet and orifice diameters (Fig. 2). Overall, the droplet diameter d_d (μm) was affected by the orifice diameter d_j (μm), impulse width I_w (ms) and exhaust vent opening, V (%) at the pressure of 35 kPa in the form of the following regression model:

$$d_d = 2.49 d_j + 13.59 I_w + 3.28V + 442.82 \text{ with } R^2 = 0.98, \text{ at } P \leq 0.01$$

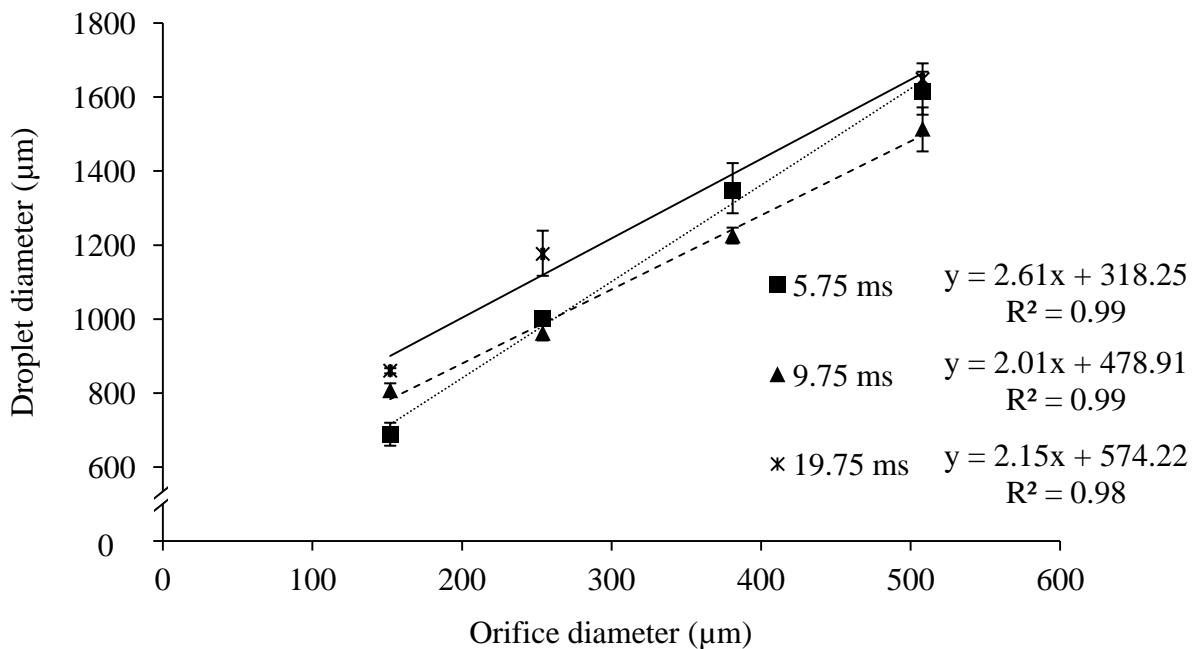


Fig. 2. Effect of orifice size on the diameter of water droplets produced at variable impulse widths (ms) and vent sizes and at a constant pressure of 35 kPa. The values are the mean diameter of the droplets produced at the upper, lower and average limit of exhaust vent opening that could generate droplets, and the vertical bars represent this range.

3.2. Effect of pressure on the diameter of water droplets

The combination of a high pressure and a short impulse width generated smaller droplets than a low pressure and a high impulse width in a defined range (Fig. 3). Chandra and Jivraj (2002) and Zabkiewicz et al. (2004) also demonstrated a reduction of droplet size with an increase in pressure. At high pressures, due to venting scope of the model, monodrops could be generated only up to 11.75 and 15.75 ms from orifice diameter of 102 μm at 185 kPa and 152 μm at 85 kPa, respectively. Likewise, we were able to generate droplets as small as 550 μm in diameter (with a 102 μm orifice diameter, 3.75 ms impulse width and 185 kPa pressure), but the size could be further decreased by increasing the pressure and venting size. Cheng and Chandra (2003) demonstrated a droplet size as small as 230 μm in diameter from a similar size nozzle for a 4.61 ms impulse width and a pressure of 138 kPa with their pneumatic droplet generator.

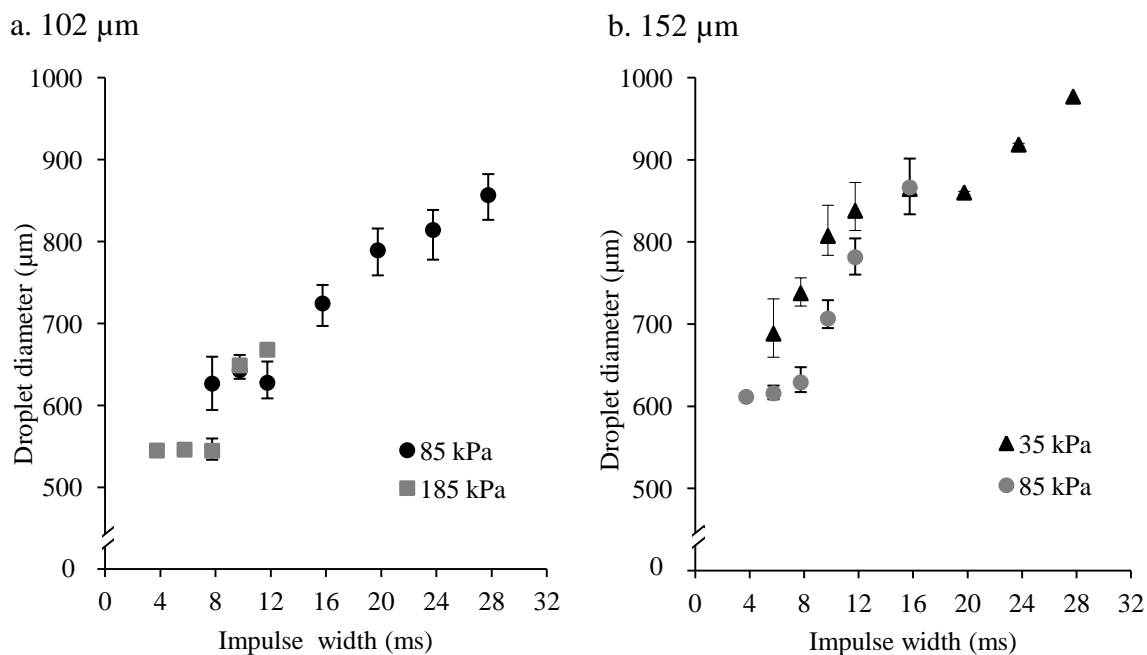


Fig. 3. Effects of pressure (kPa) and impulse width (ms) on the diameter of water droplets produced by orifices with diameters of a. 102 μm and b. 152 μm . The values are the mean diameter of droplets produced at the upper, lower and average limits of exhaust vent opening that could generate droplets, and the vertical bars represent the range.

3.3. Effect of the surface tension of glyphosate formulation on droplet diameter

Because most of commercial agrochemical products include surfactants in their formulation to affect the surface tension of the liquid, it is important to study the effect of the surface

tension on droplet generation. The surface tension of the glyphosate formulation affected both the generating condition and the size of the released droplets (Fig. 4).

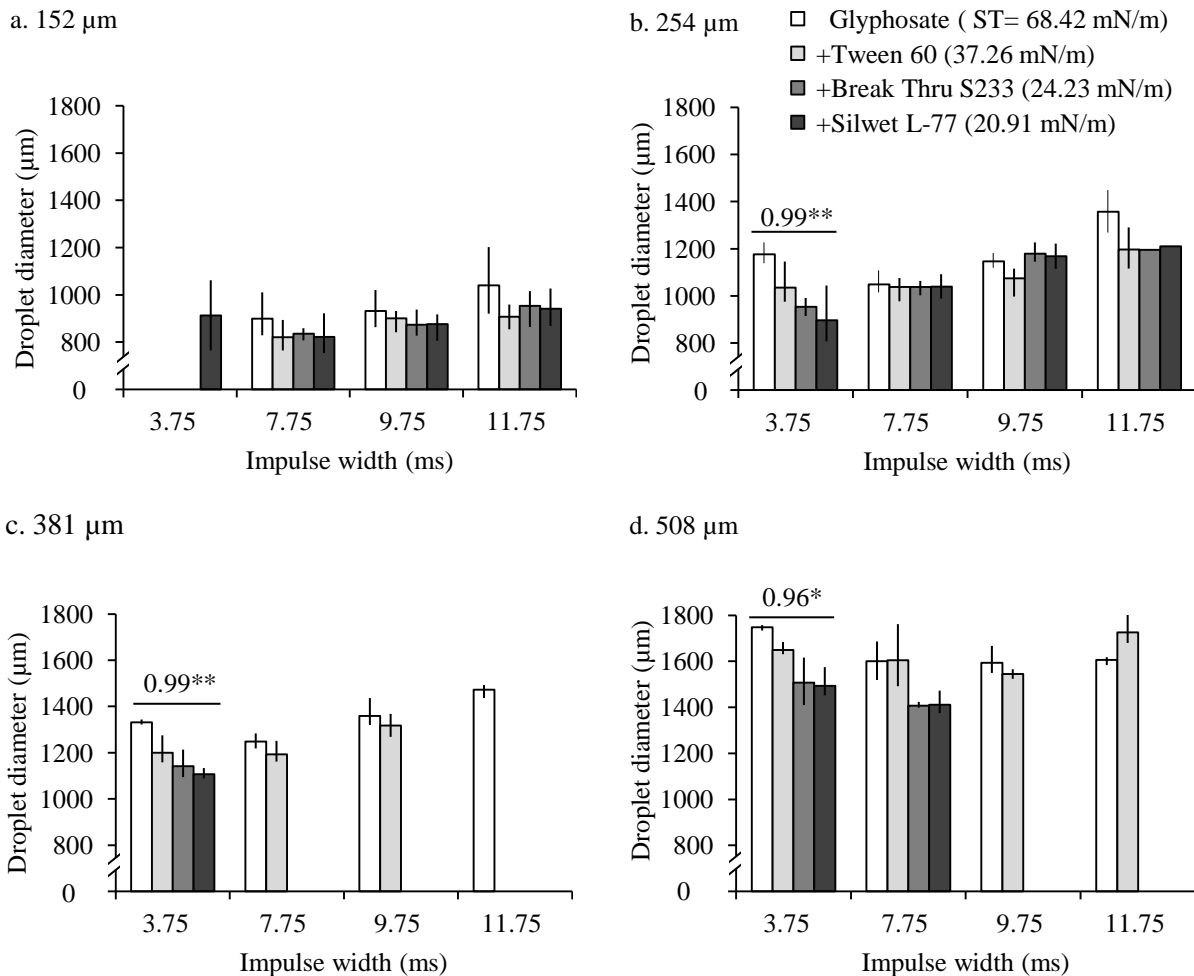


Fig. 4. Effect of the surface tension (ST) of glyphosate solutions on the droplet diameter as observed for various impulse widths (ms) at a constant pressure of 35 kPa for different orifices with the following diameters (µm): a. 152 µm b. 254 µm c. 381 µm and d. 508 µm. The values are the mean diameter of droplets produced at the upper, lower and average limit of exhaust vent opening that could generate droplets, and the vertical bars represent the range. The absence of a value represents the inability of the droplet generator to generate droplets. The droplet diameter is the mean of 5 drops for orifice diameters 508-254 µm and 10 drops for 152 mm, respectively. Horizontal bars above the mean represent the correlation between the droplet diameter and the surface tension of the liquid, and the value above it represents the Pearson's coefficient (*, **, $P \leq 0.05, 0.01$, respectively).

Solutions with a lower surface tension required less pressure to release a droplet, which could be attributed to an ease in breaking the surface tension of the liquid, as given by the Young Laplace equation (Pellicer et al., 2000). Due to the low surface tension of glyphosate + Silwet-L77 solution (20.91 mN m^{-1}), droplets were generated from a $102 \text{ }\mu\text{m}$ orifice at pressures as low as 35 kPa , while no other solutions tested could generate droplets under similar conditions. Likewise, because of the lower surface tension, a continuous stream was released at a pressure lower than that for the liquid with a higher surface tension. A strong positive correlation ($P \leq 0.05$) was observed between the mean droplet diameter and the surface tension of the solutions at low impulse (3.75 ms) for larger orifice diameters ($254\text{-}508 \text{ }\mu\text{m}$), in contrast to the insignificant relationship at higher impulse widths or smaller orifice diameters. Thus, to have less interference from the surface tension of the liquid on the droplet diameter, the system should be operated at higher impulse widths.

Overall, the orifice diameter was the major influencing factor on the droplet diameter. A combination of strong but short duration pressure bursts released smaller droplets. At low impulse widths, the surface tension of the liquids had a strong effect on the droplet diameter. With our system, we were able to produce droplets of diameters ranging from 533 to $1819 \text{ }\mu\text{m}$, although the volume could be varied by adjusting the orifice size and the pressure, amount and pulse duration. As demonstrated in the present work, at this development stage our droplet generator is suitable for use in scientific studies on droplet behaviour on selected plant surfaces under laboratory conditions.

4. References

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C. Effects of surfactants and the kinetic energy of monodroplets on the deposit structure of glyphosate at the micro-scale, and their relevance to herbicide bio-efficacy on selected weed species

1. Introduction

The efficacy of contact or systemic foliar-applied agrochemicals is influenced by their ability to penetrate the leaf cuticle. Cuticular penetration by chemicals is assumed to be largely a concentration-mediated diffusion process as explained by Fick's Law (Fick, 1855). Consequently, studies with hydrophilic active ingredients (a.i.), such as isopropylamine glyphosate, have emphasized decreasing the spread area of the solution droplet to improve its uptake in the plant (Liu, 2003). Decreased droplet spread on the one hand prolongs the drying period, and on the other hand leads to higher driving force due to the increased concentration of a.i. per unit area. And even, after the droplet dries, deposits affect the uptake of chemicals in the leaf (Liu and Zabkiewicz, 1997). Krämer *et al.* (2009) showed that the final deposit structure of glyphosate formulation droplet on the plant foliage is not always homogenous as thought in the past (Mercer, 2007). This indicates that the droplet's deposit area might be more relevant to its uptake than the assumed importance of spread area (Liu, 2003).

In the drying droplet of colloidal solution over the flat surface, the particles jam into the wedge of fluid next to the contact line, preventing it from retraction. Higher evaporation at this contact line causes capillary movement of substances to the periphery, forming an annular deposit (Deegan *et al.*, 1997). In contrast, surface tension gradient in a droplet generates the Marangoni stress, which accumulates the deposits to the centre of the droplet (Hu and Larson, 2006). Also, the inability to pin the contact surface to the droplet periphery would cause retraction of the particles from the periphery to the centre, as observed for deposition of glyphosate solution on Teflon, a hydrophobic model surface (Hunsche and Noga, 2011). Further, several other physical factors such as the dimension of the particle (Yunker *et al.*, 2011) and the gravitational field (Hampton *et al.*, 2012) are also shown to have an effect on the deposition behavior of the particle in the droplet footprint.

The kinetic energy of the applied droplet might also affect its spread and the deposit area. The impact of liquid droplets on solid surfaces can result in several outcomes, including depositing, recoil and splashing of the droplets (Bertola, 2008). In depositing the droplet, its initial momentum elicits a radial flow along the surface. The lamella grows until the initial impact energy of the droplet is dissipated. Subsequently, it retracts towards the centre of impact, depending upon competition between viscous dissipation and capillarity (Yarin,

2006). Inability to rebound causes the droplet to spread further and recoil with decreasing amplitude and frequency until the equilibrium stage is achieved (Mao *et al.*, 1997). In these sequences of events, droplet final spread area and the distribution of solutes in it, also could be affected.

Different residue patterns of foliar applied chemicals, as related to the use of adjuvants, have been reported already about three decades ago (Baker *et al.* 1983). Faers and Pontzen (2008) and Krämer *et al.* (2009) were among the few who stressed its importance in the context of efficacy of foliar applied chemicals. Faers *et al.* (2010) highlighted the advantage of an annular deposit in the droplet of lipophilic suspoemulsions containing a.i. The annular deposit enabled higher cuticular penetration due to the greater association of the particles and oil. Recently, it was shown that this association depends on the characteristics of both the treatment solution and the surface, as well as the velocity of droplet drying (Hunsche and Noga, 2011). Likewise, Krämer *et al.* (2009) concluded that, neither the deposit area nor the spread area, as affected by different adjuvants, can alone explain the bio-efficacy of hydrophilic isopropylamine glyphosate. However, the used adjuvants may have influenced not only the deposit characteristics but also other partial processes relating to penetration and bio-efficacy.

Therefore, a different approach is required to evaluate the impact of deposit properties without changing the chemistry of the substance. Accordingly, in the present experiments we aimed to change the deposit structure of the glyphosate solutions by applying droplets with or without kinetic energy. In this context, we hypothesize that the kinetic energy and the formulations of the glyphosate droplets affect its deposition pattern on the leaf surface of selected weed species. Additionally, we hypothesized that small concentrated deposits will have the strongest phytotoxic effect of glyphosate. As a methodological approach we applied droplets of glyphosate solutions having distinct surface tensions with or without (w/o) kinetic energy by a monodroplet generator or by a pipette, respectively. Scanning electron microscope (SEM) and energy dispersive x-ray microanalysis (EDX) images were used to characterize the deposit properties (Hunsche and Noga, 2008; Hunsche and Noga, 2009). Droplet residues of the glyphosate formulations were related to their respective bio-efficacy on *Stellaria media* L., *Viola arvensis* L., *Chenopodium album* L. and *Setaria viridis* L.

2. Materials and Methods

2.1. Plant materials

Four weed species with contrasting leaf surface wetting behaviour were studied: *V. arvensis* (field pansy) and *S. media* (common chickweed) with hydrophilic adaxial leaf surfaces, and *C. album* (fat hen) and *S. viridis* (green bristle-grass) with hydrophobic adaxial leaf surfaces. Seeds (Herbiseed Co., Reading, UK) were germinated in a growth chamber (day / night, temperatures and RH regimes of 20 / 15 °C, 70 / 80 % respectively; 16-h photoperiod). After germination, seedlings were transplanted to individual pots filled with a mixture of soil/sand/perlite (12/3/1) and allowed to grow for 10-12 days in growth conditions similar to those during germination, and kept constant throughout the entire experiment.

2.2. Treatment solutions

Glyphosate solutions of 43 mM (corresponding to 1.45 kg ha⁻¹ at 200 L ha⁻¹) were prepared with 62 % non-surfactant formulated isopropylamine salt of glyphosate (Monsanto Europe N.V., Antwerp, Belgium) and deionized water. Ethoxylated rapeseed oil (RSO) (Agnique RSO[®] series, Cognis Agro Solutions Co. Düsseldorf, Germany) series i.e. RSO 5, RSO 10, RSO 30, and RSO 60 with 5, 10, 30 and 60 ethylene units respectively, were used as a surfactant at 1 g l⁻¹. These surfactants were selected to affect the surface tension of the glyphosate solution, ultimately affecting its spread and deposit behaviour on the foliage. Addition of these surfactants, altered the surface tension (Mean ± S.E., n=10) of the unformulated glyphosate solution, from 75.2 ± 0.39 in the absence of surfactant to 38.4 ± 0.20, 44.8 ± 0.72, 51.8 ± 0.24 and 52.5 ± 0.37 mN m⁻¹ with increasing ethoxylation units, respectively. In addition, a commercial glyphosate product, Roundup Ultra[®] (CGP) (Monsanto Europe S.A., Antwerp, Belgium) was used at 43 mM of glyphosate having 47.3 ± 0.14 mN m⁻¹ surface tension (Mean ± S.E., n=10). Static surface tension of the solution was determined by the pendant drop method (DSA 30E, Krüss GmbH, Germany).

2.3. Micro-morphological characterization and wetting behaviour

The first two fully expanded leaves of the plants were excised and stuck on horizontal glass slides with a double-sided adhesive tape. Droplets (2 µl) of 20 % acetone solution (v: v) were applied on the right and left of the midrib, and the contact angle was measured using the droplet shape analysis system DSA 30E (Krüss GmbH, Hamburg, Germany). Thereby surfaces with a contact angle < 90°, such as *V. arvensis* and *S. media* (83.7 ± 0.88 and 67. 8 ±

1.39 respectively) were classified as easy-to-wet species while those having $> 90^\circ$, such as *C. album* and *S. viridis* (125.1 ± 1.83 and 126.9 ± 0.94 , respectively) were categorized as difficult-to-wet species. Qualitative characterization of micro roughness was done with scanning electron microscopic images taken with an XL 30 FEI ESEM microscope (FEI-Philips Co., Kassel, Germany); fresh leaves were investigated at low vacuum mode (pressure 0.3 Torr; spot size 5.5; acceleration voltage 20 keV); working distance of 10 mm; settings were held constant throughout experiment.

2.4. Application technique

Droplets (1 μ l) of the treatment solutions were applied manually by a micro pipette (Hamilton Co., Bonaduz, Switzerland) in contact with the leaf surface (kinetic energy assumed to be zero) or by a pneumatic monodroplet generator (with kinetic energy). The pneumatic monodroplet generator (Basi *et al.*, 2012) is a modified version of the droplet generator originally developed by (Cheng and Chandra, 2003). Droplets were applied from the monodroplet generator at a height of 5 cm above the leaf surface except for the droplet application of the unformulated glyphosate on the difficult-to-wet weeds which was done in close contact with the leaf surface, in order to assure droplet retention. In order to reduce the initial velocity of the droplet and simulate a free fall, the nozzle was placed at $\sim 45^\circ$ to the leaf surface. Preliminary studies showed that a low height was needed to keep the kinetic energy of the droplet below the stored energy that might rebound or shatter the droplets in the species studied.

2.5. Treatment application

Monodroplets (1 μ l) of the treatment solutions were applied to the adaxial surface of the first fully developed leaf (counted from the top) of uniform plants at 4-6 leaf stage (~ 4 weeks after sowing). Droplets were applied either by micro pipette or by the monodroplet generator and were left to dry at 20°C and 70 % RH in the climate chamber. All treatment solutions were applied to the four weed species, with exception of RSO 30 which was not applied to *C. album*. Some of the treated plants were used to quantify the droplet spread and deposit area of the a.i., whilst the remaining plants were left to grow for evaluation of the bio-efficacy.

2.6. Droplet spread and deposit area

About one hour after application, when the droplets dried, the residues from four replications of each treatment solutions were studied. Droplet residues were examined with the energy

dispersive x-ray microanalysis (EDX) system (EDAX Co., Mahwah, USA) integrated with environmental scanning electron microscope (Hunsche and Noga, 2009). The droplet spread area was determined by true image and the deposit area by a false x-ray image of phosphorous as a representative element of glyphosate, using ImageJ software (Abramoff *et al.*, 2004). The area covered by indigenous phosphorous content of the leaf was calculated from untreated areas as described above and deducted from calculated area of deposit residue.

2.7. Bio-efficacy

Ten plants were used as replications for each treatment group. After the treatment application, plants were left to grow in the climate chamber under similar environmental condition for the next ~10 days. The herbicidal injury (1, no sign of injury; 5, plant death) was noted and the biomass above ground was cut and weighed. Percent reduction of fresh weight compared to the untreated control plants defined the bio-efficacy of the treatments.

2.8. Experimental design and data analysis

Experiments were conducted in mono factorial completely randomized designs. Statistical analysis was performed with SPSS (Statistics 18.0, SPSS Inc., Chicago, IL, USA) and graphs drawn with Microsoft® Office Excel® 2007 (© 2006 Microsoft Corporation, Redmond, WA, USA) or OriginPro 8 (OriginLab Corporation, Northampton, MA01060, USA). Data were verified for their homogeneity of variance and normality by Levene's and Kolmogorov-Smirnov statistics respectively. Mean +/- standard error of the means (S.E.M.) were presented in the graph. All results were subjected to analysis of variance, and statements provided in the text are based on the difference between the mean values that were statistically significant (LSD, $P \leq 0.05$). Pearson's correlation coefficients between droplet deposits (spread area, deposit area) and bio-efficacy were identified and their significance established with a two tailed t-test.

3. Results

3.1. Droplet spread and deposit area

Deposits of unformulated glyphosate as well as the commercial glyphosate product, Roundup Ultra, (CGP), formed amorphous homogeneous residues, whilst the addition of the RSO surfactant caused the formation of amorphous heterogeneous deposits in the droplet footprint (Fig. 1 & Fig. 2).

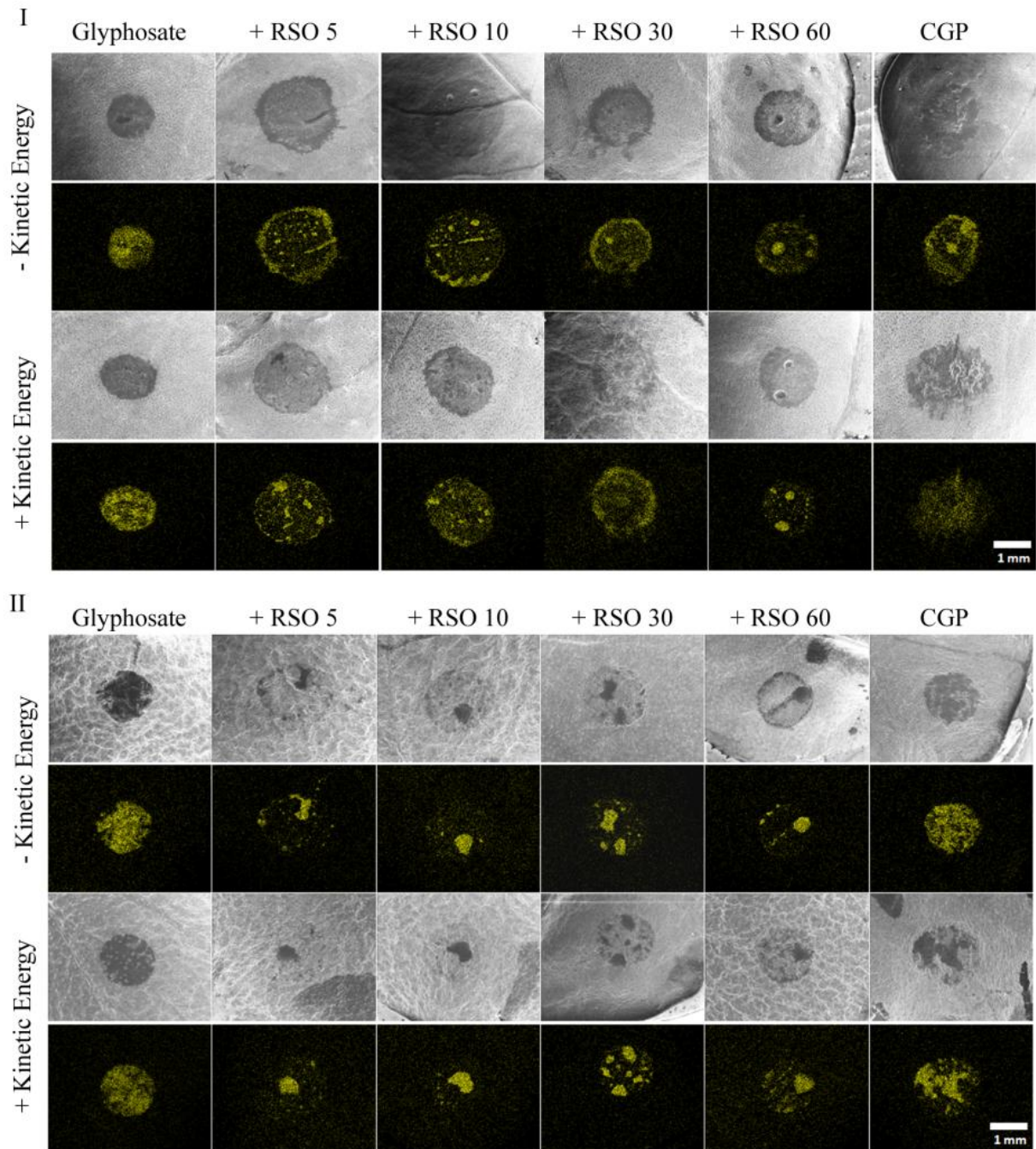


Fig. 1. Effect of kinetic energy and formulation on the deposit pattern of dried glyphosate solution droplets on easy-to-wet species I. *S. media* and II. *V. arvensis*. Droplet spread area is represented by the true grey-scaled picture followed by yellow-scaled false x-ray picture of deposit area. Residue area as influenced by formulations is shown with increasing ethoxylation (EO) units from left to right (unformulated Glyphosate; Glyphosate + selected ethoxylated rapeseed oil surfactant, RSO) followed by commercial glyphosate product (CGP). Droplets (1 μ l) were deposited on leaves with or without kinetic energy.

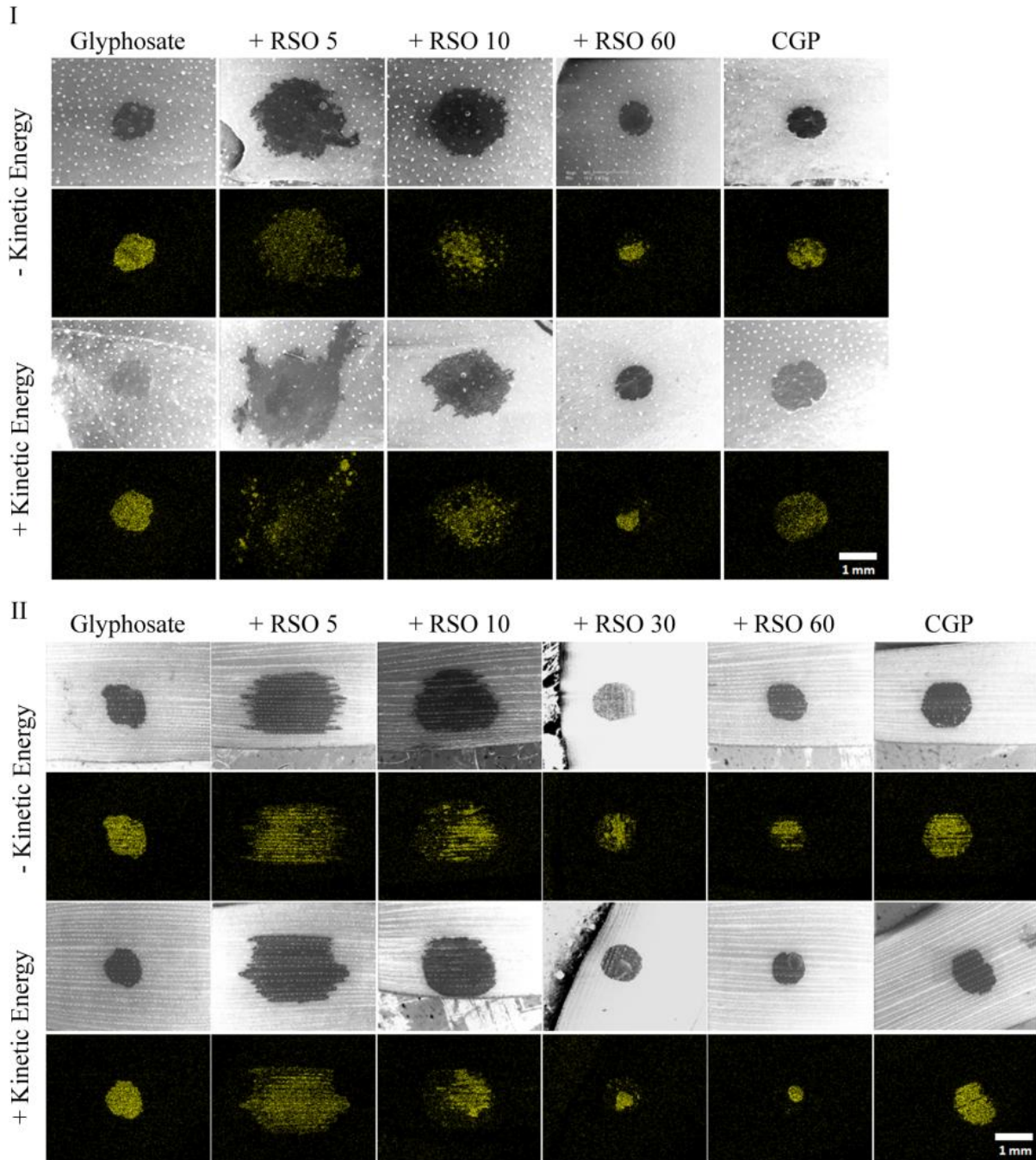


Fig. 2. Effect of kinetic energy and formulation on the deposit pattern of dried glyphosate solution droplets on difficult-to-wet species I. *C. album* and II. *S. viridis*. Droplet spread area is represented by the true grey-scaled picture followed by yellow-scaled false x-ray picture of deposit area. Residue area as influenced by formulations is shown with increasing ethoxylation (EO) units from left to right (unformulated Glyphosate; Glyphosate + selected ethoxylated rapeseed oil surfactant RSO) followed by commercial glyphosate product (CGP). Droplets (1 μ l) were deposited on leaves with or without kinetic energy.

In general, kinetic energy of the droplet did not affect the deposit area of any of the treatment solutions on easy-to-wet foliage of *S. media* and *V. arvensis* (Fig. 3). Only the RSO 10 formulated glyphosate droplets applied with kinetic energy on the foliage of *S. media* reduced its deposit area. Likewise, spread area of solution droplet was also not affected by the kinetic energy of the droplet, except when CGP was applied to *V. arvensis*. Addition of RSO 5 or RSO 10 surfactants to glyphosate doubled the droplet spread area, as compared to the unformulated control (Fig. 3A & Fig. 3C). Glyphosate formulated with RSO 5, RSO 10 and RSO 30 formed a coffee-ring deposit in *S. media* (Fig. 1I), whilst, RSO 60 formulated glyphosate formed small concentrated deposit (Fig. 1 & Fig. 3B). In contrast, on the foliage of *V. arvensis* all the RSO-formulated glyphosate solutions formed a localized deposit (Fig. 1II) with similar coverage (Fig. 3D).

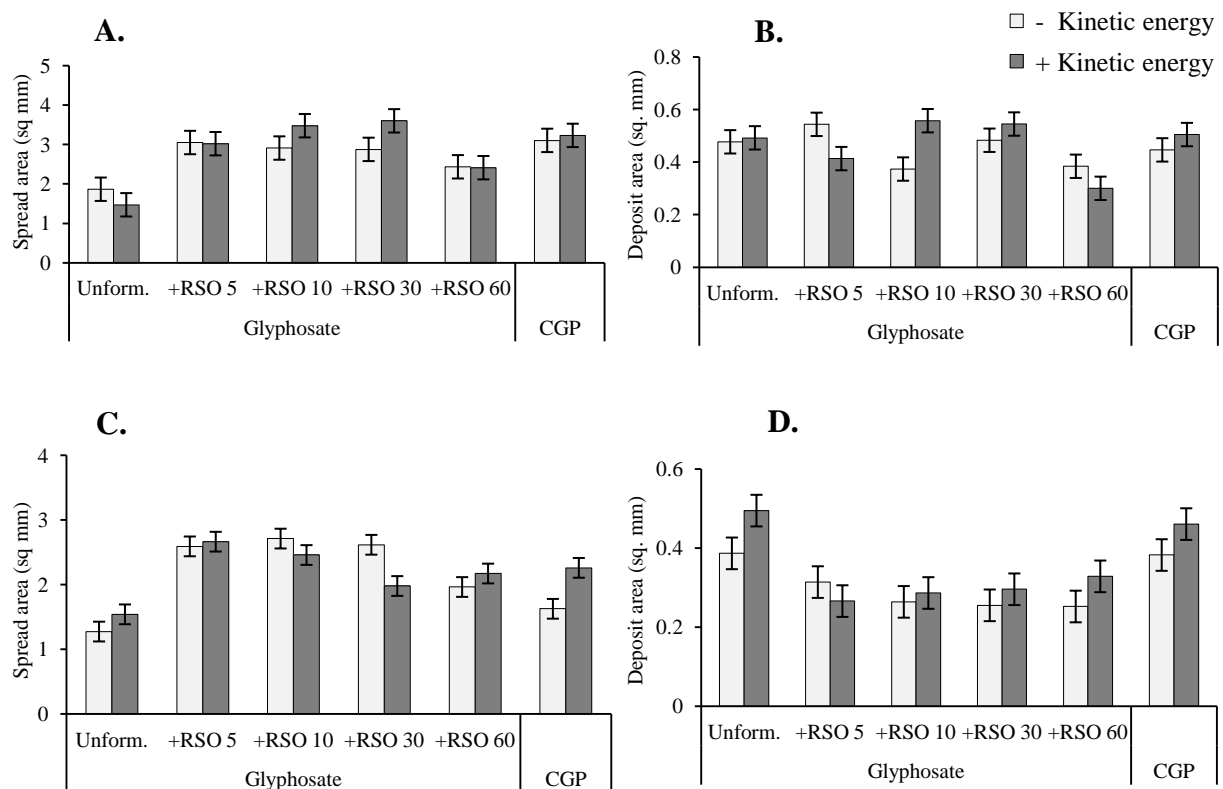


Fig. 3. Effect of droplet kinetic energy and formulations on the glyhosate droplets' final spread area (A & C) and deposit area (B & D) on the foliage of easy-to-wet species *S. media* (A & B) and *V. arvensis* (C & D). Monodroplets (1 μ l) of glyhosate formulated with or without ethoxylated rapeseed oil (RSO) surfactant and the commercial glyhosate product (CGP) were applied with(+) or without(-) kinetic energy. Error bars represent +/- SEM.

Similarly, kinetic energy of RSO formulated glyphosate droplets had no significant effect on the deposition structure when applied to the foliage of difficult-to-wet species *C. album* and *S. viridis* (Fig. 4), except for the deposit residue of glyphosate formulated with RSO 10 which slightly decreased when applied with kinetic energy (Fig. 4B & Fig. 4D). In contrast, the kinetic energy of impacting CGP droplets induced a larger deposit area on the foliage of both species. Similar to the deposit area, spread area of RSO formulated glyphosate was not affected by the kinetic energy of impacting droplets (Fig. 4A & Fig. 4C). However, the kinetic energy of the CGP droplets increased spread area in *C. album* whilst it remained unaffected on *S. viridis*. At lower surface tension of the treatment solution, e.g. with RSO 5, the droplet spread area increased nearly fivefold in *C. album* (Fig. 4A) and fourfold in *S. viridis* (Fig. 4C) as compared to the unformulated control treatment. As well as increasing the spread area, the surfactants also increased the deposit area. At higher surface tension (e.g. with RSO 60), the spread area remained unaffected compared to unformulated control, but the deposit area was reduced in both the species (Fig. 2, Fig. 4B & Fig. 4D).

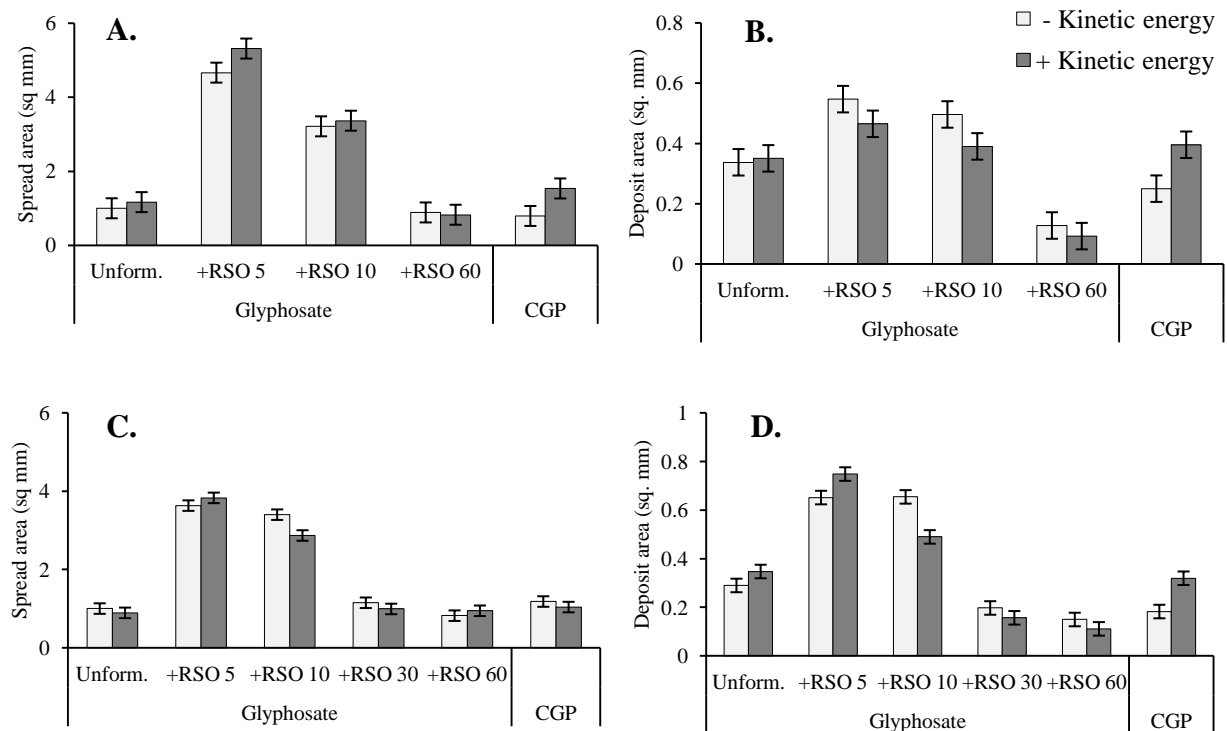


Fig. 4. Effect of droplet kinetic energy and formulations on the glyphosate droplets' final spread area (A & C) and deposit area (B & D) on the foliage of difficult-to-wet species *C. album* (A & B) and *S. viridis* (C & D). Monodroplets (1 μ l) of glyphosate formulated with or without ethoxylated rapeseed oil (RSO) surfactant and the commercial glyphosate product (CGP) were applied with(+) or without(-) kinetic energy. Error bars represent +/- SEM.

3.2. Bio-efficacy

Bio-efficacy, due to its dependence on the uptake of herbicide, was used to determine the influence of the deposit structure. As fresh weight had a strong correlation with visual injury ($r = -0.75^{**}$, $n > 110$) the former was used to calculate bio-efficacy. In easy-to-wet species, neither the kinetic energy of the droplet, nor the difference in the EO unit of the RSO surfactants led to a significant difference on the bio-efficacy of glyphosate (Fig. 5A & Fig. 5B).

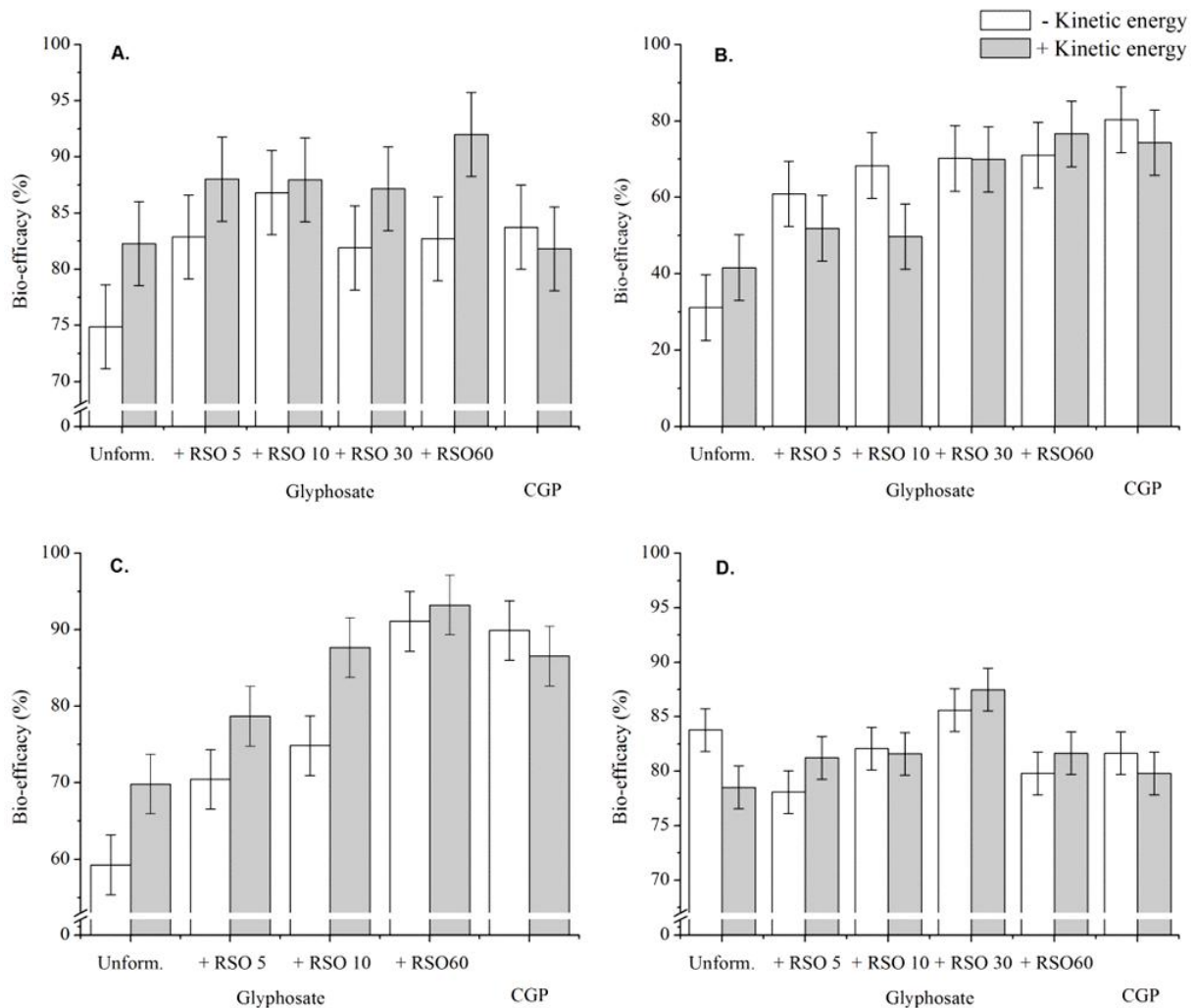


Fig. 5. Effect of kinetic energy and formulations of glyphosate droplets on its bio-efficacy on the easy-to-wet species A. *S. media* and B. *V. arvensis* and difficult-to-wet species C. *C. album* and D. *S. viridis*. Monodroplets (1 μ l) of glyphosate w/o ethoxylated rapeseed oil (RSO) surfactant as well as commercial glyphosate product (CGP) were applied with(+) or without(-) kinetic energy. Bio-efficacy is the percent reduction in fresh shoot biomass compared to the untreated control. Error bars represent +/- SEM.

Likewise, with one exception, kinetic energy of the droplet did not affect the bio-efficacy of the formulations in difficult-to-wet species (Fig. 5C & Fig. 5D). Only the RSO 10 formulated glyphosate droplet applied with kinetic energy induced higher bio-efficacy of glyphosate on *C. album* (Fig. 5C). In contrast, formulations had a significant effect on the bio-efficacy of glyphosate. The RSO surfactant ethoxylated with higher EO unit enhanced the bio-efficacy of glyphosate in *C. album*. However, the relation held true only up to 30 EO units in *S. viridis*; the further increase to 60 EO units rather decreased the bio-efficacy of glyphosate. At such highest level of glyphosate bio-efficacy, induced by RSO surfactant, the efficacy was equivalent (in *V. arvensis*, *S. media* and *C. album*) or even higher than the CGP (in *S. viridis*).

2.3. Relationship between deposit structure of glyphosate droplets and bio-efficacy

There was no consistent difference in deposition patterns (Fig. 3 & Fig. 4) or the bio-efficacy (Fig. 5) of formulated glyphosate, when droplets were applied with or without kinetic energy on any of the species observed. In contrast, surfactants significantly affected both the deposition structure and the bio-efficacy of the glyphosate on the difficult-to-wet plants (Fig. 4 & Fig. 5 respectively). Thus, only the glyphosate deposit structure as affected by the surfactant was related to its bio-efficacy. Likewise, as kinetic energy had no significant effect the data were pooled into one group. In preliminary observations, higher EO units of RSO surfactant had either no effect (in easy-to-wet species *S. media* and *V. arvensis*) or enhanced (in difficult-to-wet species *C. album*) the bio-efficacy of glyphosate. Following a different trend, in the other difficult-to-wet species *S. viridis*, the increasing EO unit of RSO surfactant up to 30 units enhanced the bio-efficacy of glyphosate whilst a further increase in the EO units (to 60) decreased glyphosate's phytotoxicity. The, RSO 60 surfactant might have affected the glyphosate bio-efficacy by other physiological mechanisms rather than simply its contact area. Thus, for further analysis and explanations of the effects of the formulations the RSO 60 surfactant was excluded in *S. viridis*.

In easy-to-wet species, surfactant differing in EO units did not induce difference in deposition structure (Fig. 3), or bio-efficacy (Fig. 5). Thus, the relation between droplet spread area or the deposit area of glyphosate with its bio-efficacy also could not be established (Table 1). Further, no relationship could be built between deposit area and spread area of the droplet in *V. arvensis*. In difficult-to-wet species, surfactant differing in EO units could affect the deposit area (Fig. 4) and also the bio-efficacy (Fig. 5), the droplet spread and deposit area both being negatively related to glyphosate bio-efficacy (Table 1).

Table 1. Pearson's correlation (r) between droplet spread area, deposit area and the bio-efficacy of RSO-formulated glyphosate solution on a. *S. media*, b. *V. arvensis*, c. *C. album*, and d. *S. viridis*.

Deposit structure	Species	Deposit area	Bio-efficacy
Droplet spread area	<i>S. media</i>	0.85**	-0.16
	<i>V. arvensis</i>	-0.21	-0.49
	<i>C. album</i>	0.91*	-0.81*
	<i>S. viridis</i>	0.99**	-0.91*
Deposit area	<i>S. media</i>		-0.49
	<i>V. arvensis</i>		0.13
	<i>C. album</i>		-0.92**
	<i>S. viridis</i>		-0.87*

*, ** - $P \leq 0.05, 0.01$ (2-tailed); $n \geq 6$

4. Discussion

A precise knowledge about the contribution of single factors to the bio-efficacy of agrochemicals is essential to optimize products and processes. As already shown, higher bio-efficacy of hydrophilic chemicals can be obtained by decreasing the droplet volume (Knoche and Bukovac, 2000) or spread area (De Ruiter *et al.*, 1992; Nalewaja and Matysiak, 1995; Liu, 2003). Even without direct proof, these results could be related to the concentration of a.i. per contact area. Existing hypotheses for uptake of hydrophilic a.i., are by diffusion processes through the 'polar pores' of cuticles (Schönherr, 1976) and/or through stomata by 'hydraulic activation' (Burkhardt *et al.*, 2009), both depending on the concentration of the solutes in the droplet. Foliar uptake is an active process which continues even after drying of the droplet (Liu and Zabkiewicz, 1997) and, since the a.i. in the droplet does not always form a homogeneous deposit (Krämer *et al.*, 2009), deposit area of the a.i. might be more relevant than the spread area of the droplet. Thus, our aim was to better understand the effect of a.i. deposit area to affect in its bio-efficacy.

On the both easy-to-wet and difficult to wet species, the kinetic energy of the droplet affected the spread and deposit residue only in isolated cases (Fig. 3 & Fig. 4). Thus, it could

be safely considered that kinetic energy of the droplet did not alter RSO formulated glyphosate's deposition pattern. In contrast, when applying CGP to the difficult-to-wet plants, the kinetic energy of the droplet induced a larger droplet spread and deposit area, probably due to the higher viscosity of the liquid (Fig. 4).

In the present experimental series, we showed that the a.i. is not always uniformly deposited in the droplet. Only the unformulated glyphosate, and the CGP droplets, formed a homogeneous deposit (Fig. 1 & Fig. 2). The distinct ethoxylation degrees of RSO surfactant did not induce significant differences in the deposit behavior of glyphosate on the hydrophilic surface of easy-to-wet plants (Fig. 3). In *S. media* a coffee-ring deposit pattern was observed with the addition of RSO surfactants; however, with RSO 60, the glyphosate deposits were slightly centralized (Fig. 1I). As observed by Singh and Sharma (2001), in absence of crystalline wax, the surfactant could not induce significant spreading of the droplet. On the foliage of *V. arvensis*, droplet periphery was unable to pin the three-phase contact line of droplet, surface and atmosphere (probably for having smooth waxy surface). Thus, all the RSO-formulated glyphosate were transported to the centre of the droplet (Fig. 1II), similar to the observation made by Hunsche and Noga (2011) on the smooth hydrophobic Teflon plate. On the rough hydrophobic surface of difficult-to-wet species, when formulated with RSO 5 or RSO 10, both the droplet spread and deposit area of glyphosate grew larger. This might be related to the enhanced capillary movement of glyphosate to the periphery by differential evaporation (Deegan *et al.*, 1997) and/or the capillary action through wax crystals driven by the Wenzel's effect (Wenzel, 1936). By contrast, with RSO 30 and RSO 60, the droplet spread was not affected, and glyphosate contracted to be deposited in the centre. Marangoni stress (Hu and Larson, 2006; Faers, 2007) caused by accumulation of lighter oil in the centre might have dictated the centralization of deposit as observed by Faers and Pontzen (2008).

Despite of having larger deposit area (above twofold) or spread area (above fourfold) with RSO 5 or RSO 10, or having smaller deposit (above twofold) or equivalent spread area with RSO 30 or RSO 60, the phytotoxicity of glyphosate on difficult-to-wet plants was equivalent, or even higher, than the unformulated glyphosate. Based on this fact, we conclude that the RSO surfactants affect not only the contact area of the deposit but also enhance the bio-efficacy of glyphosate by influencing other processes. Among the multiple causes, as hypothesized or identified with other non-ionic adjuvants, cuticle-wax fluidity (Schreiber, 1995), cuticle-water permeability (Coret and Chamel, 1995), and/or increased humectancy (De Ruiter *et al.*, 1992), might be important.

On easy-to-wet plants, increasing EO unit of RSO surfactant couldn't enhance the glyphosate bio-efficacy (Fig. 5). In contrast, on difficult-to-wet plants, RSO surfactant of higher EO units increased the glyphosate's bio-efficacy; but within an optimum limit of EO unit, similar to the observation of Coret and Chamel (1995). It is possible that, glyphosate formulated with RSO 60 surfactant might have physically damaged the cuticle in *S. viridis* foliage, reducing the glyphosate phytotoxicity (Fig. 5D). The physical damage in the cuticle might affect the uptake and translocation process (Liu *et al.*, 1996; Hunsche and Noga, 2011). At the highest level of glyphosate bio-efficacy induced by RSO surfactant with higher EO unit, the efficacy was equivalent or even higher than the CGP (in *S. viridis*). The reduced bio-efficacy of CGP in *S. viridis*, as compared to RSO 30 formulated glyphosate, might also be linked to the physical damage in the cuticle (visual observation of necrotic tissue), hindering its foliar uptake and translocation.

In easy-to-wet plants, the absence of a difference between RSO surfactants to affect the deposit area (Fig. 3B & Fig. 3D) on its foliage, reflected also the absence of significant changes in the bio-efficacy of glyphosate (Fig. 5A & Fig. 5B). But, on difficult-to-wet plants, smaller concentrated deposit on the foliage as a result of RSO surfactant with higher EO unit had a stronger effect on enhancing the bio-efficacy of glyphosate (Table 1). Here, analogous to the deposit area, the increasing droplet spread area also had a negative effect on bio-efficacy. However, once the droplet dries, only its deposit remains to affect in its foliar uptake; and such uptake process continues for several hours after its application (Nalewaja and Matysiak, 1995; Liu and Zabkiewicz, 1997). And since, deposit is not always homogeneously deposited in the droplet footprint (Fig. 1 & Fig. 2), and does not always relate to its spread area, e.g. in *V. arvensis* (Table 1), the deposit area might be more relevant to its bio-efficacy than the spread area of the droplet. The increase in the uptake or bio-efficacy of glyphosate with the increase in EO unit (up to an optimum limit depending upon the species) in its non-ionic surfactant by the difficult-to-wet (probably) species is widely accepted (Wheat: Gaskin and Holloway, 1992; Barley: Mueller *et al.*, 2001, Gauvrit *et al.*, 2007). But in easy-to-wet species (probably), some concluded a significant effect of surfactant EO unit on uptake or bio-efficacy of glyphosate solution (*Phaseolus vulgaris* L. (common bean): Gaskin and Holloway, 1992, *Solanum lycopersicum* L. (tomato) cuticle: Haefs *et al.*, 2002), whilst no effect by the others ((common bean): Holloway and Edgerton, 1992; *Abutilon theophrasti* Medik.: Hunsche *et al.*, 2007).

In overall, the deposit structure of dried droplets of isopropylamine glyphosate solutions is not always homogeneous. Surfactants altered the homogenous deposit of

glyphosate to heterogeneous larger deposits (e.g. with RSO 5) or concentrated small amorphous deposits (e.g. with RSO 60) especially on the foliage of difficult-to-wet plants. Thus, deposit area might be considered more relevant to the bio-efficacy of glyphosate than the spread area of the formulation droplet. Kinetic energy of the droplet had no consistent effect on the deposit structure or the bio-efficacy of glyphosate. On easy-to-wet species, the increase in EO unit of the RSO surfactant did not affect the deposition area of glyphosate and also to its bio-efficacy. However, in difficult-to-wet species, the increase in EO units of the RSO surfactant reduced a deposit area of glyphosate, and enhanced its bio-efficacy.

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D. Relevance of the deposit structure for the uptake and bio-efficacy of diquat, as monitored by the spatially resolved chlorophyll fluorescence

1. Introduction

It is generally accepted that the effectiveness of herbicides is enhanced when the spray solution is uniformly spread over the leaves (Puri et al., 2008). However, the uniform coverage doesn't guarantee enhanced phytotoxicity. The larger and more uniform coverage area of water-soluble glyphosate, in the presence of organosilicone surfactant, rather reduced its bio-efficacy (Gaskin and Stevens, 1993). Increased droplet spread, on the one hand reduces the active ingredient (a.i.) per unit area, reducing the concentration gradient (Fick, 1855) between the external and internal leaf surfaces, thus, expectedly antagonising the glyphosate uptake (Liu and Zabkiewicz, 1997). On the other hand, droplets with a bigger spread area evaporate faster (Xu et al., 2011) and the active ingredient has less time to penetrate while in solution. Further, adjuvants influence the properties of pesticide deposits, and result in a highly heterogeneous, centrally or peripherally concentrated deposit to a homogenous deposit in the droplet footprint (Krämer et al., 2009; Hunsche and Noga, 2011). Thus, the spread area of the droplet might not be relevant for its uptake as expected (Liu, 2003; Mercer, 2006). Rather, its concentrated form, deposited in the centre of the droplet footprint ultimately enhanced the bio-efficacy of glyphosate (Basi et al., 2013). However, to this point, it is not clear if this enhanced bio-efficacy is related to its higher uptake or the transport of the systemic glyphosate compound, or both together. Thus, studies with contact herbicides, having less complexity might be better suited to provide more precise answers to the relation between deposit properties and herbicidal activity.

Diquat dibromide is a non-selective contact herbicide with defoliant and desiccant effects (Clarke, 1989; Ritter et al., 2000). It is a highly water soluble, dipyridylum crystalline salt (Derry and Hamor, 1969) and has a mode of action similar to paraquat (Dodge and Harris, 1970). In the presence of light, it absorbs the electrons being transported from the chloroplast electron transport chain near photosystem I (PS I) (Moreland, 1967). The free radical reduces the molecular oxygen, giving rise to superoxide radical anions, ultimately forming hydroxyl radicals or singlet oxygen (Stancliffe and Pirie, 1971). Such a reactive radical causes an oxidative stress inducing lipid peroxidation and oxidation of chloroplastic components including pigments and membrane lipids (Harris and Dodge, 1972; Fuerst and Norman, 1991; Asada, 1999). Decrease of the Chlorophyll *a* (Chl *a*) content in the tissue thus reduces the fluorescence intensity (Schmitz-Eiberger and Noga, 2001). In the presence of

light, glyphosate and paraquat, though do not directly influence the dark phase of photosynthesis, could indirectly affect the Chlorophyll fluorescence (ChlF). This enables the detection even before the visual damage (Barbagallo et al., 2003). Further, diquat might reduce the efficiency of photosystem process, while accelerating the downhill electron-transfer steps in PSII as observed by Fan et al. (2009) with paraquat. Chlorophyll fluorescence is a universally accepted tool to evaluate the physiological status of the plants (Baker and Rosenqvist, 2004). Equally, it is established as a rapid and non-destructive technique to evaluate the activity of photosynthetically active herbicides on the plant tissue (Schreiber et al., 1997; Richard et al., 1983; Voss et al., 1984). The technique is proposed also for studying the penetration and translocation process of the PSII inhibitors in the plant tissues (Yanase and Andoh, 1992). Thus, the foliar uptake of diquat might also be derived indirectly, by studying its spatial and temporal effect on ChlF of the tissue.

Contrary to the laser-induced fluorescence, which enables to record the fluorescence intensity and the fluorescence lifetime in single wavelengths (Hunsche et al., 2011), the Pulse Amplitude Modulated (PAM) technique detects the chlorophyll fluorescence in a defined spectral band (usually 680-690 nm). Based on the ground and the maximum fluorescence of dark-adapted leaves, as well as the fluorescence intensity during kinetic measurements, specific parameters allow conclusions about the current photosynthetic activity and the physiological state of the tissue (Lichtenthaler et al., 2005; Baker, 2008). ChlF parameters such as the maximum efficiency of PSII (F_v/F_m) (Butler, 1978), measured after its dark adaptation or effective PSII quantum yield ($Y(II)$), measured after light induction, indicate the efficiency of PSII of the leaf tissue. In addition, $Y(II)$ explains the changes in PSII operating efficiency, whether attributable to changes in non-photochemical quenching or to the ability of an excited PSII reaction centre to drive electron transport (Baker, 2008). Likewise, the quantum yield of regulated energy dissipation $Y(NPQ)$ (Kramer et al., 2004) indicates the ability of the plant to dissipate the extra light in the form of harmless heat. As a practical application, it was proven that lower $Y(II)$ of tissues treated with diuron, a PSII inhibitor was related to its increased penetration (Habash et al., 1985).

In our previous work we related the biological efficacy of glyphosate to the deposition pattern at the micro-scale (Krämer et al., 2009; Basi et al., 2013). The systemic nature of glyphosate, and the possible impact of the adjuvants on the transport of the compound in the whole plant, might have influenced the observed results. Thus, with this study, our main objective was to analyse the relation between the deposit properties and the foliar uptake of the herbicide. The distinct deposition structures of diquat dibromide on the leaves of *Viola*

arvensis and *Chenopodium album* was enforced by a series of ethoxylated rapeseed oil (RSO) surfactants. Then, the deposit structure was related to its effect on the spatial and temporal ChlF, and to the final desiccation potential of the active ingredient. Our work is based on the hypothesis of a close relation between the deposition structures and the spatial and temporal changes of ChlF. Thereby, the larger coverage area of diquat dibromide residue reduces its uptake and its phytotoxicity as represented by its reduced spatial and temporal effect in ChlF parameter and the desiccation of the tissue.

2. Materials and Methods

2.1. Experimental plants and growth condition

The weed species *Chenopodium album* L. (common lambsquarters) and *Viola arvensis* L. (field pansy), having a contrasting wetting behaviour, were chosen as model plants. Seeds (Herbiseed Co., Reading, UK) were germinated in a tray under growth chamber conditions (day/night, temperatures and RH regimes of 20/15 °C, 70/80 % respectively; and 14-h photoperiod of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PL-L 4P, Philips Deutschland GmbH, Hamburg, Germany). After germination, seedlings were transplanted to individual pots filled with a mixture of soil/sand/perlite (12/3/1) and allowed to grow in the same environmental conditions for the entire experimental period.

2.2. Treatment solutions

Diquat solutions (10 mM) were prepared with Diquat dibromide (98 % purity, Sigma-Aldrich[®], Seelze, Germany) and deionized water. Ethoxylated rapeseed oil (RSO) surfactants (Agnique RSO[®] series, RSO 5, RSO 10, RSO 30 and RSO 60; courtesy of Cognis AgroSolutions Co. Düsseldorf, Germany) were used at 1 g L⁻¹ to affect the surface tension of the diquat solution in different ranges. Accordingly, the surface tension of unformulated diquat solution (71.01 ± 0.40) was reduced to 42.31 ± 0.84 (RSO 5), 43.28 ± 0.58 (RSO 10), 49.34 ± 0.36 (RSO 30) and 50.57 ± 0.16 mN m⁻¹ (RSO 60).

2.3. Micro-morphological characterization and wetting behaviour

First two leaves counted from the top were excised and attached on horizontal glass slides with a double-sided adhesive tape. Droplets (2 μl) of 20 % acetone solution (v:v) were applied on the right and left of the midrib, and the contact angle was measured with a droplet shape analysis system (DSA 30E, Krüss GmbH, Hamburg, Germany). Acetone solution

droplets had in average a contact angle of $83.72 \pm 0.88^\circ$ on *V. arvensis* and $125.12 \pm 1.83^\circ$ on *C. album*; on this basis the species were categorized as easy-to-wet and difficult-to-wet respectively.

2.4. Treatment application

Droplets (1 μl) of the treatment solutions were applied with a drop-on-demand (DOD) system (Basi et al., 2012) on horizontally fixed leaves. Thereby, the second leaf was treated; in each treatment group 14 uniform plants at 4-6 leaf stage (10-12 days after transplanting) were used. From those, 4 plants were used to study the deposit structure, and the remaining 10 were used to evaluate the bio-efficacy, ChlF and desiccation process. Droplets were applied from a height of 5 cm, and projected at $\sim 45^\circ$ to the leaf; with these settings the kinetic energy of the droplet was reduced and was equivalent to free fall. In case of unformulated diquat solution, the application of droplets to the difficult-to-wet *C. album* was done in contact with the leaf surface. Droplets were applied to undamaged leaves which stayed intact to the plant. Treated droplets were left to dry in the same environment as the growth condition of the plants.

2.5. Droplet spread area and deposit area

The characterization of droplet spread and deposit area was done with the energy dispersive X-ray microanalysis (EDX) system (EDAX Co., Mahwah, USA) coupled to an environmental scanning electron microscope (Hunsche and Noga, 2009). Analyses were done about 1 h after application on the dry residue of the droplets. Droplet spread area was determined using the true image, while the deposit area on the false x-ray image of Bromine as representative element of diquat. Image analysis was done with ImageJ software (Abramoff et al., 2004). Endogenous bromine content of the leaf was calculated from untreated areas as described above; in a next step the area covered was removed from the treated area.

2.6. Chlorophyll fluorescence (ChlF)

Chlorophyll fluorescence of dark-adapted (20 min) control and treated leaves was measured by Imaging-PAM chlorophyll fluorometer (Heinz-Walz, Effeltrich, Germany) at 2, 6, 26 and 51 h after treatment application. Intact plant leaves were flatly clamped, and the treated surface was placed near to the centre of the camera field. The ground fluorescence (F_0 ; blue light excitation with intensity of $0.5 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR) and the maximum fluorescence (F_m ; blue light saturation pulse of $2400 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR for 800 ms) were measured, and the

maximum quantum efficiency of PSII (F_v/F_m ; where, $F_v = F_m - F_o$) was calculated (Kramer et al., 2004). After a delay time of 10 s, actinic illumination ($205 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) was started to initiate the kinetic measurements. In the time course of 228 s, one saturation pulse ($2400 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR for 800 ms) was applied every 20 s. The tenth image, was generated and analyzed to determine the effective PS II quantum yield ($Y(II)$) (Genty et al., 1989) and quantum yield of regulated energy dissipation ($Y(NPQ)$) (Butler, 1978). While F_v/F_m measured after 'dark adapted' state estimates the maximum achievable quantum yield of PSII, $Y(II)$ measured after 'light induced' state estimates the effective quantum yield of PSII. The contributions of non photo-chemical quenching to changes in the PSII as observed in F_v/F_m measurement are not represented in $Y(II)$ measurement, thus $Y(II)$ represents the true quantum yield of PSII photochemistry (Baker, 2008).

The leaf area impaired for its ChlF by diquat was estimated on the basis of its fluorescence images. The pronounced difference in the pixel colour in the generated images of F_v/F_m and $Y(II)$, allowed the demarcation of tissue affected by diquat as against the unaffected area. As next, the pictures of F_v/F_m or $Y(II)$ were exported to ImageJ software (Abramoff et al., 2004). After conversion of the image into the 8 bit gray scale, the tissues having less than 10 % of the gray value than the unaffected area were demarcated with the freehand polygon selection. Area of the polygon was calculated to represent the tissue area affected in its ChlF as F_v/F_m impaired area and $Y(II)$ impaired area, respectively. The ChlF impaired area was measured from 2 h up to 26 h after treatment application, considering the scope of focal length of the camera, and to eliminate the possible error in its calculation after tissues start deforming due to desiccation.

2.7. Desiccation potential

The desiccation potential of the herbicide solutions was evaluated ten days after treatment application (n = 10 plants per treatment and species). Scoring of herbicidal injury was conducted in the scale of 1-5 (1, no visible injury; 5, complete plant desiccated). Plant above ground was cut, fresh biomass of plant weighed and then oven-dried (60°C) until constant weight (24-36 h). Visual injury scoring, of both the species, had the highest Pearson's' correlation coefficient ($r > 0.8$ ($P \leq 0.01$)) with the modified Relative Water Content (RWC) (Farrant, 2000) of the leaves. Modified RWC was calculated as ratio of water content of unit fresh weight of treated leaf to water content at full turgor of unit fresh weight of untreated control leaf. Later, the percent reduction of modified RWC as compared to control represented the desiccation potential of the treatment.

2.8. Data analysis

Statistical analysis was performed with SPSS (Statistics 18.0, SPSS Inc., Chicago, IL, USA) and graphs created with Microsoft Excel or OriginPro 8 (OriginLab Corporation, Northampton, MA01060, USA). Data were verified for homogeneity of variance and normality by Levene's and Kolmogorov-Smirnov test respectively, and mean was separated by Duncan's test at $P \leq 0.05$. Inability to validate homogeneity of variance or normality, in the case of droplet spread area and deposit area in *C. album*, data were transformed with Box-Cox transformation ($\lambda = -0.25$ and 0.25 respectively). Further, data unable to meet assumption even after transformation, non-parametric, Kruskal-Wallis, step-wise step down method was employed, and appropriately referred in the caption. Pearson's correlation coefficient between deposit structures (spread area, and deposit area) of RSO formulated diquat treatments with their effect on ChlF, ChlF impaired area, and desiccation potential was established with two-tailed t-test.

3. Results

3.1. Deposit structure

Diquat dibromide formed a crystalline residue after the droplet dried on the leaf surfaces. Unformulated diquat formed homogenous residues, while addition of surfactant induced heterogeneous deposits (Fig. 1). On the leaves of easy-to-wet, *V. arvensis*, surfactant had no effect on the spread area of the droplet. In some cases, the deposit area even shrank to almost half as compared to that of unformulated diquat (Fig. 2). However, within the RSO formulated treatments, with exception of RSO 10, the number of EO units had no significant effect on the deposit area of diquat residue in *V. arvensis*. In contrary, a pronounced effect of RSO surfactant on the deposit pattern of diquat was observed in the leaves of *C. album*. Inclusion of RSO 5 surfactant led the droplet spread area increase more than thrice and the deposit area more than twice, as compared to the unformulated diquat solution (Fig. 2). While, with RSO 60, deposit area of the diquat rather shrank to almost half, as compared to unformulated diquat, however maintaining the similar spread area. Additionally, diquat droplets formulated with RSO 5 caused the accumulation of a.i. sparsely in the droplet footprint. While the RSO 30 and RSO 60 induced a concentrated diquat deposit of large crystals at the centre of the droplet footprint.

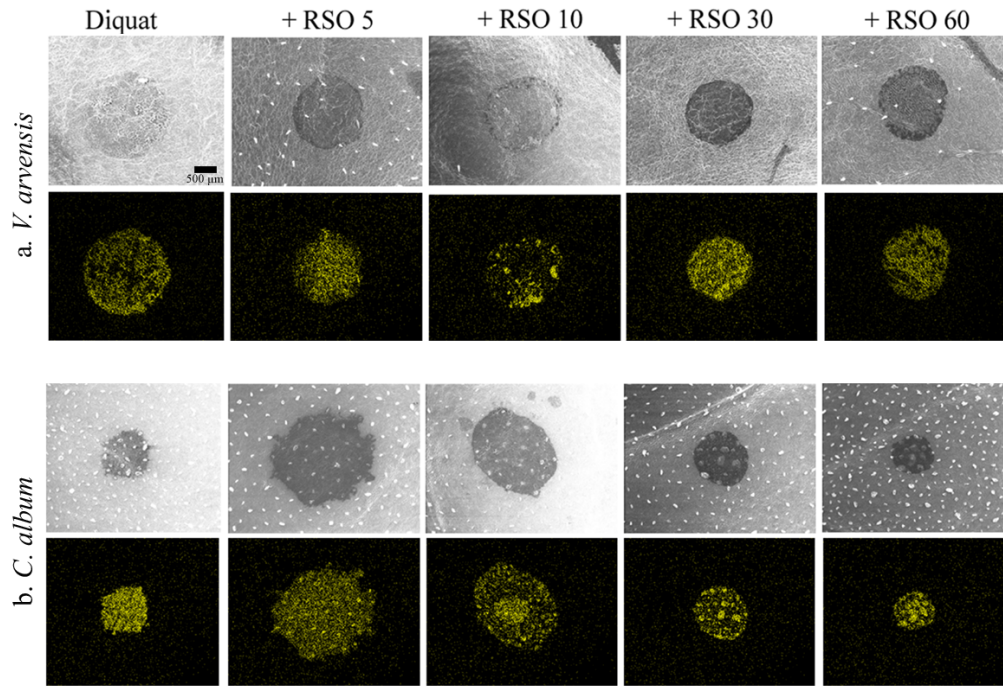


Fig. 1. Characteristic SEM micrographs demonstrating the droplet spread and the respective deposit pattern of dried residue of diquat dibromide solution droplets observed at ~ 1 h after its application. Droplets (1 μ L) of diquat with or without ethoxylated rapeseed oils (RSO) were applied on the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. The gray-scaled image taken by scanning electron microscope (SEM) represent the droplet spread area, while the yellow coloured image taken by energy dispersive X-ray microanalysis (EDX) represent the a.i. deposit area.

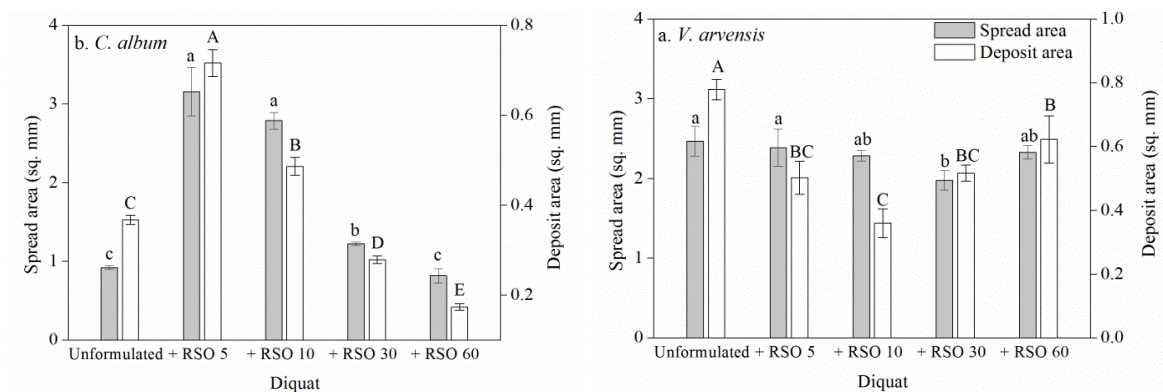


Fig. 2. Droplet spread area and deposit area of dried residue of diquat dibromide solution droplets observed at ~ 1 h after its application. Droplets (1 μ L) of diquat solution with or without ethoxylated rapeseed oils (RSO) were applied over the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. Error bars represent +/- standard error of the mean (n = 4). Similar letters (case sensitive) above the bar represent non-significant differences between the means ($P \leq 0.05$; Duncan's multiple range test).

3.2. Chlorophyll fluorescence

Maximum quantum efficiency of PS II (F_v/F_m)

The F_v/F_m was slightly reduced in the diquat treated tissues as early as 2 h after treatment (Fig. 3). The F_v/F_m continued to decrease in the time-course until the final evaluation at 51 h. There was no significant difference in F_v/F_m between the treatments in *V. arvensis*. In contrast, on *C. album*, the treatments significantly differed in their impact on F_v/F_m , which was demonstrated as early as 6 h after herbicide application. Likewise, although, RSO 30 formulated diquat had the strongest effect on decreasing the F_v/F_m values at 6 h, RSO 60 formulated diquat had the strongest effect at 51 h. Representative pictures to the impact of the treatment groups on the ChlF parameters F_v/F_m and $Y(II)$ are presented in Fig. 4.

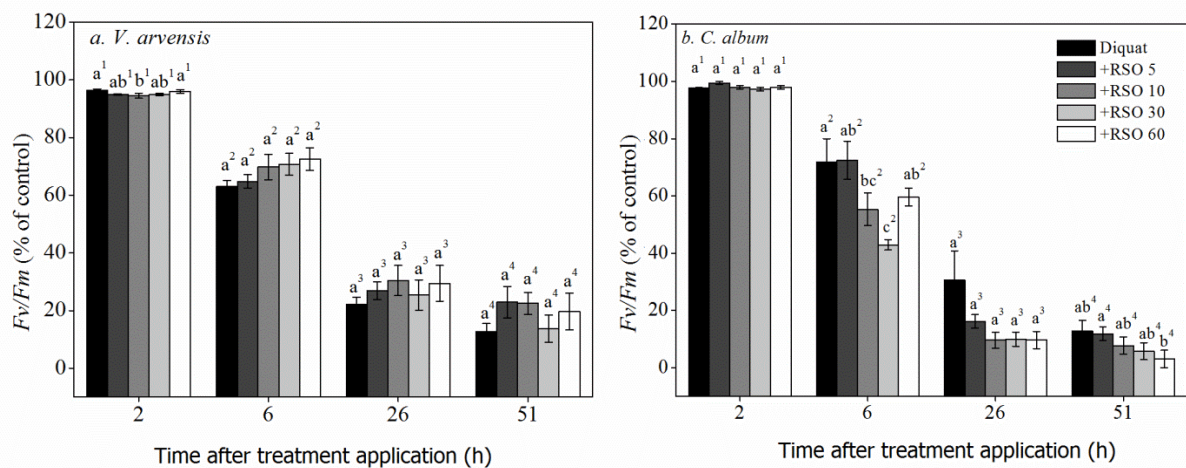


Fig. 3. Maximum quantum efficiency of PS II (F_v/F_m) as affected by diquat dibromide solution droplets. Droplets (1 μ L) of diquat with or without ethoxylated rapeseed oils (RSO) were applied over the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. Error bars represent \pm standard error of the means ($n = 10$). Similar letters (together with superscript) above the bar, represent non-significant differences between the means ($P \leq 0.05$; Kruskal-Wallis, step-wise step down).

Quantum yield of PS II ($Y(II)$) and non-photochemical quenching ($Y(NPQ)$)

The effective PS II quantum yield, $Y(II)$ of the treated leaf tissue, sharply declined with the treatment period. In contrast, quantum yield of regulated energy dissipation, $Y(NPQ)$, increased at the beginning, followed by a sharp decline. However, there was no significant difference between the treatments, either for $Y(II)$ or $Y(NPQ)$, in both the species as presented in Fig. 5.

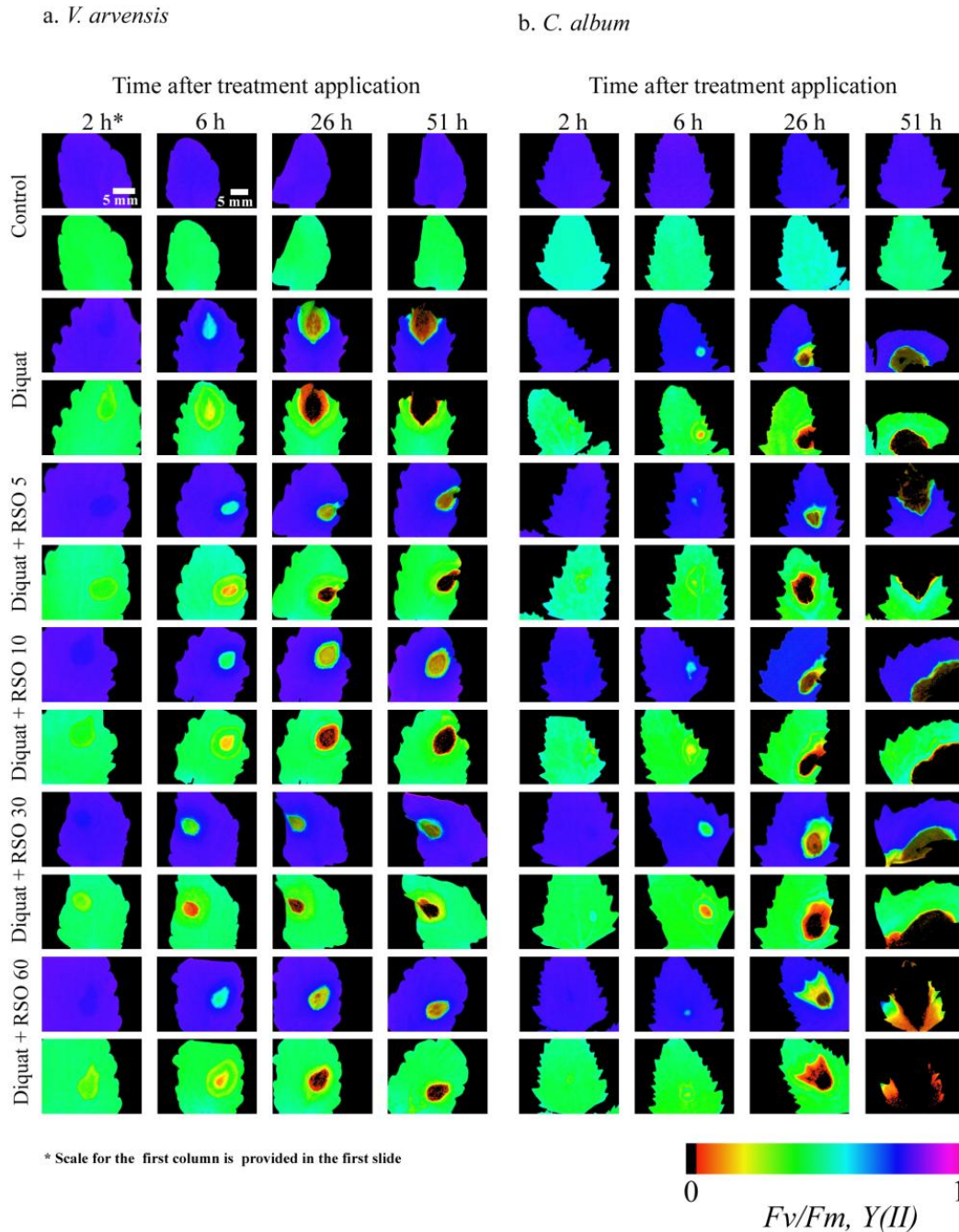


Fig. 4. Representative Pulse Amplitude Modulated (PAM) images of Chlorophyll fluorescence (ChlF) parameters, maximum quantum efficiency of PS II, Fv/Fm and the effective quantum yield of PS II, $Y(II)$, of the leaf tissue as affected by treatment of diquat formulations. Droplets (1 μ L) of diquat with or without ethoxylated rapeseed oils (RSO) were applied on the easy-to-wet leaves of a. *V. arvensis* and the difficult-to-wet leaves of b. *C. album*. The impact on ChlF was recorded at different times after treatment application. In the first row, Fv/Fm is shown, while in the second row the $Y(II)$ is presented. The shades of colour represent the value of $Fv/Fm, Y(II)$ in the range between 0 to 1 as shown in the index.

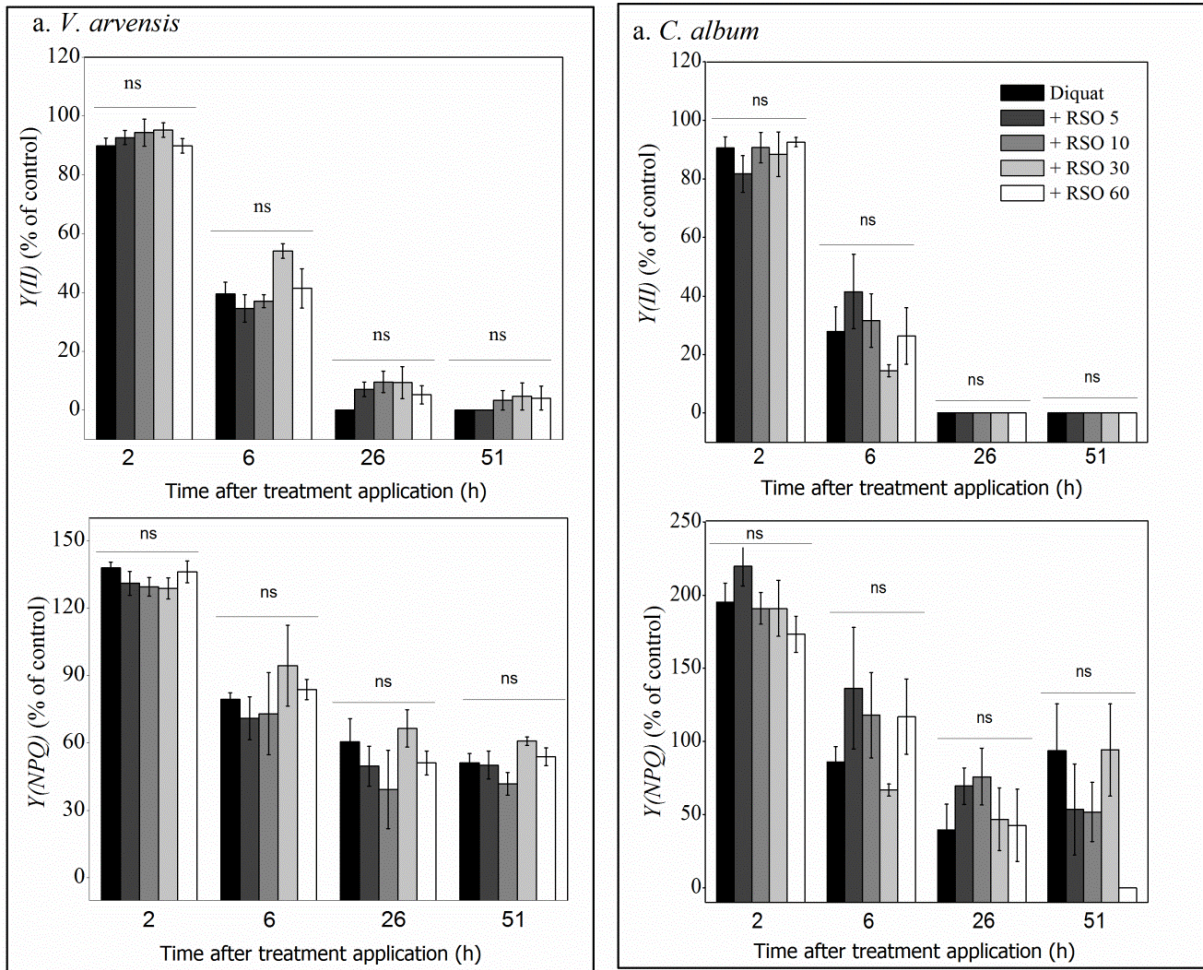


Fig. 5. Effective quantum yield of PS II ($Y(II)$) and non-photochemical quenching ($Y(NPQ)$) as affected by diquat dibromide solution droplets. Droplets ($1 \mu\text{L}$) of diquat with or without ethoxylated rapeseed oils (RSO) were applied over the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. The error bar represent \pm standard error of the means ($n = 4$). The letter 'n.s.' above the bar represent non-significant differences between the means within the group (represented by line above the bars) ($P \leq 0.05$; Kruskal-Wallis, step-wise step down).

3.3 F_v/F_m or $Y(II)$ impaired area and desiccation potential of diquat

The impaired area as affected by diquat formulations measured after light induction ($Y(II)$) was significantly larger than its dark adapted counterpart (F_v/F_m) (Fig. 6). As expected, ChlF impaired area due to diquat formulations increased with time after application. On the leaves of *V. arvensis*, unformulated diquat caused the largest F_v/F_m and $Y(II)$ impaired area. However, treatments within RSO formulated groups did not differ in terms of ChlF impaired area over the whole measurement time period. In contrary, on the leaves of *C. album*, RSO 60 formulated diquat had the largest F_v/F_m and $Y(II)$ impaired area.

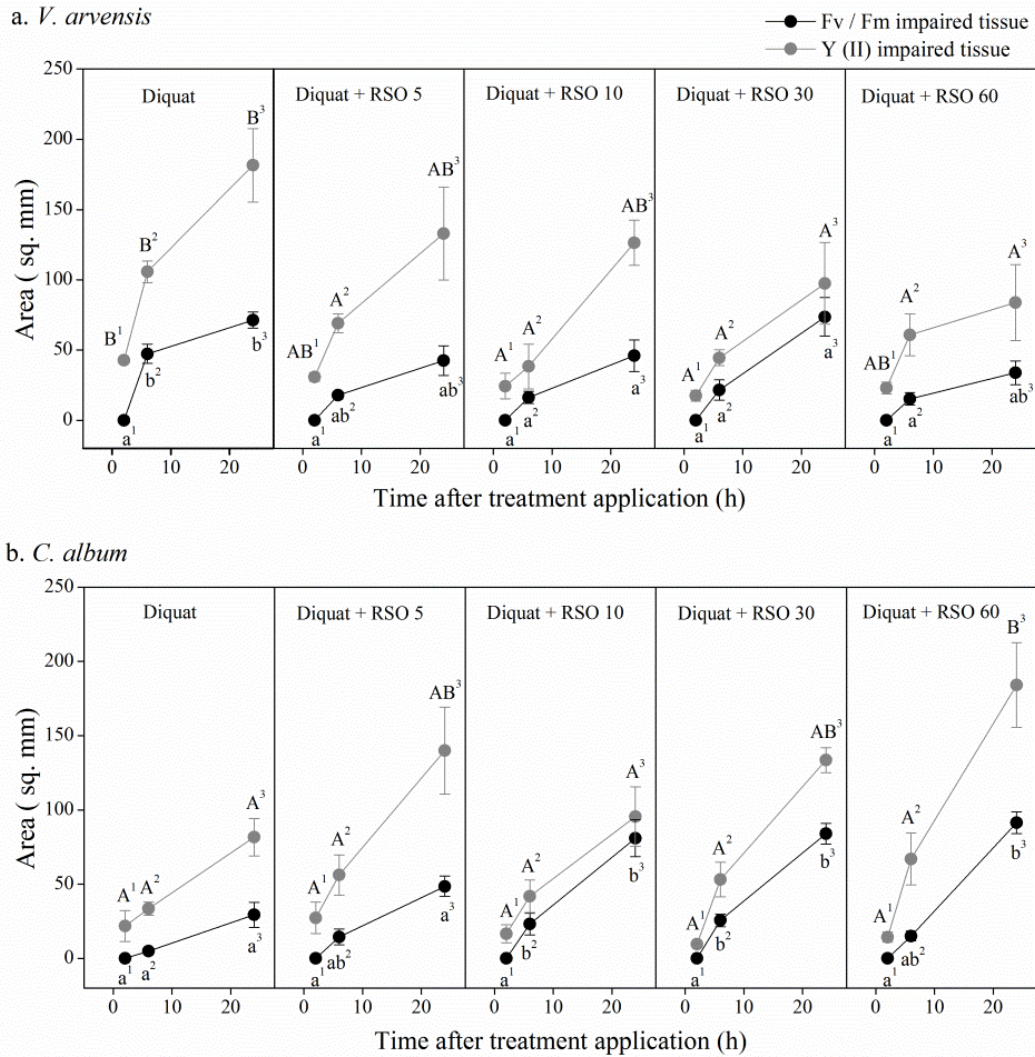


Fig. 6. Spatial and temporal effect of diquat dibromide solution droplets on impairing the tissues for its maximum quantum efficiency of PS II (Fv/Fm) and the effective quantum yield of PS II ($Y(II)$). Droplets ($1 \mu\text{L}$) of diquat with or without ethoxylated rapeseed oils (RSO) were applied over the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. The impaired area represents the tissue impaired area for its Fv/Fm or $Y(II)$. Error bars represent \pm standard error of the mean ($n = 10$, dark adapted and $n = 4$, light induced). Similar letters (together with superscript; case sensitive) above the bar, represent non-significant difference between the means ($P \leq 0.05$; Duncan's multiple range test).

As additional parameter, we evaluated the desiccation potential of diquat solutions. In *V. arvensis* the highest desiccation was obtained with diquat + RSO 30, but there was no clear relationship between the ethoxylation degree of RSO and the desiccation potential (Fig. 7). In contrast, in *C. album*, diquat + RSO 60 caused the highest desiccation of the tissue; in this

case, there was a clear relation between number of ethylene oxide units and the desiccation caused by the herbicide.

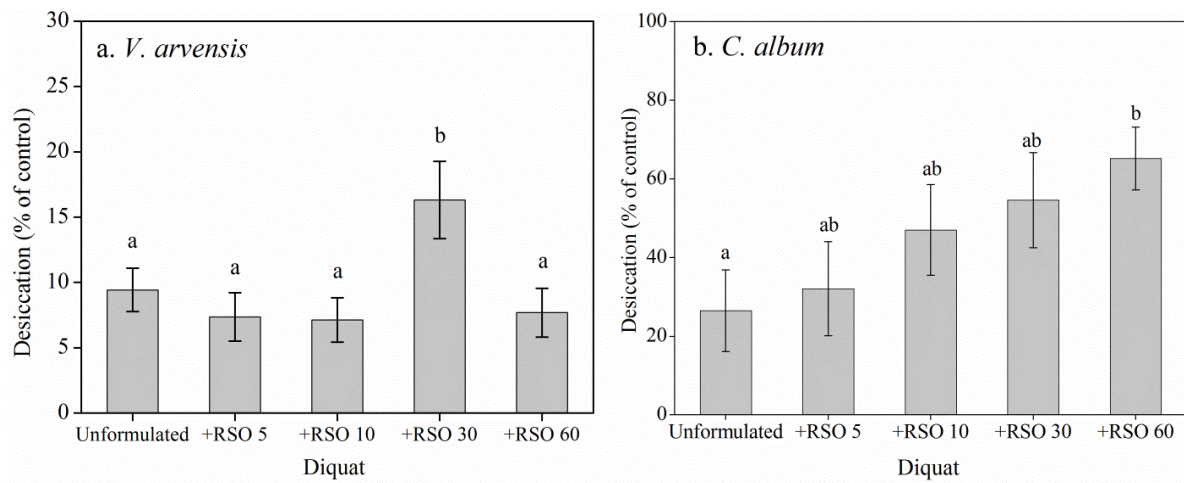


Fig. 7. Effect of diquat dibromide solution droplet on desiccation of the leaves. Droplets (1 μ l) of diquat with or without ethoxylated rapeseed oils (RSO) were applied over the easy-to-wet leaves of a. *V. arvensis* and difficult-to-wet leaves of b. *C. album*. Desiccation potential of the treatment was measured as a ratio between relative water content of treated leaf to the relative water content of untreated control leaf, measured at 10 days after its treatment application. Error bars represent \pm standard error of the mean ($n = 10$). Similar letters above the bar, represent non-significant difference between the means ($P \leq 0.05$; Duncan's multiple range test).

3.4. Relationship between deposition structure, chlorophyll fluorescence parameters and herbicide desiccation

For this analysis, only the RSO-formulated diquat treatments were used to establish the relationship. The correlation analysis confirmed for *V. arvensis*, that there was no significant relation between deposit area of diquat and its effect on the chlorophyll fluorescence parameters, ChlF impaired area, and the desiccation of the tissue (Table 1). In contrast, the analysis for *C. album* indicates a significant positive relation between the deposit area and the F_v/F_m measured 51 h after treatment application, the F_v/F_m impaired area at 26 h after treatment, and to the final desiccation of the tissue. In contrast to the deposit area, droplet spread area did not reveal any significant correlation to any of the evaluated parameters. In addition, neither the deposit area nor the spread area of the droplet were related to the reduction of the $Y(II)$ and $Y(NPQ)$.

Table 1. Relationship between deposit structure of diquat dibromide, and its bio-efficacy represented by chlorophyll fluorescence (ChlF) parameters and desiccation of the tissue. Pearson's correlation coefficient between deposition structure of diquat formulations on easy-to-wet leaves of *V. arvensis* and difficult-to-wet leaves of *C. album*, with their effect on ChlF parameters (dark-adapted *Fv/Fm* and light-induced *Y(II)*), the impaired area of the tissue at different times after application (h), and the desiccation potential.

<i>Spp.</i>	<i>Bio-efficacy</i> <i>Deposit structure</i>	<i>Dark adapted</i>				<i>Light induced</i>				<i>Desiccation potential (10 days after treatment)</i>
		<i>Fv/Fm values</i>		<i>Fv/Fm impaired area</i>		<i>Y(II) values</i>		<i>Y(II) impaired area</i>		
		<i>6 h</i>	<i>51 h</i>	<i>6 h</i>	<i>26 h</i>	<i>6 h</i>	<i>51 h</i>	<i>6 h</i>	<i>26 h</i>	
<i>V. arvensis</i>	<i>Spread area</i>	-0.42	0.49	-0.45	-0.71	-0.57	-0.65	0.57	0.05	- 0.42
	<i>Deposit area</i>	0.39	-0.66	0.31	0.18	0.60	0.34	0.01	-0.81	0.73
<i>C. album</i>	<i>Spread area</i>	0.60	0.95	-0.15	-0.91	-0.72	. ^a	-0.57	-0.63	- 0.93
	<i>Deposit area</i>	0.67	0.99*	-0.25	-0.97*	-0.84	. ^a	-0.43	-0.50	- 0.96*

* = Significant at $P \leq 0.05$; $n = 4$; .^a - Correlation analysis couldn't be conducted for *Y(II)* being '0'

4. Discussion

In this study, we aimed to better understand the relationship between deposit properties at the microscale level, and the uptake and bio-efficacy of herbicidal compounds. In previous studies, we demonstrated that while decreasing the deposit area of glyphosate, its efficacy significantly increased (Basi et al., 2013). However, to this point, it was not clear whether the enhanced bio-efficacy is related to its enhanced uptake or the systemic phytotoxicity, or both together. Thus, the diquat dibromide having a contact mode of action was selected for the sequential studies. Likewise, although, ChlF techniques has been proposed for studying the penetration and translocation process of the PSII inhibitors (Yanase and Andoh, 1992), its use for PSI inhibitor is yet to be explored. Thus, we monitored different deposit structure of diquat dibromide, influenced by surfactant by studying its indirect effect on ChlF of the tissue.

Diquat crystallized in the droplet footprint resulting in distinct distribution patterns (Fig. 1). The cause is the differential surface tension as affected by RSO surfactants,

significantly affecting the droplet spread on difficult-to-wet leaves of *C. album* but not on easy-to-wet leaves of *V. arvensis*. Unformulated diquat solution droplet formed a homogenous deposit of a salt in the droplet footprint (Fig. 1). The homogenous, spherical deposits of particles were observed when the evaporation rate in the droplet periphery was hindered (Deegan, 2000). RSO surfactants with increasing ethoxylation degree induced the diquat salts to be deposited more towards the inner of the droplet footprint, particularly on the leaves of *C. album*. The phenomenon resembles a Marangoni stress mediated deposition of solutes towards the centre of the droplet (Hu and Larson, 2006). Surfactant oil molecules being lighter, might have concentrated on top of the droplet causing a local reduction of the surface tension, resulting in a Marangoni flow of liquid current towards the centre of the droplet from periphery (Faers and Pontzen, 2008). Apart from such stresses to a forced accumulation of particle in the periphery of droplet (Deegan et al., 1997), or to the centre of the droplet (Hu and Larson, 2006), particle dimension also affects the deposition behaviour of the solutes in the droplet (Yunker et al., 2011). Likewise, while drying, after reaching super saturation concentration, diquat might have crystallized; thus, mobilization of bigger salt particle in the droplet might have been hindered, in contrast to a very strong centralized amorphous deposit of glyphosate in similar situation (Krämer et al., 2009).

Diquat affected both the maximum (Fv/Fm) and the effective ($Y(II)$) quantum efficiency of PS (II), as well as the quantum yield of regulated energy dissipation $Y(NPQ)$ (Fig. 3 and Fig. 5) as early as 2 h. Significant differences in the values of Fv/Fm as induced by the formulations were detected in *C. album* at 6 h after treatment application, but not in *V. arvensis* (Fig. 3 and Fig. 6). In addition, the RSO surfactant themselves had no effect on the different parameters of the chlorophyll fluorescence (*data not shown*). This phenomenon confirms the suitability of ChlF technique in uptake study of diquat in agreement with Habash et al. (1985), where, this technique was used to study the foliar penetration of PSII inhibitor, diuron. Apart from popularly used ChlF parameters, to determine the physiological status of the tissue, such as $Y(II)$ or $Y(NPQ)$ (Yanase and Andoh, 1992; Habash et al., 1985), we have analysed the area of the leaf tissue with herbicide-induced changes in its chlorophyll fluorescence. Over the leaves of *C. album*, diquat formulated with RSOs of higher ethoxylation degree reduced the Fv/Fm to the lowest level (Fig. 3), but not in the leaves of *V. arvensis*. This phenomenon clearly shows that the number of EO units in the RSO surfactants do not play any role for the diquat penetration in leaves of *V. arvensis*, however, does play a significant role in the leaves of *C. album*.

The relationship between the deposit area, the ChlF parameters and the respective tissue ChlF impaired area was studied with a correlation analysis (Table 1). However, amidst the evidences that surfactant might influence the cuticles, such as cuticle-wax fluidity (Schreiber, 1995) and cuticle-water permeability (Coret and Chamel, 1995), the relationship was studied only within the RSO formulated diquat formulations. Here, the non-significance of the EO units of RSO surfactant for the deposit area of diquat coincided also with the non-significance in ChlF parameter (Fv/Fm), and the ChlF impaired tissue and its desiccation potential on easy-to-wet leaves of *V. arvensis*. This confirms our previous observation with glyphosate phytotoxicity (Basi et al., 2013). In contrary, on *C. album* the decreased deposit area of diquat due to addition of RSO surfactants reduced the Fv/Fm (at 51 h after application), Fv/Fm impaired tissue area (at 26 h after application) and increased its final desiccation potential. This proves that decreased deposit area enhanced the penetration of diquat in the leaves of *C. album*. The cuticular penetration of foliar applied chemicals is assumed to be largely driven by the concentration gradient-mediated diffusion process (Fick, 1855). Additionally, droplets of larger spread area evaporates faster (Xu et al., 2011), limiting the time of the droplet to remain in the liquid phase. As result, the active ingredient has less time for cuticular penetration, while under natural conditions the stomatal uptake might happen after wetting and drying cycles (Burkhardt et al., 2012). Previously, we reported a similar phenomenon of increased bio-efficacy of the systemic herbicide glyphosate when a centralized amorphous concentrated deposit, was reached due to RSO surfactants of higher ethoxylation degree (Basi et al., 2013). Although, the enhanced concentration gradient improved uptake of both hydrophilic compounds - diquat and glyphosate, the uptake of lipophilic compounds like gibberellic acid and 2,4-D were rather negatively related to its concentration (Knoche and Bukovac, 1999). In addition, foliar uptake of lipophilic a.i. was enhanced when rather deposited in the periphery of the droplet, where the surfactant and a.i. association was the highest (Faers, 2007).

In conclusion, surfactants had no clear influence on the deposit behaviour of diquat on the leaves of easy-to-wet *V. arvensis*. Analogous to that, the spatio-temporal development of Fv/Fm and the associated impaired tissue area, as well as the desiccation potential on the leaf tissue were not affected. In contrast, on the leaves of difficult-to-wet *C. album* we observed a RSO surfactant containing more EO units, decreased a deposit area of diquat. In relation, Fv/Fm was decreased and its associated impaired tissue area, as well as desiccation potential of diquat was increased.

5. References

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E. The role of hygroscopic salts for the stomatal penetration and bio-efficacy of glyphosate

1. Introduction

The use of adjuvants in enhancing the efficacy of foliar applied chemicals dates back to the 18th century, when soap was first used to increase the toxicity of arsenic formulations against weeds (Gillette, 1888). Amongst others, adjuvants have gained an importance as penetration agents through cuticles (Manthey and Nalewaja, 1992) and the mass flow through stomata (Zabkiewicz et al., 1993). The mass flow through stomata, however, was thought impossible unless the surface tension of the liquid is drastically reduced below 30 mN m⁻¹ (Schönherr and Bukovac, 1972). But, recently, the concept of the hydraulic activation of stomata was introduced, where hygroscopic salts can enable a mass flow of liquid inside the stomata, although its surface tension is well above 30 mN m⁻¹ (Burkhardt et al., 2012). This may happen by repeated deliquescence and efflorescence cycles of hygroscopic crystalline salt particles, which leads to increased contact area and cause the spatial expansion of salt dendrite crystals (Gao et al., 2007; Burkhardt et al., 2012). Stomata are the sites of transpiration, and have the highest evaporation rate, with fluctuating relative humidity (Anders, 1878; Tyree and Yianoulis, 1980). Fluctuations in moisture at the boundary layer, also influenced by turbulent elements at this level, drive the spatial expansion of salt towards the stomata. In this way, thin water film may develop connecting the apoplast with the leaf surface along the stomatal walls, thus causing the hydraulic activation of stomata (HAS), potentially leading to stomatal penetration of liquids (Burkhardt, 2010).

Solution droplets containing small amounts of salt will form highly concentrated solutions due to water evaporation, and may alter the surface energy of the solution (Dutcher et al., 2010). Hygroscopic salts are classified according to the Hofmeister series, which originally classified ions in electrolytic solutions according to their ability to dissolve proteins (Hofmeister, 1888; Kunz et al., 2004). For anions, the series follows, SO₄²⁻ > F⁻ > Cl⁻ > Br⁻ > NO₃⁻ > ClO₃⁻ > Γ > ClO₄⁻ > SCN⁻ and, for cations, NH₄⁺ > Cs⁺ > Rb⁺ > K⁺ = Na⁺ > H⁺ (Zhang and Cremer, 2006). Anions that are considered to be kosmotropic are at the left of Cl⁻ and chaotropic ions are at the right (Zhang and Cremer, 2006). The kosmotropes (water structure makers) increase the surface tension of the solution, while, the chaotropes (water structure breakers) reduce it (Leroy et al., 2010).

Several types of salts and oils might be used as adjuvants (e.g., surfactants, humectants, spreaders, nitrogen fertilizers, and water conditioners) mostly to increase the

efficiency of foliar applied herbicides (Greene and Bukovac, 1974; Thelen et al., 1995; Ramsey et al., 2006). Organosilicone surfactants are widely adopted to drastically reduce the surface tension (ST) of spray solutions below the critical ST of the leaf surface (20 to 30 mN m⁻¹), leading to a stomatal infiltration (Greene and Bukovac, 1974; Buick *et al.*, 1992; Knoche and Bukovac, 1993; Field and Bishop, 1998). Likewise, ammonium sulfate ((NH₄)₂SO₄) is widely used for overcoming the antagonism of hard water (Suwunnamek and Parker, 1975; Nalewaja and Matysiak, 1993; de Villiers and du Toit, 1993), or neutralizing such ions within and on the leaf tissue (Thelen et al., 1995; Hall et al., 2000). Several other salts might be used as defoliant agents. Examples include sodium chlorate (NaClO₃), magnesium chlorate, potassium iodide, which are used as defoliants or desiccant contact herbicide to control a wide spectrum of weeds (Blum et al., 1983; Forbes and Pratley, 1983; Bennett and Shaw, 2000; Bond and Bollich, 2007). Although the long-term use of salts as adjuvant or as a defoliant, their possible role as penetrant enhancers needs further target-oriented investigations.

Glyphosate (N- (phosphonomethyl) glycine) (Gly) (Franz, 1974), is a broad-spectrum systemic herbicide and the worlds' most important pesticide (Copping, 2002). Chemically, it is an aminophosphonic analogue of the natural amino acid glycine. Increased Gly efficacy might result from the formation of Gly–ammonium salt complexes that are more readily absorbed by foliage than Gly–calcium or Gly–sodium complexes (Nalewaja and Matysiak, 1993). Thelen et al. (1995) concluded that the addition of (NH₄)₂SO₄ stabilize calcium in the hard water, leaving the remaining NH₄⁺ to combine with Gly to form the Gly–ammonium salt. This resulted in greater absorption of glyphosate into leaves.

The relevant pathways for the uptake of foliar applied compounds comprise the cuticle and the cuticular polar pores (Schönherr, 1976) and the stomata (Eichert et al., 1998; Burkhardt et al., 2009). Although, many authors referred to the possible involvement of stomata for foliar uptake (Sargent, 1961; Middleton, 1964; Eddings, 1967; Sands and Bachelard, 1973; Schönherr and Bukovac, 1978; Schlegel and Schönherr, 2002), there was no elucidation on how this occurs. To date only indirect evidence is available for the presence of cuticular polar pores (Koch and Ensikat, 2008). In contrast, the direct proof of stomatal uptake of fluorescent polystyrene nanoparticles having 43 nm in diameter, otherwise impossible through polar route (of estimated 0.6 (Popp et al., 2005) to 4.8 nm (Eichert and Goldbach, 2008), has been revealed (Eichert et al., 2008). As next, Burkhardt et al. (2012) showed that the presence of hygroscopic salts can enhance the stomatal uptake of foliar

uptake of chemicals. This phenomenon might have big implications in explaining the penetrant capacity of hygroscopic salts for foliar applied agrochemicals.

Thus, as continuation to our experimental series (Burkhardt et al., 2012), the role of different hygroscopic salts for the penetration of Gly on easy-to-wet leaves of *Viola arvensis* and difficult-to-wet leaves of *Chenopodium album* was studied. Real time images of salt activity around the stomata, taken by scanning electron microscopic pictures, were studied. The influence on the uptake was quantitatively studied by comparing the bio-efficacy of Gly affecting, bio-mass, and the formation of necrotic tissues. Gly solutions differing in the concentration of $(\text{NH}_4)_2\text{SO}_4$ or NaClO_3 were formulated with or without organosilicone Break-Thru® S233 to induce stomatal infiltration, and were applied over adaxial or abaxial leaf sides. Further, hygroscopic salts of sodium, with their ions specific kosmotropic to chaotropic nature as classified in the Hofmeister series, were used to evaluate their possible role on foliar uptake of Gly. We hypothesized that, both the kosmotropic and chaotropic salts lead to the formation of HAS. Compared to the kosmotropes, chaotropes would break the surface tension of the liquid to the strongest, leading to a higher stomatal uptake, and ultimately enhance the bio-efficacy of Gly to the highest.

2. Material and Methods

2.1. Trials

The experiments were conducted in two phases i.e. two trials. In the first trial, the possible role of stomatal uptake of Gly supported by hygroscopic salts, i.e. ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ of kosmotropic nature (Trial 1A) and sodium chlorate (NaClO_3) of chaotropic nature (Trial 1B), were studied. In Trial 2, different sodium salts having anions specific to kosmotropic and chaotropic natures were studied for their effect on the penetration and bio-efficacy of Gly.

2.2. Plant material

Two weed species with distinct wetting behavior, the easy-to-wet *Viola arvensis* (having amorphous epicuticular wax layer) and the difficult-to-wet *Chenopodium album* (having crystalline epicuticular waxes), were grown in the greenhouse and the growth chamber. After germination of the seeds (Herbiseed Co., Reading, UK), ~ 1 week seedlings were transplanted to individual pots filled with a mixture of soil/sand/perlite (12/3/1). Seedlings in Trial 1A were allowed to grow in greenhouse at 19.1 ± 3.4 °C and 62.2 ± 7.2 % RH,

photoperiod of 16 h ensured by additional light source ($214 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, PAR). In Trial 1B, seedlings were grown in growth chamber at $20/15^\circ\text{C}$, 70 / 80 % RH; day / night, photoperiod of 16 h ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR). In Trial 2, seedlings were grown in greenhouse at $27.0 \pm 6.4^\circ\text{C}$ and $53.1 \pm 15.9\%$ RH. Sufficient irrigation was provided through the bottom of the pots.

2.3. Stomatal density

The density of stomata was determined in microscopic images taken from the adaxial and abaxial leaf surfaces. Scanning electron microscopic images were taken with a XL 30 ESEM microscope (FEI-Philips Co., Eindhoven, The Netherlands). Fresh leaves were observed at 150x magnification, low vacuum mode with pressure 0.12 kPa; spot size 4.9 nm; acceleration voltage 15.5 kV and at working distance of 10 mm. The stomatal count of the leaf surfaces are provided in Table 1.

Table 1. Stomata density on the adaxial and abaxial surfaces of *V. arvensis* and *C. album* leaves. The variations observed between the experiments are explained by the environmental conditions and their impact on the plant development.

Trial	Species	Stomata density (number per $\text{mm}^2 \pm \text{SD}$)		P-value	Ratio Adaxial/Abaxial
		Adaxial	Abaxial		
1A	<i>V. arvensis</i>	33.9 ± 8.2	67.9 ± 11.4	**	0.5
	<i>C. album</i>	71.9 ± 12.7	122.1 ± 33.1	**	0.6
1B	<i>V. arvensis</i>	31.0 ± 8.2	52.5 ± 8.7	**	0.6
	<i>C. album</i>	55.6 ± 9.2	74.1 ± 6.4	**	0.8
2	<i>V. arvensis</i>	32.4 ± 9.5	-	-	-
	<i>C. album</i>	92.9 ± 15.5	-	-	-

** - $P \leq 0.01$ Student t-test ($n > 10$ samples)

2.4. Treatment solutions

Glyphosate (Gly) solutions (43 mM) were prepared with a 62 % non-surfactant formulated isopropylamine salt of glyphosate (Monsanto Europe N.V., Antwerp, Belgium). In Trial 1A, treatment solutions with or without (w/o) ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$; 25 and 150 mM), w/o Gly, and w/o Break-Thru® S233 (BT; 0.1 % v:v; Evonik Goldschmidt GmbH, Essen,

Germany) were prepared with deionized water. In Trial 1B similar solutions were prepared, except by replacing $((\text{NH}_4)_2\text{SO}_4)$ by sodium chlorate (NaClO_3). In Trial 2, salt solutions (sodium sulfate, Na_2SO_4 ; sodium chloride, NaCl ; sodium nitrate, NaNO_3 ; sodium chlorate, NaClO_3) in a concentration of 300 mM (99% purity) were prepared with deionized water and mixed with BT 0.01 % or BT 0.1% (v/v), and w/o Gly. None of the treatment solutions had a precipitate.

2.5. Surface tension

Surface tension (ST) of the solutions was measured by pendant drop method (DSA30E, Krüss GmbH, Hamburg, Germany). We determined the ST of Gly solutions in a concentration range (43, 215, 430 and 579.15 mM) alone or in combination with the sodium salts (300, 1500, 3000 and 4040.61 mM), and with BT (0.1, 0.5, 1 and 1.35 % v/v). The selected concentrations correspond to the expected concentrations when the droplet volume dries to 20, 10 and 7.42 % from initial volume; the initial droplet having following concentrations: Gly, 43 mM; sodium salts, 300 mM; BT, 0.1 % v/v.

2.6. Treatment application

In Trial 1A and 1B, homogenous plants having 6-8 leaf pairs (23 - 25 days after transplanting) were chosen to place individual 2 μl droplets of the test solutions over the adaxial or abaxial sides of the second fully expanded leaf. In Trial 2, homogenous plants at the 8-10 leaf stage (21 days after transplanting) were selected and six 0.5 μl droplets were applied on two opposing sides of leaf lamina. Application of droplets was done manually with a micro pipette (Hamilton Bonaduz, Bonaduz, Switzerland).

2.7. Microscopic images of salt activity around stomata

Images of stomata and dried residues of the treatment solutions were taken with an environmental scanning electron microscope (XL 30 ESEM) about 24-36 h after droplet placement. Analyses were done at accelerating voltage 15-20 kV, with the spot size 4.5- 5.8 nm. The relative humidity inside the chamber was maintained below the deliquescence point of the salts i.e., at 40-60 % RH by maintaining the pressure between 0.53-0.80 kPa, and temperature at sample level between 8-12 °C.

2.8. Bio-efficacy

The reduction of the aboveground bio-mass, as compared to the untreated control plants, was used as indicator for the bio-efficacy of the treatment solutions. Thus, higher the fresh weight of the plant, lower is the bio-efficacy of formulations. Aboveground fresh biomass was cut and weighed in Trial 1 (16 days after treatment) and Trial 2 (20 days after treatment). In Trial 2, including fresh biomass, its dry weight (oven dried at 60 °C) was also measured after achieving the constant weight.

2.9. Necrotic area

In Trial 1B, the occurrence of necrosis was evaluated 16 days after treatment (n = 10) in a visual scoring (0-10 scale), where 0 indicates no necrotic symptoms and 10 indicates necrosis on the whole leaf. In Trial 2, evaluation of necrotic area was done 20 days after treatment application; here, the presence of necrosis was scored '1' while the absence was scored '0' (n = 18), irrespective of the size of the necrotic area.

2.10. Evaluation and statistical analyses

All trials were conducted in a completely randomized setup and analyzed as single factorial design. The Trial 1A and 1B had 24 treatment levels [3 ((NH₄)₂SO₄ or NaClO₃) X 2 (BT) X 2 (Gly) X 2 (leaf side)] and 10 and 9 plants per treatment level, respectively. The Trial 2 had 7 treatment levels [Na₂SO₄, NaCl, NaNO₃, NaClO₃ (at 300 mM), 0.01 % BT, 0.1% BT (v:v) and untreated control] and 18 plants per treatment level. Untreated plants served as control treatment in all the experiments. Data on the necrotic tissue were analyzed for all the treatments, while those evaluating biomass were analyzed only when Gly was included in the treatment solution. Data were tested for normality by the Kolmogorov-Smirnov test, and homogeneity of variance by the Levene test. By failing those assumptions, data were subjected to Box-Cox transformation, and appropriately referred in the caption of the figures. ANOVA was conducted and means were separated with the least significant difference at P ≤ 0.05. For those data which did not match the prerequisites even after transformation, non-parametric - Kruskal-Wallis test was applied, and appropriately referred in the caption of the figures. All the statistical analyses were conducted in IBM SPSS Statistics 20. Graphs are prepared with OriginPro 8.0.63.988 (OriginLab Corporation, MA, USA).

3. Results

3.1. Surface tension of the solutions

The surface tension (ST) of the solutions was measured in the concentration ranges expected in a drying droplet placed on the leaf surface (Fig. 1). The ST of pure water (72.7 mN m^{-1}) was slightly affected by addition of Gly ($77.4 - 70.0 \text{ mN m}^{-1}$; as expected when it dries to 7.4 % of initial volume). Likewise, at solute concentrations, of both Gly and hygroscopic salt, equivalent to the concentrations when the solution droplet dries to 7.4 % of initial volume, the ST strongly decreased to 45 mN m^{-1} with chaotropic NO_3^- and ClO_3^- ions, while, with SO_4^{2-} and Cl^- ions, ST declined moderately to 57 mN m^{-1} . The presence of BT (0.01 % v:v) reduced the ST to almost 53 mN m^{-1} (*data not shown*) while at 0.1% the ST was lower than $\sim 20 \text{ mN m}^{-1}$.

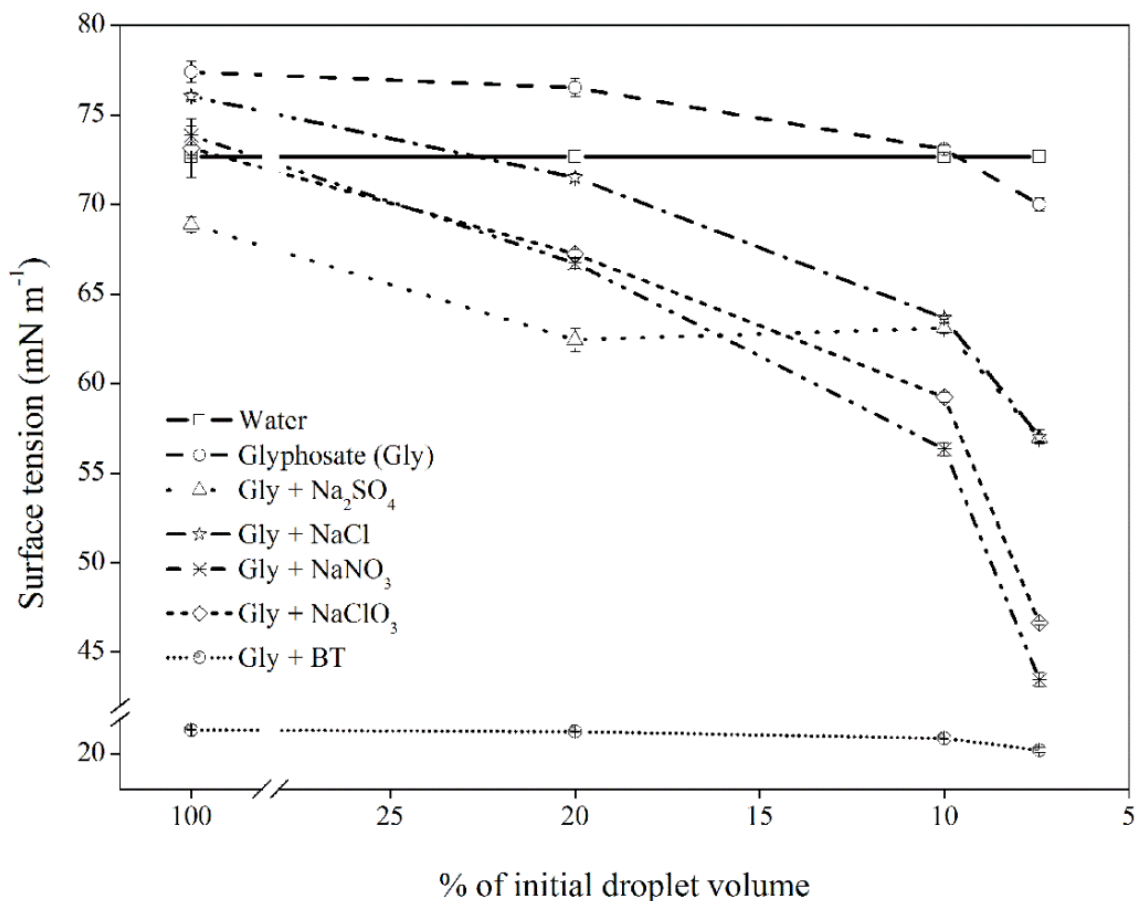


Fig. 1. Surface tension of glyphosate (Gly) solutions prepared with or without hygroscopic salts and the organosilicone surfactant Break-Thru® S233 (BT). Measurements were done at the concentrations expected in the droplet after it starts drying from the initial concentration of 43 mM (Gly), 300 mM (hygroscopic salts) and 0.1% (v:v; BT). Mean \pm S.E. (n = 10).

3.2. Microscopic images of dried deposits

Glyphosate formed a homogenous amorphous deposit on the leaf surfaces while salts were observed as structured crystals or amorphous deposits. In contrast to the dendritic crystalline structure of $(\text{NH}_4)_2\text{SO}_4$ and Na_2SO_4 , NaCl and NaNO_3 formed cubic crystalline structures with some dissolved amorphous residues; further, NaClO_3 formed a complete homogenous crust (Figs. 2 and 3).

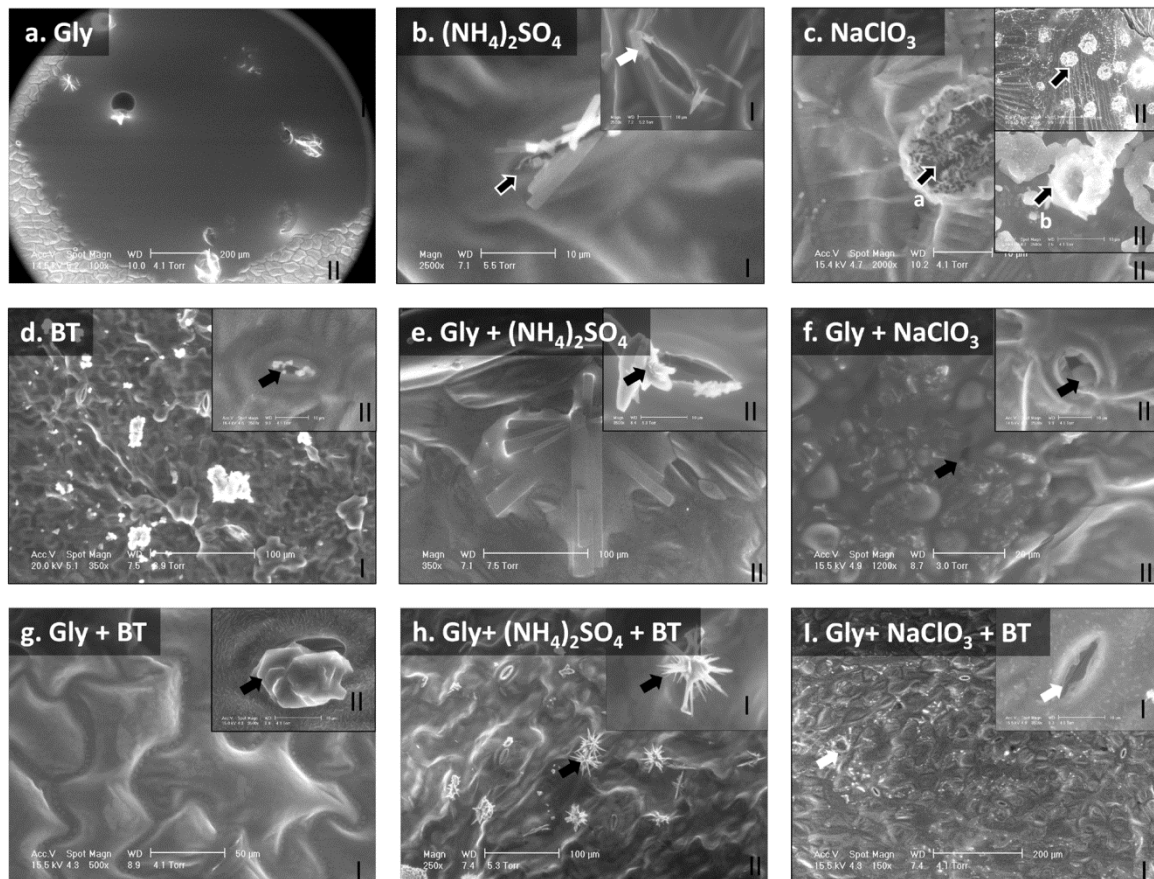
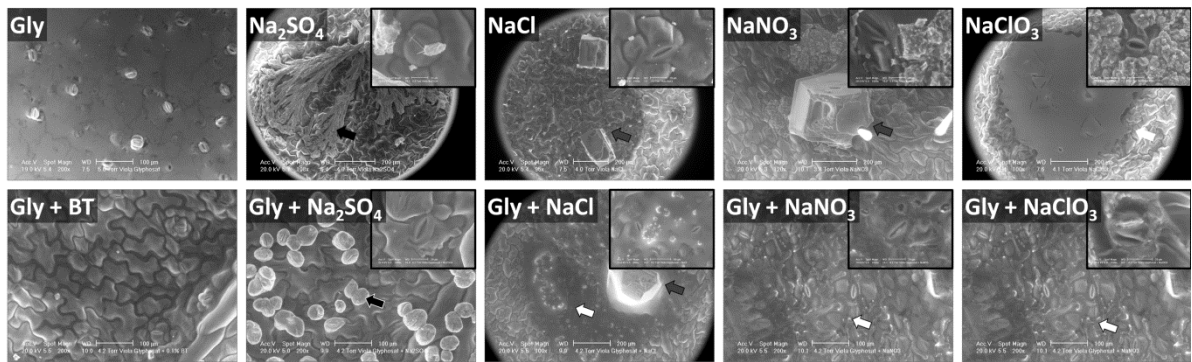


Fig. 2. Scanning electron microscope images showing representative deposits of formulations on the adaxial leaf surface of (I) *V. arvensis* and (II) *C. album*. Solutions: ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, 150 mM) and sodium chlorate (NaClO_3 , 150 mM), with or without glyphosate (Gly, 43 mM), with or without Break-Thru® S233 (BT, 0.1% v:v). The arrows indicate the interaction of the applied substances with the surface around the stomata. The arrow bar \rightarrow represent hydraulically activated stomata due to penetration of hygroscopic salts; additional letter 'a' represent the early stage of 'creeping' of salt towards the stomata, while the letter 'b' represent the later stage after penetration. The arrow bar \rightarrow , \rightarrow shows the formulation likely to be penetrating inside the stomata or has already penetrated inside the stomata respectively. ESEM Settings (Units) - Acceleration voltage, Acc V (kV); Spot size, Spot (μm); Working distance, WD (mm); Pressure (Torr), 1 Torr = 0.133 kPa.

Addition of BT enhanced the crystalline whisker growth of $(\text{NH}_4)_2\text{SO}_4$ through stomatal opening, and to disperse the homogenous crust of NaClO_3 . Salts crystallized and deposited even inside the stomata (Figs. 2b and 2c). The stomata were observed to be connected with the external environment with the thin channels of NaClO_3 (Fig. 2c). The presence of Gly led to rather amorphous deposit (Figs. 2e and 2f). Glyphosate formulated with Na_2SO_4 , formed globular structures in their deposits, while, with NaCl , NaNO_3 and NaClO_3 , deposits were of amorphous colloidal mixture of crystalline salts deposited along the periclinal walls (Fig. 3).

V. arvensis



C. album

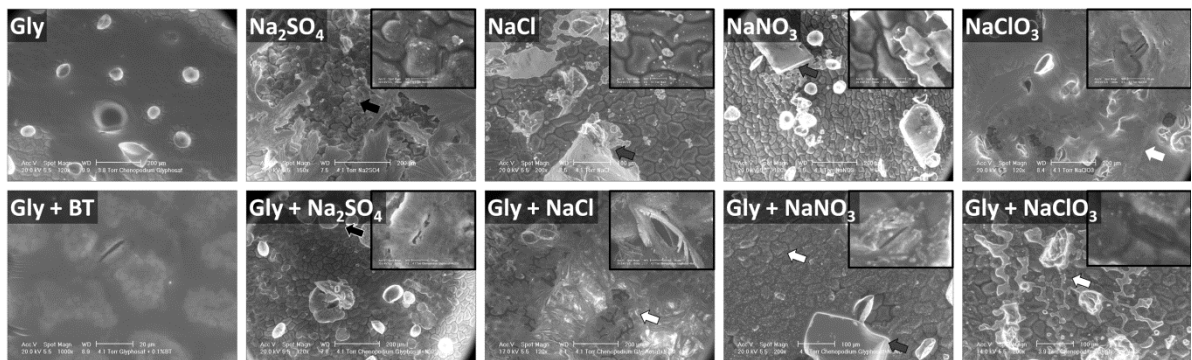


Fig. 3. Scanning electron microscope images of representative dried deposits of glyphosate (Gly) formulations on the adaxial leaf surface of *V. arvensis* and *C. album*. Glyphosate formulations (43mM) were prepared with sodium (Na) salts (300mM) of different ions from Hofmeister series ($\text{SO}_4^{2-} > \text{Cl}^- > \text{NO}_3^- > \text{ClO}_3^-$). Additionally, the organosilicone surfactant Break-Thru® S233 (BT) (0.1% v:v) was included. The inset represents the corresponding activity around the stomata. The arrow bar '→', '⇨', '⇩' shows the dendritic crystals, cubic crystals, and the amorphous homogenous crust of salt respectively. Likewise, arrow bar '⇨' shows the globular structure of Gly-salt mixture, while the '⇩' shows the amorphous colloidal Gly-salt crystal mixture. ESEM Settings (Units) - Acceleration voltage, Acc V (kV); Spot size, Spot (μm); Working distance, WD (mm); Pressure (Torr), 1 Torr = 0.133 kPa.

3.3. Tissue necrosis

In Trial 1A, $(\text{NH}_4)_2\text{SO}_4$ caused only slight tissue necrosis (*data not shown*), while in Trial 1B, NaClO_3 strongly led to a tissue necrosis. Increasing concentration of NaClO_3 induced higher frequency and size of necrotic spots in both evaluated species (Fig. 4).

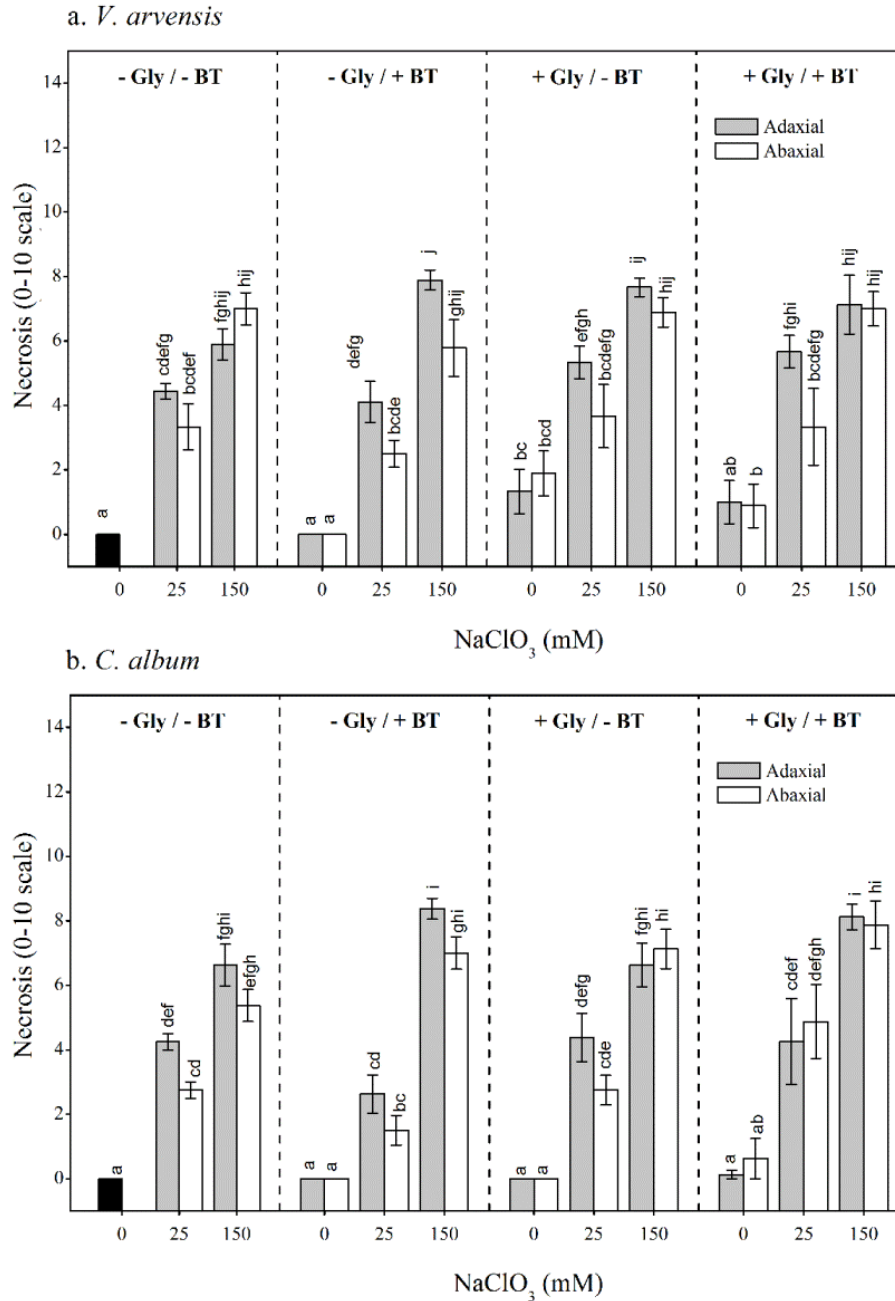


Fig. 4. Effect of the treatment solutions on the formation of necroses on the leaves. Droplets of the treatment solutions, with or without glyphosate (Gly, 43 mM), sodium chlorate (NaClO_3 ; 25 and 150 mM) and Break-Thru® S233 (BT, 0.1% v:v) were deposited on the adaxial and abaxial leaf sides of *V. arvensis* and *C. album*. The black vertical bar indicates the untreated control. Mean \pm S.E. ($n = 9$) followed by similar letter do not differ significantly (Kruskal-Wallis test, $P \leq 0.05$).

In contrast, BT had no impact on the tissue necrosis. Likewise, we observed no differences when applying droplets at the adaxial or abaxial leaf lamina. In Trial 2, only the formulations containing NaClO₃ induced a significant tissue necrosis (Table 2).

Table 2. Effects of hygroscopic salts (300 mM) (from Hofmeister series) and Break-Thru® BT S233 (BT, 0.1 and 0.01% v:v), formulated (+) with or (-) without glyphosate (Gly) (43mM), on the average frequency of necrotic spots at the treated adaxial leaf surfaces of *V. arvensis* and *C. album*.

Solutions	Average frequency of necrotic tissue (%), Mean ± S.E. (n > 18)			
	<i>V. arvensis</i>		<i>C. album</i>	
	+ Gly	- Gly	+ Gly	- Gly
Water	0	0	0	0
Na ₂ SO ₄	8.3 ± 4.5	3.7 ± 3.7	0.8 ± 0.8	0
NaCl	4.9 ± 2.4	0	0	0
NaNO ₃	0.9 ± 0.9	0	0	0
NaClO ₃	100	100	80.0 ± 5.4	81.7 ± 5.8
0.1% Bt	0	0	0	0
0.01% Bt	3.7 ± 1.7	*	16.7 ± 6.2	*

* not recorded

3.4. Bio-efficacy

In Trial 1A, (NH₄)₂SO₄, formulated without BT, significantly reduced the Gly bio-efficacy in *V. arvensis*, but had no significant effect on *C. album* (Fig. 5A). Likewise, in Trial 1B, NaClO₃, formulated without BT, also significantly reduced the Gly bio-efficacy in *V. arvensis*, but had no effect on *C. album* (Fig. 5B). However, addition of BT recovered the antagonistic effect of both salts, (NH₄)₂SO₄ and NaClO₃, on the bio-efficacy of Gly in *V. arvensis* (Figs. 5A and 5B). Addition of BT significantly enhanced the phytotoxicity of Gly ± (NH₄)₂SO₄ or NaClO₃ in both weed species. On the other hand, differences in the treatment between the leaf sides were not significant in both the species.

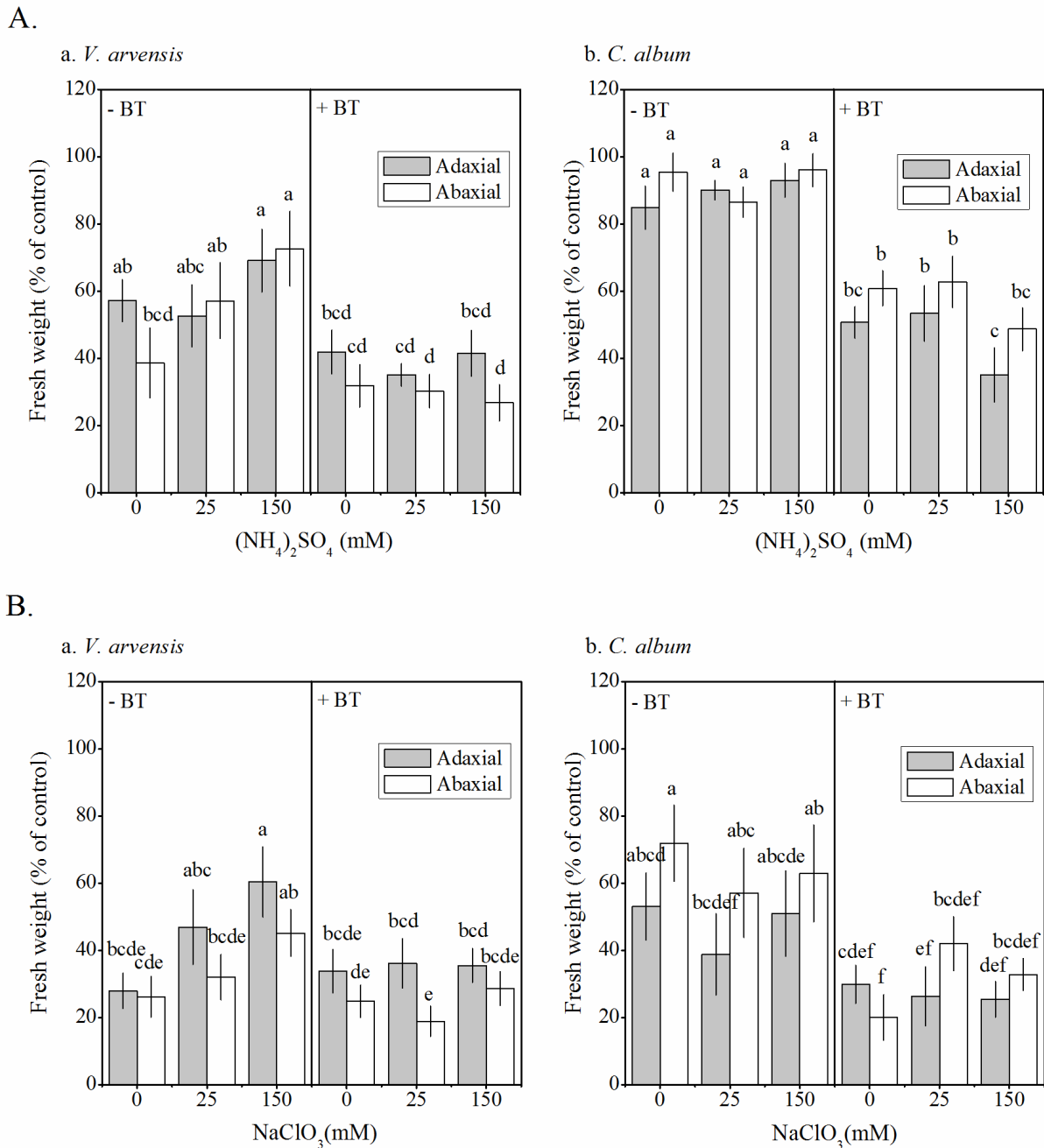


Fig. 5. Effect of A. ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$; 0, 25, 150 mM) or B. sodium chlorate (NaClO_3 ; 0, 25, 150 mM) \pm Break-Thru[®] S233 (BT; 0.1% v:v) on the bio-efficacy of glyphosate. The solution droplets were applied on the adaxial and abaxial leaf sides of *V. arvensis* and *C. album*. Mean \pm SE ($n \geq 9$) followed by same letter do not differ significantly (LSD, $P \leq 0.05$). Means were separated after Box-Cox transformation in A. (*V. arvensis*, $\lambda = 0.5$; *C. album*, $\lambda = 1.25$) and B. (*V. arvensis*, $\lambda = 0.5$; *C. album*, $\lambda = 0.75$).

In Trial 2, the bio-efficacy of Gly was not affected by addition of kosmotropic Na_2SO_4 salts of Hofmeister series (Fig. 6). But, the increase in the ionic series, NaCl to a

more chaotropic form NaNO_3 , raised the bio-efficacy of Gly (in exception to NaClO_3). Highest phytotoxicity of Gly was observed in combination with Cl^- and NO_3^- ions of sodium salt in *V. arvensis* and *C. album*, respectively. At this level of efficacy, the Gly bio-efficacy was equivalent or even higher than its combination with 0.1% of BT or 0.01% BT, respectively.

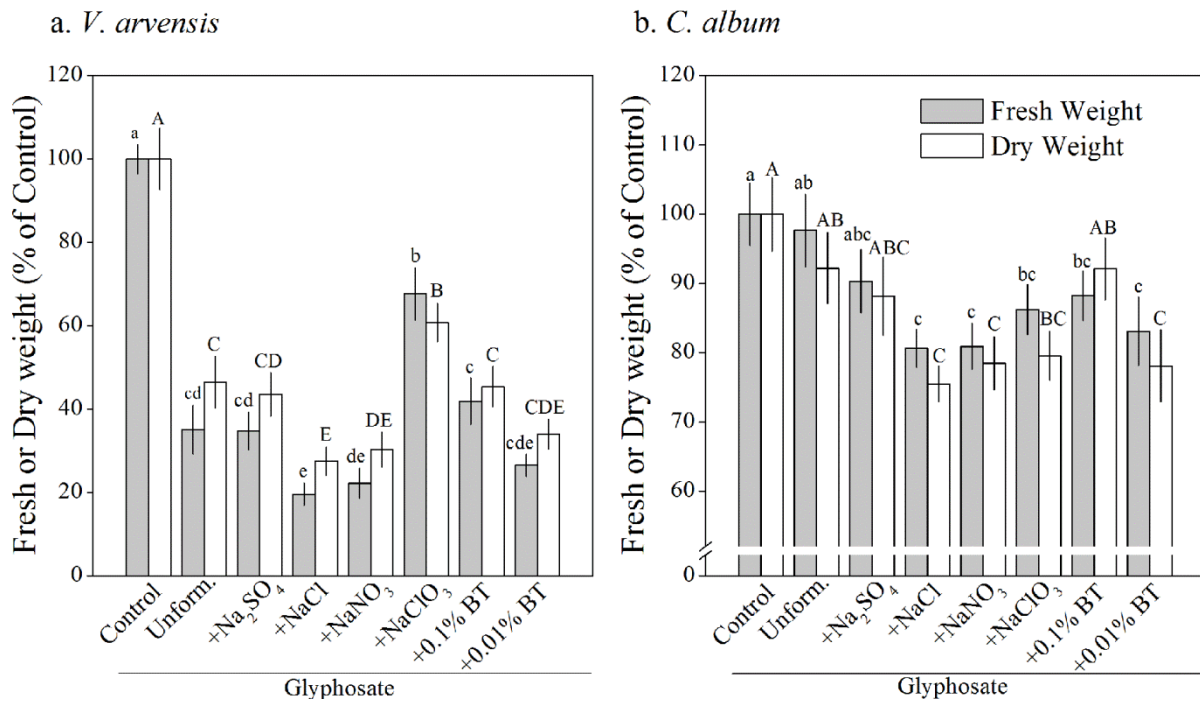


Fig. 6. Effect of different hygroscopic salts (300 mM) (from Hofmeister series) and organosilicone surfactant - Break-Thru® S233 (BT; 0.1% and 0.01% v:v) on the bio-efficacy of glyphosate (43 mM) on *V. arvensis* and *C. album*. Mean \pm S.E. ($n > 18$) followed by the same letters (case sensitive) do not differ significantly (LSD, $P \leq 0.05$).

4. Discussion

Contradictory to earlier concepts (Schönherr and Bukovac, 1972), stomatal penetration by liquids may occur in the presence of hygroscopic salts even at $\text{ST} > 30 \text{ mN m}^{-1}$ (Burkhardt et al., 2012). Thus, in this study we exploited the role of different hygroscopic salts, classified in kosmotropic or chaotropic in Hofmeister series, for the stomatal penetration of Gly. The study is based on the hydraulic activation of the stomata (HAS). In addition, the presence of chaotropes, the ST of Gly solution would be strongly reduced. This phenomenon would enhance HAS, ultimately improving the bio-efficacy of Gly.

4.1. Hydraulic activation of stomata

The kosmotropic $(\text{NH}_4)_2\text{SO}_4$, or even chaotropic NaClO_3 , even at their very high concentrations, do not decrease the surface tension of the brine solution below 30 mNm^{-1} (Burkhardt et al., 2012). Likewise with Gly, the strongest reduction of ST of solution (as expected when droplet dries to 7.5% of initial droplet volume) was nearly to 45 mN m^{-1} with the inclusion of chaotropic NaNO_3 and NaClO_3 salts. And that with Na_2SO_4 or NaCl , ST of Gly solution reduced up to 57 mNm^{-1} (presence of other solutes in Gly formulations, might have contributed for this reduction in ST, although it should have increased (Leroy et al., 2010)). In general none of the salts could reduce the $\text{ST} < 30 \text{ mN m}^{-1}$, that could have allowed the stomatal infiltration as explained by Schönherr and Bukovac (1972). However, the presence of formulations inside the stomata (Figs. 2b and 2c), confirms the stomatal penetration even at such high surface tension of the liquid. The dendritic growth of salt crystals due to fluctuation in humidity increases the contact area of the salt crystals to the leaf surface (Burkhardt et al., 2012). The fluctuation of moisture, provided by the stomata, led to dendritic growth of salt crystals near the stomata and linked the internal of stomatal chamber to the external environment leading to HAS (Fig. 2c). Further, brine solution with Break-Thru®S233, having $\text{ST} < 30 \text{ mN m}^{-1}$, induced the growth of large crystals from the stomata (Fig. 2h), resembling the ST gradient mediated growth of crystalline whiskers (Sears, 1957). Usually, solutes in a drying droplet are deposited either in the form of coffee-ring, central or the homogeneously deposited in the droplet footprint (Hunsche and Noga, 2011). However, while reaching the supersaturated concentrations, salt crystallized homogeneously towards the droplet footprint, counteracting the capillary or Marangoni force (Deegan et al., 2000, Hu and Larson, 2006).

Likewise, addition of Gly in the brine solution induced the formation of amorphous deposits (Figs. 2 and 3). Alteration of the crystalline deposition form of salts, due to presence of Gly, to a globular form when mixed with Na_2SO_4 (Fig. 3), appear to partially hinder the HAS. However, presence of NaCl , NaNO_3 or NaClO_3 in Gly solution formed a colloidal mixture. They were observed, flowing through the periclinal cell wall and even inside the stomata, thus promoting the stomatal infiltration of formulations (Fig. 3).

4.2. Bio-efficacy of glyphosate

Addition of $(\text{NH}_4)_2\text{SO}_4$ reduced the bio-efficacy of Gly (Fig. 5A). A similar observation was made by Burkhardt et al. (2012) when droplets of Gly + $(\text{NH}_4)_2\text{SO}_4$ were applied to the stomatal abaxial surface of apple leaves. Increasing concentration of NaClO_3 , raised the size

of the necrotic tissue in both the species (Fig. 4), leading to a decrease of the Gly bio-efficacy particularly in *V. arvensis* (Fig. 5B). This localized tissue death might have hindered the Gly movement of out of this zone (McKinlay et al., 1972; Merritt, 1982; Sherrick et al., 1986; Geiger and Bestman, 1990; Liu et al., 1996; Kirkwood et al., 2000). In contrast to larger droplet spread on the leaf of *C. album*, absence of crystalline wax on the leaf surface of *V. arvensis* might have reduced the capillary movement of the liquid, leading to a limited spread (Basi et al., 2012). The larger droplet spread in *C. album* leaf might have allowed faster stomatal penetration of Gly before localized tissue death. This is further supported by the similarity in Gly bio-efficacy between the formulation having different concentration of $(\text{NH}_4)_2\text{SO}_4$ or NaClO_3 in combination with BT. There are significant differences between adaxial and abaxial leaf surfaces. In addition to differences in stomata number or its sensitivity between the leaf sides (Wang et al., 1998), differences in the cuticle permeability might also exist (Karbulková et al., 2008). However, despite of all those differences, the foliar uptake and bio-efficacy of Gly formulations, adaxially or abaxially treated, did not differ (Figs. 5A and 5B). In contrast, Gly uptake increased when treated on stomatous abaxial treatment of Gly as compared to astomatous adaxial treatment on apple leaves (Burkhardt et al., 2012). This shows the significance of stomata and not other differences between the leaf surfaces for the higher uptake of foliar applied chemicals when treated abaxially.

Presence of different sodium salts significantly affected the bio-efficacy of glyphosate. The sodium salt containing the kosmotropic SO_4^{2-} had no effect on the bio-efficacy of Gly in *V. arvensis* and *C. album*. In contrary, the chaotropic NO_3^- enhanced the bio-efficacy of Gly in both the species (Fig. 6). Although the brine solution of Na_2SO_4 led to the HAS (Fig. 3), addition of Gly led to the formation of globular amorphous deposits (Fig. 3), probably hindering the stomatal uptake of Gly. However, with the chaotropic NaNO_3 salt, the surface tension of the Gly solution is highly reduced (Fig. 1) and the crystal-liquid colloidal solutions appeared to move along the periclinal cell walls and inside the stomata. This enhanced stomatal penetration, favored by humidity fluctuations around stomata, is assumed to induce the higher bio-efficacy of Gly.

At the highest level of Gly phytotoxicity i.e., with NaNO_3 , the bio-efficacy was equivalent to bio-efficacy reached with 0.01% or 0.1% BT. The larger spread area (de Ruiter et al., 1992; Liu, 2003), or the deposit area (Krämer et al., 2009), is antagonistic for foliar uptake (Basi et al., 2013). Equivalently, larger coverage area of formulation in presence of organosilicone, might have reduced bio-efficacy of Gly. Reduced foliar uptake of commercial formulation (Roundup) due to higher surfactant concentration was reported (Field et al.,

1992; Gaskin and Stevens, 1992). Likewise, while, increasing the ionic series in the Hofmeister series, SO_4^{2-} , Cl^- , NO_3^- and ClO_3^- , the chaotropic ClO_3^- caused the drastic increase in the necrosis of the treated tissue (Table 2). This spatial isolation of formulation due to tissue necrosis might have hindered the Gly movement of out of this zone, reducing the Gly bio-efficacy (Fig. 6) as explained elsewhere.

Thelen et al. (1995) reported on the addition of $(\text{NH}_4)_2\text{SO}_4$ to stabilize calcium Ca^{+2} of the hard water to CaSO_4 . The remaining ammonium ion, combine with Gly to form the Gly-ammonium salt, and thus enhance the herbicidal bio-efficacy. In our study, only SO_4^{2-} containing sodium salts formed the globular structure with Gly, but not the Cl^- , NO_3^- and ClO_3^- ion. And equivalently, SO_4^{2-} couldn't enhance the bio-efficacy of Gly, but the Cl^- and NO_3^- enhance its bio-efficacy. From this new perspective, replacing SO_4^{2-} by other chaotropic Cl^- and NO_3^- in $(\text{NH}_4)_2\text{SO}_4$ might lead to higher bio-efficacy of Gly.

In conclusion, addition of hygroscopic kosmotropic and chaotropic salts induced the Hydraulic Activation of Stomata. The kosmotropic Na_2SO_4 , in combination with glyphosate, slightly reduced the surface tension of the solution and formed globular glyphosate-salt residue on the leaves when it dried. In contrast, chaotropic NaNO_3 , NaClO_3 , strongly reduced the surface tension of solution and formed colloidal mixtures of glyphosate-salt residues on the leaves, and were flowing through the periclinal walls and even inside the stomata. Coincidentally, kosmotropic Na_2SO_4 couldn't enhance the bio-efficacy of glyphosate. In contrast, chaotropic NaNO_3 enhanced the bio-efficacy of glyphosate that was equivalent or even better than that with organosilicone Break-Thru® S233. Thus stomatal pathway is relevant for foliar uptake of agrochemicals.

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F. Summary and Conclusions

In our experiments, the deposit residues were studied in terms of enhancing either the cuticular or the stomatal uptake of agrochemicals. The formulations of glyphosate or diquat were prepared either with surfactants or the hygroscopic salts. The ethoxylated rapeseed oil (RSO) surfactant of different number of ethylene oxide (EO) units, were included, to affect the liquid surface tension. Likewise the hygroscopic salts were included in the tank-mix formulation to have differential surface tension and crystallization behaviour. The formulation droplets were applied to plant leaves with kinetic energy (from monodroplet generator), and/or without kinetic energy (from micropipette). The easy-to-wet species, *Stellaria media* and/or *Viola arvensis*, and the difficult-to-wet species, *Setaria viridis* and/or *Chenopodium album*, were used. The formulation residues were observed using a scanning electron microscope. Deposit residues, and its relevance for the cuticular uptake and the hydraulic activation of stomata, were discussed in relation to uptake of foliar applied chemicals. The major results can be summarized as follows:

1. The need to study the effect of kinetic energy of droplet on deposit residue formation led us to construct the pneumatic droplet generator. As shown, the orifice diameter was the major influencing factor for the droplet diameter. A combination of strong but short duration pressure bursts released smaller droplets. At low impulse widths, the surface tension of the liquids had a strong effect on the droplet diameter. With this system, we were able to produce droplets of diameters ranging from 533 to 1819 μm . As demonstrated in the present work, at this development stage our droplet generator is suitable for use in scientific studies on droplet behaviour on selected plant surfaces under laboratory conditions.

2. The deposit structure of dried droplets of isopropylamine glyphosate solutions is not always homogeneous. Surfactants altered the homogenous deposit of glyphosate to heterogeneous larger deposits (e.g. with RSO 5) or concentrated small amorphous deposits (e.g. with RSO 60) especially on leaves of difficult-to-wet plants. Thus, deposit area might be considered more relevant for the bio-efficacy of glyphosate than the spread area of the formulation droplet. Kinetic energy of the droplet had no consistent effect on the deposit structure or the bio-efficacy of glyphosate. However surfactants had significant effect on deposit structure of glyphosate. On easy-to-wet species, the increase in EO unit of the RSO surfactant did not affect the deposition area of glyphosate nor its bio-efficacy. However, in

difficult-to-wet species, the increase in EO units of the RSO surfactant reduced the deposit area of glyphosate, and enhanced its bio-efficacy.

3. Diquat dibromide having a contact mode of action was selected for the studies on the relevance of deposit properties and bio-efficacy. The non-significance of the EO units of RSO surfactant for the deposit area of diquat coincided also with the non-significance in ChlF parameter (F_v/F_m), and the ChlF impaired tissue and its desiccation potential on easy-to-wet leaves of *V. arvensis*. In contrary, the decreased deposit area of diquat due to addition of RSO surfactants reduced the F_v/F_m (at 51 h after application), F_v/F_m impaired tissue area (at 26 h after application) and increased its final desiccation potential on the leaf of difficult-to-wet *C. album*.

4. In our studies on the hydraulic activation of stomata (HAS) causing a mass flow of liquid inside the stomata, we observed that, addition of hygroscopic kosmotropic and chaotropic salts induced the HAS. The kosmotropic Na_2SO_4 , in combination with glyphosate, slightly reduced the surface tension of the solution and formed globular glyphosate-salt residue on the leaves when it dried. In contrast, chaotropic NaNO_3 , NaClO_3 , strongly reduced the surface tension of solution and formed colloidal mixtures of glyphosate-salt residues on the leaves. These flowed through the periclinal walls and even inside the stomata. Kosmotropic Na_2SO_4 couldn't enhance the bio-efficacy of glyphosate. In contrast, chaotropic NaNO_3 enhanced the bio-efficacy of glyphosate that was equivalent or even better than that obtained with the organosilicone Break-Thru® S233.

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Name:

SABIN BASI

Publications

Unpublished thesis:

- 2006:** Basi, S. Morphological and cytogenetic effect of gamma ray on indica rice var. Radha-4. Unpublished Master Thesis. Institute of Agriculture and Animal Science, Nepal.
- 2008:** Basi, S. Molecular and histological study of root tissue in relation to phosphorous deficiency and drought tolerance in rice. Unpublished Master Thesis. INRES Crop Science and Plant Breeding, University of Bonn, Germany.

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- 2011:** Basi, S., Hunsche, M., Damerow, L., Schulze Lammers, P. and Noga, G. Potential use of monodroplet generator for a precise weed control. In: *Poster presentation, S. Basi (Presenter)*, 63rd International Symposium on Crop Protection, Ghent University, Belgium.
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Co-authored:

- 2008:** Chin, J.H., Lu, X., De Leon, T., Basi, S., Peñarubia, M., Haefele, S., Bernier, J., Ulat, V.J., Ismail, A.M., Wissuwa, M., Heuer, S. Association studies and development of molecular markers for Pup1, a major QTL for phosphorus deficiency tolerance. In: *Poster presentation, J.H. Chin (Presenter)*, International Rice Research Institute, Philippines.
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