Retention, Uptake, and Translocation of Agrochemicals in Plants

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Fundamental understanding of the uptake, translocation, and distribution of agrochemicals is of great interest among scientists in industry and academia, because biological activities of pesticides against their target species can be significantly influenced by the biokinetics of the pesticides. Biological activity of pesticides is initially identified during the courses of *in vitro* bioassays, but the active molecules often lose their biological activity in greenhouse tests. The lack of translation of activity between *in vitro* assays and greenhouse tests is generally associated with many factors, including poor retention on plant surface, lack of foliar or root uptake, and limited systemicity within plants. Therefore, a clear understanding of the factors that govern the effectiveness of pesticides is key to overcome certain barriers for the expression of biological activity, and this can lead to a strategy to improve biological performance.

This ACS symposium book is based on a symposium that was held at the 246th American Chemical Society National Meeting & Exposition in Indianapolis, Indiana from September 8-12, 2013. Although uptake, translocation, and distribution of agrochemicals in plants have been extensively studied over the years, there are still many unanswered questions that need to be addressed. This book aims to update current knowledge with new studies that contain new findings on the uptake, translocation, and distribution of agrochemicals in plants as well as provide review-style chapters that summarize existing information on specific subjects.

It is hoped that this book will serve as a valuable resource for researchers who study uptake, translocation, and distribution of pesticides in plants. As researchers involved in discovery and development of agrochemicals want to understand a broad range of biological factors, it is also hoped that this book promotes researchers in other scientific disciplines to generate new ideas and technologies in the process of new product development. Knowledge of the biokinetics will help us further understand the use of agrochemicals on our planet.

We thank the presenters and authors for their invaluable contributions to the symposium and this book. We gratefully acknowledge ACS division of agrochemicals and Dow AgroSciences for financial assistance for the symposium. We would like to express our sincere thanks to many other colleagues who reviewed chapters for their timely and critical assessment. We are also thankful for excellent supports of staff members in ACS Books Division.

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Kyung Myung received his B.S. and M.S. degrees in Horticulture from Kyung Hee University, South Korea and Pennsylvania State University, respectively, and his Ph.D. degree in Plant Physiology from University of Kentucky. Since then he has worked for Northern Illinois University and USDA-ARS and then joined Discovery Research Department at Dow AgroSciences. He is currently engaged in research in biokinetics of pesticides in biological matrices. He has published over 20 research articles in the field of agricultural biology and chemistry.

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Norbert M. Satchivi obtained his Bachelor degree in Plant Physiology from the Université d'Abidjan (Cote-d'Ivoire) and his Doctorate from the Université de Perpignan in France (Agrochemistry). He was a Postdoctoral Scientist at the University of Illinois at Urbana-Champaign where he worked on the development of computer simulation models for the movement of xenobiotics in plant. He also worked at the University of Guelph (Ontario, Canada) as a Research Scientist focusing on the mechanisms of herbicide antagonism. He is currently a Senior Research Scientist with Dow AgroSciences with a focus in herbicide discovery. Publications included the areas of herbicide characterization, xenobiotic uptake and transport, and modeling xenobiotic movement.

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Coleen K. Kingston obtained her Bachelor degree from the University of Wisconsin (Biochemistry, Math) and her Doctorate from Purdue University in Indiana (Analytical Chemistry). Subsequent posts have included research and development scientific positions within the pharmaceutical and agrochemical industries. She is currently a senior scientist with DuPont Crop Protection with a specialty in crop residue human safety assessment. Publications over the years have included the areas of pharmaceutical analysis method development in human matrices and the prediction of residues in food and feedstuffs yielded following foliar and systemic delivery application of crop protection chemicals.

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

Chapter 1

Spray Retention of Crop Protection Agrochemicals on the Plant Surface

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Retention efficiency of crop protection products, one of the important attributes for delivering biological efficacy, is mainly determined by the physicochemical properties of the spray formulations and the surface characteristics of target plants. In this chapter, effects of plant leaf surface characteristics, developmental stages and canopy density on leaf wettability and spray retention are reviewed. Factors modifying the physicochemical properties of spray liquids, including active ingredients, formulation types, and a variety of adjuvants are also discussed. Using a quantitative structure-activity relationship (QSAR) analysis, we have developed a model based on four calculated physicochemical properties of a training set of 17 fungicides to predict compound retention rates on wheat seedling plants. The model was validated by a strong correlation between experimentally determined retention rates and predicted values of a small test set, which included six additional fungicide compounds. Retention efficiency of three epoxiconazole formulations was also evaluated, and significant differences in retention were observed for the products Ignite (8 % EC, w/w), Opus (12.1% SC, w/w), and a generic lab SC (10% w/w).

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

Introduction

High efficiency spray retention on the plant surface is required for maximizing activity delivery of crop protection agrochemicals because it increases the amount of active ingredient potentially available for reaching the biological site of action. Retention is the overall capture of spray droplets by a plant and determines the amount of active ingredient on a plant. It is dependent on the complex interfacial interaction of spray droplets and the plant surface. The factors considered to be important for spray liquid adhesion and retention include: 1) physicochemical properties of the spray solutions; 2) diameter spectra and impaction velocity of spray droplets; and 3) characteristics of plant surface, shape and orientation of the target leaves and density of plant canopy. While the impact of adjuvants on the interaction of spray liquids and leaf surface has been extensively studied and reviewed (*1–4*), specifically the effect of major additives in the spray formulations such as solvents, surfactants, oil and polymer adjuvants, attention to the active ingredients has not been the focus for the majority of the studies. For plants, wettability of the leaf surface is typically governed by surface roughness caused by different microstructures (trichomes, cuticular folds and wax crystals), together with the hydrophobic properties of the epicuticular wax. Hydrophobicity of epicuticular wax and the microstructures can efficiently reduce the deposition and retention of spray droplets by increasing contact angles and reducing contact area with plant leaf surface (*4*, *5*). Greenhouse grown seedlings and young plants have been the major targets in earlier studies. Surface properties and plant canopy structures change dramatically as plants grow, and ultimately affect the quantity of spray deposited and redistributed over the entire plant canopy (*6*, *7*).

In this paper, we present the results of our investigations on the effect of the physicochemical properties of fungicides on retention by seedling wheat plants, and the development and validation of a QSAR model to predict fungicide retention. The impact of three epoxiconazole formulations on retention and the relationship to fungicidal activity were also investigated. The relationship between retention of spray droplets and the wetting characteristics of target plants and their canopy structure, as well as the physicochemical properties of spray liquids, are reviewed based on the published literature.

Effect of Plant Surface Characteristics and Canopy Structures on Spray Retention

The Plant Cuticle and Leaf Wettability

All aerial surfaces of terrestrial plants are covered by a cuticle, which serves as the interface of plants to their above ground environment. The primary function of the cuticle is to prevent water loss from plants so that physiological processes can proceed under water-limiting conditions. In addition, the cuticle acts as an effective barrier to the entry of xenobiotics and microorganisms into the plants.

The plant cuticle consists of two major components: cutin and waxes. Cutin is a polymer complex consisting of many long-chain fatty acids that are attached to one another by ester linkages, which create a rigid three-dimensional network (*8*).

Cutin is hydrophobic, but contains some hydrophilic moieties attached to the chain, such as hydroxyl or epoxide groups. Waxes are hydrophobic complex mixtures of long-chain acyl lipids. The most common components of waxes are straight-chain alkanes and alcohols of 25 to 35 carbon atoms. Long-chain aldehydes, ketones, esters, and free fatty acids are also found in waxes. The waxes of the cuticle are synthesized by epidermal cells, which are embedded in the cutin polymer matrix (intracuticular) and also deposited on the surface of cuticular layer (epicuticular). The epicuticular waxes often crystallize in an intricate pattern of rods, tubes, or plates (*9–11*).

Table 1. Leaf Surface Characteristics of Selected Plant Species

Common name	*Latin name*	*Wettability*	*Surface morphology*
Rice	*Oryza sativa*	Super hydrophobic	Micropapillae superimposed by waxy nanobumps
Wheat	*Triticum aestivum*	Difficult	Crystalline plates, trichomes
Barley	*Hordeum vulgare*	Difficult	Crystaline plates, trichomes
Corn	*Zea mays*	Difficult	Crystalline plates
Oilseed rape	*Brassica napus*	Difficult	Crystalline tubes, plates and dendrites
Pea	*Pisium sativum*	Difficult	Dense arrangement of crystalline plates
White clover	*Trifolium repens*	Difficult	Dense arrangement of crystalline plates
Strawberry	*Fragaria ananassa*	Difficult	Thick film overlaid by fine wax ribbons
Soybean	*Glycine max*	Difficult	Crystalline, trichomes
Sugar beet	*Beta vulgaris*	Easy	Thin film; occasional wax mounds
Dry bean	*Phaseolus vulgaris*	Easy	Extremely thin film, trichomes
Curly dock	*Rumex crispus*	Easy	Smooth
Apple	*Malus domestica*	Easy	Smooth
Cucumber	*Cucumis sativus*	Easy	Thin film, trichomes

Leaf surface characteristics are critical factors affecting leaf wettability and retention of spray droplets (*4*, *12–14*). Leaf surface wetting is dependent on microroughness of epicuticular wax crystals (*5*, *9*, *15–17*). Table 1 lists the leaf

surface characteristics of selected plant species often used in spray retention and deposition studies of crop protection products. Generally, smooth leaf surfaces without crystalline epicuticular waxes or hydrophobic trichomes are easy-to-wet, with a water contact angle less than 110 degrees (*15*). Leaf surfaces covered with crystalline epicuticular waxes are difficult-to-wet and give a contact angle with water droplets greater than 110 degrees (*15*). An easy to use and quick way to determine leaf surface wettability is to pipette a 2-μL water droplet onto the plant surface; plants with easy-to-wet surfaces are very receptive to the placement of the water droplet, but plants with microscopically rough surfaces make placement of the water droplet extremely difficult (Yao, unpublished data).

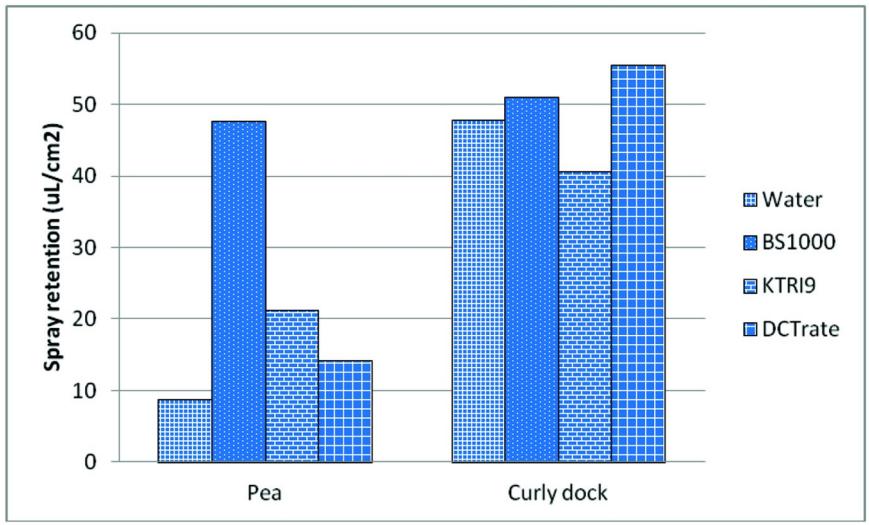

Figure 1. Retention of herbicide metsulfuron by difficulty-to-wet pea and easy-to-wet curly dock plants, with or without the addition of an adjuvant. Data from Nicholls et al. (18).

In track spray applications, Nicholls *et al.* (*18*) (Figure 1) observed that easy-to-wet curly dock retained the herbicide metsulfuron methyl very efficiently, and inclusion of three adjuvants in the spray solutions showed little effect on spray formulation retention. In contrast, the same three adjuvants, BS1000 in particular, improved the herbicide deposition significantly on the difficult-to-wet field pea plants. Many investigations have observed the same general trend: on plants with smooth, easy-to-wet leaf surfaces, retention is high and not greatly affected by spray application and solution properties (*19–21*). For monocotyledonous plant leaves with crystalline vertical wax plates such as barley and maize, 95% of the outmost leaf surface consisted of air because the exposed edges of wax plates only represented approximately 5% of the leaf surface (*5*). The presence of air at the interface between the droplet and the hydrophobic crystalline epicuticular wax on the leaf surface causes large contact angles and poor spray droplet adhesion (*5*, *13*, *15*). The contact area of droplets on difficult-to-wet plants, such as oilseed rape,

maize, and strawberry, was smaller than the contact area on plants with smooth surfaces, such as sugar beet and dry bean (Figure 2) (*12*). Baker *et al.* (*12*) have observed that the impaction diameter of droplets on oilseed rape is approximately one sixth of those on dry bean (Figure 2). To achieve good retention on micro-rough surfaces, a low dynamic surface tension at the moment of impact has to be guaranteed so not only the exposed edges but also the largest possible surface area of the wax is wetted (*5*).

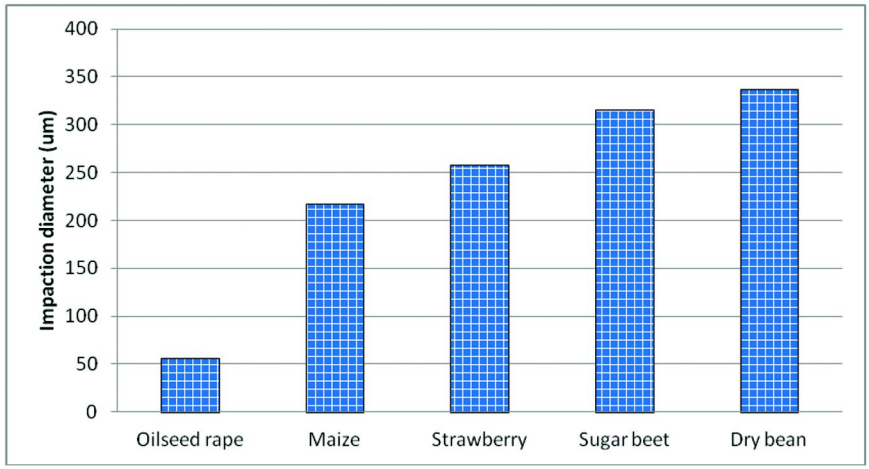

Figure 2. Impaction diameters for droplets (in-flight diameter 175 µm) of 0.3% aqueous solution of Uvitex 2B on to leaves of five plants. Data from Baker et al. (12).

Development Stage and Canopy Density

Greenhouse grown plants are more difficult to wet due to the intact epicuticular wax when compared to plants grown outdoor, which are subjected to the effects of physical wax abrasion caused by rain waters and leaves rubbing against each other, and other environmental conditions (*16*). Taylor (*4*) has shown that outdoor grown spring barley plants retained more surfactant solutions than those grown in the greenhouse. Scanning electron micrographs of the sprayed leaves showed that all of the deposits observed were in close contact with at least one of the wax-abraded areas.

Another factor influencing leaf wettability is plant developmental stage. Leaves at different growth stages may have different wettability. Moran Puente and Baur (*7*) found that soybean leaves at growth stage (GS) 16 were 30 times more wettable with water than leaves at GS 11. A dense layer of epicuticular wax crystals was observed uniformly on the adaxial and abaxial leaf surfaces from GS 11. However the uniform layer of dense wax crystal was only observed in the abaxial leaf surface but not on the adaxial surface in the GS 15 to 17, which were found to be only partially covered by epicuticular wax crystals.

In another study, Moran and Baur (*22*) showed that soybean leaves developed at early growth stages have low wettability and also the lowest tebuconazole penetration, indicating that good uptake of crop protection products requires good contact of the spray droplets to the leaf surface. This was true even when using spray solutions with added wetting agents (*22*). It has also been demonstrated that maize leaves prior to GS 15 were densely covered with crystalline waxes, with water retention at practically zero. In contrast, at GS 18, maize leaves were covered with an amorphous wax film due to wax biosynthesis modifications at later growth stages, resulting in a water retention value of 85% (*23*).

Ellis *et al.* (*6*) have shown that wheat plant size and canopy density have a significant impact on spray retention of three spray liquids- tallow amine, EC blank, and water. When the plant density was at 1540 stems/m^2, there was a clear ranking of the retention ability of the three spray liquids, with tallow amine retention higher than EC, and EC retention higher than water (Table 2). At the highest canopy density of 2640 stems/m^2, with a leaf area index higher than seven, the effects of liquid properties on whole plant retention became very small and retention rates were almost identical for the three liquids (Table 2). This indicated that a high percentage of the spray is retained on the plants, with little liquid deposition bouncing off the plants to the ground. Because plants at high densities and more advanced growth stages have significant more biomass, it was not surprising to observe that larger plants or those grown in high densities received less spray liquid when retention rates were based on a unit rather than the total plant dry weight (Table 2).

Table 2. Retention Rates (µL/g) of Three Spray Liquids on Outdoor-Grown Wheat Plants at Different Growth Stages and Densities. The Number in the Parenthesis Is the Standard Error of the Mean. Data from Ellis *et al.* (*6*).

Liquid	*1540 stems/m^2 (GS 22 to 26)*	*1540 stems/m^2 (GS 30)*	*2640 stems/m^2 (GS 22 to 26)*
Tallow amine	53(±4.32)	34(±2.35)	37(±3.10)
EC	48(±3.13)	27(±1.93)	37(±1.52)
Water	38(±2.01)	20(±0.94)	35(±1.30)

For field crops, the top canopy will most likely intercept more spray liquids than the lower canopy, either due to more receptive leaf surface characteristics or closer proximity to the spray nozzles, resulting in less canopy penetration and less available spray formulation for lower leaves. This is not a big issue for crop protection products which have good systemicity, particularly those with phloem translocation. For products with limited or no systemicity, however, less canopy penetration may lead to unsatisfactory commercial performance. More efforts should be directed to better understand the interception of spray droplets on leaves at different positions in the plant canopy. This information would be of practical use in catering the formulation of crop protection products to specific field situations and specific active ingredients.

Effect of Physicochemical Properties of Spray Solutions on Spray Retention

The physicochemical property of a spray formulation is the most important factor determining the outcome of the interaction between plant surface and spray solutions. Formulation types and the components such as active ingredients, adjuvants, and additives, define the overall spray solution properties. While the effect of adjuvants and formulation types on spray droplet deposition and adhesion has been extensively investigated, little effort has been directed toward understanding the impact of active ingredients.

Active Ingredients

Experimental Study of the Retention Rates

We have investigated the retention rate of fungicides on greenhouse grown wheat seedlings and the relationship of retention rates with physicochemical properties of the compounds. Fungicides as technical materials were formulated as 10% EC formulations (N-methyl pyrrolidone:Aromatic 200:Agrimul Lipo-D, 47:47:6 by volume). Spray formulations were applied onto 9-day old wheat seedlings (variety 'Yuma') using a track sprayer fitted with a TeeJet 8003E nozzle operated at 32 psi. The fungicide rate was 100 gai/Ha with an application volume of 200 L/Ha. Each fungicide treatment had four pots of five wheat plants, each pot representing an individual replicate. One hour after spraying, plants were cut right above the soil level and weighed. The compound was washed from the plant surface by immersing the plants in chloroform for 1 min. Plants were removed from the chloroform, allowed to dry, and frozen for future use. The chloroform was evaporated from the vials with nitrogen gas, then 1 mL of acetonitrile was added to each vial. The samples were quantified with LC-DAD/MS to determine fungicide retention rate in ng/mg fresh weight of plants.

The initial retention study included 17 fungicide compounds: epoxiconazole, azoxystrobin, tebuconazole, myclobutanil, isopyrazam, penthiopyrad, cyproconazole, picoxystrobin, fluxapyroxad, pyraclostrobin, fluoxastrobin, fenbuconazole, bixafen, and four Dow AgroSciences experimental fungicides (Compounds I to IV). The 17 fungicide compounds exhibited different levels of retention rate on wheat seedlings (Figure 3).

After the compounds were removed from the plant surface by the chloroform wash, the plants treated with epoxiconazole, azoxystrobin, and Compounds I, II and IV were extracted with acetonitrile to determine the amount of material partitioned into the plant tissue. The amount of fungicide detected inside the plants was minimal, and would not significantly affect the surface retention rate (Table 3).

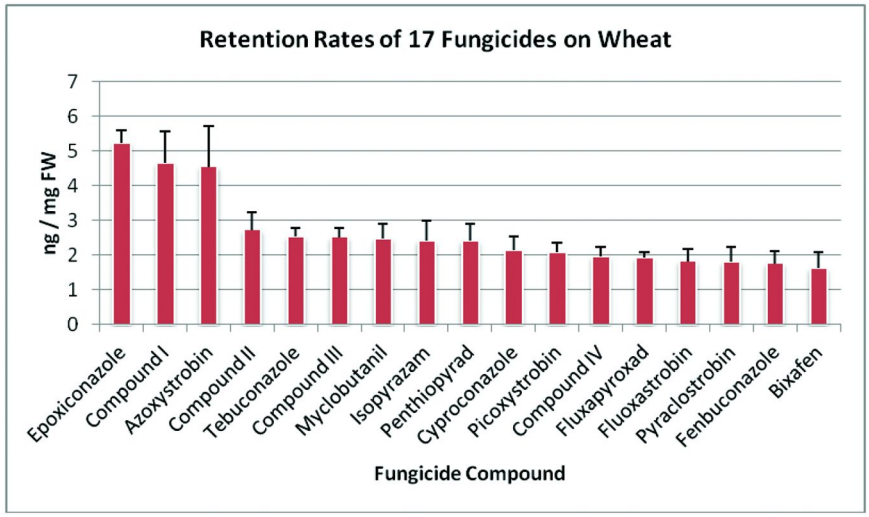

Figure 3. Retention rates of 17 fungicide compounds on wheat plants. Error bars represent one standard deviation.

Table 3. Amount of Fungicides Extracted from Treated Plant Tissues. Number in Parenthesis Is the Standard Deviation.

Fungicides	*ng/mg fresh weight*
Epoxiconazole	Not detected
Azoxystrobin	0.14 (±0.04)
Compound I	0.1 (±0.02)
Compound II	0.14 (±0.04)
Compound IV	0.07 (±0.01)

QSAR Analysis of Physicochemical Properties Contributing to the Retention

A QSAR study was carried out to understand how the physicochemical properties of a fungicide affect its retention on the plant surface. For a more accurate comparison, the retention rates by weight were transformed into a logarithmic scale of molar number ($\log_{10}$(Retention/MW)). The genetic algorithm (GA) method implemented in Molecular Operating Environment (MOE, Chem. Comp. Group Inc.) was used to identify the optimal combination of molecular descriptors that were most likely to contribute to the surface retention using

$\log_{10}$(Retention/MW) as the dependent variable and the calculated descriptors as independent variables. A leave-one-out (LOO) procedure was employed to validate the model (Q^2). The best fitting QSAR equation showed that four descriptors accounted for 92% of the variation in fungicide retention rates for all 17 compounds: the aqueous solubility (CIQPlogS: conformation-independent predicted aqueous solubility calculated with Qikprop [Schrodinger Inc.]), the number of hydrogen bond acceptors, the molecule polarizability, and the passive membrane permeability, which was determined with an atomistic physical model (*24*) (Figure 4). Fungicide retention was positively correlated with the aqueous solubility and the number of hydrogen bond acceptors, but negatively correlated with the passive membrane permeability and the polarizability of the molecule.

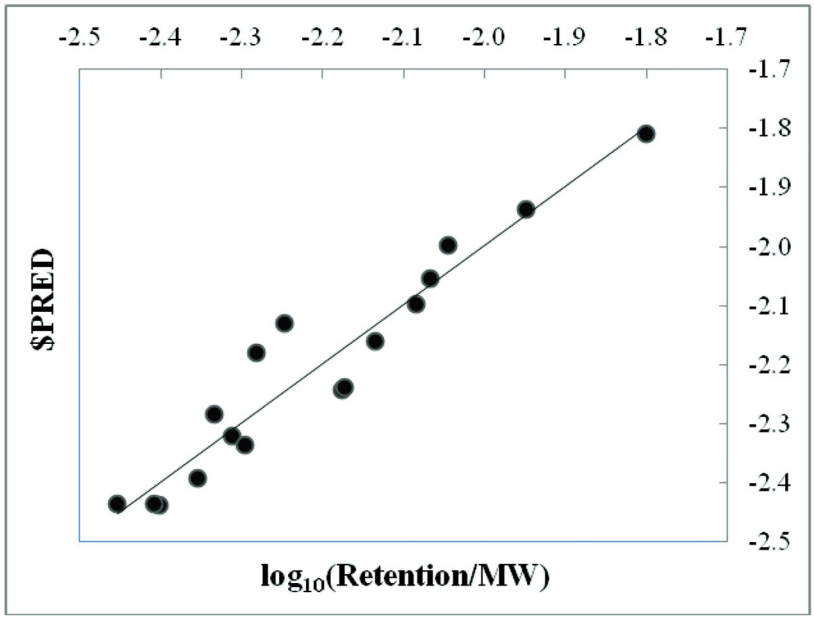

Figure 4. Correlation between the experimental and predicted fungicide retention rates on wheat. $R^2 = 0.92$, $Q^2 = 0.86$. $PRED is the predicted retention value based on the following equation: $PRED = -2.0 + 0.090 * LogS – 0.013 * SMR + 0.097 * AccptHB - 0.028 * Mem_HDLD$, where LogS = CIQP aqueous solubility, AccptHB = the number of HB acceptors, SMR = the polarizability of molecule, and Mem_HDLD = the passive membrane permeability. Log_{10}(Retention/MW) is the experimentally determined retention value.

Among the four descriptors, the aqueous solubility and the molecule polarizability were the most important contributing factors for fungicide retention on plants, with each factor producing an R^2 value of ~0.3 when plotted against the retention rates. We used predicted water solubility data for the analysis

because experimental solubility data was not available for all compounds. When the predicted solubility value was plotted against the experimental solubility values (only available for 13 fungicides), there was a strong correlation between the predicted value and published data with an R^2 value of 0.71 (Figure 5). This suggests the predicted solubility value is a reasonable representative of a molecule's experimental solubility, and can be reliably used in the analysis.

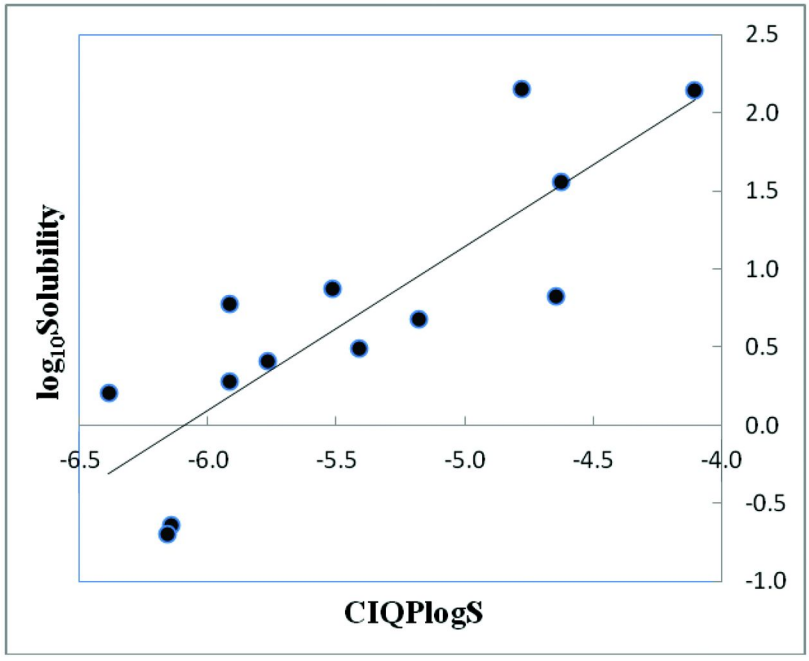

Figure 5. Correlation between the experimental solubility (log_{10}Solubility) and the predicted solubility (CIQPlogS) for 13 compounds.

To further validate the model, the retention rates of six additional fungicides, including prochloraz, propiconazole, fluopyram, penflufen, metconazole, and flutriafol, were predicted with the established QSAR model. All six fungicides share the same modes of action as some of the fungicides in the training set of 17 fungicides. The predicted retention rates were then compared to the experimentally determined retention rates of these compounds. The experimental and predicted values had a high level of correlation, with an R2 value of 0.84, showing the retention rate of a fungicide was reliably predicted by the QSAR model (Figure 6).

In summary, a clear trend can be observed from this QSAR analysis, despite the relatively small dataset. Physical properties have a significant impact on the retention rate of a fungicide on the plant surface. Generally speaking, the more soluble and less polarizable the fungicide, the more retention the fungicide on a plant surface. The underlying mechanism for this phenomenon is likely related to the effect of the two physical properties on the partition of the fungicide molecules into different phases of the spray formulation: higher water solubility results more active ingredients dissolved in the water phase, and higher polarizability leads to more fungicide molecules retained in the emulsion droplets. In the current study, the spray formulation consisted of 99.5% water and 0.5% organic solvents; more compound present in the water phase would provide larger contact area of fungicide molecules and leaf surface.

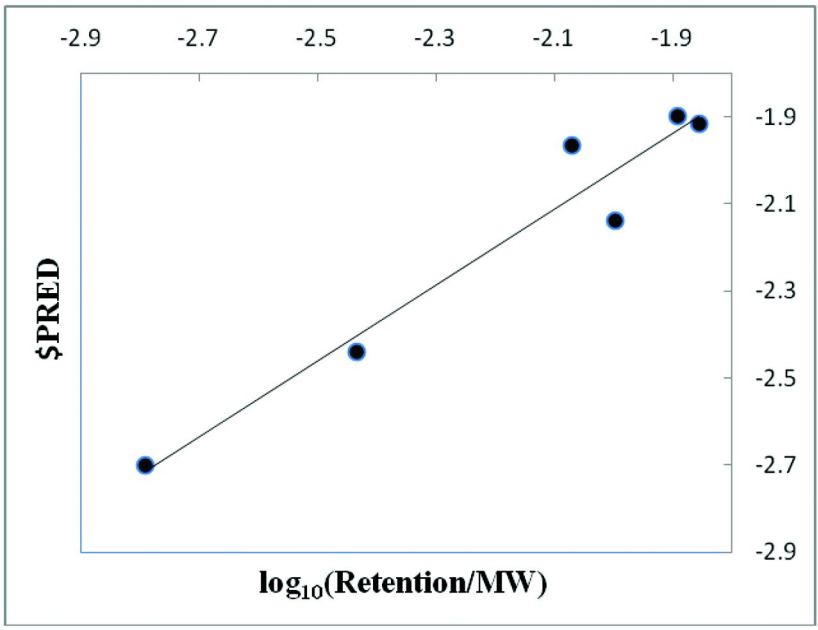

Figure 6. Correlation between the predicted retention rates and experimentally determined retention rates for six additional fungicide compounds.

Adjuvants

Using adjuvants to enhance the performance of crop protection agrochemicals, either as a tank mixture or built-in coformulates, is a very common practice. There are a large number of generic and proprietary adjuvants available in the market. The most important classes of adjuvants are listed in Table 4.

Table 4. Major Classes of Adjuvants

Class	*Example*
Surfactants	Nonylphenol polyethoxylates
	Tallowamine polyethoxylates
Emulsifiable oils	Mineral oil
	Vegetable oil and derivatives
Polymers	Polyvinyl alcohols (PVA)
	Polyacrylamides
	Synthetic latex
Polymer-forming compounds	Terpenes
Inorganic salts	Ammonium sulfate

Surfactants

Surfactants are surface-active compounds consisting of a hydrophobic tail and a hydrophilic head. The hydrophilic group is typically polyoxyethylene (EO), with EO contents in the range of 2 to 20. By keeping the hydrophobe constant and varying the EO content, a series of surfactant products with different hydrophile-lipophile balance (HLB) values can be produced. The products can vary in appearance from viscous liquids to waxy solids, with water solubility increasing with higher HLB values. Because of their surface-active properties, most surfactants will increase spray deposition on difficult-to-wet foliage. Spray droplets containing surfactants adhere to leaf surfaces because the hydrophobic head of the molecule attaches to the epicuticular waxes on foliage, while the hydrophilic EO group remains in the water phase of the droplets, resulting in a larger contact area and a smaller contact angle. Holloway *et al.* (*25*) compared sodium fluorescein retention efficiency on field bean, pea and barley using nine adjuvants that represent the major classes of adjuvants. They found that three EO-based surfactants, tallow amine, nonylphenol and organosilicone, showed significant retention enhancing ability on two difficult-to-wet plants: pea (Figure 7) and barley. For the easy-to-wet plant, field bean, all adjuvants and water had a similar level of retention.

The retention efficiency of surfactants is also related to their composition and concentration in spray liquids. For the maximum effect, surfactant concentrations need to be well above the critical micelle concentration (CMC) of the surfactants. Retention enhancement by EO-based surfactants is directly affected by their EO content. For surfactants with the same hydrophobe, lower HLB products (EO<6) often provide retention inferior to those with higher HLB (*26*). Although the influence of surfactant structure on spray deposition efficiency has not been studied systematically, the shape and size of the hydrophilic and/or hydrophobic groups could be important. Taylor and Chambers (*27*) compared the retention

of two surfactants with different hydrophilic head groups on winter barley. The surfactant with the smaller hydrophilic head group was more effective at a low concentration. Surfactants with a shorter hydrophobic carbon chain may diffuse faster to the surface of the droplets, as diffusion through liquids varies inversely with the square root of molecular weight (*28*). Surfactants with higher HLB, being more hydrophilic, also may diffuse more readily through the water to the liquid/air interface of the droplets (*29*). Moreover, surfactants with very low HLB may separate from the water phase, giving an uneven coverage of the droplet surface (*29*).

The majority of surfactants decrease the volume median diameter (VMD) of droplets in the spray spectrum when compared to pure water (*25*, *26*, *30*, *31*), as illustrated by tallow amine and nonylphenol in the Figure 8. The droplets with smaller VMD are retained better by target plants because of their lower mass and kinetic energy at the impaction site. However, for less water soluble surfactants (lower HLB), an increase in VMD has been observed (*26*). Organosilicone surfactants can reduce the surface tension of spray droplets more significantly than other surfactants, but their effect on spray droplet VMD is not consistent in the published literature. Stevens *et al.* (*32*, *33*) reported that the VMD of spray droplets with organosilicone surfactants can be reduced to a significantly greater degree than conventional surfactants, but Holloway *et al.* (*25*) actually observed the opposite effect in that VMD of droplets increased significantly when organosilicone surfactants were used. The different spray nozzles and spray pressure, as well as other ingredients in the spray solution, could be the cause of the observed discrepancy. Because of their exceptional surface activity, organosilicone surfactants can infiltrate into plants through the stomata. The efficiency of stomatal infiltration is strongly influenced by stomatal status (*34*) and surfactant concentration (*35*).

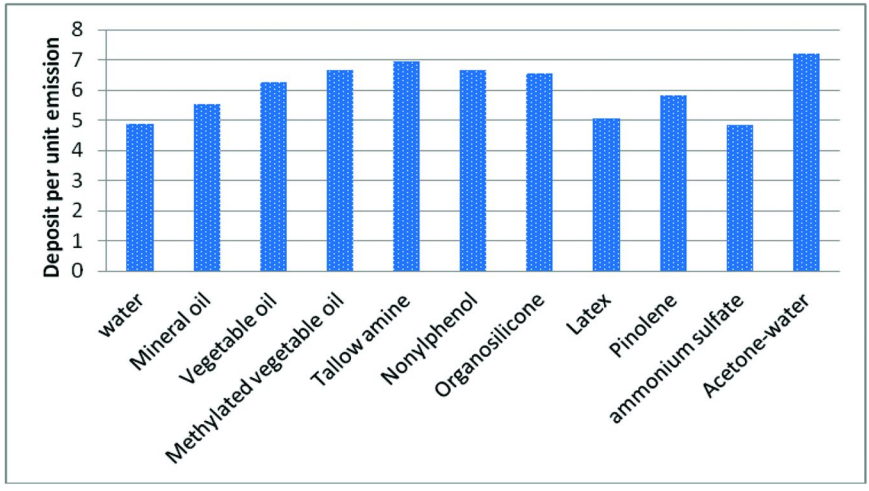

Figure 7. Adjuvant effects on fluorescein retention by pea foliage (averaged results from two experiments). Data from Holloway et al. (25).

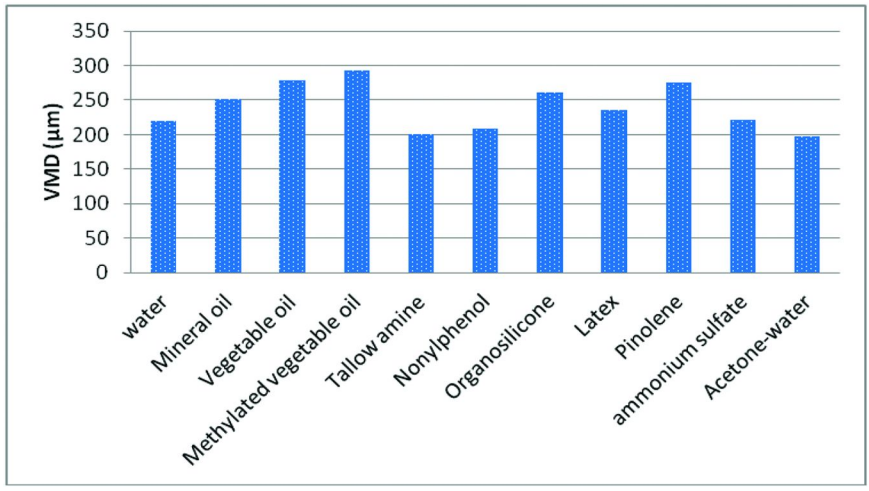

Figure 8. Adjuvant effects on spray droplet volume median diameter (µm) from an even fan spray nozzle. Data from Holloway et al. (25).

In the retention studies of tebuconazole on maize leaves, a wide range of tebuconazole formulation types were evaluated for their dynamic surface tension (DST). From these studies, a strong linear correlation between tebuconazole retention and liquid DST has been demonstrated (*5*). Anderson and Hall (*36*) and Green and Green (*37*) also showed that spray retention, or biological activity, is strongly correlated with DST, but not the equilibrium surface tension. Flight time of a droplet from a spray nozzle to the leaf surface is very short, typically in the range of 50 to 100 ms (*13*), so the droplet surface is generally far from equilibrium when it reaches the plant surface (Figure 9). For most surfactants, a concentration of at least 0.1% is needed before a decrease in surface tension causes a reduction in droplet size in such a short time interval. Higher concentration of a surfactant provides more desirable DST in the short flight of the droplets (Figure 9).

Acetone and alcohols are routinely used to prepare lab formulations for evaluating biological activity of early discovery compounds. These solvents alter the bulk properties of water and thus show no dynamic surface tension change when compared to the equilibrium surface tension of the solution (Figure 9). These types of solutions have ideal surface-active properties. *N*-propanol-water mixtures exhibited approximately 30mN/m optimal surface tension, which allows maximum retention on a difficult to wet target, without run-off (*26*). When acetone-water (1:1 v/v) was compared to all major classes of adjuvants in terms of retention efficiency on field bean, pea and barley, none of the adjuvants tested were superior in performance to the aqueous acetone benchmark (Figure 7) (*25*).

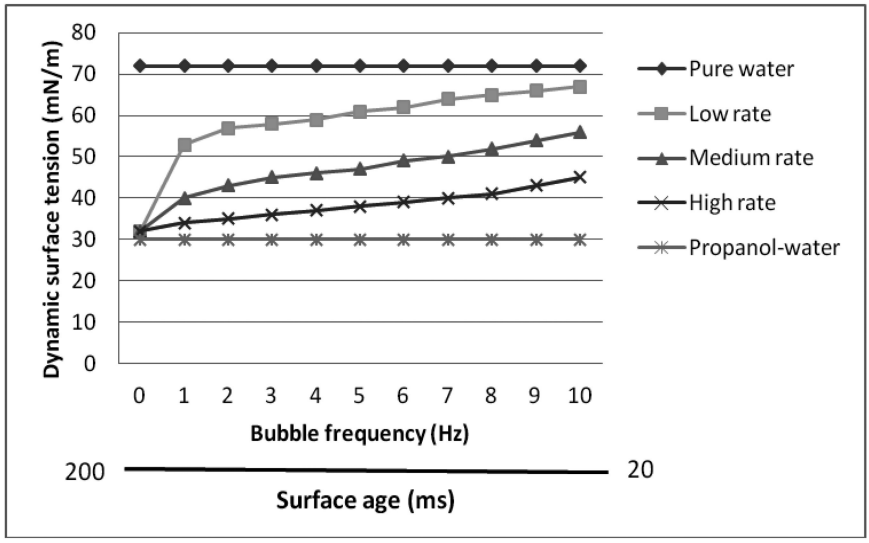

Figure 9. Schematic diagram of dynamic surface tension profiles of pure water, a hypothetical EO-based surfactant at three concentrations, and propanol-water mixture. The equilibrium surface tension is given at zero bubble frequency for reference.

Both retention and DST are concentration dependent well above the CMC of the surfactants, which helps explain the large surfactant amounts often needed for the optimization of biological performance (*5*). Pathan *et al.* (*38*) also demonstrated a strong correlation between the concentration of an organosilicone superwetter Silwet L-77 and the amount of glyphosate retained on barley and broccoli. In addition, Taylor (*4*) showed there was a strong correlation between retention and dynamic contact angles of the surfactant solutions on barnyard grass, fat hen and black nightshade.

Retention rate is inversely related to the VMD of droplets, droplet velocity and the dynamic contact angle (*4*, *39*, *40*). VMD and droplet velocity are interrelated and contribute to the kinetic energy of droplets when they impact a plant surface. The kinetic energy of a falling droplet at terminal velocity varies as the seventh power of its diameter, which requires significant adhesional force at the impact site to prevent the droplet from bouncing off. This phenomenon makes it very challenging to retain droplets with very large VMD (*41*). Small droplets generate lower kinetic energy at the point of contact, and also have a higher surface area to volume ratio; therefore, plant surfaces can more readily retain them. For droplet sizes in the range of 100 to 400 μm, retention on difficult to wet plant surface is mainly affected by the DST of spray solutions (*40*). While DST is a major factor affecting the deposition of spray liquids, there are other factors that are also important for developing a reliable model to predict agrochemical retention on plant surface. These factors include: droplet velocity and VMD, spray liquid viscosity, advancing and receding contact angles on plant surfaces, leaf surface characteristics, and canopy structure and density.

Oil Adjuvants

Emulsifiable oils are mainly used as tank mixtures and derived from three sources: 1) mineral oil from petroleum, 2) vegetable oil from oilseeds (soybean, canola, etc.), and 3) esterified vegetable oils such as methyl oleate. Mineral oils are mixtures of various hydrocarbons with carbon numbers in the range 16 to 30. Vegetable oils are mixtures of triacylglycerols, usually with a high degree of unsaturation. Oil-based adjuvants are viscous liquids containing varying amounts of surfactants as emulsifiers, which form an oil-in-water dispersion once mixed with spray formulations. VMD increases substantially for oil emulsion, with the droplet size of methylated vegetable oil being the largest, followed by vegetable oil and mineral oil (Figure 8). Interestingly, methylated vegetable oil contained the most emulsifiers, while the mineral oil had the least amount of emulsifiers (*25*). Compared with pure water, the VMD of vegetable oils increases ranging from 5 to 22% in flat fan applications (*42*, *43*). Improvement in spray retention by tank mixing with adjuvant oils was quite effective when compared to surfactants (Figure 7). Oil composition, however, is not a major factor of retention enhancement, because retention enhancement is affected mainly by the emulsifier contents and concentrations. Hall *et al.* (*44*) observed that on young foliage of barley and peas, spray retention from the emulsifier alone was equivalent to the spray retention of whole emulsions, but on old leaves, emulsions gave superior performance to the emulsifiers. It is impossible to determine the DST of adjuvant oil-in-water emulsions because they are two-phase systems. Even with enlarged VMDs, oil adjuvants have shown high efficiency in retention of spray droplets, which is clearly different from the requirement of surfactants for high retention efficiency.

Polymers and Other Adjuvants

Water-soluble polymers, polyvinyl alcohols (PVAs) and polyacrylamide, as well as insoluble, dispersed polymers such as synthetic latex, form a thin film once in contact with the target plant surface. These adjuvants have little surface activity, but some of them have very good retention-enhancing ability. PVAs do not significantly influence surface tension, spray solution viscosity, and VMD of the spray droplets, however, they have very good retention-enhancing abilities, sometimes being superior to some of the commonly used EO-based surfactants (*5*, *26*). The retention enhancement of polymeric adjuvants such as PVAs cannot be predicted either from DST or droplet VMD. Clearly this type of adjuvant has a different mechanism for enhancing spray retention when compared to surfactants. PVAs exhibit a reduced surface elasticity of spray droplets, making them less prone to bouncing off the plant surface. Since water-soluble polymers such as polycrylamides can increase droplet size, and simultaneously do not compromise spray retention, some of them have been mainly used as drift retardants, which can increase the VMD from 45% to more than 125% (*3*). Polymer concentrations do

affect droplet spectrum: the higher the concentration, the larger the increase in the droplet VMD; however, synthetic polymer latex has shown a limited effect on the spray retention and the size of the spray droplets because it is not a water-soluble polymer, and does not impact the spray solution viscosity (Figures 7 and 8) (*25*).

Polymer-forming adjuvant terpenes polymerize on contact with air, forming an adhesive and protective film on the plant surface. The effect of terpenes on the spray droplet spectrum depends on the adjuvants used. Among three menthene-based adjuvants evaluated, Chapple *et al.* (*45*) found the VMD of spray droplets varied from -19 to 7%, when compared to pure water. Another polymer-forming adjuvant, pinolene, had a positive effect on spray retention and VMD (Figures 7 and 8) (*25*).

Inorganic salts, such as ammonium sulfate, are mainly used in tank mixtures with glyphosate formulations. Holloway *et al.* (*25*) evaluated a spray solution containing 3% ammonium sulfate and found there was no change in the droplet VMD and the spray deposition efficiency relative to water (Figures 7 and 8).

It should be noted that many retention studies were performed using the adjuvant solutions alone. Other components in the formulation, such as active ingredients, emulsifiers and solvents, will further modify the physicochemical properties of the spray liquids. The potential interactions of all the components in the agrochemical formulations can render spray retention studies with the adjuvant alone to be a lesser representation of a realistic situation. Our fungicide retention work has clearly demonstrated that the physicochemical properties of an individual fungicide can affect the amount of compound retained by wheat plants.

Formulation

Spray droplet retention is also influenced by the commercial formulations that are used to deliver biological activity of crop protection agrochemicals, because each component in a formulation may have an effect on the physical and chemical properties of the final spray solution. The most common formulations include: soluble concentrate for water soluble compounds, emulsifiable concentrate for compounds soluble in organic solvents, and wettable powder and suspension concentrate for water insoluble chemicals. The major types of crop protection agrochemical formulations are shown in Table 5. When surfactants are added as a tank mixture above CMC, they likely play a predominate role in modifying spray droplet behavior. For formulations used as stand-alone products, the quantity and property of emulsifiers, wetting agents, and active ingredients will determine the retention efficiency on the plant surface. In this case, spray volume could become an important factor; a decrease in spray volume at a constant use rate of agrochemicals will increase the concentration of surfactants and emulsifiers in the final spray formulation, thus improving droplet retention.

Table 5. Major Types of Formulations for Crop Protection Products

Formulation type	*Code*
Granule	GR
Solution concentrate	SL
Emulsifiable concentrate	EC
Wettable powder	WP
Suspension concentrate	SC
O/W emulsion	EW
Suspoemulsion	SE
Water-dispersible granule	WG

In our studies three formulations of epoxiconazole, including the commercial formulations Ignite (8 % EC) and Opus (12.1% SC), and a 10% generic lab SC, were used to determine epoxiconazole retention efficiency on wheat seedling plants. Spray application and compound recovery followed the same procedures as described above in our fungicide retention studies. The commercial formulation Ignite showed the best retention of epoxiconazole on the wheat surface (Figure 10). The other two epoxiconazole formulations did not show a significant difference in their retention rates. One hour after compound application, the amount of epoxiconazole uptake into the plants for each formulation was minimal relative to the amount detected on the plant surface.

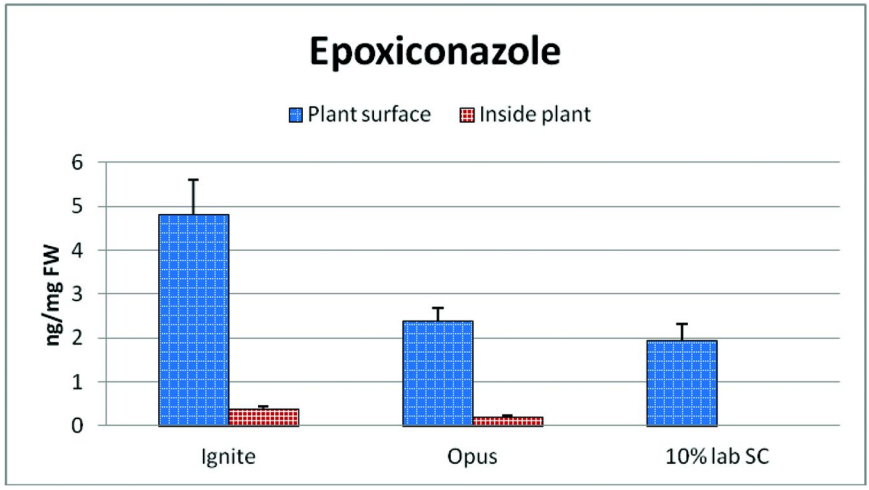

Figure 10. Effect of three formulations on epoxiconazole retention and uptake one hour after spray application. Error bars represent one standard deviation.

Whole plant activity of the three epoxiconazole formulations was evaluated in a dose-response analysis vs. wheat brown rust (*Puccinia triticina*). Plants were inoculated with *P. triticina* two hours after compound application, and disease severity was assessed on the primary leaves once the rust was fully developed on untreated plants. ED_{50} and ED_{80} values and 95% confidence intervals were calculated (Table 6). Even though no significant difference in protectant efficacy was detected for the three formulations at 95% confidence level, there was a clear trend based on ED_{50} and ED_{80} values, Ignite, with the best retention of epoxiconazole on plant surface, also delivered the best protectant activity, followed by Opus, and 10% generic lab SC.

Table 6. ED_{50} and ED_{80} Values in gai/Ha with 95% Confidence Intervals for Three Formulations of Epoxiconazole versus *Puccinia triticina*

Formulation	ED_{50}	ED_{80}
10% SC	4.93 (±2.12)	10.5 (±7.74)
Opus	2.75 (±0.54)	5.16 (±1.03)
Ignite	2.35 (±0.49)	3.77(±1.38)

In a BASF's technical report (*46*), Ignite has shown faster drying time on the plant surface than Opus (10 *vs*. 16 min.), therefore providing better rainfastness and retention. In field evaluations, it delivered better efficacy vs. Septoria leaf blotch and wheat brown rust than Opus (*46*). Wirth et al. (*5*) compared the retention rates of four experimental tebuconazole formulations on barley leaves. The results indicated EC and EW formulations provided much better retention of the fungicide than two WP formulations, particularly when the surfactant loading was reduced. Therefore, taken together with our findings, formulation types can significantly influence retention rates of agrochemicals, and ultimately biological activity.

Conclusions

Retention efficiency of spray droplets is mainly influenced by surface characteristics of the target plant and the physiochemical properties of the diluted spray formulation. For plants with an easy-to-wet surface, the inclusion of adjuvants in the spray solution is not needed because these types of plants are very receptive to the deposition of water droplets. For plants with a difficult-to-wet surface, addition of adjuvants in the spray formulation is essential for better droplet adhesion and retention. Performance of surfactants used to enhance retention is mainly determined by DST, VMD and velocity of spray droplets. As a result of reduced DST and smaller droplet size, a large contact area at the impaction site and less kinetic energy are both generated at the interface of the plant and droplets, allowing high retention efficiency of spray formulations. Oil

adjuvants form an oil-in-water emulsion and typically increase the size of droplets, but still show high efficiency in spray retention. Retention efficacy is mainly influenced by the content and concentration of emulsifiers, with much less effect from oil composition. Polymers and polymer-forming adjuvants don't reduce DST and typically increase the size of droplets; however, some of the adjuvants in the group have shown retention enhancing abilities comparable to commonly used surfactants. A series of fungicides, when formulated as ECs, have shown significant variation in their ability to be retained by wheat seedling plants. QSAR analysis established a model based on four physicochemical properties of the 17 fungicides evaluated, which predicted fungicide retention quite reliably, and the model proved that the active ingredient can be a major factor influencing droplet retention. There is a limited amount of information on the impact of formulations on spray retention; however such information could be useful to understanding the performance of formulations in commercial situations. We have shown that the Ignite formulation of epoxiconazole provided the best fungicide retention among the three formulations evaluated, and also delivered the best protectant activity *vs.* wheat brown rust. The majority of retention investigations in the open literature have utilized greenhouse grown seedlings or small plants. While using this type of plants is very helpful to gain an understanding at the factors affecting spray retention, plant surface wettability changes at different growth stages and plant canopy structure and density also significantly affect how the spray droplets are intercepted. More efforts should be directed toward studies using plants with comparable size and density to field situations to better understand the complicated interaction between spray droplets and plants.

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Chapter 2

Co-penetration of Actives and Adjuvants and Its Significance for the Matched Pair Liaison

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Most formulants and adjuvants are low molecular weight solutes and can principally be sorbed in the surface lipids and deeper layers of the cuticle. This can have huge impact on the sorption potential of actives and their mobility in the rate limiting barrier of the cuticle. Factors like volatility, photostability, plant compatibility, selectivity, salt compatibility, rainfastness, speed of action, weed control, residual efficacy, the possibility of product combinations etc. depend often primarily on the relative absorption and penetration of active and adjuvant and their interaction. However, many factors affect the manifestation of these effects in practice like separation of active and adjuvant from the respective dispersion during evaporation, unmatched speed of penetration, or precipitation of active or adjuvant, respectively. Typically, adjuvants have several functions and – ignoring wetting effects in this contribution – they can act in the dry spray deposit as much as in the cuticle, for example they can solubilize actives in the former and mobilize them in the latter. For best results a timely and rate fit is needed, robust enough to withstand practical variability. Important commercial formulations show a perfect match of active(s) and adjuvant penetration or non-penetration, respectively. Examples on interactions among formulation or spray ingredients will be shown for a model compound and leaf cuticle system. On the

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

other hand, a striking example will be given that shows that plant structure can change the picture completely. The latter is in the authors opinion a missed cause for the difficulty of proper product optimisation under field and particularly greenhouse conditions.

Introduction

There is hardly any crop protection agent that is made up only by one or more active ingredients with no content of other ingredients or so called formulants (*1–4*). The use of many of these ingredients is related to physical and chemical stability of the active and preparation, respectively, that affects biological performance by proper delivery. However, there are further ingredients that improve biological performance of a physically and chemically stable and applicable product. These adjuvants can manifest their effects by very different means and there are several classifications that distinguish the action as spray modifiers (e.g. buffer, conditioner, drift retardants) from not directly spray related activation (*5–7*). This has to do with effects on volatility, photostability or bioavailability on the leaf surface and the speed of penetration through the plant leaf cuticle.

The activation effects of adjuvants beyond spray modification depend basically on the effects on bioavailability in the spray deposit (*8–14*) and the modification of the leaf cuticle transport properties (*12*, *15*, *16*). Now, there are hundreds of different agrochemicals and hundreds adjuvants, and the principle questions arise whether there are particular matched pairs that have to be identified case by case or whether there are universal adjuvants that can be used with numerous agrochemicals or classes, e.g. systemic fungicides for cereals. The answer to both questions is clearly yes. For the latter case one can refer, for example, to the most popular and effective methylated seed oil adjuvants that are globally recommended at concentrations of 0.25% to 2% for fungicides, herbicides and insecticides according to the labels of the hundreds of products. The main reason for this wide use and suitability of methylated seed oils is that, similar to a wetting agent that works robustly with any spray system, they swell the leaf cuticle of practically all plant surfaces after contact to the leaf surface (*17*). Often however, there are antagonistic effects with such good products like crop selectivity and for robust and good residual performance relatively high threshold concentrations are often needed with methylated seed oils.

The answer to the first question, do matched pairs exist, is also yes. This applies particularly to so called ready-to-use formulations where all ingredients, one or more actives and one or more adjuvants, are included in the formulation. For the formulator and the optimization of formulations the identification of the matching partner adjuvant or the matching adjuvant system is related to the integration of bio-performance, plant compatibility, cost, content and in-can use in a stable formulation that can be passably formulated. Looking at the global market it is striking that most of the globally meaningful products and generally most products in Europe are such ready-to-use formulations or occasionally

twin packs or other combinations of agrochemical active and activator. Ignoring the numerous individual reasons for that we find matched pairs in the sense that a particular combination is superior for whatever reason. For example, IPA-glyphosate formulations work extremely well with tallow amine alkoxylates and other alkyl amine alkoxylates, glufosinate formulations contain practically only alkylether sulfates, aryloxyphenoxyproprionate herbicides, azole and strobilurin formulations come very often with long alkyl chain alkoxylates, and there are also several sophisticated and complex formulation systems like oil dispersions of sulfonylurea herbicides or mesotrione suspension concentrates that contain at least three components with adjuvant function. In all these cases there is hardly a tankmix adjuvant that gives any biological effect when the ready-to-use products are used at the recommended rates and not adversely over diluted.

The products in these examples are all wetting agents but replacing just this wetting function with another product typically gives a lower level of performance. So there are other features that are more specific for the best fit of an adjuvant to a particular active in a particular formulation. Besides the fit to the specific formulation this has to do with the action of an adjuvant or adjuvant system to increase bioavailability in the spray deposit and its action as penetration enhancer on the level of the plant cuticle (*12*). Obviously, these are two different sites of action, i.e. on and in the cuticle or epidermis, respectively, and the questions arise about the time course of adjuvant absorption into the cuticle, how this is affected by the other ingredients and the impact on solute (diffusing active ingredient) penetration. Most adjuvants (and formulants) are low molecular weight solutes and depending on whether or not they carry charges they can principally be absorbed in the surface lipids and deeper layers of the cuticle. To keep it simple, in this contribution we consider only interactions of non-ionic alcohol ethoxylates and non-ionic actives or model compounds in the spray and deposit. Strong interactions will be shown with impact on penetration kinetics obtained with plant cuticles (*17*, *18*) and we will consider also the opposite, non-interactions and non-relevance of adjuvants with special primary plant organs.

Foliar Penetration Proceeds from the Deposit

Homogeneous dispersion of the agrochemical in the spray solution is essential for good application practice and delivery to the crop. This includes the prevention of adverse processes like foaming, filter blockage, drift and other adverse effects. However, while good spray delivery is an obvious prerequisite for good performance on the plant surface it is more important for herbicides and all systemic agrochemicals that good penetration is provided from the spray deposits that forms often only minutes after application (*13*, *19*). Very often dispersions of poorly water soluble actives are used and they appear as more or less even distributed particles on the leaf surface as indicated in Figure 1. This spray deposit may contain one or more dispersing agents, other formulation ingredients like anti-freezing agents, salts from the spray water and the adjuvants. The spray deposit merges more or less with the surface waxes depending on their

physical state. The epicuticular wax crystals have been found to be in most cases irrelevant for foliar penetration from the deposit as they do not add any barrier on top of the transport limiting skin below the surface waxes (*12*, *20*, *21*). However, it cannot be excluded that with some products the bioavailability or driving force of actives in the deposit is affected by the mass ratio and degree of dissolution of waxes and wax crystals by the the formulation or adjuvant, respectively.

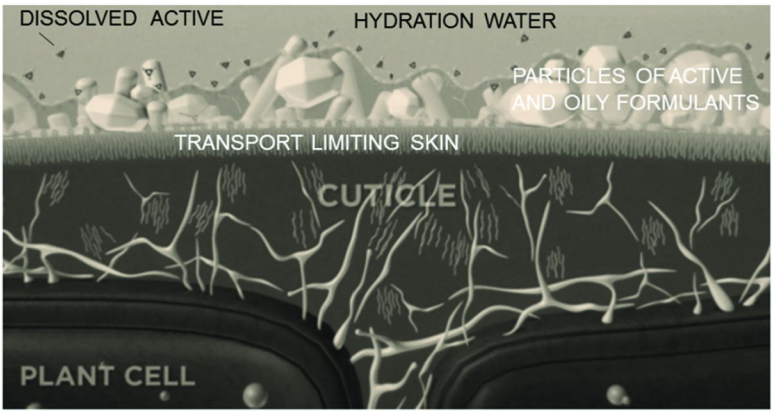

Figure 1. Schematic drawing of a spray deposit on the leaf cuticle with particles of active and layer of oily formulants. If the deposit hydrates active may dissolve in the aqueous phase.

As indicated the active particles are even thicker than the transport limiting skin and it is clear that agrochemicals have to dissolve from the particles in order to be absorbed as molecules into the leaf surface. For that reason we speak about solutes in the forthcoming chapters. The schematic drawing of Figure 1 indicates also that hydration of the spray deposit will enable some active molecules to dissolve in the aqueous phase and this fraction is available for sorption in the leaf cuticle and penetration. If it is a highly water soluble active the amount of water will affect the fraction dissolved and the partitioning into the cuticle (*22*). Similarly, the often paste like complex mixture in the dry spray deposit changes its physical state with temperature. This affects the available or dissolved fraction of the active and has sometimes a huge impact on the foliar penetration characteristics (*19*). The interactions are complex and in this paper we consider only an average and constant scenario of temperature and humidity and consider other possible interactions that are more related to the availability of the chemicals and penetration characteristics. While the below results were obtained from laboratory studies and are affected by certain environmental conditions we consider them as not misleading since they apply to typical (average) conditions and conditions that limit performance under non optimum conditions for agrochemical bioavailability.

Self-Penetration of Surfactants and Its Relationship to Agrochemical Activation and Penetration

Many non-ionic surface active adjuvants are linear low molecular weight substances with moderate to high lipophilicity and have all properties to be rapidly absorbed in the leaf surface. When comparing solute mobility in Citrus x aurantium leaf cuticles it was found that aliphatic solutes have about 10-fold higher mobility than cyclic solutes (*22*). Figure 2 shows the penetration of radiolabelled octaethylene glycol dodecyl ether (C12E8) across pear leaf cuticles to be very rapid with 60% of the applied amount having penetrated within 2 hours. This agrees with earlier reports based on the measurement of penetration across isolated cuticles (*23*), or based on surface recovery, i.e. wash-off from the leaf surface after certain time periods (*24*). This surfactant shows fastest absorption and penetration of about 60-85% within one day with all plant species investigated, with only slight kinetic differences (*23*). Surfactant penetration was fastest during and immediately after droplet water evaporation and, except with Citrus CM, where penetration was constant and followed a first order kinetics, the time course of penetration ceased after 5 hours similar to the time course of C12E8 in Figure 2.

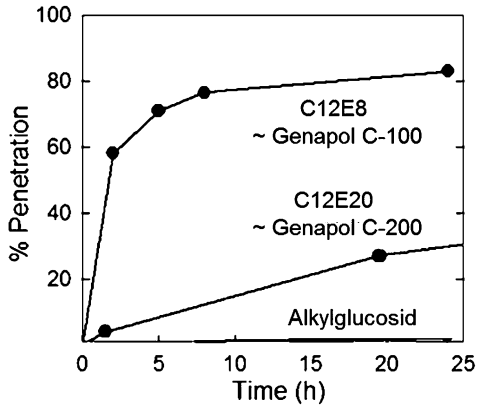

Figure 2. Time course of self-penetration across leaf cuticles of pear of C12E8 (corresponds to average formula C12.5E8 of Genapol C100), C12E20 (Genapol C200) and C8-10 Alkylglucosid. (geometric mean of 10-15 leaf cuticles, 25°C/56% rh). Data are partly from reference (18).

The monomer C12E20 showed a contrary time course of penetration that was relatively constant over time and while it was 10 times slower than C12E8 for the first two hours after application, one day later more than 30% penetration had accurred and was still increasing. While after 8 hours 75% of the C12E8 had

penetrated with almost no increase thereafter. Similar findings were reported by others for other radiolabelled ethoxylates and plant species like wheat, oat or field bean, i.e. C12E16 was found to be slightly behind C13E6 (*24*) but a C16-18E22 did not show measurable penetration (*25*). Figure 2 shows also that a radiolabelled alkylglucosid did not penetrate at all within 24 hours.

What is the consequence of this differential potential and speed of self-penetration? The technical adjuvant Genapol C100 is a coconut based product with an average formula of C12.5E8.1 and thus close to C12E8, while Genapol C200 equals more a C12E20. We compared the self penetration of these adjuvants and an alkylglucosid with the penetration of a hydrophilic model compound, methylglucose, in the presence of the adjuvants at 1.0 g/l spray concentration. The result is shown in Figure 3 and it almost looks like a copy of Figure 2 with respect to the shape of the three curves. Methylglucose alone does not show measurable penetration above 1% at 24 hours with a relative humidity below 60% (see Figure 4). In the presence of 1.0 g/l Genapol C100 in the spray, methylglucose penetration is promoted particularly at the beginning while 5 hours after application penetration almost stopped. This is a very strong correlation with the surfactant (C12E8) penetration and the impact of co-penetration becomes visible. Genapol C100 (~C12E8) is able to swell the leaf cuticle effectively (*12*) and increases the mobility of solute. Due to the rapid absorption of C12E8, the cuticular mobility of methylglucose and the hydrophilicity of the leaf cuticle are increased and this allows absorption of methylglucose during evaporation of the droplet water.

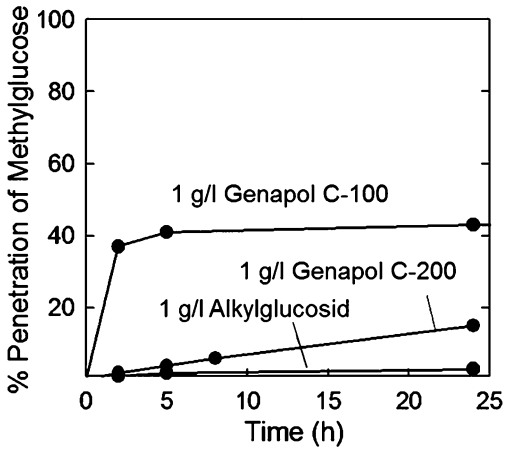

Figure 3. The effect of 1 g/l Genapol C100, C200 and C8-10 Alkylglucosid on penetration of methylglucose across leaf cuticles of pear. (geometric mean of 10-15 leaf cuticles, 25°C/56% rh. Data are partly from reference (18).

After 5h about 75% of the Genapol C100 penetrated (Figure 2) and both, reduced swelling of the cuticle due to depletion of the adjuvant and no reservoir on the leaf surface assisting in re-dissolving the methylglucose cause the ceasing of methylglucose penetration. In this case, the adjuvant has been fully absorbed and penetrated the cuticle leaving the active behind on the surface. A higher use rate of adjuvant can prolong the existence of a sufficient amount of adjuvant on the surface but this is just one option and applies only if the biological system can cope with rapidly developing peak concentrations of active and adjuvant, respectively. The penetration of methylglucose with the other two adjuvants equals as well the time course of the adjuvant penetration: no penetration of alkylglucosid and no effect on methylglucose penetration. In fact, an alkylglucosid can be antagonistic for hydrophilic actives as it is not increasing permeability due to the lack of affinity; and the partitioning to the lipophilic cuticle from a hydrophilic to aqueous deposit might be even reduced (*23*). For the Genapol C200 there was a slow but constant penetration of methylglucose in full agreement with the slow absorption of Genapol C200 in the cuticle. It helps re-dissolving methylglucose in the deposit only slightly as it becomes wax-like under the experimental conditions and it does not enhance penetration on the level of the plant cuticle. In conclusion, the matched pair that has to be identified is clearly related to the co-penetration of the active and adjuvant but the definition of perfect match depends obviously on the targeted time course and intended effect (e.g. rainfastness vs. residual efficacy). The term co-penetration so far has to be interpreted not as migration of a pair but of two distinct compounds at the same time. They are not linked like in a chelate but via the timely interactions within the spray deposit and leaf cuticle.

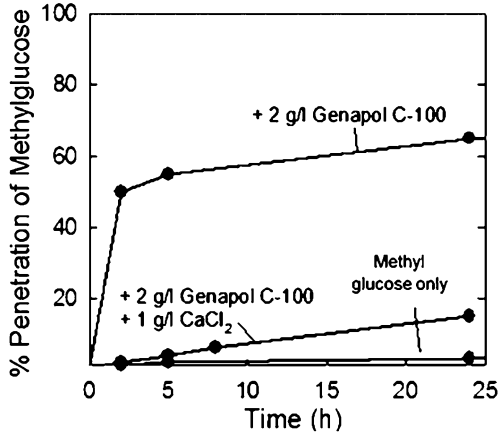

Figure 4. The effect of 2 g/l Genapol C100 with or without the addition of 1 g/l $CaCl_2$ on penetration of Methylglucose across leaf cuticles of pear. (geometric mean of 10-15 leaf cuticles, 25°C/56% rh. Data are partly from reference (18).

Foliar Penetration Is Affected by Interactions of Adjuvants and other Inerts in the Deposit

The whole complexity and probably most often underestimated multiple interactions in typical spray liquids having blends of several agrochemicals is addressed in this section. As suggested above, a higher (2-fold) use concentration of Genapol C100 increased methylglucose after one day from 40% (in Figure 3) to more than 60% penetration. This results from both, an even more immediate penetration during the first 2h and also due to longer lasting rate constant of penetration after 5h (Figure 4).

This picture changes drastically when the salt calcium chloride is added at a concentration of 1 g/l to the spray. In effect, no measurable penetration until the first sampling time about 2h after application indicates antagonistic interaction of Ca ions with the Genapol C100 starts immediately in solution. Chlorine appears not to be involved as alkaline chlorides showed no effect (data not shown). The bulk water of the droplet has evaporated after one hour but as shown in Figure 4, penetration was also much slower from the dry spray deposit. The result is a Genapol C100 deposit that resembles the more wax-like deposit of Genapol C200 and indeed the resulting methylglucose penetration with 1 g/l Genapol C-200 (Figure 3) is practically the same as that with 2 g/l Genapol C100 plus 1 g/l $CaCl_2$ (Figure 4). It appears that the $CaCl_2$ has solidified the usually paste-like spray deposit at the relative humidity of 56% rh and this would hinder the absorption of Genapol C100. However, $CaCl_2$ has a deliquescense point of 28-31% rh and it should dissolve completely at 56% rh making the deposit more liquid. In fact, the deposit was fully transparent on a polystyrene surface. The deposit was not waxy but obviously, the high concentration of $CaCl_2$ appears to be still antagonistic for penetration of the surfactant. The nature of these interactions is unknown and studies on interactions of monodisperse alcohol ethoxylates in micellar solution are not giving a hint. For example, Schick (*26*) found that for C12E8 the micellar aggregation number increases from 123 to 149 in the presence of 0.5 M $CaCl_2$ while that of C12E18 decreased from 51 to 44. In any case there is an interaction and the site of action can be either in the spray deposit or in the cuticle (or both). The effect of $CaCl_2$ on the Genapol C100 action agrees with the previously reported antagonistic effect of $CaCl_2$ on penetration of C12E8 (*23*). At 1g/l $CaCl_2$ the initial rate constants of C12E8 penetration decreased about 3-4-fold, demonstrating a strong interaction (Figure 5). However, after 2h rate constants were even higher suggesting that the main interaction manifests not in the spray deposit but has to do with lack of absorption of C12E8 during and immediately after droplet water evaporation. This interaction that is obviously very effective at a concentration as low as 0.2 g/l $CaCl_2$ did also give a strong decrease of C12E8 penetration at 25°C/56% rh (Figure 5).

The time course of C12E8 penetration with 0.2 g/l is practically equal to the one at 1 g/l $CaCl_2$. At lower humidity of 11% the initial rate constants at 1 g/l have been reduced even 20-fold (*23*) while at high relative humidity of 93% the affect vanished. However, after one day, absolute penetration at the concentrations initially antagonistc at 56% rh, was no different from the control without $CaCl_2$. Increasing the concentration of C12E8 from 0.04 to 2 g/l did not

change the antagonistic effect. This concentration of 0.2 g/l is much lower than the use concentration of foliar fertilizer, may come with the formulation and even moderately hard water has a Calcium content in that range. So we consider these interactions as highly relevant for practical conditions. The interaction of Ca ions with the ethylenoxide chain in aqueous solution is known (*26*). So we assume that these interactions start in solution and last for the deposit formation. During the bulk water free "deposit phase" the antagonism depends on the salt and, as very similar results to $CaCl_2$ have been obtained with $MgCl_2$, we assume that the dry salt affects deposit and active availability as well.

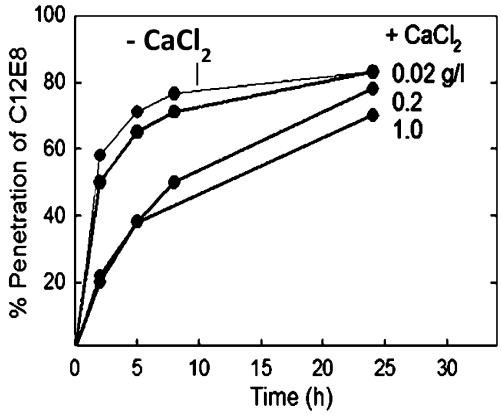

Figure 5. Time course of self penetration of C12E8 across leaf cuticles of pear as affected by increasing concentrations of $CaCl_2$ (geometric mean of 10-15 leaf cuticles, 25°C/56% rh. Data are partly from reference (23).

Different Pathways for Different Plant Organs: Coleoptile and Hypocotyl versus Mature Leaves

Mature leaves and other primary organs have to withstand the strong gradient in water potential between plant tissue and the non-water saturated ambient air and a low water permeability of the air interface is an essential function of the cuticle for terrestrial life of plants (*27*). In contrast, seeds germinate typically under good conditions of water supply from water located in the free space surrounding soil or substrate. In this phase, the young seedling gets organic nutrients essentially from the reservoir of the storage bodies while minerals and water are taken up by the first root organ, the radicle, which has not a large surface at that stage. The primary stem and distinct above root organs hypocotyl and coleoptile grow at least partly (non-aerial) in the soil and one may expect uptake of minerals and water by these organs until the root system has further developed. Early reports on the uptake of soil applied agrochemicals suggest uptake via the coleoptile and hypocotyl (*28*, *29*). The coleoptile of monocotyledons can be considered a hypocotyl as well, although some call it the first leaf (*30*, *31*). We have recently found (*32*) that

the non-aerial coleoptile of wild oat is a very poor barrier against the diffusion of organic solutes, and for example the penetration of radiolabelled imidacloprid was almost quantitative after one day only. In Figure 6 we compare imidacloprid penetration across the non-aerial coleoptile with the results for the penetration obtained with aerial coleoptile and mature leaves, respectively, that underlines the striking difference.

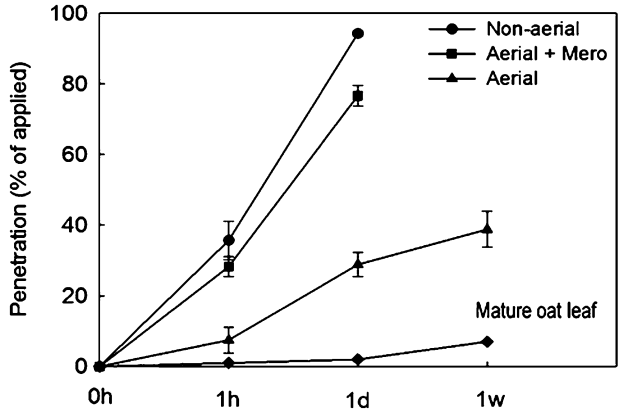

Figure 6. Time course of penetration of imidacloprid across mature leaves, and the aerial and non-aerial hypocotyl of wild oat (Avena fatua L.). (26°C, 70% rh, 12h photoperiod at 2.2 kLux; n = 10, geometric means with 95% CI). Reproduced with permission from reference (32). Copyright 2010 ISAA Society.

In contrast to the quantitative penetration with the non-aerial coleoptile, there is hardly any penetration across the mature oat leaf. Actually, the non-aerial coleoptile acts more like an organ optimized for efficient uptake, like a root, while the mature leaf has a cuticle with a high resistance for water permeability (*33*). Interestingly, the penetration result for the aerial coleoptile is exactly between those obtained with the non-aerial coleoptile and the mature oat leaf. The addition of adjuvants like methylated seed oils (MSO, like Mero, Bayer CropScience, in Figure 6) did not change the kinetics of penetration across the non-aerial coleoptile (data not shown), but enhanced it for the aerial coleoptile to give the same level as with the non-aerial one without adjuvant. From comparisons with an adjuvant that is similar to the methylated seed oil type an increase of imidacloprid penetration across the mature leaf of monocots gives about 50% penetration within one day ((*20*), data not shown), i.e. a level similar to that with the aerial coleoptile. The relative effect of the methylated seed oil adjuvant (related to the control without adjuvant) was of course much higher (>10-fold) with the mature leaf compared to the aerial hypocotyl (about 2.5-fold).

The exceptional high permeability of young organs was also found for the penetration of the rynaoid active flubendiamide across the hypocotyl of mung bean (*Vigna radiata* L.). Flubendiamide (Belt, Bayer CropScience) is a phthalic acide diamide insecticide that is applied onto mature leaves for the control of lepidopterous pests. While the uptake is usually via ingestion it shows

translaminar activity that gives local systemic protection. This indicates that even though flubendiamide is a big molecule there is some foliar penetration. The McGowan molecular volume is about 480 cm^3/mol (*34*) and for molecules of that size the fraction penetrated into the leaf after one day is very low, generally less than 1% penetration within one day without the addition adjuvants (*12*). This low penetration was also found for the penetration of flubendiamide across the leaf cuticle of mature mung bean leaves as indicated in Figure 7. Again, this contrasts with about 20-fold faster penetration across the non-aerial hypocotyl although there was not a quantitative penetration within one day as in the case of the non-aerial oat coleoptile.

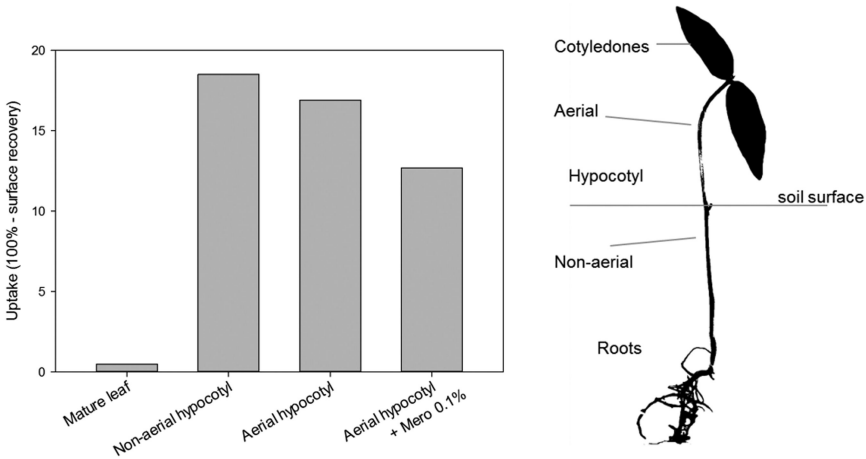

Figure 7. Penetration of flubendiamide after one day into the aerial and non-aerial hypocotyl and mature leaf of Vigna radiata. (26°C, 70% rh, 12h photoperiod at 2.2kLux, geometric mean n=10).

Flubendiamide has an almost three times higher McGowan's molar volume than imidacloprid. Flubendiamide belongs to the 1% actives with highest molecular weight and the rest 99% of all other systemic agrochemicals have a molecular volume at least 30% lower, such that the result for the non-aerial hypocotyl may appear surprising. However, it agrees well with recent findings for the same species (*35*) that show that PEG600 with a similar molecular weight or molar volume, respectively, was also penetrating as indicated by osmotic studies. The same holds for the results with radiolabelled ATP which is even a charged molecule. The radiolabelled studies showed also that urea, an organic solute 8-fold smaller than ATP is not much faster penetrating than ATP. In the osmotic studies it was just PEG1000 that was absolutely not absorbed by the hypocotyl surface and from these studies a cut-off diameter of about 1.5nm was suggested for molecules being able to rapidly penetrate via pore like openings. The flow of water in the osmotic flow tests was very rapid (reaction time of water loss from hypocotyl into the hyperosmotic solutions in the range of hours) and suggested further a high water permeability of the hypocotyl.

Besides the radiolabelled diffusion studies and the osmotic experiments (*35*) we also used staining techniques with a hydrophilic cationic dye, methylene blue, that stains hydrophilic and particularly anionic surfaces and indicates also the particular surface characteristics of the hypocotyl. The non-aerial hypocotyl and a bit less so the aerial, stained intensively blue, similar to the primary root surface while the mature leaf is not stained at all. Similar to the results in the coleoptile trials (Figure 6) penetration was also fast across the aerial hypocotyl but the level was even closer to the non-aerial hypocotyl. The addition of the MSO adjuvant Mero did not give any further increase in penetration and was even decreasing penetration. Obviously, due to the low water solubility and high molecular size of the active, faster penetration can not be realized due to subsequent limitations in the low to negligible translocation. This may also explain that the observed penetration of flubendiamide for the non-aerial was not much higher than for the aerial hypocotyl and is also a difference to the result with imidacloprid which is rapidly translocated (*20*, *32*). Being a lipophilic molecule with a log P of 4.2 (*36*) flubendiamide may also not partition well into the hypocotyl from the methylated seed oil emulsion and lipophilic spray deposit with the adjuvant Mero.

The above results suggest that young stems like the hypocotyl of mung bean as well as the coleoptiles of monocotyledonous plants are very open organs due to pore like openings. So called polar or hydrophilic pathways that are established by aqueous channels that traverse the cuticular barrier have been postulated by others for mature plant organs based mainly on studies with cuticles from selected species (*37–39*). However, the methods employed do not allow these conclusions undoubtedly and if different methods were applied the use of different species prevents better evidence. The indirect evidence for the existence of pores in the leaf surface of selected species that are formed at high relative humidity and link to a network of water channels is just one of several explanations for the obtained data. In addition, the estimated pore size of 0.3-2.4 nm for the mature leaves is in a range not different from that existing for all lipophilic actives which, according to the authors, take the lipophilic route. One may wonder, if the diameter in the water filled channels is just the same as the free volume space allowing diffusion between the lipophilic chains of the cutin/wax composite layer in the limiting skin of the cuticle. However, there is a significant difference in the nature of the movement. In such narrow channels there is only restricted diffusion possible (*40*). In our own studies we could not get evidence for polar pathways across cuticles of mature leaves and findings like fast penetration at high relative humidity through water filled channels could always be explained by other reasons related to e.g. increased contact area or higher dissolved fraction of active. Further, water filled channels with such a small diameter do not enhance solute mobility nor solubility, and while we estimated similar pore size of 1.5 nm for the hypocotyl of mung bean but not for mature leaves, our conclusion is different. The surface of young plant organs acts as a filter with a cut-off size around 1.5 nm, allowing entrance into it with simply no further limiting skin, or only a very thin one below the surface. As mentioned above, an aqueous channel with the dimension of the quoted 0.3 to 2.4 nm is too narrow to allow free diffusion as most agrochemical molecules have diameters of at least 1 to 1.5 nm. This means that the average distance to the channel wall is much smaller than the mean free path length of 1 nm for diffusion

in aqueous systems (*40*). The result is a largely restricted diffusion and in fact, one may assume that the restricted diffusion for both hydrophilic and lipophilic solutes is an essential part of the barrier besides the thickness and tortuosity of this layer.

In contrast, the non-aerial hypocotyl and coleoptile behave more like a cuticular membrane where the cuticular waxes have been quantitatively extracted. These polymer matrix membranes have properties of a pore membrane (*41*). It appears reasonable that the primary organs hypocotyl and coleoptile start without such waxes in a moist soil environment and in a later stage of growth, after reaching the air space, waxes are produced that block the pores and increase the thickness of the barrier layer. A lot has been learned about the biosynthesis of waxes, species dependent differences, ontogenetic changes, erosion of the surface waxes (*42*, *43*). However, while the existence of intra- and epicuticular waxes is known and the formation of epicuticular wax crystal structure in vivo and in vitro recrystallization are quite well understood (*42*, *44*, *45*) this is not yet the case with the intracuticular waxes. The lack of knowledge is about the controlled transport of waxes to their target sites. It is suggested that as result of their inclusion in the cuticle and intercalation in the voids, the adding of the waxes increases viscosity and both the solid state characteristics and the barrier thickness, and as a consequence the apparent solute mobility decreases. The size selectivity (change of solute mobility with molecular volume) was very similar for the same lipophilic active ingredients in intact cuticles and polymer matrix membranes (*12*, *22*). These actives were all moderately to very lipophilic and removal of waxes was essentially reducing the path length for diffusion while the molecular environment was the same. This was also suggested by an equal free energy enthalpy relationship for intact cuticles and polymer matrix membranes that did apply also for different species, suggesting that solutes diffuse in a similar network of the plant cuticle (*12*). In contrast, size selectivity for hydrophilic and lipophilic molecules differed in poplar membranes and was also lower for hydrophilic solutes in mung bean (*32*). The decrease in size selectivity was not independent of absolute permeability but correlates with an increase in permeability. As essentially the same pore size (0.3 to 2.4nm in the literature, 1.5nm in our studies) exists the decreased selectivity is obviously linked to lower viscosity (of a thinner barrier). This difference does not need diffusion in channels but may exist simply due to these openings and it might be speculated that the very thin barrier itself is the reason.The interested reader is referred to Aponte and Baur (*35*) for more details on permselectivity of the hypocotyl and a discussion of the transport mechanisms.

We consider the pore-like openings in hypocotyl and coleoptile, particularly in the non-aerial parts, as preferred sites of uptake in spray applications onto seedlings and maybe also young plant organs. Under greenhouse trials these structures may exist throughout the duration of the biological test. Since this uptake route does not respond to or depend much on the common adjuvant systems proper optimization of formulations or spray systems, with adjuvants appears impossible in such tests. This may add to the well known discrepancy of greenhouse and field results and can mislead with respect to the right selection of candidates.

Conclusion

Co-penetration of actives and adjuvants is a situation that typically applies in practice, and very often proper co-penetration is behind the identification of optimized systems which have, of course, to consider other needs as well. Considering the number of active and adjuvant combinations and the influence of rate and ratios there is not much mechanistic information found in the literature nor can it be deducted from patents. It is suggested that this is due to the complexity of the interactions and the fact that the industry prefers to keep robust systems as a trade secret.

There are striking correlations between the self-penetration potential of adjuvants and their impact on foliar penetration. In fact, if fast penetration is wanted due to e.g. fast rain fastness, according choice of rapidly co-penetrating adjuvants is useful for modern typically large molecular weight agrochemicals. The suitability and choice of a particular adjuvant depends on the molecular size, rate and selectivity of the agrochemical and the basic fit to the environmental conditions has to be considered. As shown in this paper for a salt, the self-penetration and physical state of an adjuvant can be manipulated by such salts. This has immediate impact on the adjuvant power as penetration enhancer and there are many formulants that behave as such third compounds.

Coleoptiles and hypocotyls of weeds and crops appear to be poor barriers against the penetration of typical low molecular weight agrochemicals. This holds particularly for the non-aerial part of it and other adjuvants than those typically used to enhance foliar penetration of agrochemicals.

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Chapter 3

Modeling Xenobiotic Uptake and Movement: A Review

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The complex relationship between xenobiotic physicochemical properties and formulation, plant physiological and biochemical processes and, the environmental factors that affect these processes constitute the cornerstone of the effectiveness of foliar-applied xenobiotics such as pesticides. Permeation of plant cuticle, short-distance transport to the mesophyll cells and long-distance transport in the vascular tissues are among the multitude of processes involved in the movement of a foliar-applied xenobiotic from the leaf surface to the meristematic tissues. In the past decades several experimental and theoretical advancements in the understanding of cuticular permeation and phloem mobility have been accomplished. The development of several models has fostered our knowledge of the complexity of the interplay between the different physical, physiological and biochemical process involved in xenobiotic uptake and translocation in a plant. This chapter describes the processes governing penetration of foliar-applied xenobiotics in and movement through plant cuticles and the mathematical relationships describing the cuticle permeation of non-polar and polar xenobiotics. The movement of a xenobiotic from the point of contact with the leaf surface to distant sites of action was also described in relation to phloem mobility with an emphasis on phloem loading from sources, long-distance transport in the sieve elements and phloem unloading into the sink regions. An overview and utility of the ERMESSE model was also discussed.

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

Introduction

The efficacy of a foliar-applied xenobiotic depends upon a complex interplay of factors including the physicochemical properties and formulation of the xenobiotic compound, physiological processes within the plant and environmental factors affecting plant processes (*1*, *2*). Any systemic postemergence xenobiotic must first permeate the cuticle, and then subsequently move through short-distance transport to mesophyll cells, long-distance translocation that includes loading into and transport within the vascular tissues, and unloading into the sites of action located in the new growth region of the roots, stems and leaves (*3*).

The movement and behavior of a systemic postemergence xenobiotic being a highly complex phenomenon, simulation models were developed to better understand the interplay between the different physical, physiological and biochemical processes involved in xenobiotic uptake and translocation in a plant. In the past decades several simulation models have also been developed to address either the cuticular movement of xenobiotics, or the mobility of xenobiotic molecules through plants vascular tissues. The extensive work of Schönherr and Riederer (*4–8*) on the permeation of xenobiotic molecules through isolated, reconstituted and intact leaf cuticles has established a relationship between xenobiotic octanol/water partition coefficient, cuticle/water partition, xenobiotic molar volume and cuticle permeability. The models developed for mobility through the vascular tissues have focused primarily on the role of long distance transport phenomena (xylem and phloem) in this process. The model developed by Tyree *et al.* (*9*) related the phloem mobility of non-electrolyte xenobiotics to their ability to passively permeate the plasma membrane. This concept was further developed by Lichtner (*10*) who related the phloem mobility of a xenobiotic to its octanol/water partition coefficient. Because the mobility of xenobiotic molecules within the phloem can also be affected by the presence of Brönsted acid groups on the molecules (*11*), Kleier (*12*) has developed a model that predicted xenobiotic phloem mobility based on membrane permeability and the acid dissociation constant (pK_a) of the xenobiotic.

While previous models have contributed to a better understanding of cuticular penetration or the phloem mobility of a xenobiotic, these models did not integrate appropriate plant physiological and anatomical characteristics, environmental factors that affected the interaction between xenobiotic and plants, and the analysis of effects on xenobiotic translocation resulting from plant metabolic activity (*3*). Moreover, the interaction of environmental factors such as relative humidity and temperature on cuticle penetration (*13*), phloem translocation (*13*), and xenobiotic metabolism (*14*) has not been considered in earlier models.

The nonlinear dynamic simulation model ERMESSE, which was developed at the University of Illinois, considers xenobiotic physicochemical properties (MV, pK_a, and $logK_{ow}$), plant physiological, anatomical and biochemical processes (e.g. xylem/phloem connections, cuticle thickness, membrane permeability, apoplast, symplast and vascular sap pH), and relevant soil and environmental parameters (relative humidity, temperature and soil water potential). Because the model integrates a large number of plant parameters as well as xenobiotic and environmental parameters in a complex mathematical network (*15*), the

ERMESSE model more accurately predicts xenobiotic uptake and translocation (*16*, *17*). Such a model may prove useful in rational pesticide design as well as for understanding effects of environmental factors in determining xenobiotic effectiveness. However, the use of models for the prediction of xenobiotic uptake into plants is not limited to the design of novel pesticides. Modeling the uptake of chemicals into plants have proven to be useful in the assessment of human exposure to pesticides (*18–21*), the assessment of the effects and impact of air and soil pollutants (*22*, *23*), the evaluation of chemical residues in foods (*24*, *25*) and for phytoremediation (*26*, *27*).

In this book chapter, the process of cuticle permeation by foliar-applied xenobiotics such as pesticides is reviewed as well as the phloem mobility of such chemicals. An overview of the ERMESSE model is described in addition to providing the utility of the model in simulating xenobiotic uptake and translocation. Furthermore, the relationship between xenobiotic physicochemical properties and xenobiotic uptake and translocation was analyzed.

Modeling Uptake of Foliar-Applied Xenobiotics

Uptake of foliar-applied agrochemicals is a complex process that depends on the interplay of several interrelated parameters. Extensive work on the cuticle penetration of water, non-polar lipophilic compounds and polar ionic compounds have contributed to a better understanding of the cuticular permeation process and to the development **of** models that can be useful in predicting xenobiotic diffusion and movement in plant cuticles.

The Plant Cuticle

The plant cuticle, referred also as cuticular membrane is a non-cellular, non-living and lipoidal membrane covering all aerial parts (leaves, stems, flowers and fruits) of the higher terrestrial plants. The primary function of the cuticle is to protect plants against uncontrolled loss of water. The plant cuticle is exposed to both abiotic factors such as light, wind, rain, etc…, and also to biotic factors like microbes, fungi and insects (*28–30*). With regard to foliar-applied agrochemicals, the cuticle is the first point of contact between the formulation and the plant; it represents the major barrier that xenobiotic compound has to overcome for penetration and movement through the plant-atmosphere interface.

The cuticular membrane, with a thickness ranging from 0.10 to 20μm (*31*, *32*) is so heterogeneous that there is no typical morphological structure. Regardless to the plant taxonomy, Holloway (*33*) has proposed six general morphological types based on the fine structure of the cuticular membrane.

The predominant structural model of the cuticle is a bilayer cuticular membrane in which the two layers are distinguishable by their ontogeny, ultrastructure and chemical composition (*34*). The bilayer cuticular membrane consists of two regions, the cuticle proper and the cuticular layer. The cuticle proper, which bears a superficial layer of soluble epicuticular waxes, constitutes

the outer region of the cuticle and consists mainly of soluble and polymerized aliphatic lipids (including intracuticular waxes), whereas the cuticular layer represents the inner layer, which forms by impregnation of the cell wall and contains large amount of various cell-wall polysaccharides. Adjacent to the cuticular layer is a pectinaceous layer of pectin-rich cell-wall polysaccharides (*35*, *36*).

The main structural component of the cuticular membrane is the lipid polymer cutin. Within the cutin, also called cutin framework or cutin polymer matrix, are found lamellae which may contain the intracuticular waxes and fibrillae, which are composed of polysaccharides (*37*). The amount of plant cutin varies considerably between species, ranging from 20 to 80% (*37*, *38*). The basic cutin matrix composition is polyester of hydroxylated fatty acids (C_{16} or C_{18}) (*33*). The -OH and -COOH groups confer to the cutin its hydrophilic property whereas its lipophilic property is due to the $-CH_2$ and $-CH_3$ groups. Because of its chemical composition, plant cutin is a polyelectrolyte carrying negative and positive charges with an isoelectric point around 3 (*39*). Due to this isoelectric point, plant cuticles are negatively charged at physiological pH. The cuticular waxes, which are produced in the epidermal cells (*40*), comprise the epicuticular waxes located at the outer surface of the cutin polymer matrix and the intracuticular waxes embedded into the cutin framework (*37*, *41*). Removal of the cuticular waxes with organic solvents leads to an increase of cuticular permeability by several orders of magnitude, demonstrating the importance the cuticular waxes as the transport-limiting barrier of the cuticle (*42*, *43*). The epicuticular waxes, whose physical nature depends on plant species, can be amorphous, semi-crystalline or crystalline (*44*). This physical structure plays an important role in the barrier property of plant cuticles (*6*, *45*). Although the epicuticular waxes play an important role in the wettability of leaf surfaces and affect spray deposition, distribution and retention (*35*, *46*, *47*), their impact on the rate of penetration of a foliar-applied xenobiotic into leaves is limited (*36*, *48*). The main barrier to the penetration of foliar-applied xenobiotic consists of the cutin matrix and the embebbed intracuticular waxes (*49*, *50*). The transport-limiting properties of the intracuticular wax are related to its solid and crystalline aggregate state that greatly influences both diffusion and solubility of foliar-applied xenobiotics (*51*, *52*). In addition to the cutin matrix, pectin fibers, which are polysaccharide polymers, may extend from the apoplast (cell wall) into the cutin framework at the inner surface of the cuticular membrane (*53*, *54*).

Cuticle Penetration

The penetration of foliar-applied xenobiotics through the cuticle occurs by a process of diffusion which consists of three steps: sorption into the cuticular membrane, diffusion through the cuticular membrane and desorption into the apoplast of the epidermal cells (*55*, *56*). The xenobiotic compounds are first sorbed into the epicuticular wax aggregates at the leaf surface, then diffuse through the epicuticular waxes into the cutin polymer matrix and enter in contact with the intracuticular waxes. Equilibrium between the xenobiotic formulation,

the epicuticular waxes and the cutin polymer matrix including the intracuticular waxes is established within 30 min (*55*). After the equilibrium is reached, the amount of xenobiotic in these compartments no longer increase and desorption into the apoplast of the epidermal cells concludes the process of xenobiotic transfer across the cuticle.

Over the past decades studies dealing with cuticular permeability have been conducted. Water permeability of cuticles has been extensively studied (*30*, *57–59*). The interest for improved efficacy of foliar-applied agrochemicals mainly pesticides has led to extensive research in the understanding of cuticular penetration of these xenobiotic compounds (*5*, *35*, *60–63*). Plant cuticles are permeable to water, non-polar lipophilic compounds, ionic and other polar compounds, and it has been suggested that movement in the cuticular membrane follows two parallel pathways for lipophilic and hydrophilic xenobiotics, respectively (*64*, *65*). Experimental data gathered over the past decades using isolated cuticles, reconstituted wax and whole leaves led to the development of a very well-established theory and a series of experimental techniques to predict and measure the permeability of the cuticles to water and non-polar lipophilic compounds (*5*, *7*, *8*, *43*, *59*, *66*). Diffusion through the lipophilic pathway required two important parameters, *i.e.*, lipophilicity and mobility. Lipophilicity, which is commonly described as the partition coefficient between the plant cuticle or wax layer of the cuticle and the xenobiotic aqueous formulation phase (*43*), characterizes the solubility of the penetrant within the cuticular wax barrier of the cuticle (*55*, *67*). Mobility describes the diffusion of the penetrant across the cuticular membrane, and as such parametrically depends on the size of the diffusing molecules (*7*). Water, as a small polar non-ionic molecule diffuses through the cuticular membrane using cutin polymer matrix and the cuticular waxes domains as transport path (*67*).

The second cuticular pathway i.e. the hydrophilic polar pathway has been hypothesized for decades to be the preferential vessel for movement of polar non-electrolyte molecules as well as for ionic compounds (*64*, *68*, *69*). However, there was little evidence of the existence of this pathway in the cuticle until recently. Based on extensive experimental studies, the aqueous or polar pathway in cuticles has been characterized (*67*, *70–75*). In these experiments strong evidence of the diffusion of electrolyte molecules across isolated cuticles was demonstrated. The movement of ionic polar compounds occurs through aqueous pores in the cuticle, which are located in the cuticular ledges, at the base of trichomes and in cuticles over anticlinal walls. Average pore radii varied from 0.45 to 1.18 nm (*73*).

Modeling Xenobiotic Penetration of Plant Cuticles

Before a foliar-applied xenobiotic can perform its biological activity, it must be transported from the leaf surface through the cuticle to reach distant sites of action. Several factors related to the xenobiotic chemical itself, properties of the leaf cuticle, and environmental conditions at the time of application may influence xenobiotic penetration through the cuticular membrane. Schönherr and colleagues (*43*, *48*) have developed a model that relates xenobiotic cuticular permeation

to lipophilicity, mobility cuticle characteristics, xenobiotic physicochemical properties and environmental factors. Xenobiotic diffusion in the cuticular membrane can be estimated from the following equation:

$$\log D = [(\log D^{o} - \beta' * MV) - E_D/(RT)]$$

where D ($m^2\ s^{-1}$) is the xenobiotic diffusion coefficient through the cuticle, D^o ($m^2\ s^{-1}$) represents the diffusion coefficient of a hypothetical molecule having a molar volume of 0 $cm^3\ mol^{-1}$, MV ($cm^3\ mol^{-1}$) is the molar volume estimated according to Abraham and McGowan (*76*), β′ (mol cm^{-3}) is the size selectivity of the barrier and is related to the free volume available for diffusion (i.e. $\beta^{-1} = 2.303*\beta'$), E_D (kJ mol^{-1}) is the activation energy of diffusion, R (J $mol^{-1}\ K^{-1}$) is the universal gas constant and T is the temperature in K. In this equation, the diffusion coefficient is independent of the xenobiotic partition coefficient. Indeed, due to the fact that plant cuticles are heterogeneous membranes, and xenobiotic formulations are rarely aqueous (because of the adjuvants and surfactants), the diffusion coefficient is independent of the differential solubility of the xenobiotic. However, the xenobiotic permeance, which is related to the diffusion coefficient, is dependent on the xenobiotic partition coefficient and on the thickness of the transport barrier. Permeability coefficient or permeance P ($m\ s^{-1}$) of a foliar-applied xenobiotic, which is proportional to the partition coefficient (K), the diffusion coefficient (D) and inversely proportional to the membrane thickness (ΔX, m) can be estimated by:

Once a xenobiotic is sorbed into the cuticle it can be retained in the cuticular waxes, passed into the apoplastor enter the mesophyll cells. Cuticular retention may be enhanced by high lipophilicity (*77*) or by the ability of the weak acid xenobiotic to create covalent bonds with the cuticle polymer (*78*). Xenobiotic desorption into the apoplast depend on the cell wall permeability and the differential concentration of the xenobiotic between the cuticular membrane and the apoplast.

Modeling Translocation of Foliar-Applied Xenoiotics in Plants

Xenobiotic compounds that enter the epidermal cells from the cuticle can either enter the mesophyll cells (symplast) or move directly into the phloem sieve tube/companion cell complex. Movement into the phloem from the mesophyll is also possible. Short distance transport between cells in the symplast occurs via the plasmodesmata whereas long-distance transport of foliar-applied xenobiotics occurs via the phloem.

Short-Distance Transport into the Mesophyll Symplast

Xenobiotics that enter the apoplast from the cuticle can either enter mesophyll cells (symplast) or move directly into the phloem sieve tube/companion cell complex. Movement into the phloem from the mesophyll is also possible. The movement of a xenobiotic through the plasma membrane depends on its lipophilicity (*79*), pK_a, and the solution pH as membrane permeability is greater

for an undissociated molecule than its dissociated form (*80*). Consequently, the undissociated molecules will permeate the plasma membrane more readily than will the dissociated (anionic) moiety. Penetration of undissociated molecules should follow Fick's first law of diffusion, whereas the movement of the ionized moities (dissociated) follow the Nernst-Planck equation. The movement of the undissociated and anionic moieties across the membrane will continue until equilibrium is reached. Because undissociated xenobiotic movement through membranes will tend to follow Fick's first law (*81*, *82*) the absorption rate depends parametrically on plasma membrane permeability which is $\log K_{ow}$-dependent:

$$\log P = a \log K_{ow} + b$$

where P ($m\ s^{-1}$) is the plasma membrane permeability coefficient, a and b are constants that depend upon the membrane thickness and viscosity.

Long-Distance Transport in the Phloem

Long-distance transport in the phloem occurs in the sieve tubes. It is generally accepted that the pressure flow hypothesis proposed by Münch (*83*) is the principle of the transport mechanism in the phloem. According to this hypothesis, the mass flow in the phloem is osmotically driven by a differential pressure gradient generated by accumulation of sugars and other solutes at the sources (loading) and their release (unloading) at the sinks. The sources are mainly leaves and the sinks are energy-demanding or storage tissues such as roots, fruits and meristematic tissues (*84*).

Phloem Anatomy

Phloem tissue consists of three different interconnected compartments *i.e.* the parenchyma cells, the companion cells and the sieve elements. The sieve tube elements or sieve elements are elongated living cells, usually without nuclei in which transport actually occurs (*85–87*). They are connected end to end with pore-filled sieve plates, forming long cellular aggregates called sieve tubes (*88*).The companion cells constitute the second components of the phloem. The companion cells, which are closely associated with the sieve elements, have dense cytoplasm and distinct nuclei. There are numerous plasmodesmata in the walls between sieve elements and their companion cells (*88*) and almost all plasmodesmata joining the SE-CC complex to bordering cells lead into the companion cells rather than the sieve elements (*89*). The companion cells have also the same osmotic potential as the sieve elements to which they are associated (*90*), which may provide additional evidence of the involvement of the companion cells in the phloem loading process (*91*). The third component of the phloem tissue is the parenchyma cells, which are thin-walled cells similar to other parenchyma cells, except that some are elongated.

Because phloem tissue consists of three different interconnected compartments (parenchyma cells, companion cells and sieve elements), phloem transport of photoassimilates, nutrients, amino acids and other xenobiotics

involves several different, yet interrelated processes. Collectively, these processes are associated with phloem loading into the sieve element/companion cell complex (SE-CC), translocation toward the sinks within sieve elements, and unloading from the sieve element/companion cell complex to growing and storage cells (*92*, *93*).

Phloem Loading

Phloem loading, which represents the initial step of the long-distance translocation of photoassimilates and other solutes in the phloem, is a complex process that comprises the entire pathway from the mesophyll to the sieve tube via a series of transport events through several different cell types (*94*). Three phloem loading mechanisms are know and more than one mechanism can be used by a single species (*92*, *94–97*). Each loading strategy involves a specific type of companion cells in the SE-CC complex (*98*, *99*). In the apoplastic loading, which is an active (energy-driven) loading process, sucrose produced in the mesophyll cells enters the apoplast and is then pumped across the plasma membrane into the companion cells by phloem transporters. The apoplastic loading is a thermodynamically active process that requires a proton gradient as a source of energy and sucrose transporters (*96*, *98–102*). The apoplastic loading involves specialized companion cells called transfer cells (*103*), which are characterized by cell wall ingrowths that facilitate uptake from the apoplast (*99*) and presence of few plasmodesmata which connected them to the mesophyll cells (*84*).

Polymer trapping is another loading strategy which involves specific companion cells called intermediary cells. These intermediary cells have abundant plasmodesmata connecting them to the adjacent bundle sheath cells (*104*). In the polymer trapping loading process, sucrose is diffuse into the intermediary cells through the numerous plasmodesmata and is then converted into raffinose and stachyose in the companion cells (*105*, *106*). Polymer trapping is an active mechanism, although it does not involve active transport in the formal sense of moving ions or molecules across a membrane. This phloem loading process is thermodynamically active since energy is utilized to create a concentration gradient between the mesophyll and the phloem (*96*).

The symplastic loading does not involve crossing the plasma membrane. In this passive loading process, sucrose and other solutes move simply by diffusion through the abundant plasmodesmata connecting the mesophyll cells and the SE-CC (*96*, *107*).

Mobility in the Phloem

Once photoassimilates and other solutes are loaded into the SE-CC complex, long-distance transport in the phloem sieve tubes occurs by mass flow driven by a gradient in hydrostatic pressure between sources and sinks. The hydrostatic pressure is a function of the phloem water potential and the osmotic pressure

(*108*). According to Münch (*83*), mass flow in the sieve elements occurs as long as the hydrostatic pressure in the phloem is higher at the source than in the sinks. The solutes move in the osmotically translocation stream from the sources to the sink along the vascular pathway. The osmotic pressure gradient is generated and maintained by loading and unloading of the photoassimilates and other solutes at the sources and sinks tissues, respectively (*109*). In other words, water enters the phloem due to the low water potential, keeping the hydrostatic pressure above that in the sink. Bulk flow of water carries sucrose, and other solutes, from the source leaf to sink tissues where it unloads into sink cells, either through plasmodesmata or via the apoplast.

Phloem Unloading

The transport events from the sieve elements to the sites of utilization within the sink cells contribute to phloem unloading. Phloem unloading plays an important role in the transport and distribution of photoassimilates and other solutes into the sink organs. The phloem unloading pathway depends mainly on the receiving organs, not only on the species (*110*). As such three possible pathways are identified: apoplastic, symplastic, and a combination of both *i.e.* a symplastic route interrupted by an apoplastic step (*111*, *112*). However, for most sinks the symplastic route is considered to be the common mode for unloading (*111*, *113*, *114*). Vegetative apices (shoots and roots apices) typically receive phloem-unloaded photoassimilates through the symplastic route which involve the plasmodesmata that link the sieve elements and the meristematic cells of the apices (*115–117*). The symplastic unloading pathway assumes that photoassimilates and other solutes move passively through plasmodesmata canals by diffusion (*106*). In the stems of various species, phloem unloading to the sinks is apoplastic (*112*, *116*). The apoplastic unloading requires the photoassimilates to move across the SE-CC plasma membrane which invokes an active process (*84*).

Modeling Phloem Translocation of Xenobiotics

Translocation from the point of entry into plant to distant site of action located in the meristems is an integral part of the effectiveness of foliar-applied xenobiotics mainly herbicides. Long-distance transport occurs via the phloem for most postemergence xenobiotics. As with photoassimilates and other endogenous solutes, the movement of xenobiotics in the phloem includes also loading into the phloem SE-CC complex at the source (generally leaves), transport in the sieve elements and unloading into the sink tissues (root and leaf apices). With regard to xenobiotic molecules such as herbicides, little is known about their loading into the phloem. However, it is generally assumed that xenobiotic transport in the phloem follows the direction of sucrose flow (*118*, *119*). Hence, in species where sucrose molecules are loaded through the apoplastic pathway, it is conceivable that xenobiotic molecules may be loaded similarly. The apoplastic loading of xenobiotic depends on SE-CC complex plasma membrane permeability,

xenobiotic physicochemical properties, and the apoplast and phloem sap pH. In species or tissues with a symplastic sucrose loading, xenobiotic molecules could be transferred directly along with the sucrose.

Two theories have been proposed for the movement of xenobiotics in the phloem: weak acid theory proposed by Crisp and colleagues (*11*, *120*) and the intermediate permeability or diffusion theory developed by Peterson and colleagues (*121–123*). Xenobiotic movement through the plasma membrane depends upon lipophilicity (*79*), acidity and the solution pH as membrane permeability is greater for an undissociated molecule than its dissociated form (*80*). The uptake of lipophilic neutral compounds depends strongly on their ability to partition into the plasma membrane and is independent of the pH (*79*). In contrast, absorption of weak acids (a common form for herbicides) is strongly related to the solution pH. Weak acids can either be in an anionic (dissociated) or neutral (undissociated) form. The weak acid theory proposes that compounds with a free carboxylic acid functionality will be in the undissociated form in the apoplast because of the low pH of the apoplast (pH 5 to 5.5), consequently enter the phloem in the undissociated form but once in the phloem they tend to dissociate. The ratio of dissociated and undissociated forms is governed by the solution pH and the xenobiotic pK_a according to the Henderson-Hasselbach equation:

$$pH = pKa + \log ([A^-]/[AH])$$

where $[A^-]$ and [AH] are the concentration of the dissociated and undissociated forms, respectively. The lipophilicity of weak acid molecules is pH-dependent and consequently the partition coefficient decreases dramatically with an increase of the ionization of the weak acid molecules (*81*). In general, a $\log K_{ow}$ of a dissociated form of a weak acid is lower by 3.7 to 4.0 units compared with that of the undissociated molecules (*124*). Therefore the dissociated (anionic) moiety of the xenobiotic, which is less lipophilic, will be less able to permeate the plasma membrane in comparison to the undissociated form (*125–127*). Due to the fact that the dissociated form cannot readily cross the plasma membrane, it will tend to accumulate in the phloem because of the ion trapping mechanism. Accumulation of weak acids in this manner can be predicted by the following equation (*128*):

$$\frac{[HA]_i+[A]_i}{HA]_o+[A]_o} = \frac{(1+10^{pHi\,-pKa})*[P_{HA}/P_A+\{(FE/RT)/(1e^{-FE/RT})\}*10^{pHo\,-pKa}}{(1+10^{pHo\,-pKa})*[P_{HA}/P_A+\{(FE/RT)/(1e^{-FE/RT})\}*10^{pHo\,-pKa}*e^{-FE/RT}]}$$

where ‘i’ and ‘o’ refer to concentration in the symplasm and apoplasm, respectively, P_{HA} and P_A are the permeabilities of the undissociated and dissociated forms of the xenobiotic through the plasma membrane, F is Faraday constant, E is the charge on the membrane. Although the weak acid theory can explain the mobility of compounds with acid functionality in the phloem, it can’t be used to explain and predict the mobility of lipophilic compounds which are neutral (nonelectrolytes) at physiological pH.

The "intermediate permeability" states that the most critical determinant of phloem mobility is the optimum membrane permeability coefficient of a molecule (*9*, *123*). Lipophilic xenobiotics with "intermediate permeability" are slowly absorbed into the phloem, but efflux even more slowly, leading to their retention in the phloem for sufficiently long so they are transferred with the phloem stream (*3*). The "intermediate permeability" theory was developed into a mathematical model that predicts the optimum membrane permeability P (m s^{-1}) of non-weak acid xenobiotics (*9*):

$$P = rV/2l * \ln (1-1/0.9L)$$

where r = radius of the sieve tubes (m), V = average daily translocation velocity (m s^{-1}), l = length (m) of source into which the chemical is being loaded, and L = length (height, m) of the plant. Thus P is not constant for a given xenobiotic but depends on the plant characteristic and growing environment.

Both phloem mobility theories are not exclusive of each other (*125*), and Kleier has developed a unified model that combined the weak acid mechanism and the "intermediate permeability" mechanism (*12*). Kleier's model, which describes phloem mobility in terms of both the acidity (pKa) and lipophilicity (membrane permeability), is as follows:

$$C_f = \left[\frac{[H^+]_i + K_a}{[H^+]_o + K_a}\right] * \left[\frac{(a[H^+]_o P_{HA} + P_A K_a)}{[H^+]_i(P_{HA} + b) + K_a(P_A + b)}\right] * \exp\left[\frac{-c([H^+]_i P_{HA} + P_A K_a)}{([H^+]_i + K_a)}\right]$$

where C_f is the concentration factor, Ka is the acid dissociation constant, $[H^+]_i$, and $[H^+]_o$ are the hydrogen ion concentration inside and outside the sieve tube, respectively, P_{HA} and P_A represent the permeability coefficient of the undissociated and dissociated forms of the herbicide through the plasma membrane, and a, b and c are parameters that describe the application zone, the sieve tube radius, plant length and the phloem sap velocity.

As it is well known and documented, the effectiveness of foliar-applied xenobiotic dependent on their penetration of the cuticle, permeation of the cell wall, plasma membrane and long-distance translocation in the phloem. Models have been developed to understand either cuticular penetration or the phloem mobility of a xenobiotic, however there is no model that integrates cuticle permeation and phloem mobility. The dynamic, non-linear, simulation model ERMESSE was developed for whole plant transport and allocation of foliar-applied xenobiotics.

The ERMESSE model has been extensively described previously (*15*, *16*, *129*). The model describes the movement of a systemic postemergence xenobiotic from the point of entry (leaf surface) to distant sites of action (apical meristems of new growth regions of roots, stems and leaves) as affected by cuticular penetration, vascular translocation, metabolism and delivery to subcellular organelles according to a block diagram in 9 steps (Figure 1). The mathematical equations describing the physical, physiological and biochemical processes considered by the model ERMESSE have been extensively discussed previously

(*15*). Many of the mathematical relationships developed for the model and used to simulate xenobiotic movement into different compartments or across barriers are based upon Crank's postulate (*108*) where equations are in the form:

Flow rate = Proportionality coefficient * Driving force

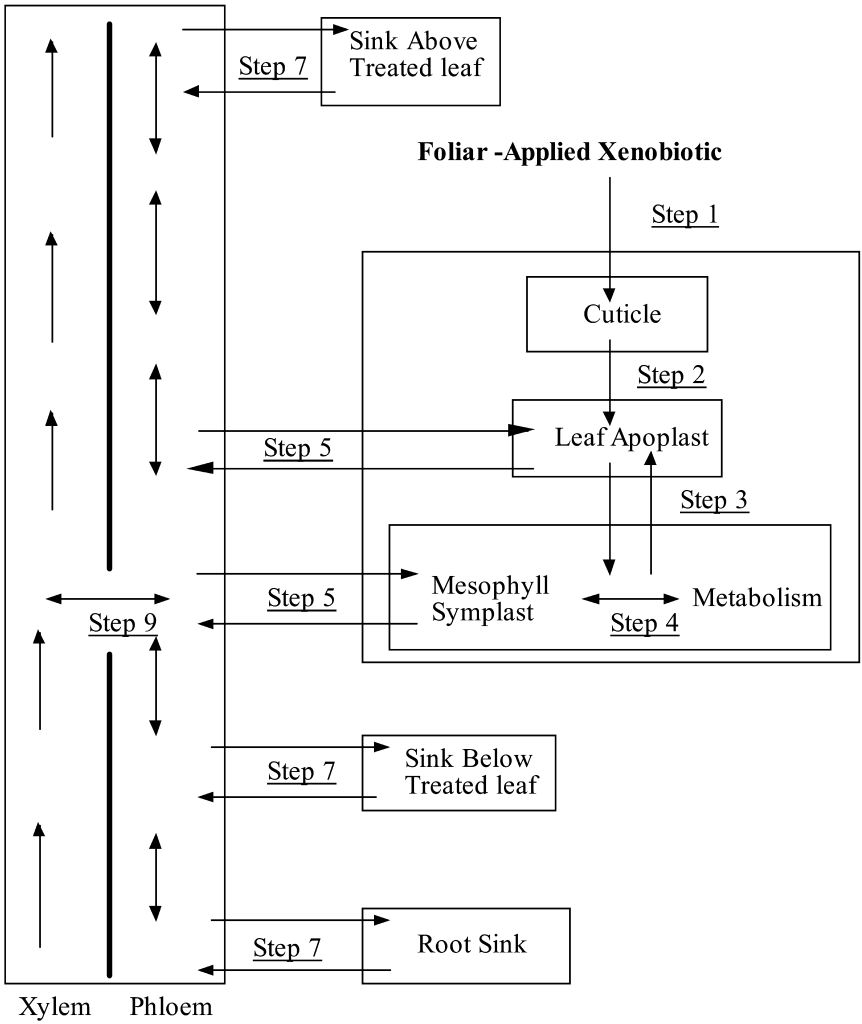

Figure 1. Block diagram describing the pathway of xenobiotic transport from the site of foliar application to final deposition in sink regions. Steps indicated in the diagram correspond to text discussion regarding the development of specific mathematical relationships describing each process. See Satchivi et al. (15) for more details.

While the proportionality coefficient characterizes the properties of any barriers to solute flux, the driving force is generally the concentration gradient at the interfaces of the barrier or compartment boundary.

Predicting Xenobiotic Uptake and Translocation with the Ermesse© MODEL

Plant Parameters

The hypothetical plant parameters used in the simulation of the uptake and translocation of systemic xenobiotics are a total plant length of 20 cm, a cuticular membrane thickness of 2 μm, a cell wall thickness of 5 μm, an apoplast and symplast sap pH of 5.5 and 7.5, respectively. The xylem and phloem sap pH were 5.5 and 7.5, respectively. It was suggested that the resistance of the cell wall will not exceed 5×10^4 s m^{-1} (*43*), therefore the model assumes a cell wall permeability coefficient of 2×10^{-5} m s^{-1}. A sieve tube length of 3 mm and a xylem vessel length of 10 mm (*108*) were assumed for the simulations. The phloem sap velocity and the transpiration rate, which depend upon the environmental conditions at the time of xenobiotic application, were 2.1×10^{-4} m s^{-1} and 4.1×10^{-4} mol m^{-2} s^{-1}, respectively. A root and leaf water potential of -0.48 and -1.442 MPa, respectively, were also assumed.

Xenobiotic Parameters

Relevant physicochemical parameters ($\log K_{ow}$, pK_a and MV) for the pesticides considered in the simulation studies are listed in Table 1. The metabolism kinetic parameter (half-life), when it is available, has been used directly as an input in the model. But in most instances, these parameters have been estimated (or extrapolated) from literature reports of herbicide metabolism. Thus herbicide half-life in the plant was generated by plotting the percent of herbicide metabolized over time. When simulating the effects of physicochemical properties ($\log K_{ow}$, pKa and molar volume) on xenobiotic uptake and translocation, the following hypothetical xenobiotic inputs were used: a $\log K_{ow}$ ranging from –5.0 to 5.0 (increment 0.5); a pK_a of 0 to 14 (increment 1.0); and a molar volume of 100 to 450 mol cm^{-3} (increment 25.0 mol cm^{-3}). The cuticle/water (K_{cw}) and wax/water (K_{ww}) partition coefficients were estimated from the octanol/water (K_{ow}) partition coefficient of the undissociated form of the hypothetical xenobiotic by the following equations:

$$\log K_{cw} = 0.057 + 0.97 \log K_{ow}$$

$$\log K_{ww} = 0.889 \log K_{cw} - 0.576$$

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

While the first equation was from Schönherr and Riederer (*43*), the second equation (r=0.99) was generated using actual data from Schreiber and coworkers (*8*, *55*). These partition coefficients play an important role in the xenobiotic uptake, retention and movement through the cuticular membrane because they determine its solubility in the cuticular waxes and cutin (*55*). The wax/water partition coefficient is always lower than the cuticle/water partition coefficient and this indicates that low xenobiotic solubility in wax layers accounts, in part, for the low permeability of the cuticular membrane (*6*).

The xenobiotic permeability through the cells plasmalemma and the sieve tube membranes, which is considered to be a function of the xenobiotic $\log K_{ow}$ (*10*, *130*), was estimated according to the equation described by Grayson and Kleier (*130*):

$$\log P = 0.33 \log K_{ow} - 8.05$$

This equation was also used to calculate the permeability of the dissociated form of the xenobiotic molecules as previously described (*15*).

Table 1. Physicochemical Properties of the Xenobiotic Considered in the Simulation Studies

Xenobiotic	*Molar Volume (cm^3 mol^{-1})*	*Partition coefficient ($LogK_{ow}$)*	*Dissociation constant (pK_a)*
2,4-D	138	2.9	3
2,4-DB	166	3.9	4.8
Acephate	134	-0.85	8.4
Acifluorfen	209	5.4	2.5
Bentazon	167	2.8	2.9
Chlorimuron	270	1.5	4.2
Chlormequat chloride	120	-3.8	-
Chlorsulfuron	230	1.8	3.6
Choline chloride	114	-3.7	-
Clethodim	324	4.2	4.2
Clopyralid	114	1.2	2.3
Dicamba	138	3.1	1.9
Difenzoquat	206	0.6	7.0
Fluroxypyr	145	2.04	2.9
Halosulfuron	278	1.7	3.5
Haloxyfop-methyl	235	4.33	-

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Table 1. (Continued). Physicochemical Properties of the Xenobiotic Considered in the Simulation Studies

Xenobiotic	*Molar Volume (cm^3 mol^{-1})*	*Partition coefficient ($LogK_{ow}$)*	*Dissociation constant (pK_a)*
Imazapyr	194	0.2	3.6
Imazaquin	231	1.9	3.7
Imazethapyr	222	1.2	3.9
Metalaxyl	230	1.7	0
MSMA	92	0	4.1
Nicosulfuron	278	-0.4	4.3
Phenylurea	107	0.83	-
Picloram	136	1.8	2.3
Primisulfuron	249	1.58	5.1
Prosulfuron	265	1.5	3.8
Pyridate	287	5.4	-
Rimsulfuron	280	2	4
Sethoxydim	303	4.61	4.58
Thifensulfuron	246	1.8	4
Triadimefon	215	3.3	2.3

Environmental Parameters

Environmental parameters (relative humidity and temperature) for the simulations were the same as those used in each reported study (see appropriate references). The conditions for each simulation are indicated in Table 2. When relative humidity and temperatures values were not indicated, a relative humidity of 50% and air temperature of 23°C were assumed. The soil water potential of -0.40 Mpa was utilized for the simulations.

Simulations

The simulations of xenobiotic uptake and translocation and the effects of various plant parameters, environmental factors and xenobiotic physicochemical properties were performed with Stella® modeling software (High Performance Systems, Lyme, NH) using a IBM® ThinkPad computer (1.83 GHz). The time-dependent mathematical relationships in the format of finite difference equations were evaluated by fourth-order Runge-Kutta methods.

Table 2. Surfactant Physicochemical Property and Environmental Conditions Used in the Simulations

Surfactant	*CMC (% v/v)*	*C (% v/v)*	*Temperature (°C)*	*Relative Humidity (%)*	*Reference*
Ethylan TU	0.001	1	18	75	(*61*)
X-77	0.008	0.3	25	50*	(*147*)
none	none	none	25	70	(*136*)
COC	0.04	0.6	24	68	(*148*)
COC	0.04	1	25.5	50*	(*142*)
COC	0.04	1	26.5	50*	(*138*)
Tween-20	0.06	0.5	18	65	(*139*)
COC	0.04	1	22	50*	(*154*)
MSO	0.04	1	22	50*	(*154*)
none	none	none	28	50*	(*134*)
none	none	none	28	50*	(*134*)
X-77	0.008	0.5	24.5	50*	(*135*)
X-77	0.008	0.3	24	65	(*152*)
Tween-20	0.06	0.5	19	50*	(*150*)
Tween-20	0.06	0.5	19	50*	(*150*)
Silwett L-77	0.002	0.01	24	70	(*151*)
X-77	0.008	0.3	27.5	60	(*131*)
X-77	0.008	0.25	22.6	50*	(*140*)
X-77	0.008	0.2	24	70	(*137*)
Tween-20	0.06	0.3	25	50*	(*144*)
Tween 20	0.06	0.1	21.5	80	(*141*)
Tween-20	0.06	0.3			(*143*)
Tween 20	0.06	0.25	23	50*	(*145*)
Octoxynol	0.02	0.5	19	40	(*132*)
X-77	0.008	0.3	26.5	50*	(*149*)
Tween-20	0.06	0.5	21.5	65	(*133*)
Tween-20	0.06	0.3	28	50*	(*146*)
Atplus	0.0005	1	18	40	(*153*)

The model predictions of uptake (expressed as % of applied herbicide) and translocation (expressed as % absorbed herbicide) were compared to the actual data from published studies in the literature. The duration of each simulation, expressed as hours after treatment (HAT), parallels the duration of each experiment from the actual study. One of the major uses of simulation models is in the design of new molecules. The xenobiotic physicochemical properties and their interactions constitute a major source of interest for Discovery chemists and biologists. The ERMESSE model was utilized to investigate the relationship between physicochemical properties and also to determine combination of properties required to provide optimum activity. In this regard, the effect of the physicochemical properties of a systemic xenobiotic on its uptake was simulated by varying the molar volume and partition coefficient while the pK_a was held constant at 4.0. This value has been chosen because most plant cuticles have an isoelectric point around 3.0 (*39*); therefore with a pK_a of 4.0 the model assumes that the cuticular sorption involved mainly the undissociated form of the xenobiotic. The absorption of xenobiotic for a hypothetical duration of 24 hours after treatment (24 HAT) was simulated for each individual value of $logK_{ow}$ while MV was varied. A total of 165 simulations were conducted the effects of MV and $logK_{ow}$ on xenobiotic absorption. To evaluate the physicochemical property requirements for an optimum foliar uptake, the absorption rate was held constant, while the $logK_{ow}$, MV and pK_a of the hypothetical xenobiotic were varied. For each value of $logK_{ow}$ a total of 285 simulations were conducted with each simulation corresponding to a unique value of MV times a unique value of pK_a. With regard to translocation, the relationship between the hypothetical amount of xenobiotic translocated and the xenobiotic physicochemical properties was simulated by maintaining the molar volume and $logK_{ow}$ constant while the xenobiotic pK_a was varied from 0 to 14. For each hypothetical molar volume a total of 21 simulations were run. Each simulation corresponds to a unique value of $logK_{ow}$. The physicochemical parameters required to achieve an optimum translocation was determined by holding constant the translocation rate while the hypothetical xenobiotic molar volume, dissociation constant and partition coefficient were changed.

When the theoretical absorption and translocation predictions from the model were compared to empirical data from the literature, the amount of absorbed and translocated xenobiotic 24 h after application was used. The observed absorption and translocation were expressed as percent of applied and as the percentage of absorbed, respectively. The relative closeness between actual data from the literature and simulated data was estimated by the ratio of predicted over observed. A ratio of 1.0 means that both the actual and predicted data are equivalent.

Comparison between Model Prediction of Xenobiotic Uptake and Actual Uptake from the Literature

The model predictions of xenobiotic uptake are fairly consistent with the actual data from the literature. As shown in Table 3, most simulations closely predicted actual xenobiotic absorption. Indeed, over 102 comparisons between model prediction of xenobiotic uptake and actual uptake from the literature, 69% of the predicted xenobiotic uptake had a ratio of predicted/observed that varied between 0.7 and 1.4. Several model predictions of xenobiotic uptake were exactly identical to actual data (Table 3). . For example the model predicted 37% absorption of MSMA 24 hour after application which was exactly the percent of MSMA absorbed by *Xanthium strumarium* (*131*). Similar equivalency was observed for pesticides such as 2,4-DB (*134*), bentazon (*136*), chlorimuron (*137*), clethodim (*138*), clopyralid (*139*), fluroxypyr (*140*), dicamba (*141*), haloxyfop-methyl (*142*), imazapyr (*143*), thifensulfuron (*149*) and many other pesticides (Table 3). In some instances the model predicted more than 3 times the amount of xenobiotic absorbed in comparison to the actual absorption. The model predictions of absorption were overestimated for herbicides such as acifluorfen (*135*), imazaquin (*145*), imazethapyr (*147*), nicosulfuron (*150*), prosulfuron (*152*),sethoxydim (*153*) and Halosulfuron (*154*). These overestimations of xenobiotic absorption represent only 14% of the simulations (Table 3). The tendency to overestimate xenobiotic absorption could result from an overestimation of processes involved in xenobiotic movement into the leaf and its associated cellular compartments. Indeed, a permeability coefficient was generated for each xenobiotic simulated, therefore any disparities in estimates of the permeability coefficient would lead to an over or underestimation of cuticular permeation, especially when the uncertainty of cuticle structure and thickness is involved.

Table 3. Comparison of Predicted and Actual Xenobiotic Absorption

Herbicide	*Hat*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*Reference*
Acephate	24	21-99	68	0.7-3.2	(*61*)
Chlormequat Chloride	24	44-88	53	0.6-1.2	(*61*)
Choline Chloride	24	46-69	53	0.8-1.1	(*61*)
Metalaxyl	24	26-93	76	0.8-2.9	(*61*)
Phenylurea	24	13-89	93	1.0-7.2	(*61*)
Triadimefon	24	35-97	92	0.9-2.6	(*61*)

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Table 3. (Continued). Comparison of Predicted and Actual Xenobiotic Absorption

Herbicide	*Hat*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*Reference*
Msma	24	35-38	37	1.0	(*131*)
Difenzoquat	24	41-47	63	1.3-1.5	(*132*)
2,4-D	24	56	86	1.5	(*133*)
Chlorsulfuron	24	38	43	1.2	(*133*)
2,4-Db	48	82-96	72	0.8-0.9	(*134*)
Pyridate	48	62-74	51	0.7-0.8	(*134*)
Acifluorfen	168	41967	22	0.9-2.0	(*135*)
Bentazon	192	18-21	17	0.8-1.0	(*136*)
Chlorimuron	192	25-31	27	0.9-1.1	(*137*)
Clethodim	24	62	80	1.3	(*138*)
Clopyralid	24	98-99	95	1.0	(*139*)
Picloram	24	98	98	1.0	(*139*)
Fluroxypyr	144	79	78	1.0	(*140*)
Dicamba	120	72	70	1.0	(*141*)
Halosulfuron	24	52-62	54	0.9-1.0	(*154*)
Haloxyfop-Methyl	96	98-99	90	0.9	(*142*)
Imazapyr	24	25	32	1.3	(*143*)
Imazaquin	72	21-61	55	0.9-2.6	(*145*)
Imazethapyr	24	28-46	67	1.4-2.4	(*147*)
Thifensulfuron	24	50	52	1.0	(*149*)
Nicosulfuron	24	13-58	44	1.3-4.4	(*150*)
Rimsulfuron	24	20-37	54	1.5-2.7	(*150*)
Prosulfuron	24	3-21	32	1.5-10.8	(*152*)
Sethoxydim	24	33-70	81	1.2-2.5	(*153*)

Table 4. Comparison of Predicted and Actual Effects of Surfactants on Absorption of Difenzoquat

	Absorption (% applied)		*Ratio*	*Absorption (% applied)*		*Ratio*
Octoxynol (%)	*8 HAT*			*32 HAT*		
	Observed	*Predicted*		*Observed*	*Predicted*	
0.01	10	20	2	22	53	2.4
0.05	19	25	1.3	53	59	1.1
0.1	20	29	1.5	66	63	1
0.2	31	35	1.1	68	67	1
0.3	32	38	1.2	76	68	0.9
0.4	35	39	1.1	77	69	0.9

The model ERMESSE can also be useful in the evaluation of the effects of adjuvant on xenobiotic absorption (Table 4). Indeed, when the effect of the adjuvant octoxynol on the absorption of difenzoquat was simulated, it appeared that the model predictions were quite close to the actual data observed by Sharma *et al* (*132*). These data showed that the model ERMESSE can accurately predict xenobiotic uptake when the compound is applied with or without an adjuvant.

Comparison between Model Prediction of Xenobiotic Translocation and Actual Translocation from the Literature

The ERMESSE model simulated xenobiotic translocation out of the treated leaf is presented in Table 5. The model simulations showed a pattern of simulated xenobiotic translocation comparable to actual translocation from the literature. Although some deviations from the actual xenobiotic translocation pattern were observed for some tested pesticides, the results shown in Table 5 demonstrate the accuracy of the model prediction of xenobiotic translocation. The majority of simulations (63%) yielded a ratio of predicted/actual between 0.7 and 1.4. For example the model prediction of the amount of 2,4-D translocated within 24 HAT was identical to that reported by Wall *et al.* (*133*). Similar observations were noticed for the simulated translocation of triadimefon, acifluorfen, picloram or fluroxypyr (Table 5). Although some deviations from actual translocation pattern were observed for some simulated xenobiotic, the ERMESSE model accurately predicts translocation of foliar-applied xenobiotics.

Table 5. Comparison of Predicted and Actual Xenobiotic Translocation

Herbicide	*Translocation Actual*	*Translocation Predicted*	*Ratio*	*Reference*
2,4-D	33	35	1.1	(*133*)
2,4-DB	22-30	28	0.9-1.3	(*134*)
Acephate	21-45	9	0.2-0.4	(*61*)
Acifluorfen	78-90	79	0.9-1.0	(*135*)
Bentazon	12-15	16	1.1-1.4	(*136*)
Chlorimuron	23-28	25	0.9-1.1	(*137*)
Chlorsulfuron	10	13	1.3	(*133*)
Clethodim	17	21	1.2	(*138*)
Clopyralid	46-51	36	0.7-0.8	(*139*)
Dicamba	16	14	0.9	(*141*)
Difenzoquat	17-32	36	1.1-2.1	(*132*)
Fluroxypyr	46	50	1.1	(*140*)
Halosulfuron	13	16	1.2	(*154*)
Haloxyfop-methyl	12-21	22	1.0-1.8	(*142*)
Imazapyr	26	19	0.7	(*143*)
Imazaquin	24-26	36	1.4-1.5	(*145*)
Imazethapyr	25-28	29	1.0-1.2	(*147*)
Metalaxyl	9-43	23	0.5-2.6	(*61*)
MSMA	25-26	20	0.8	(*131*)
Nicosulfuron	15-40	18	0.4-1.2	(*150*)
Phenylurea	19-43	30	0.7-1.6	(*61*)
Picloram	46-57	46	0.8-1.0	(*139*)
Pyridate	33-51	21	0.4-0.6	(*134*)
Rimsulfuron	15-30	26	0.9-1.7	(*150*)
Sethoxydim	6-30	22	0.7-3.7	(*153*)
Thifensulfuron	7	6	0.8	(*149*)
Triadimefon	8-14	12	0.9-1.4	(*61*)
Prosulfuron	7-47	10	0.2-1.4	(*152*)

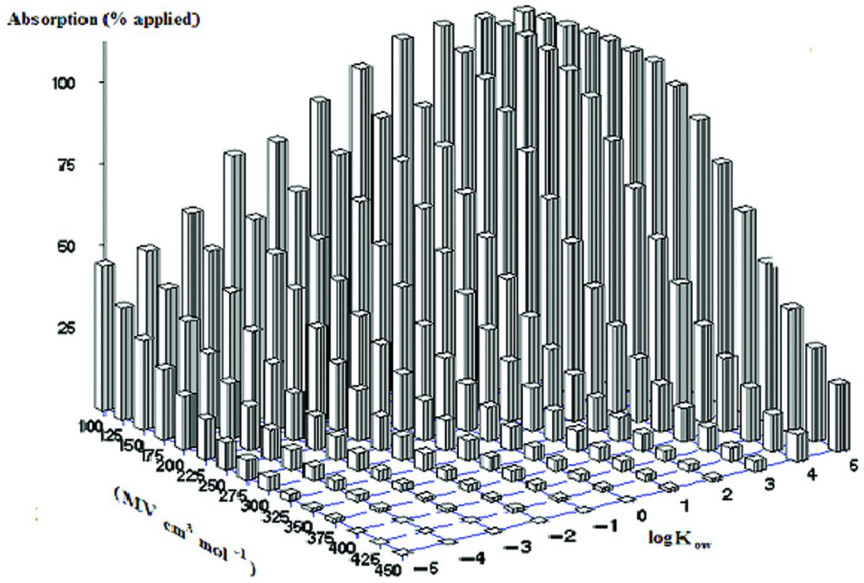

Figure 2. Effect of different hypothetical physicochemical properties (molar volume, MV; partition coefficient, log Kow) on xenobiotic foliar absorption expressed as percent of applied 24 hour after simulated application. Model sensitivity to changes in MV and log Kow was tested while dissociation constant pKa was held constant at 4.0, plant and environmental parameters were also held constant (see xenobiotic, plant, environmental and simulation parameters). See Satchivi et al. (17) for more details.

Predicting Xenobiotic Absorption As Affected by Molar Volume and Partition Coefficient

The relationship between absorption, molar volume and partition coefficient is important to understand the activity of any foliar-applied xenobiotic. To determine the physicochemical requirements for an optimum absorption, the simulated xenobiotic absorption was plotted against hypothetical molar volumes and partition coefficients. The model output showed that a correlation between absorption and both the xenobiotic $\log K_{ow}$ and MV. For any partition coefficient, the rate of absorption decreased as the molar volume increased (Figure 2). The decrease in the absorption rate is related to a decline of the predicted xenobiotic diffusion coefficient. For example, for hypothetical xenobiotics with a constant partition coefficient of -5.0 and molar volumes of 100 or 450 cm^3mol^{-1}, the model predicted a diffusion coefficient of 4.7 X 10^{-5} m^2s^{-1} and 5.1 X 10^{-7} m^2s^{-1}, respectively. Several authors have also reported a correlation between the decrease of the diffusion/permeability coefficient and the increase of the molar volume of the compound (*7*, *45*). The model output also showed that for any

hypothetical molar volume the predicted amount of xenobiotic absorbed was a function of the partition coefficient (Figure 2). For example, when the molar volume was held constant at 100 cm^3mol^{-1} and the partition coefficient was varied between -5.0 and 5.0, the predicted xenobiotic absorption varied from 45 to 100%. This differential absorption was due to the fact that the predicted permeability of the hypothetical xenobiotic through the wax layer was much smaller at a $logK_{ow}$ of -5.0 (2.5×10^{-16} ms^{-1}) than at a $logK_{ow}$ of 5.0 (1.7×10^{-7} ms^{-1}). Previous studies with intact leaves and isolated cuticles have shown similar results and provided evidence that the lipophilicity of a compound greatly affects its permeability coefficient (*8*, *55*).

The utility of the ERMESSE model in the design of novel pesticides was demonstrated by determining the physicochemical properties requirements for an optimum absorption of a foliar-applied xenobiotic. In this regard, simulations were performed to determine the combinations of molar volumes and partition coefficients that will provide xenobiotic absorption of 50% or greater 24 HAT. The model simulations predicted that for hydrophilic compounds ($logK_{ow}$ <0) absorption of 50% or more was achieved when the molar volume was ≤ 200 cm^3 mol^{-1} (Figure 3).

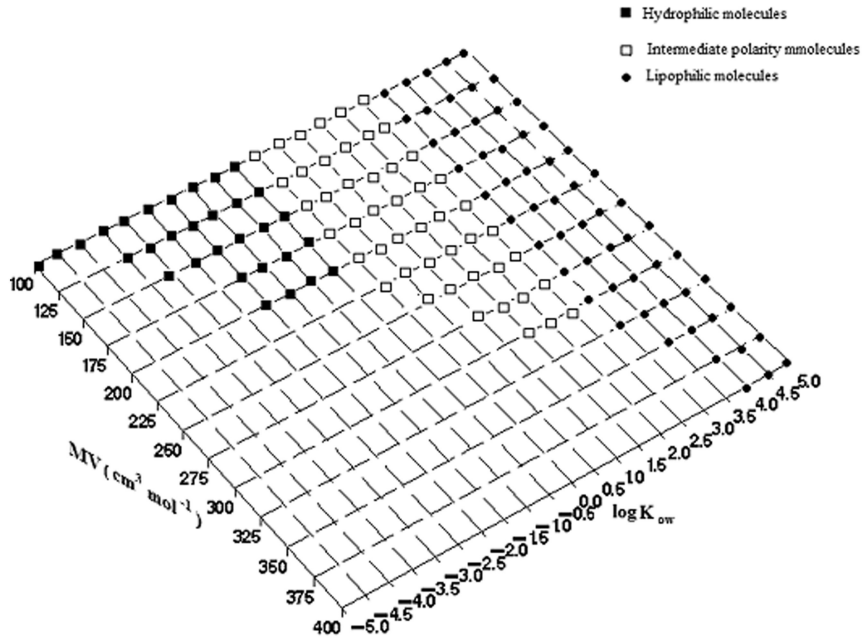

Figure 3. Correlation between molar volume (MV) and partition coefficient (log Kow) required to provide a theoretical xenobiotic absorption ≥ 50% after 24 hour of treatment. Xenobiotic absorption was set constant while the physicochemical parameters varied (see xenobiotic, plant, environmental and simulation parameters). See Satchivi et al. (17) for more details.

Table 6. Predictions of Absorption of Hydrophilic Xenobiotic As Affected by Molar Volume 24 h after Treatment

Xenobiotic	*Molar Volume (cm^3 mol^{-1})*	*LogK_{ow}*	*p-K_a*	*Plant species*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*References*
Chlormequat chloride	120	-3.8	-	*Zea mays*	67.0	53	0.8	(*61*)
				Brassica napus	87.7	53	0.6	
				Fragaria annanassa	44.1	53	1.2	
				Beta vulgaris	57.8	53	0.9	
Choline chloride	114	-3.7	-	*Zea mays*	46.3	53	1.1	(*61*)
				Brassica napus	69.1	53	0.8	
				Fragaria annanassa	57.0	53	0.9	
				Beta vulgaris	51.1	53	1.0	
Acephate	134	-0.85	8.4	*Zea mays*	21.0	68	3.2	(*61*)
				Brassica napus	89.2	68	0.8	
				Fragaria annanassa	98.8	68	0.7	
				Beta vulgaris	56.2	68	1.2	
Nicosulfuron	278	-0.4	4.3	*Amaranthus retroflexus*	10.0	44.2	4.4	(*150*)
				Ambrosia artemisiifolia	15.0	44.2	2.9	
				Avena fatua	15.0	44.2	2.9	
				Panicum miliaceum	25.0	44.2	1.8	
				Digitaria ischaemum	35.0	44.2	1.3	

Table 7. Predictions of Absorption of Intermediate Xenobiotic As Affected by Molar Volume 24 h after Treatment

Herbicide	*Molar Volume (cm^3 mol^{-1})*	*LogK_{ow}*	*pK_a*	*Plant species*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*Reference*
Difenzoquat	206	0.6	7.0	*Hordeum vulgare*	41.0	62.7	1.5	(*132*)
				Avena fatua	47.0	62.7	1.3	
Imazapyr	194	0.2	3.6	*Imperata cylindrical*	25.0	31.9	1.3	(*143*)
Phenylurea	107	0.83	-	*Zea mays*	12.8	93	7.3	(*61*)
				Brassica napus	89.4	93	1.0	
				Fragaria annanassa	77.2	93	1.2	
				Beta vulgaris	69.5	93	1.3	
Metalaxyl	230	1.7		*Brassica napus*	92.6	75.6	0.8	(*61*)
				Fragaria annanassa	88.5	75.6	0.9	
				Beta vulgaris	26.2	75.6	2.9	
Imazethapyr	222	1.2	3.9	*Amaranthus retroflexus*	97.0	84.5	0.9	(*148*)
Clopyralid	114	1.2	2.3	*Brassica napus*	98.3	94.7	1.0	(*139*)
				Helianthus annum	98.6	94.7	1.0	
Picloram	136	1.8	2.3	*Brassica napus*	97.6	98.2	1.0	(*139*)
				Helianthus annum	97.3	98.2	1.0	

Continued on next page.

Table 7. (Continued). Predictions of Absorption of Intermediate Xenobiotic As Affected by Molar Volume 24 h after Treatment

Herbicide	*Molar Volume (cm^3 mol^{-1})*	*LogK_{ow}*	*pK_a*	*Plant species*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*Reference*
Prosulfuron	265	1.5	3.8	*Abutilon theophrasti*	57.0	49.1	0.9	(*154*)
				Abutilon theophrasti	65.0	49.1	0.8	
Halosulfuron	278	1.7	3.5	*Abutilon theophrasti*	52.0	54	1.0	(*154*)
				Abutilon theophrasti	62.0	54	0.9	
Rimsulfuron	280	2	4	*Avena fatua*	20.0	54.1	2.7	(*150*)
				Ambrosia artemisiifolia	25.0	54.1	2.2	
				Amaranthus retroflexus	27.0	54.1	2.0	
				Digitaria ischaemum	35.0	54.1	1.5	
				Panicum miliaceum	37.0	54.1	1.5	
Fluroxypyr	145	2.04	2.9	*Apocynum cannabinum*	56.0	54	1.0	(*140*)
Imazethapyr	222	1.2	3.9	*Glycine max*	54.2	66.6	1.2	(*149*)
Thifensulfuron	246	1.8	4	*Glycine max*	50.1	52.1	1.0	(*149*)
2,4-D	138	2.9	3	*Silene vulgaris*	56.0	86.1	1.5	(*133*)

Herbicide	*Molar Volume (cm^3 mol^{-1})*	*LogK_{ow}*	*pK_a*	*Plant species*	*Absorption Actual*	*Absorption Predicted*	*Ratio*	*Reference*
Imazaquin	231	1.9	3.7	*Xanthium strumarium*	88.0	66.3	0.8	(*146*)
				Glycine max	91.0	66.3	0.7	
				Arachis hypogaea	93.0	66.3	0.7	
				Cassia obtusifolia	95.0	66.3	0.7	
				Desmodium tortuosum	97.0	66.3	0.7	
Clethodim	324	4.2	4.2	*Setaria glauca*	62.0	80	1.3	(*138*)
Sethoxydim	303	4.5	4.6	*Cynodon dactylon*	56.0	81	1.4	(*153*)

Baker et al (*61*) reported that hydrophilic compounds such as acephate (MV = 134 cm^3 mol^{-1}, $logK_{ow}$ =-0.9), chlormequat chloride (MV = 120 cm^3 mol^{-1}, $logK_{ow}$ =-3.8) and choline chloride (MV = 114 cm^3 mol^{-1}, $logK_{ow}$ =-3.7) exhibited more than 50% absorption when applied to *Brassica napus* or *Beta vulgaris* (Table 6). At higher molar volumes, hydrophilic xenobiotics are too large to permeate the polar components of the cuticle leading to poor absorption. For example, with nicosulfuron (MV = 278 cm^3 mol^{-1}, $logK_{ow}$ =-0.4), Mekki et al. (*150*) showed an absorption of less than 40% in multiple weed species. These examples confirmed the predictions from the ERMESSE model.

For compounds with intermediate lipophilicity *i.e.* $logK_{ow}$ higher than 0 and lower than 3, the model predicted that a molar volume of 300 cm^3 mol^{-1} or less was required to provide an absorption rate of 50% or more 24 HAT. Larger xenobiotic compounds i.e. MV > 300 cm^3 mol^{-1} require a partition coefficient above 3 to achieve an absorption 50% or more 24 HAT (Figure 3). In general, the model predictions of xenobiotic absorption were close to the actual data of most published intermediate compounds (Table 7).

Predicting Xenobiotic Translocation As Affected by Partition Coefficient, Acid Dissociation Constant, and Molar Volume

The mobility of foliar-applied xenobiotics in the vascular tissues, especially in the phloem, represents one of the most important characteristics of today's herbicides. Understanding the physicochemical parameters that would favor translocation of foliar-applied xenobiotics from the point of entry to distant site of action located in the meristems has become an integral part of the design and discovery of novel pesticides. To determine the combination of physicochemical properties required to obtain optimum xenobiotic translocation, simulations were performed with various combinations of molar volume, acid dissociation constant and partition coefficient that will provide a hypothetical xenobiotic translocation rate of 25% or more 24 HAT. The simulations predicted that hydrophilic ($logK_{ow} \leq -0.5$) weak acidic xenobiotic ($5 \leq pK_a \leq 8$) exhibited 25% or more translocation only when their molar volume is ≤ 225 $cm^3 mol^{-1}$ (Figure 4). The insecticide acephate can be classified in this first group of molecules (Group I). The second group (Group II) corresponds to lipophilic weak acid compounds with a molar volume not exceeding 225 cm^3/mol. The acid dissociation constant of these hypothetical xenobiotic should be between 4.0 and 6.5 and their $logK_{ow}$ greater than 2.5 (Figure 4). The third group of molecules corresponds to compounds with a molar volume not exceeding 225 cm^3/mol and a partition coefficient between 1.25 and 2.5. These compounds should also be weakly or strongly acidic with a pKa between 0.0 and 4.0 (Figure 4). This group III includes auxinic herbicides such as clopyralid, picloram, or fluroxypyr and many others pesticides. Finally, the fourth group of xenobiotic that may meet the 25% or more translocation threshold includes only weak acidic molecules ($4.0 \leq pK_a \leq 8.0$) with an intermediate lipophilicity ($-0.5 \leq logK_{ow} \leq 2.5$) and a molar volume of 225 to 300 cm^3/mol, Xenobiotic molecules such as prosulfuron, nicosulfuron or sethoxydim could be included in this third group.

Overall, the ERMESSE model, which incorporates plant, environmental and xenobiotic parameters, satisfactorily simulated observed foliar absorption and translocation patterns of slow-acting systemic xenobiotic molecules. The model enables quantitative prediction of maximum uptake and translocation based on the combination of the molar volume, dissociation constant and the partition coefficient of the molecule. Indeed, the theoretical predictions from the ERMESSE model showed that an optimum absorption and translocation can be expected for weak acid molecules with a molar volume which does not exceed 300 cm^3/mol and a partition coefficient in the range of -1.5 to 2.5. The model offers promise for future use in the design of molecules that exert good mobility through the cuticular membrane as well as acceptable translocation pattern within the vascular system. Although the ERMESSE model provided absorption and translocation predictions of significant accuracy for slow-acting systemic postemergence xenobiotic, it is limited in predicting the movement of fast-acting, contact xenobiotics. The structural design of the model as well as the various mathematical equations used in the model, which were developed from data collected from systemic foliar applied pesticide, would not allow at this point an accurate prediction of the uptake and translocation of contact xenobiotics.

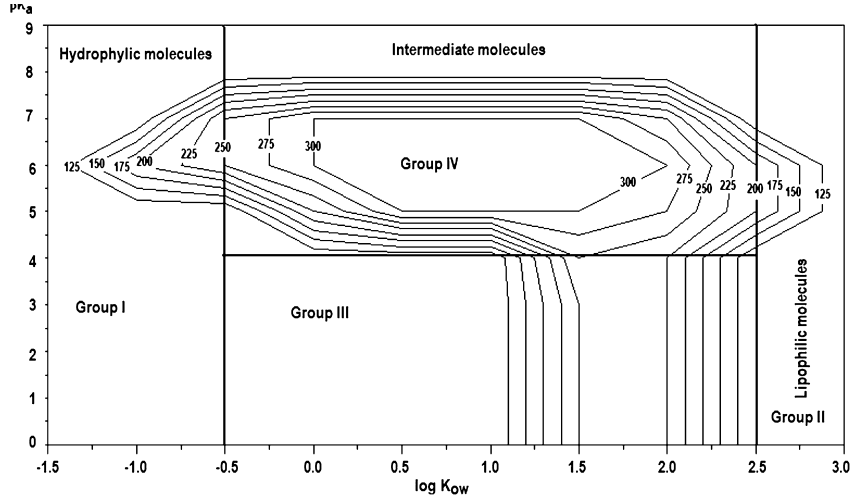

Figure 4. Contour graph illustrating the physicochemical parameters requirements for a xenobiotic to provide 25% or more translocation 24 h after simulation. The theoretical translocation rate as well as the plant and environmental parameters were set constant while the hypothetical pKa, MV and log K_{ow} varied. See Satchivi et al. (17) for more details.

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Chapter 4

Comparison of Translocation Properties of Insecticides versus Herbicides That Leads To Efficacious Control of Pests As Specifically Illustrated by Isoclast™ Active, a New Insecticide, and Arylex™ Active, a New Herbicide

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An important aspect of agrochemical effectiveness is the ability of a pesticide to translocate in the plant to the relevant site of action, for herbicides movement to the meristem for control of weeds and for insecticides movement to the plant tissue used by insects as a food source. For modern synthetic insecticides, the most effective mode of translocation in plants is via xylem movement, allowing for even, uniform distribution throughout the foliage that is being attacked by sucking or chewing insect pests. These insecticides when applied to roots or when sprayed and contact the plant stem are called systemic as they protect all parts of the plant from insect damage. In contrast, for modern synthetic herbicides, phloem mobility is far superior to xylem mobility as it allows for targeted concentration of the active material in the rapidly dividing tissue of the meristem. This chapter will illustrate general translocation properties of xylem translocated commercial insecticides and xylem or phloem translocated commercial herbicides and then focus on specific attributes of two new agrochemicals from Dow AgroSciences. Isoclast™ active, a xylem mobile insecticide for control of sucking insects, which illustrates well the uniform distribution that leads to good control of aphids in the field. Arylex™ active,

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

a new cereal-selective herbicide, which in contrast to Isoclast™ active, is translocated via the phloem where the active herbicide is concentrated to an effective dose in the meristematic tissue of the plant.

Introduction

Due partly to no-till and other forms of reduced tillage most commercial pesticides are generally developed for foliar (postemergence) application of the active ingredient (*1*, *2*). Therefore, translocation of the applied compound and the resulting distribution of the active ingredient within the plant are important for successful activity delivery (*3*, *4*). Long distance translocation within the plant proceeds via the phloem and xylem tissues. The phloem transports assimilates out of the leaf towards the growing shoot and root tips. The xylem carries ions and water with the transpiration stream from the root into the leaf (*5*). Systemic pesticides are translocated throughout the plant via these routes. How well a given compound is transported by the xylem and the phloem will determine its overall distribution throughout the plant (*6*). Volatile compounds may achieve total plant coverage independent of plant transport by movement through the air from the site of application to untreated areas (*7–9*). Distribution and mobility requirements differ widely for herbicides and insecticides. It is generally accepted that foliar active herbicides have to be phloem mobile which ensures that the active molecule arrives at, and controls new growth. In contrast, insecticides may be active by contact or by ingestion. Therefore, mobility within the plant is not a prerequisite for efficacy but represents a competitive advantage. For instance, xylem mobility supports seed treatment or root drench application, and phloem transfer enhances sap feeder efficacy (*10*). Attributes like local diffusion or volatility support translaminar transfer and help in the control of cryptic feeders.

Phosphor imaging allows a comprehensive visual documentation of whole plant distribution of radioactivity after application of ^{14}C-labeled compounds to stem, leaf and root (*11*). Stem application reveals whether a compound is translocated upwards with the transpiration stream in the xylem tissue. Redistribution of label below the site of application points to phloem transport or volatility. Phloem mobility is highlighted if a compound accumulates in the shoot tip, unfolding leaves or root tips. Leaf application allows for the assessment of symplastic movement, whether a compound is exported out of the leaf in the phloem tissue towards new growth. Transport in the xylem only provides for acropetal distribution along with the transpiration stream. For instance, after stem application a xylem-mobile compound will distribute to transpiring tissue only (*12*).

Translocation involves the movement of a pesticide from the point of application (leaf, stem, roots etc) to other parts of the plant. The extent and characteristics of the pesticide movement can been described as occurring in six predominant ways: local, phloem, xylem, ambimobile, translaminar and systemic with the first three illustrated in Figure 1.

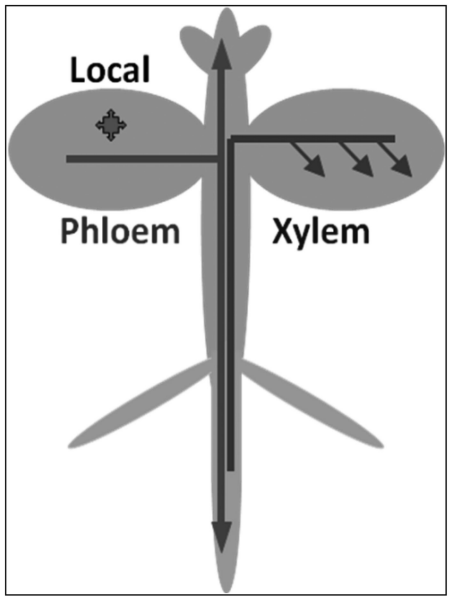


Figure 1. Schematic representation of local diffusion, phloem and xylem flow patterns in a plant. Local diffusion occurs when the applied compound is distributed to the margin of the treated leaf whereas a phloem translocation involves both acropetal and basipetal movement and xylem transport follows the transpiration stream from the point of entry (root or stem) to the leaf.

Local movement involves limited movement from the point of application with a volatile compound producing diffuse application spots. Phloem translocation would be characterized by a pesticide being acid trapped in the phloem to such an extent that it moves out of a leaf (source tissue) and eventual concentrates at the meristem (sink tissue) (*13*). Xylem movement occurs when the neutral pesticide enters the xylem and is moved with the transpiration stream with unloading into surrounding plant tissue giving a uniform distribution and eventual concentrating at the leaf margins (*4*, *14*, *15*). If a pesticide is ambimobile it will display both phloem and xylem movement with most phloem mobile pesticides being ambimobile (*16*). Translaminar movement is defined as movement through a plant leaf, a characteristic important for insecticidal activity (*17*). Finally, systemic movement is used to describe movement of a pesticide throughout the whole plant, particularly for insecticides when applied to roots or plant stems and produces complete uniform plant protection from insects (*18*, *19*).

Methods

Plant Material

Seeds of vine (*Vitis vinifera*), spring wheat (*Triticum aestivum*), cabbage (*Brassica oleracea*), pepper (*Capsicum annuum*), cotton (*Gossypium hirsutum*), rice (*Oryza sativa*), bird's-eye speedwell (*Veronica persica*) and purple deadnettle (*Lamium purpureum*) were planted in a soil-less potting mix (Sun Gro MetroMix® 306 Growing Media, Sun Gro Horticulture, Bellevue, WA) contained in a 81 square centimeters (cm^2) plastic pot. MetroMix has a pH of 6.0 to 6.8 and an organic matter content of about 30%. Plants were between BBCH 11 and BBCH 13 (1^{st} to 3^{rd} true leaf) growth stage at the time of treatment and were kept in a growth chamber with a photoperiod of 16 hours light and 8 hours darkness. The average temperature was 20 °C. For stem and leaf application the plants remained in the pots whereas for root application, plants were transplanted and grown hydroponically in an Erlenmeyer flask containing 150 mls of hydroponic solution and were covered with aluminum foil. The solution consisted of 1:100 dilution of a modified Peters 20:20:20 fertilizer previously described (*20*).

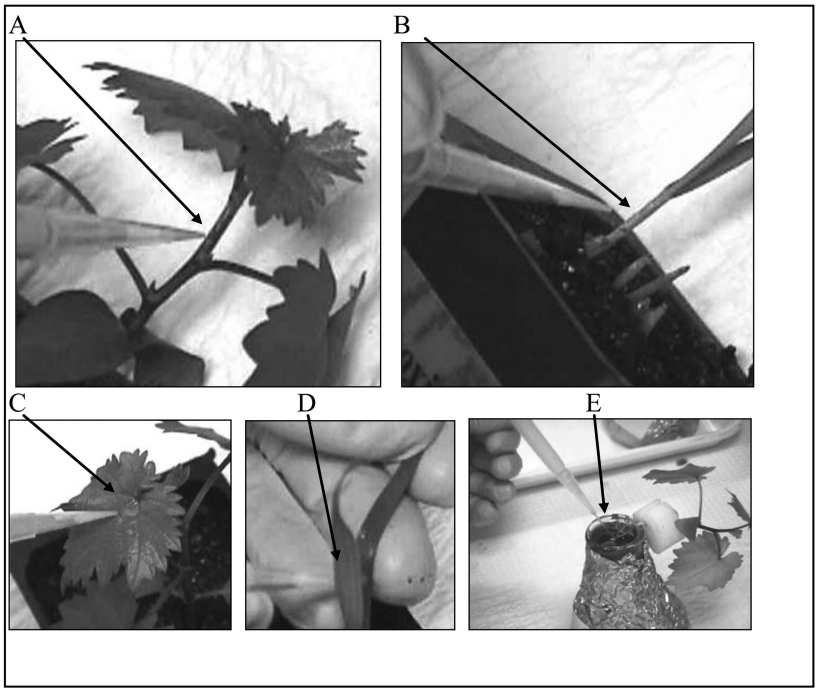


Figure 2. Pesticide application to stem, leaf and root with arrow pointing to application site (A= vine stem, B= wheat stem, C= vine leaf, D= wheat leaf and E= vine root).

Application of Commercial ^{14}C-Pesticides and Phosphor Imaging

For both stem and leaf application, the pesticides were formulated at 25 µg/µL in blank emulsifiable concentrate (EC) (N-methylpyrrolidone 47%, Aromatic 200 47%, Sponto 500T 4.8% and Sponto 300T 1.2%). The compounds were applied in 0.5 µL droplets using an electronic multi-pipetting syringe, three droplets to stems (Figures 2A and 2B) and six droplets in a vertical line across the leaf (Figures 2C and 2D). For root uptake, 0.2 µCi of ^{14}C-labeled pesticide was directly applied to the hydroponic solution (Figure 2E). Plants were harvested at 3, 4 or 5 days after application. The application rate in each case was below a toxic rate.

Phosphor Imaging

Roots were briefly washed in water to remove adhering ^{14}C-pesticide in the case of root application, and plants were subsequently arranged between 1 layer of filter paper and sandwiched between multiple layers of newspaper. Cardboard on both sides finalized the stack, which was pressed together with strong rubber bands. After freezing at –80 °C, the plants were lyophilized for 2 days in a Virtis Genesis freeze drier at 33 mbar with the shelf temperature at –10 °C. Dried plants on filter paper were transferred to phosphor imaging cassettes. To prevent radioactive contamination of the phosphor screen, a layer of Mylar film was placed between the plant and the screen prior to exposure. After appropriate exposure, ^{14}C distribution was visualized using a Molecular Dynamics 'STORM' Phosphor Imaging system.

Insecticides

Six insecticides (4 broad spectrum, 1 sap feeding and 1 *Lepidoptera* chewing) were selected to visualize representative whole plant distribution patterns. Five compounds were neutral and one compound was a base. The Log K_{ow} values range from 6.9 to 1.4 and water solubility ranged from 0.009 to 510 mg/L (Table 1). The broad spectrum compounds include the pyrethroid cypermethrin which modulates sodium channels (*21*), the benzoylurea hexaflumuron a chitin synthesis inhibitor (*22*), the organophosphate chlorpyrifos an acetylcholine esterase inhibitor (*23*), and fipronil which affects the gamma aminobutyric acid (GABA)-gated chloride channel (*24*). The sap-feeding neonicotinoid imidacloprid is an acetyl choline receptor agonist (*25*). Spinosad is active against *Lepidoptera* species via a novel mode of action (*26*).

Herbicides

Eight ^{14}C-labeled herbicides were selected to visualize typical whole plant distribution patterns displayed by commercial herbicides. The compounds represent several modes of action and span a wide range of physical properties with log K_{ow} values ranging from 4.5 to -4.0 and water solubility ranging from 0.4 to 1010 mg/L (Table 2). The selection includes two neutral compounds, trifluralin, a microtubule inhibitor (*27*), and isoxaben, a cell wall biosynthesis inhibitor (*28*) and

the base atrazine, a photosynthesis inhibitor (*29*) which is neutral at physiological pH. Furthermore, the following compounds were tested which are phloem-mobile due to an acid functionality: cloransulam-methyl, a triazolopyrimidine acetolactate synthase (ALS) inhibitor (*30*), the auxinic compound 2,4-D (*31*), and the haloxyfop-acid an acetylCoA carboxylase (ACCase) inhibitor (*32*). Glyphosate, which inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP synthase (*33*) and imazethapyr, an imidazolinone ALS inhibitor (*34*) have more than one ionizable functionality and their translocation may not be completely explained by the classic acid trapping mechanism (*35*).

Table 1. Physical Properties of Commercial Xylem Mobile Insecticides

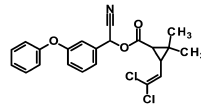
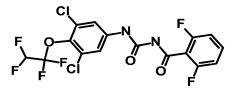
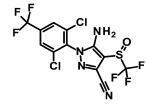
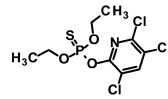
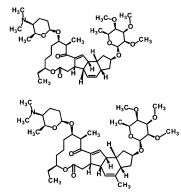
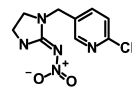
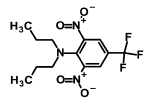
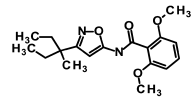
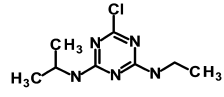
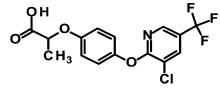
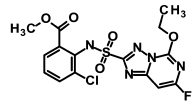
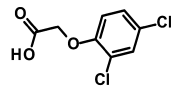
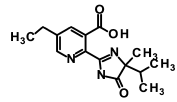
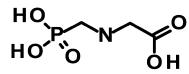
Insecticide	Structure	pKa	Log K_{ow}	Water Solubility (mg/L)
Cypermethrin		Neutral	6.94	0.009
Hexaflumuron		Neutral	5.68	0.027
Fipronil		Neutral	3.45	1.9
Chlorpyrifos		Neutral	4.70	2.0
Spinosad		7-8, basic	4.31	235
Imidacloprid		Neutral	1.4	510

Table 2. Physical Properties of Commercial Xylem and Phloem Mobile Herbicides

Herbicide	Structure	pKa	Log K_{ow}	Water Solubility (mg/L)
Trifluralin		Neutral pH 2-10	4.55	0.4
Isoxaben		Neutral pH 2-10	3.61	1.04
Atrazine		1.7 Basic	2.64	28.0
Haloxyfop-acid		4.33 Acidic	2.79	43.3
Cloransulam-methyl		4.84 Acidic	1.85	184.0
2,4-D		2.88 Acidic	2.80*	900.0
Imazethapyr		3.71 Zwitterionic	1.49	140.0
Glyphosate		pK_2=2.29 pK_3=5.96 Zwitterionic	-4.0	1010.0

* Compound is ~1.5% ionized and the Log Kow value could be slightly lower than reported.

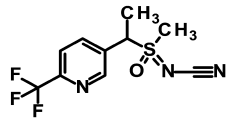
Application of ^{14}C-labeled Isoclast™ Active

The dose applied was 125 ppm (25 g ai/ha field dose) at a volume of 200 L/ha (125 µg/1000 µl in 1% emulsifiable concentrate (EC) formulation blank). A stock solution of 21.5 µCi of sulfoxaflor was dissolved in 1000 µl of 1% EC blank. To the 1st true leaf on cabbage, pepper and cotton, five 1-µl drops were applied across the center of each leaf.

Isoclast™ Active

Isoclast™ active (sulfoxaflor) controls economically important and difficult to control sap-feeding insect pests including certain species of aphid, jassids, plant bugs, plant hoppers, scales, stink bugs, and whiteflies. This new insecticide exhibits complex and unique interactions with the insect nicotinic acetylcholine receptors that are distinct from those observed with neonicotinoids. Isoclast™ active has physical properties (Table 3) like Log K_{ow} and water solubility that are similar to imidacloprid (Table 2).

Table 3. Physical Properties of Isoclast™ Active Insecticide

Insecticide	Structure	pKa	Log K_{ow}	Water Solubility (mg/L)
Isoclast™ active		> 10	0.802	670

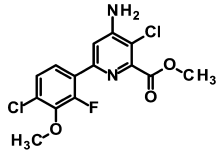
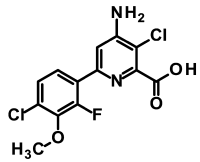
Application of ^{14}C-labeled Arylex™ Active

Arylex™ active was solubilized in an aqueous solution of 1.25% volume per volume (v/v) Agri-Dex crop oil concentrate (COC) to form a concentration of 18 ppm with activity levels of 1 µCi ml^{-1}. A total of four microliters of the formulation was applied as 1-µl droplets to the adaxial surface of the second fully expanded leaf.

Arylex™ Active

Arylex™ active is de-esterified in all plants to the active and mobile form, halauxifen-acid (halauxifen, Table 4) which controls broadleaf weeds with utility in multiple crops especially wheat where selectivity was due to a slower rate of de-esterification than was found in susceptible weeds (*36*). Arylex™ active is a new member of the synthetic auxin class of herbicides (HRAC group O, WSSA group 4) with physical properties illustrated in Table 4.

Table 4. Physical Properties of Arylex™ Active (Halauxifen-Methyl) and Halauxifen (Acid) Herbicides

Herbicide	Structure	pKa	Log K_{ow}	Water Solubility (mg/L)
halauxifen-methyl		2.84	3.76	1.67
halauxifen		4.90	1.23	121.3

Results

Insecticides

Cypermethrin did not show long distance transport in the plant after application to vine and wheat leaves (Figure 3). A limited amount of xylem mobility was observed for cypermethrin into wheat shoots from a wheat root application (Figure 4), but the majority of the radioactivity was retained in the roots likely due to a high Log K_{ow} of 6.94 (Table 1). Hexaflumuron seemed to remain at the site of application also when applied to vine and wheat leaves (Figure 3). Transfer into the shoot was limited also for hexaflumuron after stem or root application (Figure 4) and also is likely due to a high Log K_{ow} of 5.68 (Table 1). Fipronil, when applied to the vine leaves shows very little movement, but xylem translocation was evident when fipronil was applied to wheat leaves or stem (Figures 3 & 4). In comparison, chlorpyrifos which is also neutral molecule so can not be trapped in the phloem appears systemic from a leaf application

on vine and wheat (Figure 3). Therefore the observed radioactive material in plant parts outside of the treated leaf indicative of phloem movement can only be reconciled by its volatile nature (*37–39*). Chlorpyrifos moved up into the vine leaf after stem application (Figure 4), however unloading from the vein was limited likey due to a high Log K_{ow} of 4.70 (Table 1). Imidacloprid with a low Log K_{ow} of 1.4 was xylem-mobile (*40*) and showed excellent redistribution after stem and leaf application in vine and wheat (Figure 3 & 4). In wheat, imidacloprid clearly accumulated in the tip of the leaf (Figure 3). In comparison, spinosad with a high Log K_{ow} of 4.31 (Table 1) was poorly mobile with no label being transferred from the root to the shoot and no redistribution evident after stem application (Figure 4); however, some local redistribution occurred when spinosad was applied to the leaf of vine and xylem redistrubution when applied to a wheat leaf (Figure 3).

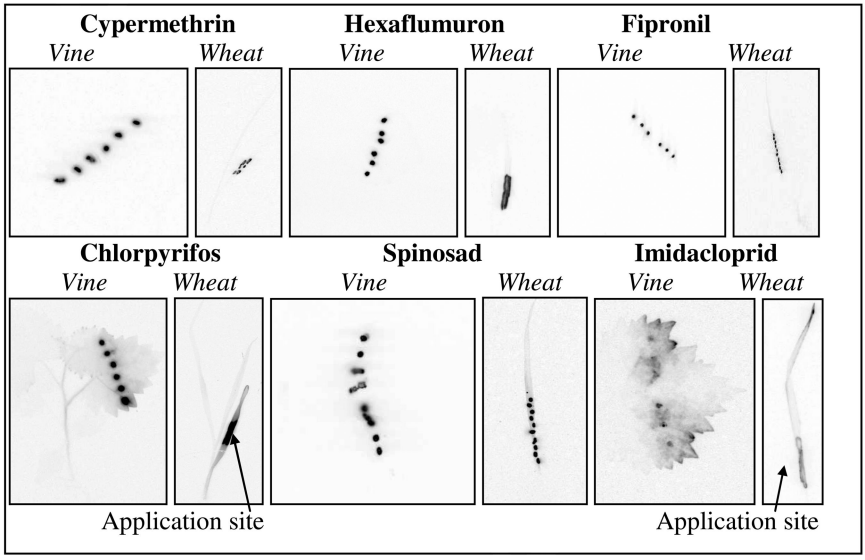


Figure 3. Phosphor images of commercial insecticides 4 days after foliar leaf application with arrow pointing to ambiguous application site.

Consistent with findings in the literature (*41*), insecticides with Log K_{ow} values above 4, cypermethrin, hexaflumuron, chlorpyrifos and spinosad are are limited in their xylem mobility. However, fipronil with a Log K_{ow} of 3.5 and a water solubility of 19 mg/L was also not well distributed via the xylem. Only, imidacloprid with a Log K_{ow} of 1.4 and water solubility of 510 mg/L showed good xylem mobility, which may be an important component of its high efficacy against sucking pests. All other compounds are mainly delivered via contact and ingestion and do not rely on redistribution within the plant.

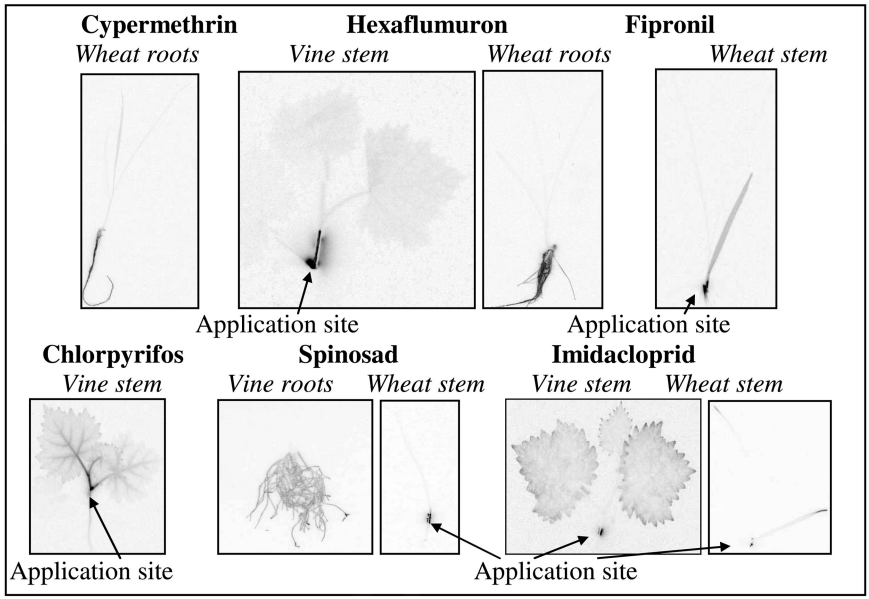


Figure 4. Phosphor images of commercial insecticides 4 days after root or stem application with arrow pointing to ambiguous application site.

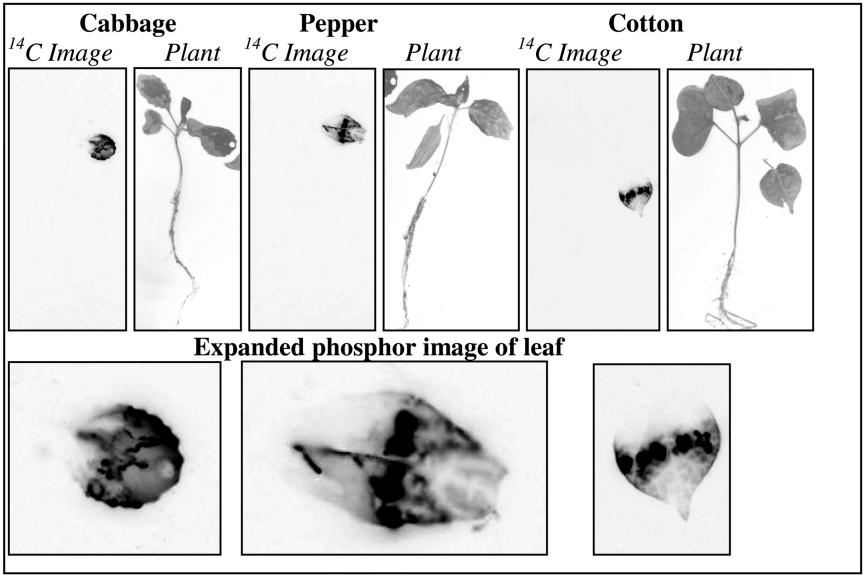


Figure 5. Plant translocation of Isoclast™ active in cabbage, pepper and cotton 3 days after foliar application.

Isoclast™ active when applied to plant leaves illustrates classical xylem mobility with no phloem translocation out of the leaf but clear movement with the transpiration stream in the cabbage, pepper, cotton and rice leaves (Figure 5 & 7). When applied to the stem of cabbage, pepper, cotton, or roots of rice, Isoclast™ active uniformly distributes throughout the plant above the application point with no concentration at the meristem as would occur for a phloem mobile material (Figure 6 & 7).

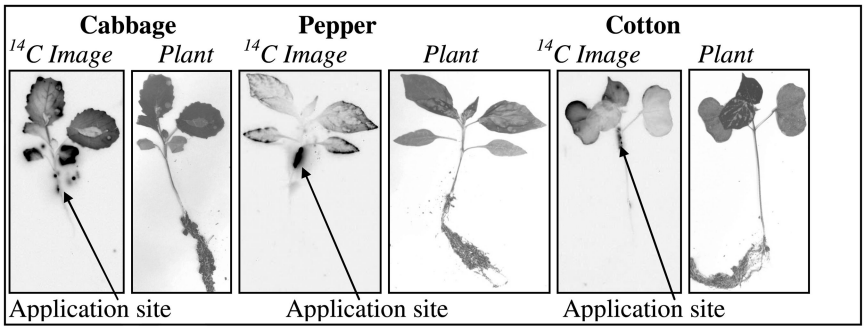


Figure 6. Plant translocation of Isoclast™ active in cabbage, pepper and cotton 3 days after stem application with arrow pointing to application site.

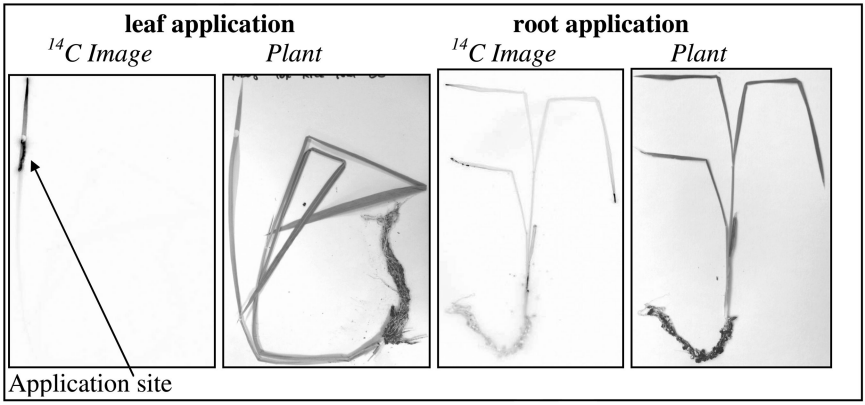


Figure 7. Plant translocation of Isoclast™ active in rice 5 days after leaf or root application with arrow pointing to ambiguous application site.

Herbicides

Phosphor imaging of plants treated with trifluralin, isoxaben and atrazine revealed a whole plant distribution typical for xylem mobile compounds (Figures 8 & 9). After stem application, the compounds were translocated up the stem into the transpiring leaf. In the case of trifluralin, imaging of the vine plants

revealed that this compound accumulated in the veins which is an indication for poor vein unloading (Figure 8). In contrast, isoxaben and atrazine were more evenly distributed throughout the whole leaf (Figure 8). Only a small amount of trifluralin moved away from the application spot, and was equally distributed across the vine lamina (Figure 8). This pattern could be explained by redistribution through the vapor phase rather than translocation within the water stream of the leaf (*41*). Volatility may also explain faint labeling of wheat roots (Figure 9). For comparison, isoxaben and atrazine vine leaf application resulted in a typical xylem movement pattern away from the application spot acropetally towards the tip of the leaf (Figure 8). Poor vein unloading of trifluralin may be attributed to its high Log K_{ow} of 4.5, compared to isoxaben and atrazine with Log K_{ow} values of 3.6 and 2.6, respectively, properties well suited for xylem mobility (*42*).

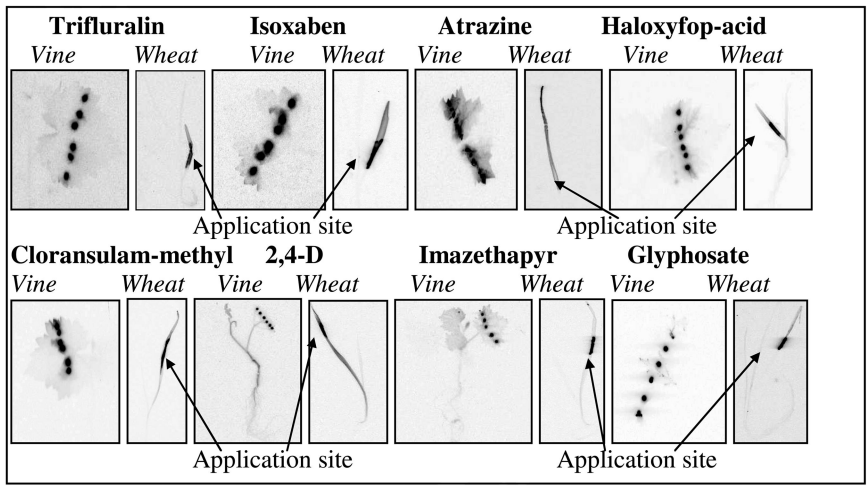


Figure 8. Phosphor images of commercial herbicides 4 days after foliar application with arrow pointing to ambiguous application site.

The classic ion trapping hypothesis suggests that the neutral species – favored in the more acidic cell wall space - permeates into the phloem sieve tube elements and dissociates to the ionized species in this more alkaline environment. The fully ionized species is trapped inside, accumulates within the sieve tube elements and will be carried along with the assimilate stream towards new growth (*43*). Analysis of whole plant distribution of ^{14}C-labeled acidic herbicides confirmed the phloem mobility of these compounds. Haloxyfop-acid clearly accumulated in the newly unfolding leaf after application to the vine stem (Figure 9). However, export after application to a vine leaf is poor (Figure 8), probably due to limited transfer through the cuticle. On the other hand, some export out of the wheat treated leaf was evident (Figure 8). In vine and wheat after leaf application of haloxyfop-acid, movement was observed to the leaf edges typical of acropetal transport within the xylem (Figure 8). In principle, cloransulam-methyl showed a

similar pattern (Figures 8 & 9), but phloem mobility was limited when compared to haloxyfop-acid. For instance, cloransulam-methyl was hardly exported out of the wheat leaf at all (Figure 8). The auxinic compound 2,4-D showed an exclusive phloem distribution pattern (Figures 8 & 9). After stem and leaf application in vine, 2,4-D moves via the phloem into new growth. However, transfer into fully grown, transpiring leaves via the xylem was limited (Figures 8 & 9). Imazethapyr showed good phloem mobility and some xylem movement in vine (Figures 8 & 9). Again, export out of the wheat leaf appeared limited (Figure 8). All these compounds have one weak acid functionality ranging in pKa from 2.9 (2,4-D) to 4.8 (cloransulam-methyl).

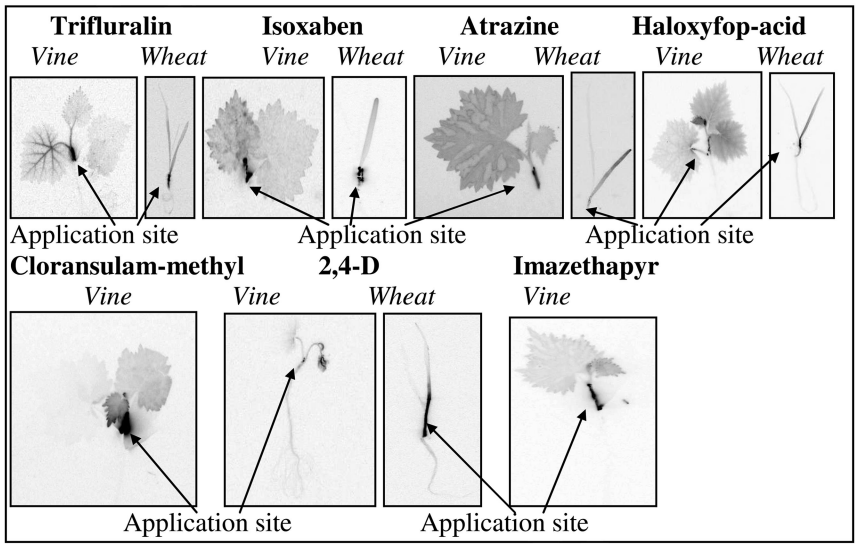


Figure 9. Phosphor images of commercial herbicides 4 days after stem application with arrow pointing to application site.

According to the ion trapping theory, one would expect that cloransulam-methyl and haloxyfop-acid with pKa values above 4 might possess superior phloem mobility. At the cell wall pH of 5, much more neutral species would be available for permeation through the membrane, as compared for instance to 2,4-D. However, the results suggest that phloem mobility increases with decreasing pKa. For imazethapyr, the situation was even more complex due to the fact that this compound is zwitterionic at acidic pH. Log K_{ow} values have been used to factor in membrane permeability (*44*), but, haloxyfop-acid and 2,4-D share almost identical Log K_{ow} values (Table 4) with clear differences in their overall phloem mobility. Additional information is needed to predict the extend of herbicide phloem transport in a quantitative manner. Due to multiple ionization sites, glyphosate exists in monovalent or divalent anionic state over a wide pH range. Therefore, glyphosate cannot accumulate in the phloem due to

ion trapping and its excellent phloem mobility is attributed to carrier-mediated transport (*45*). Poor penetration was evident when glyphosate was applied to vine and wheat leaves (Figure 8) because the ^{14}C-glyphosate was not specifically formulated to assist in cuticle penetration of this highly charged molecule.

Arylex™ active was very rapidly de-esterified in plants to the acid (halauxifen) and the acid was both the active herbicidal agent and the transported entity in plants (*36*). When Arylex™ was applied to wheat leaves both phloem and xylem mobility was observed. In wheat, Arylex™ (as the acid) moved with the transpiration stream up the leaf in the xylem and a small amount of movement out of the leaf in the phloem. In wheat after uptake, translocation and metabolism studies it was found that Arylex™ active was metabolized rapidly to inactive metabolites achiving wheat selectivity and reducing amount of halauxifen available for movement in the phloem (*36*). In contrast, both deadnettle and speedwell metabolized halauxifen (Arylex™ acid) slowly allowing for phloem movement out of the treated leaf towards growing points of the plant (Figure 10).

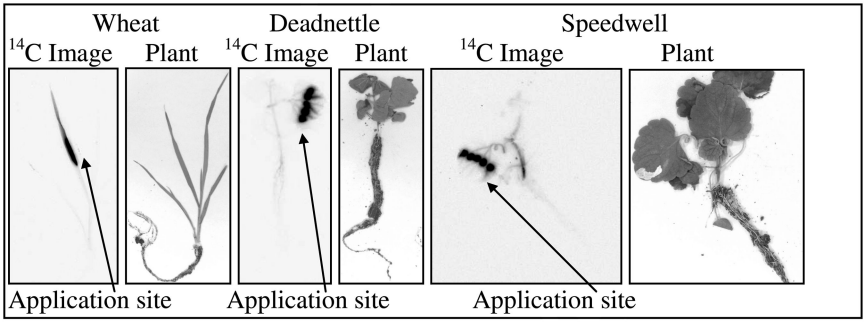


Figure 10. Plant translocation of Arylex™ in wheat, deadnettle and speedwell 3 days after leaf application with arrow pointing to application site.

Summary

Translocation and redistribution of pesticides in plants is important to their ultimate effectiveness. Representative whole-plant redistribution patterns were visualized for ^{14}C-labeled herbicides and insecticides in vine and wheat. Application of labeled compounds to leaves and stems allowed for qualitative analysis of long distance redistribution via phloem and xylem.

Insecticides like cypermethrin, hexaflumuron, fipronil and spinosad showed limited mobility in plants. Imidacloprid was efficiently distributed within the plant by the xylem and the redistribution of chlorpyrifos was supported by volatility. Acidic herbicides showed classical phloem-mobility distribution patterns. In contrast, the neutral herbicides trifluralin, isoxaben and atrazine were only redistributed through the xylem. Based on phosphor imaging, overall phloem export and mobility could be qualitatively ranked. Haloxyfop, 2,4-D,

imazethapyr and glyphosate were all exported to the growing shoot with high efficiency. Leaf cuticle penetration proved to be limiting for some compounds, especially glyphosate.

Physical properties like Log K_{ow} and pKa allow a preliminary, qualitative prediction of a compound's ability for passive membrane permeation and the resulting use of phloem and xylem transport pathways. For instance, all weak acids with Log K_{ow} values below 4 are assumed to be phloem mobile and neutral compounds with Log K_{ow} values ranging from -3 to 4 are considered xylem mobile (*44*). However, based on the findings in the current study as well as reports in the literature (*46*), no reliable quantitative predictions are possible based on Log K_{ow} and pKa alone, especially when analyzing whole plant distribution which results from substance flow through both xylem and phloem.

Isoclast™ active is primarily a xylem translocated insecticide based on classical movement profiles that followed the transpiration stream. It was confirmed by leaf application that Isoclast™ active was translocated in the xylem versus the phloem. Stem application also illustrated how systemic the material is as it moves in the transpiration stream. Isoclast™ active has the right combination of plant uptake, xylem translocation and metabolic stability characteristics to achieve excellent sucking pest insect control.

Arylex™ active showed only a slight amount of xylem mobility in wheat indicating that Arylex™ as the acid form is ambimobile. Arylex™ as the acid form, was primarily translocated in the plant via the phloem as illustrated by movement out of the treated leaf of deadnettle and speedwell towards the meristem. Arylex™ has the right combination of plant uptake, phloem translocation and wheat selectivity to achieve excellent control of key weeds in cereals.

To achieve reliable control in today's insecticidal market, xylem mobility is a key attribute especially for sucking pest control. Isoclast™ active has those right attributes as has been illustrated here visually after both foliar and stem application of ^{14}C formulation to important crops.

In contrast, new herbicides need to exhibit phloem translocation as has been shown to be the case for Arylex™, a new wheat selective herbicide that controls select broadleaf weeds by killing the meristem after post emergence application, a direct result of phloem translocation.

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Chapter 5

Fungicide Mobility and the Influence of Physical Properties

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Redistribution is an important and complex facet of the field performance of fungicides. Physical properties of the active ingredient strongly influence mobility; this review focuses on factors affecting redistribution (particularly translaminar movement) and methods for measuring and modeling fungicide mobility. Surface and vapor-phase redistribution, local and translaminar redistribution, and long-distance systemic movement in xylem and phloem may all affect fungicide performance to varying degrees, and the physical properties of the active ingredient strongly influence the relative importance of each mode of redistribution. Mobility can be measured using biological activity as an indicator, with radiolabeled molecules, or using analytical techniques; each method has advantages and disadvantages for early-stage fungicide discovery. Redistribution is best considered a continuum in which active ingredients can partition into most leaf tissues to varying degrees and the proportion in each determines the utility of a fungicide product.

Redistribution in plants is a valuable attribute for fungicides, insecticides, and herbicides. Spray applications rarely provide complete coverage and movement of the active ingredient from the spray droplet to untreated areas improves the performance and reliability of pesticide products. Pesticides applied by seed treatment or ground applications may also be more effective if taken up by roots

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

and distributed through the plant vascular system. Redistribution is a complex attribute of a plant protection product that is influenced by formulation, plant characteristics, and environment as well as by the physical properties of the active ingredient (*1*).

Movement of the active ingredient after application has been categorized in a variety of ways and terminology can be confusing. For example, Neumann and Jacob (*2*) distinguished "mobility" from "systemicity," using "mobility" to describe transport of any substance while "systemicity" described ability of a pesticide to provide activity in another part of the plant. Most authors do not make this distinction. "Systemicity" is often used (and is used here) to refer to longer-distance redistribution involving phloem or xylem transport, while "redistribution" and "translocation" are more general terms applicable to both local and long-distance mobility. In reality, redistribution is a continuum (*2*) that can be manipulated with formulation, and most products with systemic properties probably exhibit at least minor amounts of local, translaminar, xylem, and phloem redistribution. Indeed, xylem-mobile compounds may partition into the phloem and move symplastically for a distance that is concentration-dependent before leaking out again, in some cases resulting in limited control of disease basipetal to the application zone (*e.g.,* (*3*)). A further challenge with categorizing the systemic properties of a pesticide product is that mass transport, key to the physics of redistribution, is concentration dependent, so a product may act systemically at high application rates but not at low application rates. Difficulty in classifying redistribution has led scientists to propose novel redistribution categories for new active ingredients, including quasi-systemic, surface systemic, and mesostemic (*4*, *5*).

This chapter focuses on the redistribution of fungicides and testing methods applicable to early-stage fungicide discovery. Other chapters cover related topics such as modeling the penetration of the leaf cuticle, phloem redistribution, systemicity from seed or root applications, and the influences of adjuvants and formulations on deposition and redistribution.

Types of Systemicity/Redistribution

Vapor-phase systemicity ("molecular redistribution by air" is considered more accurate by Bartlett et al., (*6*)) may be the easiest type of redistribution to experimentally confirm because the active ingredient deposit (on another plant, on aluminum foil, in a glass dish) can be physically separated from the target plant. Accumulation on the target plant can be detected with a bioassay (illustrated in (*6*)) or analytically. Formation of vapors depends on physical properties of the active ingredient (particularly volatility and lipophilicity) and environmental conditions, but reliable models of volatility from plant surfaces have proved difficult to develop (*7*). To complicate prediction further, formulation has been shown to significantly affect vaporization rates (*8*). Further, some compounds with low vapor pressure, such as quinoxyfen (1.2×10^{-2} mPa at 20°C) and picoxystrobin (5.5×10^{-3} mPa at 20°C) (*9*), have vapor-phase redistribution that

contributes to field performance, so testing new active ingredients for volatile activity early in the development process is important regardless of predictions from volatility models. Sometimes vapor phase movement is suspected when a fungicide is applied to one part of a plant and activity is seen in another part of the same leaf or plant, but other factors such as translaminar redistribution or stimulation of a systemic host defense response could cause a similar result so this conclusion should be considered preliminary until confirmed with definitive testing.

Surface redistribution may include vapor phase redistribution but usually refers to redistribution on the surface of the leaf without partitioning into the epidermal or mesophyll plant cells. Depending on the physical properties of the active ingredient and the formulation, surface redistribution may occur when water on the surface of the leaf solubilizes or suspends the compound and it moves by diffusion or water movement. Local redistribution over the leaf surface with dew or rain is likely important to the robustness of foliar fungicides, evening out particle distribution in the same environment required for most fungi to infect. Aqueous redistribution is likely important to the performance of protectant multisite fungicides such as mancozeb and chlorothalonil (*10*). For highly lipophilic fungicides such as trifloxystrobin and kresoxim-methyl, partitioning into the waxy cuticle and migrating through the waxes across the leaf could be of equal or greater importance to aqueous redistribution. Cuticular redistribution likely also occurs with fungicides that are capable of partitioning into the leaf tissue since they accumulate in the cuticle first, but the relative importance of surface redistribution may be less when a fungicide is well-distributed within the plant tissue. Ypema and Gold (*4*), for example, noted that kresoxim-methyl has cuticular redistribution as well as local redistribution in plant tissue and referred to the combination as "quasi-systemic transport."

Once a compound has partitioned into the leaf tissue in sufficient titer to control fungal infection, redistribution tends to be categorized based on fungicidal utility. Translaminar redistribution, for example, is defined as movement from one side of the leaf to the other and may be considered both a method of measuring "local redistribution" and an indicator of a valuable fungicidal property. That redistribution could be a function of the compound partitioning into the cell walls and apoplast, into the cytoplasm, plasmadesmata and symplast, into the air volume of the leaf interior, or all three, with relative proportions driven by physical properties and the inner leaf environment. Depending on the method of measurement, translaminar activity may not require movement of a compound to the opposite epidermis at all; if translaminar activity is measured by inoculating a pathogen on the opposite side of the leaf from compound application, and the fungus ramifies through the mesophyll rather than being limited to the epidermis, translaminar activity is probably indistinguishable from local redistribution. Although "local" redistribution generally implies that the compound diffuses a short distance from the entry point into the leaf, and that loading into xylem or phloem elements changes the redistribution to "systemic," any local mass transport within the plant (other than in the vapor phase) invariably requires partitioning into the apoplast or the symplast (with the proportion in each driven by physical properties) (*2*). Since the apoplast is a continuum with the xylem elements, as

is the symplast with phloem elements, local redistribution vs. systemicity is a matter of degree rather than separate phenomena. For example, Kemmitt et al. (*11*) showed that myclobutanil demonstrated both local redistribution and long-distance systemicity and concluded that both contributed to field control of Asian soybean rust. Lehoczki-Krsjak et al. (*12*) examined the effect of plant tissue type and location on local-to-systemic redistribution of prothioconazole and tebuconazole. When applied to roots or stems these fungicides demonstrate clear xylem systemicity (*3*). When applied to wheat florets, tissues that act as a "sink" for assimilates, both tebuconazole and desthio-prothioconazole were exported from the florets, implying symplastic redistribution. Symplastic redistribution was not observed when fungicides were applied to the flag leaf blade. Clearly, the type of redistribution demonstrated by a fungicide product depends on factors beyond the physical properties of the active ingredient; in this example plant tissue had a significant effect.

Measurement of Redistribution

Screening compounds for a complex attribute such as redistribution in a fungicide discovery program is more difficult than screening for activity against target pathogens. Assessment of both local and long-distance systemicity in screening programs frequently uses control of disease development to indicate compound movement, although this has limitations as discussed below. If a compound has low activity on the target pathogen, as is often true for weakly active compounds from early-stage discovery programs, translocation is difficult to determine without a radiolabel. Re-synthesis of compounds to incorporate a radiolabel is time-consuming and expensive, so it is impractical for screening of early-stage compounds. Analytical methods such as liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) can be used to assess redistribution of unlabeled compounds (*12*, *13*) and equipment with ever-greater sensitivity is becoming increasingly accessible.

Biological Activity as an Indicator of Redistribution

Measuring redistribution using selective application and inoculation and observing fungicidal activity is the most common method of demonstrating redistribution and many examples are found in literature. It is simple, fast, inexpensive, and fits easily into the regimens of fungicide testing programs. Dahmen and Staub (*14*), for example, used control of wheat powdery mildew to demonstrate redistribution of difenoconazole. Wheat leaves were treated with several drops of difenoconazole formulation arranged in a 1-cm-wide zone, plants were inoculated with powdery mildew spores, and the size of the disease-free zone outside the application zone was measured. Similarly, Ypema and Gold

(*4*) used control of powdery mildew on pumpkin and control of rust on beans to show translaminar redistribution of kresoxim-methyl. The pattern of activity (larger disease-free zones in the direction of the leaf tip or margin vs. towards the leaf base) may indicate the dominance of xylem vs. phloem transport (*3*, *14*, *15*). Disease organisms that create a fine, uniform lawn of sporulation, such as powdery mildews, or small numerous lesions such as wheat leaf rust, are particularly suitable for this technique (*16*, *17*). When examining translaminar redistribution, powdery mildews have the additional advantage that mycelia colonize only the outermost cells of the leaf surface, providing a more definitive model than diseases that colonize the interior of leaves.

Despite these clear advantages, use of biological activity as an indicator of redistribution has several pitfalls. The most obvious is the influence of fungicidal potency. When comparing fungicides for relative redistribution in plants, the application rates need to be normalized for inherent activity against the pathogen. Inherent activity can be determined *in vitro* for necrotrophic fungi but for biotrophic pathogens, the best measure of potency may be the foliar IC_{50} in a short (1 day or less) protectant, high-volume assay (*18*). Using other organisms or target site activity to normalize rates may be better than no normalization but since structure-activity relationships within a fungicide series differ from one pathosystem to another, there is a real danger that the normalization is irrelevant for the pathosystem used as the biological indicator. Normalization may produce its own artifacts, in that compounds applied at high rates (because of low potency) may partition into the plant to a greater extent because of a higher diffusion gradient; the higher concentration in the leaf could influence comparative redistribution. Experimental evidence that higher concentrations produce greater total partitioning, however, is lacking, with some researchers showing no effect or an inverse effect of concentration on cuticle partitioning (*19*, *20*).

A second problem with a biological indicator is that it can only be used with fungicidally active compounds. This can be an issue if improved redistribution is a goal of an early-stage fungicide invention project. Determining the effects on redistribution of physical property or structural differences between analogs is impossible when the compounds critical to the comparison have weak activity. If the compound is a profungicide with fungicidally active metabolites, bioassays do not distinguish between the movement of the prodrug and the movement of the metabolite; quantitative analysis is needed to sort out the chemical structure of the translocated active compound.

A third limitation is the poor precision of the technique. Because expression of fungicide control is heavily influenced by the environment, the physiology of the plant, and the fitness of the fungal isolate, biological tests are inherently variable and obtaining sufficient precision for statistical analysis or modeling requires highly controlled conditions and likely a large number of samples. This biological variability makes reproduction of the test results at another time or between labs challenging.

Despite these limitations, for practical purposes, demonstrating redistribution in the greenhouse using disease control as an indicator is a straightforward technique that gives the scientist confidence that systemicity is likely to occur in the field and contribute to product success.

Quantitative Analysis of Compound Concentration

Given the limitations of biological assessment, quantitative measurement of the applied compounds is an appealing approach for comparing the redistribution of compounds. Inherent activity is irrelevant and (at least on first glance) the technique should be less variable with better precision. LC-MS/MS is an example of an analytical technique used to document redistribution of compounds following seed treatment or foliar application in greenhouse and field studies (*12*, *13*). It provides the selectivity and sensitivity needed for quantitative identification of xenobiotic compounds in complex biological matrices such as leaf tissue extracts. Because LC/MS/MS is used routinely to detect residues of applied compounds (*e.g.,* (*21*)) and study compound metabolism (*e.g.,* (*22*)), the equipment is available at many institutions. In LC/MS/MS, liquid chromatographic separation prior to MS/MS quantitation separates the fungicide from plant matrix components that may suppress its ionization response. MS/MS analysis requires 1) formation of a molecular ion specific to the compound of interest, 2) selection of the molecular ions, 3) formation of compound-specific fragment ions from the isolated molecular ions, 4) detection of the compound-specific fragment ions. The intensity of the signal of the compound-specific fragment ions is directly related to the concentration of the fungicide in the sample being analyzed. The eluent from the LC column is introduced into the MS, which continuously performs the four steps in a millisecond timeframe (*13*).

Klittich et al. (*13*) used LC/MS/MS to compare the redistribution of 23 fungicidal compounds from a dropline application (similar to the application method used in (*14*)) with the goal of developing a moderately high-throughput method to compare compounds for xylem redistribution in a leaf regardless of potency. The technique successfully distinguished compounds determined to be systemic or non-systemic in biological assays, but several limitations were found for this method. Although the distance between the application zone and the non-contiguous distal zone was not a significant factor, the time from application to sampling was important; picoxystrobin and thifluzamide concentrations doubled in the distal zone between 24 and 48 hours. A second limitation was the extreme care needed to avoid cross-contamination when harvesting and handling samples, since even a tiny amount of compound transferred from the application zone to the distal zone on forceps or cutting surface would skew the results. Between the requirement for meticulous avoidance of cross-contamination and the importance of collecting samples in a relatively short time period, this analytical technique would not be suited to high-throughput screening of analogs for systemicity.

Another limitation of the analytical technique is that the compound must be completely extractable from ground tissue with routinely used solvents and then produce a signal that can be detected and quantified by the instrument. A single solvent mixture is unlikely to extract every compound in the same proportion and multiple solvents and washes may be needed to obtain complete extraction (*23*). Further, unless the technique is modified to identify metabolites as well as parent compound, the rapid metabolism of the fungicide will skew the results.

In our study (*13*), two of the 23 compounds that showed systemic activity in biology testing (UK2A and flutolanil) had little accumulation of compound in the distal zone and considerable variation between replications. The causes were not determined but could include incomplete detection by MS, metabolism by the plant, or poor extraction from plant tissue caused by binding to plant tissue or poor solubility in acetonitrile.

Despite these limitations and relatively low through-put, quantitative analysis can provide accurate detection of redistribution with good quantification. Because tissue is destructively harvested for analysis and contamination of nearby areas close to the application zone is hard to avoid, the technique is probably not useful for determining local systemic movement such as translaminar activity but is well-suited for examining longer distance transport in the xylem and phloem.

Use of Radiolabels for Measuring Redistribution

Incorporation of ^{14}C labels into synthetic fungicides is a well-documented technique for detecting and quantifying movement of a compound in a plant (e.g., (*5*, *24*)), typically through phosphorimaging or autoradiography or through combustion and scintillation analysis, and the techniques will not be reviewed here. For fungicides in a development pipeline, radiolabeled active ingredient is essential for the precise quantification of fungicide and metabolite residues in complex matrices and thus radiolabeled compound is likely to be available for biological redistribution studies. At earlier stages in fungicide development when multiple analogs are being compared, the radiolabeling of many compounds is generally too cost-prohibitive and time-consuming.

Even in the cases where radiolabeled fungicides are available for redistribution testing, the technique has some disadvantages. When using phosphorimaging or autoradiography to visualize redistribution of a fungicide in a plant, the high concentration of radioactivity at the application site can obscure local redistribution although long-distance systemicity can be easily detected. Quantifying the amount of compound in distal zones is not easily done with radioimages. Redistribution can be accurately quantified if plant tissue is harvested and combusted but the need to control cross-contamination and difficulty in detecting translaminar and very local redistribution are limitations similar to the LC/MS/MS technique. A third challenge is that the radiolabel methods detect the presence of the radiolabeled atom rather than the intact compound, and if the fungicide is metabolized in the plant the technique may be measuring redistribution of an inactive metabolite rather than the compound of interest. A thorough understanding of the metabolism of the active ingredient is a necessary prerequisite to radiolabel studies (*24*). Kemmitt et al. (*11*) used a combination of methods to examine the redistribution of myclobutanil in soybean plants, including phosphorimaging and extraction/combustion with radiolabeled compound, extraction and quantitative analysis of unlabeled myclobutanil and its metabolites, and biological assay with Asian soybean rust. The result was a clear picture of myclobutanil systemicity, demonstrating strong xylem redistribution from a stem application but no movement out of the leaf from a leaflet application.

Despite its cost and limitations that render the technique unsuitable for most early-stage compounds, the value of visualizing redistribution throughout a plant with a single image can be very high and the technique provides redistribution information difficult to obtain with any other method.

Effects of Adjuvants on Redistribution

Although formulations and adjuvants can influence significantly the initial stages of fungicide redistribution, particularly leaf surface redistribution through vapor, surface systemicity, and cuticle penetration, adjuvants generally do not affect redistribution of active ingredients once partitioning into the epidermis is accomplished (*8*, *24*). Formulations and adjuvants may be correlated with increased translocation if they increase the amount of compound that has partitioned into the plant and is available for redistribution, although that assumption has little published validation (*20*). Forster and Kimberley (*19*), for example, found that the increased uptake provided by adjuvants did not necessarily lead to increased translocation or increased biological activity and found no effect of either adjuvant or dose on translocation of epoxiconazole. Most studies on adjuvants and formulations have shown that adjuvants do not penetrate beyond the cuticle or (at most) the epidermal cell layer, although some can penetrate sufficiently to cause phytotoxicity (*20*). Once the active ingredient is in the plant, redistribution appears to be driven by the physical properties of the active ingredient and the physical environment provided by the plant.

Comparisons of compounds for relative redistribution and systemicity frequently involve non-standardized commercial formulations (*14*, *25–27*). Because formulation and adjuvancy can significantly affect foliar uptake of pesticides (*15*, *27–30*), direct comparison of active ingredients for local redistribution or translaminar activity cannot be made when the formulations differ.

Effects of Physical Properties on Redistribution

Cuticular Penetration

The movement of xenobiotics through plant cuticles has been extensively modeled and reviewed (e.g., (*15*, *20*, *31*)), and is considerably more complex than predicted by simple laws of mass transfer (*32*). Adjuvants, formulation type, ratio of active ingredient to adjuvant, chemical and physical structure of the cuticle, spread of the droplet on the leaf surface, drying time, and concentration of active ingredient in the spray droplet are among the many application parameters that influence uptake of the active ingredient (*23*, *29*, *30*, *32–35*). Among physical properties of the active ingredient, lipophilicity is frequently considered the parameter most predictive of cuticle penetration (*20*, *28*). Wang and Liu (*20*) considered lipophilicity to be critical to foliar uptake because of its influence on transcuticular movement, but challenged the opinion that foliar penetration can be

explained by only one or two parameters. Water solubility also has an important influence on cuticle penetration (*15*, *24*) and xylem systemicity (*13*). Molar volume has been considered a key predictor of movement of a compound across a cuticular membrane (*31*, *34*). Briggs and Bromilow (*15*) considered melting point to be a key property affecting cuticular penetration because it controls the solubilization of a compound on the leaf surface, the first step in leaf penetration. They also considered melting point to be tightly linked to water solubility, and used melting point and log P_{oct} to predict water solubility. Other investigators, however, failed to detect a statistical correlation between measured solubility of weakly soluble compounds and melting point (*36*). More details on the effects of physical properties on cuticular penetration and modeling are found in the chapter, "Modeling xenobiotic uptake and movement – a review."

Physical Properties and Redistribution

Published reports examining the effects of physical properties on redistribution of fungicides after the cuticle penetration step are few, likely because cuticle effects are easier to measure using artificial cuticles, because cuticular penetration is influenced by formulation, and because in the field only the aggregated effect is important. As with cuticular penetration, lipophilicity is considered the most critical physical property to redistribution within plant tissues (*20*, *28*). In fact, Bromilow and Chamberlain (*37*) stated that the systemicity of compounds can be predicted by lipophilicity alone: compounds with log K_{ow} less than 3 were expected to have both xylem and translaminar mobility while those with log K_{ow} of 3-4.5 should have no xylem mobility but might show translaminar movement. Briggs and Bromilow (*15*) and Sauter (*38*) proposed that melting point is also an important parameter affecting redistribution. The effects of physical properties on longer-distance xylem systemicity and local redistribution may differ and will be discussed separately. The physical properties needed for long-distance symplastic transport are well-defined and discussed in detail in another chapter.

Translaminar and Local Redistribution

Klittich and Ray (*18*) studied the effects of physical properties on translaminar redistribution of fungicides using cucumber powdery mildew control to measure translaminar activity. Over 60 fungicidal compounds from three chemical classes and a range of physical properties were compared in a consistent, simple formulation system with minimal adjuvant. Application rates were normalized based on LC_{50} values calculated from dose response curves of high-volume foliar applications with the same simple formulation. Translaminar movement (driven by physical properties) could be differentiated in the models from potency-influenced translaminar control. Translaminar movement (the ratio between the foliar LC_{50} and the minimum concentration delivering translaminar activity) was significantly correlated with lipophilicity. A second measure of

translaminar activity, the average diameter of the translaminar disease-free zone, was also significantly correlated with lipophilicity, accounting for 22-26% of the variation in diameter. In all models greater lipophilicity predicted decreased translaminar activity.

Water solubility also significantly affected translaminar activity in this study, although it accounted for less of the variability than did lipophilicity. In all significant models, higher water solubility predicted increased translaminar movement and translaminar disease control. Water solubility is also known to be an important factor influencing cuticle penetration (*15*) and xylem systemicity (*13*).

Molar volume is a key parameter in models of cuticular penetration, with large compounds penetrating less than smaller compounds of the same lipophilicity and pK_a (*31*, *35*). Molar volume should influence the partitioning of the compound into the leaf but should have little influence on redistribution once the compound is past the cuticular path (*39*). In the Klittich and Ray study, molar volume was a poor predictor of translaminar movement and translaminar control in our study, consistent with other models.

Melting point had no significant effect on any of the Klittich and Ray models of translaminar activity, despite deliberate selection of compounds with a broad range of melting points. Briggs and Bromilow (*15*) considered melting point to be an important driver of solubilization on the leaf surface, the step initiating cuticular penetration. Other investigators, however, failed to detect a statistical correlation between measured solubility of weakly soluble compounds and melting point (*36*).

Multiple regression models explained 60-61% of the variability in average translaminar diameter, with foliar IC_{50}, water solubility, and lipophilicity contributing significantly. The remaining 39-40% of the variability in translaminar control was unexplained and must be attributed to factors other than the fungicidal activity and physical property parameters included in the study. Examples of additional physical properties that could affect redistribution could include dipole moments, three-dimensional shape characteristics, or free energy of insertion (*40*). Determining the additional parameters influencing translaminar redistribution would be a valuable follow-up to this study.

Other published models of translaminar activity or local systemicity are few. Baker et al. (*24*) modeled the uptake and translocation of 26 radiolabeled pesticides into leaves of four plant species using a uniform formulation. They concluded that water solubility and lipophilicity were correlated with pesticide uptake but that their predictive utility was inadequate, accounting for less than 15% of the variation in the regression model. Melting point and molecular weight were not correlated with uptake. Translocation beyond the application zone was strongly influenced by the rate of uptake; compounds that penetrated rapidly and in large amounts were the ones most likely to be translocated out of the application zone. Solel and Edgington (*41*) compared several commercial benzimidazole fungicides for both transcuticular and translaminar activity on apple leaves. They found that compounds with transcuticular activity were not necessarily translaminar. Although transcuticular movement is the necessary first step for translaminar movement, it was not sufficient to predict translaminar movement.

Long-Distance Redistribution

As with local and translaminar redistribution, few published studies are available assessing the influence of physical properties on xylem redistribution. In most studies a compound or mixture of compounds is applied to one part of a plant and a distal area of the plant is assessed for disease control or analyzed for accumulation of compound or radiolabel (*e.g.,* (*3*, *11*)). The analyses rarely include determination of physical property effects, but can provide insights into the redistribution continuum. Lehoczki-Krsjak et al. (*12*), for example, treated wheat ears or flag leaves with a commercial formulation of prothioconazole + tebuconazole and analyzed translocation to other plant parts using LC/MS/MS. They found that over an 8-day period neither tebuconazole nor the main metabolite of prothioconazole, prothioconazole-desthio, moved in the symplast from the flag leaf (source) into the ears (sink). Interestingly, when spikelets on one side of the ear were treated, both tebuconazole and prothioconazole-desthio were translocated to untreated spikelets and to the flag leaf. While local redistribution in the apoplast could contribute to redistribution within the ear, redistribution to the flag leaf implies short-distance symplastic redistribution of these two compounds. A similar result was reported by Augusto and Brenneman (*3*) who found that prothioconazole painted onto peanut leaves controlled disease development on stems and leaves below the treated leaves as well as above them, while azoxystrobin, tebuconazole, and flutolanil provided acropetal protection only. Commercial formulations were compared so the effects of physical properties independent of formulation effects could not be determined.

A broader study of xylem mobility by Klittich et al. (*13*) examined the redistribution of 23 fungicides and experimental compounds from the base of a wheat leaf toward the tip in an NMP-based 10% EC formulation. Compounds were known to have long-distance systemicity, local systemicity, or no systemicity based on published and internal data. Redistribution in the leaf was measured analytically using LC/MS/MS and correlated with physical properties of the fungicides. Compounds that accumulated at 1% or more in the distal zone were all known to be systemic (e.g., picoxystrobin, epoxiconazole, nuarimol). Most compounds that accumulated at less than 0.1% in the distal zone were known to be non-systemic or locally systemic (e.g., trifloxystrobin, fludioxonil, pencycuron). Compounds that accumulated at 0.1-0.9% in the distal zone ranged from locally systemic (dimethomorph) to systemic (azoxystrobin, metalaxyl). No compounds that were known to be non-systemic accumulated to more than 0.05%.

Regression analysis was used to determine the influence of physical properties on accumulation in distal zones. Water solubility and lipophilicity were both correlated with measured translocation but did not account for the majority of the variability between compounds. Still, the lipophilicity model was as effective at separating systemic compounds from locally systemic or non-systemic compounds as the method of measuring accumulation in the distal zone. Because the analytical method tended to classify locally systemic molecules as systemic, the best use of the analytical method was detection of systemicity at any level rather than distinguishing between locally systemic and systemic compounds. For this use,

however, the lipophilicity model was equally predictive and considerably simpler than the LC-MS/MS technique.

Conclusions

Redistribution is a valuable attribute of most successful fungicides, mitigating the challenges of incomplete spray coverage and imperfectly timed spray intervals. Modeling the redistribution of fungicides is challenging but valuable to fungicide discovery programs that strive to reduce application rates and improve the reliability of fungicide products. Although redistribution effects are usually categorized as one type or another, redistribution is better considered a continuum in which active ingredients can partition into most leaf tissues to varying degrees and the proportion in each determines the utility of a product. Fortunately, successful commercial fungicides demonstrate a wide variety of redistribution patterns that have driven the development of products uniquely suited to individual crops and diseases. The continued development of models based on physical properties will provide additional tools to the fungicide invention process.

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Chapter 6

The Relative Influence of Retention, Uptake, and Translocation on the Bioefficacy of Glyphosate

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Extensive experimentation in recent decades has assisted in developing a better understanding of the individual processes (spray adhesion, retention, uptake and translocation) that control the biological efficacy of applied herbicides. Various models are available, each usually limited to predicting the efficacy of an individual process. In this study, spray retention, uptake and translocation of glyphosate (as influenced by an organosilicone surfactant Silwet L-77®) on three plant species was determined. Non-linear regression models were used to describe the relative effects of retention, uptake and translocation processes on bioefficacy. All three variables were highly correlated, making it difficult to distinguish their relative influence on bioefficacy. Uptake and translocation were highly correlated (> than 0.98 for all species studied), with about 40-45% of the glyphosate taken up translocated for all three species. Overall the best model, explaining 85-99% variance, used retention and uptake together. It appeared that the bioefficacy of glyphosate towards barley and broccoli was 30-50% related to retention and 50-70% related to uptake, while glyphosate efficacy towards bean was entirely related to uptake and not directly related to retention. For practical purposes, it is reasonable to expect that experimental results from retention

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and uptake studies (or predictions of each from available models) considered together will give a good indication of the relative bioefficacy of different formulations for a given AI, across a wide range of systemic active ingredient (AI) and species, minimising the need for translocation measurements.

Introduction

The challenges that are faced by agrichemical users have been increasing in complexity over recent years. On one hand regulators insist on reducing risk to the operator or environment (*1*), while on the other, increased efficiency is pursued by growers to maximise profits. These demands have the prerequisite that the biological efficacy of agrichemicals is at least retained, if not improved.

The primary factors involved in the biological efficacy of pesticide sprays are: (1) deposition (as a proportion of the amount applied within the target area less drift), (2) retention (the overall capture by plants of spray droplets either on initial or subsequent impact, and after loss due to run-off), and in the case of systemic pesticides (3) uptake into the plant and (4) translocation to the site of biological action. Bioefficacy can be adversely affected by poor efficiency in any one step of the deposition–plant retention–uptake–translocation process. In order to optimise the biological efficacy of sprays, each of these factors need to be maximised, without having a detrimental effect on the other factors. For example, deposition to the target area can be maximised by eliminating drift using very large droplets, but this can lead to very low levels of spray retention, particularly on moderate through to difficult-to-wet targets (crops or weeds). Similarly, a formulation may maximise spray retention, but may have a detrimental effect on uptake of a particular systemic pesticide. It must be remembered that all of these factors are inextricably linked to determine the overall biological efficacy of sprays. Traditionally, expensive empirical field trials have been performed to determine the spray application parameters and/or formulations that provide the best overall biological efficacy, with no real underlying understanding of the role or extent to which the individual factors are affecting the result. It is now more common that laboratory screening methods are used to determine the retention, uptake and possibly translocation of agrichemical formulations, to make at least informed decisions on the best spray application parameters and/or formulations to test in field trials, reducing the need for large factorial experiments in the field. Nevertheless, there is a practical need to develop mathematical and computational models to help predict biological efficacy in terms of the complex interactions of spray deposition, retention (and coverage), uptake and translocation.

Models for the individual factors involved in spray formulation efficacy do exist, but the usefulness of these models vary widely. Models for spray deposition from aerial application (*2*) are well advanced and well validated (*3*, *4*), although their focus has been on spray drift. Equivalent models from ground application are less advanced, and the focus of ongoing studies (*5*, *6*). While empirical models for adhesion (a component of retention) (*7*, *8*) and retention

(*9*, *10*) aid our understanding, more universally useful process-driven models for spray adhesion (*11*, *12*) and retention are currently being advanced (*13–16*). Although a range of useful models are available to help understand and predict uptake and translocation (*17–31*) they tend to be either too specific (in terms of active ingredient, surfactant, species), too simplistic or too complex and much more work is required before models are available that are both easy to use and universal.

The key question is, if advanced process-driven models for these individual factors were available, how would they be integrated together to predict biological efficacy? The development of a universal spray formulation efficacy model (SFEM) is a challenging and resource intensive task that would require numerous experiments to cover, for example with herbicides, a diverse range of weed:herbicide formulation interactions. In addition, surfactant effects can be species specific. Individual surfactants may interact differently with herbicide formulations on different plant species (*32*). Add to that the diverse range of responses to environmental conditions (temperature, relative humidity, light intensity, water availability and nutrient levels (*33–40*), the variation in susceptibility to a particular herbicide among plant species (*41*), and within species variation due to plant growth stage (*42–45*), and the task appears daunting. These issues, the high diversity in intrinsic plant factors (e.g. dynamic surface and canopy characteristics) together with the availability of a range of formulations and application techniques demand complex experimental designs and high resource requirements. Despite the perceived complexities and known limitations, attempts are needed to generate an in-depth understanding of the relative importance of retention, uptake and translocation processes in order to efficiently direct resources towards a universal SFEM.

The development of process-driven models (driven by physical processes and their associated physiological parameters), have the advantage over empirical models (derived from experimental measurements) in that they allow for extrapolation into new circumstances, which must be the ultimate goal. However they require that the underlying mechanisms that affect the physical and physiological parameters are well understood. In the current work, we have chosen to develop empirical models, aiming to increase our understanding of the relative importance of retention, uptake and translocation on biological efficacy. There are no such models in the literature, except for a small study by Pollicello *et al.* (*46*) who developed a simple equation relating retention and uptake to bioefficacy but did not consider translocation.

The current study determined the bioefficacy of glyphosate, as it is effected by Silwet L-77® surfactant - a superspreader organosilicone adjuvant frequently used to increase the efficacy of glyphosate on a range of plant species (*47*), on three different plant species ranging in plant surface wettability and canopy characteristics.

The objectives of the current study were (1) to study the effect of adjuvant and glyphosate concentration on spray retention, uptake, translocation and bioefficacy, (2) to relate spray retention, uptake and translocation of glyphosate to its biological performance on different plant species, and determine their relative influence on glyphosate bioefficacy and (3) to generate experimental data that can subsequently

be used to (a) validate individual process-driven models such as retention and (b) aid in the development of an integrated spray formulation efficacy model (SFEM) for predicting the bioefficacy of glyphosate on a range of plant species.

Materials and Methods

Plant Material

Plant materials used were barley (*Hordeum vulgare* L.), broccoli (*Brassica oleracea* L. var. Italica) and field bean (*Vicia faba* L.). These three species were chosen as model plants as they provide a good range of plant surface wettability and canopy characteristics for the spray retention, uptake, translocation and bioefficacy studies. Barley and field beans were raised from seeds in individual pots for a period of four weeks. Two week old broccoli seedlings, purchased from a local garden centre, were transplanted in individual pots and raised for a further 2-3 weeks. The three plant species were raised in individual pots, in Bloom seed raising mix (Yates NZ Ltd.), in a controlled environment facility at 20°C/15°C day/night temperature, 70% RH, in a 12h photoperiod with a photon flux density of approximately 500 μmol m^{-2} s^{-1}.

Chemicals

For the retention and bioefficacy experiments, glyphosate (relative molecular mass = 169.1, Log P = -2.69) concentrate carrying 62% w/w glyphosate (technical grade, unformulated glyphosate-IPA salt, Nufarm, Australia) was applied in the absence or presence of Silwet L-77® (an organosilicone surfactant supplied by Momentive Performance Materials, Tarrytown, NY, USA) providing a range of dynamic surface tensions (23-73 mN m^{-1}) in spray solution. In Experiment 1, 12 different treatments were applied to all three species: glyphosate at 6.2, 62 and 620 g ae ha^{-1}, in the absence or presence of Silwet L-77 at 3 different concentrations (0.01, 0.025 and 0.1% w/v). In Experiment 2, eight different treatments were applied to broccoli and bean: glyphosate at 6.2, 62, 310 and 1,240 g ae ha^{-1}, each in the presence of 0.2% (w/v) L-77, and 1,240 g ae ha^{-1} glyphosate in the absence or presence of L-77 (0.01, 0.025 and 0.1% w/v), while 10 different treatments were applied to barley: glyphosate at 6.2, 62, 310 and 620 g ae ha^{-1}, each in the presence of 0.2% (w/v) L-77, 310 g ae ha^{-1} glyphosate in the presence of L-77 (0.01, 0.025 and 0.1% w/v), and 62, 310 and 620 g ae ha^{-1} glyphosate applied alone. Tartrazine dye (9 g L^{-1}) was incorporated as a tracer in all spray treatments used for retention and bioefficacy studies.

For the uptake and translocation assessments, an appropriate amount (equivalent of ca. 6.67 kBq; ca. 0.1% of total glyphosate mass in treatment solution) of radiolabelled glyphosate (phosphono-methyl-^{14}C; ARC, Inc.; specific activity 1.924 GBq $mmol^{-1}$; 99% purity) was incorporated into treatment solutions just prior to use. Equimolar concentrations of glyphosate acid (Monsanto, NZ) and isopropylamine (Sigma®, Sigma-Aldrich, Germany) were mixed together to form water-soluble glyphosate IPA salt (equivalent to that used in the retention

experiments, and made up at the same concentrations), and appropriate quantities of the surfactant (to give the same concentrations as for the retention experiments) were added before incorporating into vials containing radiolabelled glyphosate.

The same treatment solution concentrations (glyphosate and L-77) were used for all studies, ie. retention, uptake, translocation and bioefficacy.

Retention, Uptake, and Translocation Experiments

For retention experiments, all species (15 replicate plants per treatment) were sprayed with treatment solutions at a nominal application rate of 200 L ha^{-1} through a hollow cone nozzle (TXVS-6 (Spraying Systems Co., Wheaton, USA); 220 kPa) mounted 0.5 m above 2/3 mean plant height of each species using a calibrated moving head track sprayer. Artificial targets (stainless steel plates x 6), mounted horizontally under the sprayer, were sprayed in all treatments to accurately confirm the spray volume delivered. After spraying, the plants were harvested at soil level and both plants and targets were washed immediately in a known volume of de-ionised water to recover retained dye. Absorbance of each solution (from plant or stainless steel plate) was measured using a Shimadzu 1240 UV/VIS spectrophotometer (tartrazine λ=427 nm) to determine total dye recovery. Individual plant surface areas were measured (using a LI-COR LI-3100 area meter; Li-cor Inc., Lincoln, Nebraska, USA) and retention was expressed as µl dye solution per cm^2. This volume of spray contained a known amount of glyphosate. Percent plant retention was worked out from the amount of dye recovered per plant area devided by the known amount applied per unit area.

For uptake and translocation experiments (five replicate plants per treatment), droplets (17 x 0.24 µl, ca. 770 µm diameter) were applied by a microsyringe to the central region on the adaxial (upper) surface of the youngest, fully-expanded leaf on individual plants within four hours of the start of the photoperiod. The droplet density and applied volume simulated a spray application rate of 200 L ha^{-1} (2 µl cm^{-2}). The quantity of radiolabelled glyphosate applied to leaves in each treatment (5 plant replicates) was determined by dispensing 17 droplets directly into scintillation vials (5 replicates). Barley and broccoli are extremely difficult-to-wet plant targets. Hence, glyphosate treatments not containing L-77 were made in aqueous acetone (50% v/v, surface tension 30 mN m^{-1}) so that droplets could be deposited on the leaf surface in uptake experiments. Similarly, the treatment consisting of 0.01% L-77 applied to barley was also made in aqueous acetone. The use of 50% acetone:water is considered to have no significant effect on the uptake of the active ingredient (*48*). After application, plants were returned to the controlled environment chambers (conditions as described above under "Plant material") and harvested at 24 hours after treatment. The treated leaf was excised and washed with 2 x 4 ml 50% aqueous ethanol to recover unabsorbed glyphosate. Recoveries of radiolabelled glyphosate applied to plant surfaces, after droplet dry down, are greater than 96% using this method (*49*). Scintillant solution (13 ml ACS II; Amersham) was added to washings and radioactivity was quantified by liquid scintillation counting (Packard Tricarb 2100 TR). Foliar uptake was defined as the radioactivity not recovered from washing the treated leaves and was calculated as a percentage of the applied dose. Glyphosate mass

uptake and translocation per unit plant leaf area (μg cm^{-2}) was calculated on the basis of total glyphosate mass retention per unit plant leaf area (μg cm^{-2}), i.e. % uptake from the uptake (or translocation) experiments multiplied by μg cm^{-2} retained = μg cm^{-2} taken up (or translocated).

The washed and excised treated leaf was stored frozen in sealed plastic bags until processed in a Biological Oxidiser (Harvey OX500). Radiolabelled glyphosate within plant tissues was determined by combusting/oxidising the leaf and absorbing the $^{14}CO_2$ generated in scintillant solution (Carbon-14 scintillant; RJ Harvey Corporation), which was then quantified by liquid scintillation counting. Combusting treated leaves (after surface washing) determined the amount of glyphosate which was absorbed into, but not translocated out of the treated leaf. By combining the radioactivity recovered from washings and treated leaf combustions, the amount of translocation out of the treated leaf was determined by difference. These calculations are possible because all glyphosate applied can be recovered when the tissues of whole plants are analysed ((*50*); uptake studied over 72 hours into *Abutilon theophrasti*).

Bioefficacy Assessments

One set of 15 plants per treatment was sprayed for bioefficacy assessments, as per the retention experiments. After spraying, plants used for bioefficacy experiments were returned to the controlled environment facility for 48 hours to allow the uptake process to occur under controlled conditions. The plants were then taken either to a glasshouse, to protect plants from frost damage and keep them in relatively warm conditions during winter months, or to an open nursery during warmer parts of the year. Bioefficacy was assessed on a 0-10 scale (0 being a healthy plant and 10 being a dead plant) by recording visible herbicide-induced damage on plants at approximately weekly intervals for up to 7 weeks. The score taken 2 weeks after spraying showed treatment effects most clearly and this score (converted to percent) was therefore chosen for analysis. The other variable used to assess bioefficacy was plant dry weight. Individual plants were excised at ground level after final scoring and placed in paper bags in a 65°C oven for 48-72 hours prior to final dry weight determinations. In addition to the sprayed plants, 15 untreated control plants per species were assessed using the same procedures in each experiment.

Statistical Analysis

All of the glyphosate x L-77 treatment combinations (4 x 5 factorial design) were tested for each species in the retention, uptake and translocation trials, with an additional control treatment in the bioefficacy trial. Because of the logistical limitations of handling all treatments in a single trial, the treatment combinations were split between two experiments conducted at different times for each species, using a 4 x 3 factorial design in the first experiment and a 5 x 4 factorial design in the second experiment. The two trials were essentially disconnected designs, apart from having a common control treatment for the bioefficacy assessments, and having two treatment combinations from the first trial repeated in the second

experiment for barley only. Means of retention, uptake and translocation (μg cm^{-2}) and of the bioefficacy score and final dry weight were calculated for each treatment combination. These means were then plotted against L-77 and glyphosate concentration to determine the forms of the responses. Based on these plots, appropriate nonlinear regression models were then fitted to the treatment means using the SAS (Version 9.1) NLIN procedure. Separate models were fitted for each plant species. The statistical significance of the effect of glyphosate and L-77 concentration was determined by testing relevant model parameters at the 5% level of significance. To account for differences between experiments (e.g., due to differences in plant size), the models included separate intercept parameters for each experiment fitted using dummy variables.

Models To Explain the Effect of Glyphosate and L-77 Concentration on Retention, Uptake, and Translocation

The following regression model was used for modelling retention, uptake, and translocation (Y; μg cm_{-2}) as a function of glyphosate concentration (G; %):

$$\ln(Y) = a_i + b \times \ln(G) \qquad \text{(Model 1)}$$

The model contains separate intercept parameters a_1 and a_2 for each experiment fitted using dummy variables. These allow for differences in leaf size or shape between experiments. The b parameter indicates the form of the relationship. For example, if b does not differ significantly from zero, it can be concluded that there is no significant relationship with glyphosate concentration. If b is close to one, it can be concluded that Y increases proportionately with glyphosate concentration, while if b is greater than 1 it can be concluded that Y increases disproportionately with glyphosate concentration.

Next, the effect of L-77 concentration was incorporated into Model 1 as a multiplier (M) of the following form:

$$M = 1 + c \times L + d \times L^2 \qquad \text{(Model 2)}$$

where, L is L-77 concentration (%).

The coefficients in Model 2 indicate whether the L-77 enhances (if $c>0$) or diminishes (if $c<0$) the effective concentration of glyphosate. A non-zero value of d is indicative of a non-linear L-77 effect. The parameter d was only included if it was found to be statistically significant ($p<0.05$).

M was multiplied by G giving the following model:

$$\ln(Y) = a_i + b \times \ln(M \times G) \qquad \text{(Model 3)}$$

Modelling Bioefficacy

Both plant dry weight and bioefficacy score at two weeks were regarded as potential measures of bioefficacy. Dry weight W (g) generally declined exponentially with glyphosate concentration and the following model was therefore used to model this behaviour:

$$W = a_i \times \exp(b_i \times G) \qquad \text{(Model 4)}$$

Note that different a and b coefficients were fitted for each experiment to account for differences in plant size and morphology between experiments.

For bioefficacy score S, the following model was used:

$$S = a_i + b_i \times G^f \qquad \text{(Model 5)}$$

Models 4 and 5 were then modified to incorporate any influence of L-77 concentration by incorporating the multiplier (Model 2):

$$W = a_i \times \exp(b_i \times M \times G) \qquad \text{(Model 6)}$$

and,

$$S = a_i + b_i \times (M \times G)^f \qquad \text{(Model 7)}$$

The effect of retention, uptake and translocation X (μg cm^{-2}) on bioefficacy was then modelled using:

$$W = a_i \times \exp(b_i \times X) \qquad \text{(Model 8)}$$

and,

$$S = a_i + b_i \times X^d \qquad \text{(Model 9)}$$

Finally, to test the relative effects of retention and either uptake or translocation on bioefficacy, the following models were used:

$$W = a_i \times \exp(b_i \times (c \times R + (1-c) \times X) \qquad \text{(Model 10)}$$

and,

$$S = a_i + b_i \times (c \times R + (1-c) \times X)^d \qquad \text{(Model 11)}$$

where R is retention and X is uptake or translocation. In these models, if the effect on plant weight or bioefficacy score is solely determined by glyphosate retention, it would be expected that the parameter c should be close to one. On the other hand, if it is the amount of glyphosate taken into the leaf rather than the amount retained on the surface of the leaf that is important, the estimate of c should be close to zero.

Results and Discussion

A total of 62 treatments were studied in terms of the spray retention, uptake, translocation and bioefficacy of glyphosate. The interactions were complex, and different for each of the three species studied, making it difficult to gain an overall understanding from the large amounts of data without looking at it in a statistical manner. However, prior to looking at the results from models, the 12 treatments applied in experiment 1 to the difficult-to-wet species barley and the easy-to-wet species bean will be used to illustrate the data being modelled, and therefore aid understanding. Overall, trends for difficult-to-wet broccoli were similar to those discussed in this section for barley.

The retention, uptake and translocation of glyphosate, applied to barley and bean in the absence and presence of L-77, along with overall biological efficacy (in terms of both bioefficacy score and dry weight), for experiment 1, are given in Tables 1 and 2.

It can be seen that spray retention of glyphosate to both barley (Table 1) and bean (Table 2) increases approximately 10-fold for every 10-fold increase in glyphosate concentration. Spray retention to barley increases with increasing concentration of L-77, while L-77 concentration has little effect on retention to bean. It is interesting that although the dose of glyphosate retained by barley increases substantially with increasing L-77 concentration (average of about 28% retention in the absence of L-77, to an average of about 75% in the presence of 0.1% L-77), the amount of glyphosate taken up at 24 hours actually reduces. It is known that the uptake of glyphosate has generally plateaued by 24 hours (*51*), and yet the treatments having the highest L-77 concentration (0.1% L-77) are the most biologically efficacious (Table 1) on barley. The highest glyphosate concentration, applied in the presence of the highest L-77 concentration studied, provides the best overall bioefficacy towards barley, despite the fact that although about 86% of the glyphosate applied was retained, only about 18% of that applied was taken up, and only about 6% of that applied was translocated at 24 hours. This indicates that uptake had in fact not plateaued by 24 hours. The results imply that retention, uptake, and translocation viewed alone are insufficient to describe bioefficacy. This will be covered in the next section of this work. In contrast to barley, uptake into bean generally increased with increasing concentration of L-77, with about 34% of the highest concentration of glyphosate (in the presence of the highest L-77 concentration) applied taken up, and 17% translocated. These results illustrate not only the robustness of pesticides (a small proportion of applied reaching the site of biological activity can actually do the job), but also that there is huge room for improvement across the factors influencing biological efficacy, which if achieved should lead to a reduction in the amount of active ingredient required.

In Experiment 2 a higher concentration of glyphosate (1,240 g ae ha^{-1}, in the absence and presence of L-77), was applied to both bean and broccoli, as 620 g ae ha^{-1} glyphosate failed to kill those plants (Table 2, bean). In contrast, 620 g ae ha^{-1} glyphosate was efficacious against barley (Table 1), and therefore an intermediate concentration (310 g ae ha^{-1}) was considered. All three species were also sprayed with 6.2, 62, 310 and 1,240 g ae ha^{-1} glyphosate in the presence of 0.2% L-77, to investigate the effect of increased or decreased retention and uptake on bioefficacy.

Table 1. Retention, Uptake, and Translocation of Glyphosate (Experiment 1), Applied to Barley in the Absence and Presence of L-77, along with Overall Biological Efficacy

Treatments		*Retention*	*Uptake*	*Tranlocation*	*Bioefficacy Score*	*Dry weight*
[a]Gly g ae ha^{-1} [b](µg ae cm^{-2})	*L-77 (%)*	*µg cm^{-2} (%)*	*µg cm^{-2}*	*µg cm^{-2}*		*(g)*
6.2 (0.061)	0	0.02 (33)	0.004	0.001	0.8	4.0
62 (0.675)	0	0.18 (26)	0.059	0.021	1.1	4.1
620 (6.70)	0	1.70 (25)	1.085	0.409	7.2	0.02
6.2 (0.057)	0.01	0.02 (35)	0.004	0.001	1.0	3.88
62 (0.619)	0.01	0.14 (23)	0.056	0.019	0.8	4.1
620 (5.96)	0.01	1.48 (35)	1.184	0.498	8.3	0.24
6.2 (0.055)	0.025	0.03 (55)	0.002	0.001	1.1	4.06
62 (0.541)	0.025	0.29 (54)	0.030	0.011	2.1	3.40
620 (5.48)	0.025	2.98 (54)	1.129	0.493	9.5	0.10
6.2 (0.054)	0.1	0.04 (74)	0.002	0.000	1.4	3.94
62 (0.598)	0.1	0.39 (65)	0.034	0.007	3.5	2.91
620 (5.12)	0.1	4.39 (86)	0.897	0.295	9.8	0

Treatments		*Retention*	*Uptake*	*Tranlocation*	*Bioefficacy Score*	*Dry weight*
[a]Gly g ae ha^{-1} [b](µg ae cm^{-2})	*L-77 (%)*	*µg cm^{-2} (%)*	*µg cm^{-2}*	*µg cm^{-2}*		*(g)*
control					0.7	3.59

[a] Nominal g ae ha^{-1} glyphosate applied. [b] Actual µg ae cm^{-2} glyphosate applied

Table 2. Retention, Uptake, and Translocation of Glyphosate (Experiment 1), Applied to Bean in the Absence and Presence of L-77, along with Overall Biological Efficacy

Treatments		*Retention*	*Uptake*	*Tranlocation*	*Bioefficacy Score*	*Dry weight*
[a]Gly g ae ha^{-1} [b](µg ae cm^{-2})	*L-77 (%)*	*µg cm^{-2} (%)*	*µg cm^{-2}*	*µg cm^{-2}*		*(g)*
6.2 (0.073)	0	0.04 (55)	0.007	0.002	0.0	3.71
62 (0.878)	0	0.40 (46)	0.112	0.023	0.1	3.52
620 (9.16)	0	3.93 (43)	0.835	0.188	0.4	3.17
6.2 (0.060)	0.01	0.03 (50)	0.009	0.002	0	3.91
62 (0.634)	0.01	0.34 (54)	0.089	0.017	0.3	3.62
620 (6.72)	0.01	3.63 (54)	1.367	0.569	2.2	2.81
6.2 (0.059)	0.025	0.03 (51)	0.007	0.001	0.1	3.69
62 (0.602)	0.025	0.33 (55)	0.122	0.043	0.4	3.39
620 (5.69)	0.025	3.06 (54)	1.451	0.480	2.8	2.87
6.2 (0.048)	0.1	0.03 (63)	0.014	0.010	0.0	3.59
62 (0.519)	0.1	0.31 (60)	0.128	0.073	0.4	3.77
620 (5.12)	0.1	3.29 (64)	1.730	0.876	2.4	3.26

Treatments		*Retention*	*Uptake*	*Tranlocation*	*Bioefficacy Score*	*Dry weight*
[a]*Gly g ae ha^{-1}* [b]*(μg ae cm^{-2})*	*L-77 (%)*	*μg cm^{-2} (%)*	*μg cm^{-2}*	*μg cm^{-2}*		*(g)*
control					0.0	4.1

[a] Nominal g ae ha^{-1} glyphosate applied. [b] Actual μg ae cm^{-2} glyphosate applied

The Effect of Glyphosate and L-77 Concentration on Retention, Uptake, and Translocation

The effect of glyphosate and L-77 concentration on glyphosate retention, uptake and translocation (results of fitting Model 3) are given in Tables 3-5.

Retention (Table 3; μg cm^{-2} glyphosate, not spray volume) was predicted to be significantly higher in all cases at the same glyphosate and L-77 concentrations for Experiment 1, which used larger plants (data not presented), than Experiment 2 (cv. a_1 and a_2). The primary reason that a larger plant would retain more glyphosate on the same unit area of leaf compared to a smaller plant is expected to be due to the recapture of bouncing or shattering droplets. After initial impact, any droplets that shatter or bounce move away from the point of impact, and hence the larger the leaf surface area, the greater the proportion of droplets likely to be recaptured (*14*). A further reason would be that the younger (smaller) plants may have been more water repellent (*52*). The largest difference in plant size between the two experiments was for barley, which was over twice the size (area) in Experiment 1 compared to the plants used in Experiment 2 (corresponding to the biggest difference in parameter estimates for a_1 and a_2).

Glyphosate retention increased in proportion to its concentration for a given application volume (and L-77 concentration) for all species (*b* did not differ significantly from one ($p>0.05$)). It makes sense that for a given spray volume, simply doubling the a.i. concentration would double the a.i. concentration retained, as long as the a.i. doesn't alter parameters relevant to retention (e.g. dynamic surface tension) or change the spray pattern (droplet size, swath). This research was set up to allow glyphosate concentration to be studied by using unformulated glyphosate, to eliminate the effect of concurrently increasing co-formulant concentration. In reality, increasing the concentration of commercial glyphosate formulations containing surfactants will also increase the concentration of surfactants used in those formulations, which may lead to an increase or decrease in spray (volume) retention, depending on plant wettability.

L-77 had a significant influence on retention (c parameter, $p<0.05$), the effect being positive (i.e., retention increased with L-77 concentration) for barley and broccoli, but negative (presumably due to increased runoff) for bean. This is in agreement with a plethora of published literature stating that the addition of adjuvant is beneficial for retention to difficult-to-wet species, while it may be of no benefit, or even disadvantageous for retention to easy-to-wet species (eg. (*53–56*)).

The relative effect of L-77 reduced with its increasing concentration (i.e. the *d* parameter was significant ($p<0.05$) for all species).

As with retention, uptake (Table 4) was also predicted to be significantly higher in Experiment 1 for barley and bean but not for broccoli. This may in part be due to much less difference in retention to broccoli between the experiments compared to barley and bean. The same trend was seen for translocation (Table 5) as for uptake, but the differences between the experiments were not significant for any species.

In contrast to retention, uptake and especially translocation increased disproportionately with glyphosate concentration (ie. $b > 1$). This effect was apparent for all species (although not significant for uptake into broccoli), but especially for barley (Figure 1).

Table 3. Details of the Regression Model Describing *Retention* (μg cm^{-2}) from Glyphosate and L-77 Concentration. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2 for Model 3.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	1.14 (0.12)	2.06 (0.05)	1.00 (0.09)
a_2	0.01 (0.10)	1.46 (0.05)	0.80 (0.09)
b	0.994 (0.026)	1.012 (0.013)	0.996 (0.020)
c	24.8 (6.3)	-3.64 (0.94)	28.7 (5.5)
d	-83.9 (31.7)	13.8 (5.0)	-104 (27)
R^2	98.9	99.8	99.6

Table 4. Details of the Regression Model Describing *Uptake* (μg cm^{-2}) from Glyphosate and L-77 Concentration. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2 for Model 3. For All Species the Parameter *d* in Model 2 Was Not Significant and Was Not Included in the Final Fitted Model.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	0.80 (0.24)	0.91 (0.10)	-0.02 (0.24)
a_2	-0.70 (0.23)	0.56 (0.11)	0.23 (0.28)
b	1.252 (0.063)	1.073 (0.026)	1.040 (0.067)
c	-1.04 (1.01)	2.48 (1.02)	-2.09 (1.18)
R^2	96.3	99.3	95.7

Despite increasing retention on difficult-to-wet barley and broccoli, L-77 did not increase uptake or translocation (the c parameter was significant only for bean) at 24 hours. L-77 enhances spreading of individual droplets on leaf surfaces resulting in a reduction of glyphosate dose per unit leaf surface area. Cuticular uptake of glyphosate is dose dependent and a reduction in dose per unit area is known to reduce glyphosate uptake on a range of plant species (*51*). In contrast, although L-77 caused a slight reduction in retention for bean, it caused a significant increase in uptake and translocation. This was as expected for bean since L-77 is known to promote rapid stomatal infiltration due to low surface tensions of formulations containing the surfactant (*49*, *57*, *58*).

In contrast to retention, *d* was not significant for translocation or uptake for any species (i.e. the relative effect of L-77 concentration was linear).

Table 5. Details of the Regression Model Describing Translocation (μg cm^{-2}) from Glyphosate and L-77 Concentration. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2 for Model 3. For All Species the Parameter d in Model 2 Was Not Significant and Was Not Included in the Final Fitted Model.

Parameter	*Barley*	*Bean*	*Broccoli*
a^1	-0.41 (0.43)	-0.39 (0.20)	-0.95 (0.25)
a^2	-0.76 (0.34)	-0.69 (0.24)	-0.72 (0.30)
b	1.387 (0.111)	1.110 (0.053)	1.189 (0.075)
c	-0.56 (1.48)	12.5 (4.4)	0.57 (2.28)
R^2	93.7	97.3	96.4

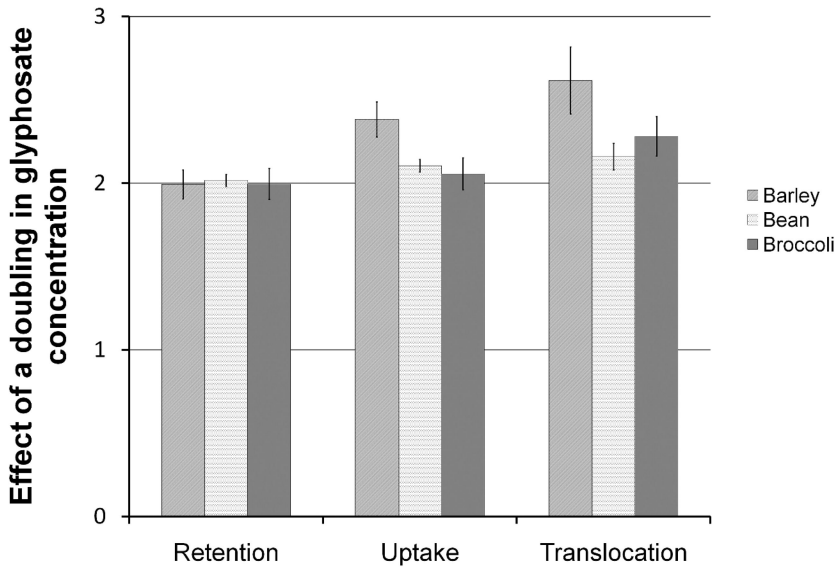

Figure 1. Multiplier effect of doubling the concentration of glyphosate on retention, uptake, and translocation. These effects were calculated using 2b where b is the parameter estimate from Model 3. Error bars show standard errors.

These results are summarised in Figure 2 which shows the estimated value of the multiplier *M* (Model 2) for L-77 at 0.1% concentration. L-77 can have very different effects on retention and uptake (Figure 2). It strongly increases retention for barley & brocolli (by about 2.6-2.8 times for L-77 at 0.1% concentration - Figure 2). Despite this, L-77 reduces uptake for these species. The opposite is true for bean where L-77 reduces retention but increases uptake. However, bean is also an extremely easy-to-wet target with an additional uptake pathway (stomatal infiltration) that responds well to L-77 for uptake, but incurs losses in retention. A balancing act may be required in the optimisation of retention and uptake or translocation to achieve an overall increase in bioefficacy when using L-77 (or other surfactants). Although an adjuvant may not enhance all of the primary factors involved in herbicide bioefficacy (ie. retention, uptake and translocation), enhancing a single factor may result in enhanced bioefficacy. Additionally, since retention and uptake may be enhanced by different types of adjuvants, the use of adjuvant mixtures may achieve optimal biological performance (*59*). All of this highlights a potential risk with focussing solely on a single primary factor (retention, uptake or translocation) involved in bioefficacy when attempting to find the best adjuvant for use with an active ingredient.

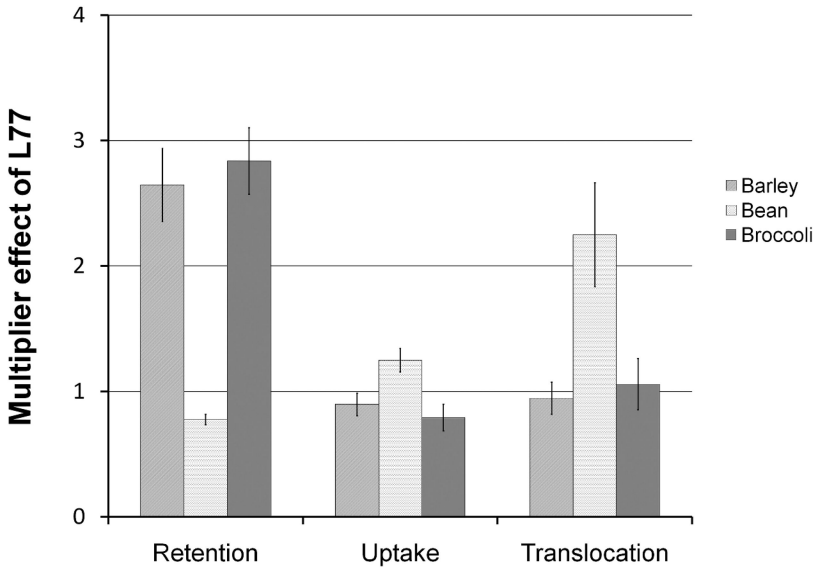

Figure 2. Multiplier effect of L-77 at 0.1% concentration on retention, uptake, and translocation of glyphosate. Error bars show standard errors.

Table 6. Details of the Regression Model Describing *Plant Dry Weight* (g) from Glyphosate and L-77 Concentration (%). Shown Are Parameter Estimates with Standard Errors in Parentheses for Regression and R^2 for Model 6, and t-test of the Significance of Parameter *c*.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	4.19 (0.15)	3.74 (0.11)	2.47 (0.10)
a_2	3.91 (0.21)	5.37 (0.19)	2.30 (0.16)
b_1	-4.39 (0.75)	-0.387 (0.117)	-1.78 (0.34)
b_2	-3.41 (0.49)	-0.512 (0.069)	-0.40 (0.11)
c	4.04 (2.06)	2.52 (1.39)	23.7 (9.3)
R^2	96.1	87.7	91.7
Test of significance of *c*	t_{19}=2.49, p=.022	t_{17}=2.22, p=.040	t_{17}=5.37, p<0.0001

Table 7. Details of the Regression Model Describing *Bioefficacy Score* from Glyphosate and L-77 Concentration (%). Shown Are Parameter Estimates with Standard Errors in Parentheses for Regression and R^2 for Model 7, and t-test of the Significance of Parameter c.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	7.1 (3.7)	-2.0 (2.9)	0.0 (5.6)
a_2	-0.4 (3.2)	1.3 (3.9)	1.6 (5.2)
b_1	122.3 (12.2)	24.9 (6.5)	62.4 (7.1)
b_2	14.1 (7.2)	19.9 (5.0)	32.4 (6.2)
c	4.6 (2.0)	2.9 (2.9)	6.7 (4.7)
f	0.778 (0.157)	0.664 (0.353)	0.352 (0.084)
R^2	95.6	75.6	89.6
Test of significance of c	t_{19}=3.20, p=0.0046	t_{17}=1.25, p=0.23	t_{17}=2.11, p=0.050

Models To Determine Bioefficacy

The Effect of Glyphosate and L-77 Concentration on Bioefficacy

For all three species, there was a highly significant association between both measures of bioefficacy (dry weight and bioefficacy score) and glyphosate concentration (parameter b_i, Tables 6 & 7). Plants became less healthy (dry weight

decreased: - b_i; bioefficacy score increased: + b_i) as glyphosate concentration increased. L-77 increased the bioefficacy of glyphosate for all species (the parameter c was statistically significant ($p<0.05$) for all species with respect to dry weight and for Barley & Broccoli in the case of bioefficacy score; Tables 4 & 5; Figure 3).

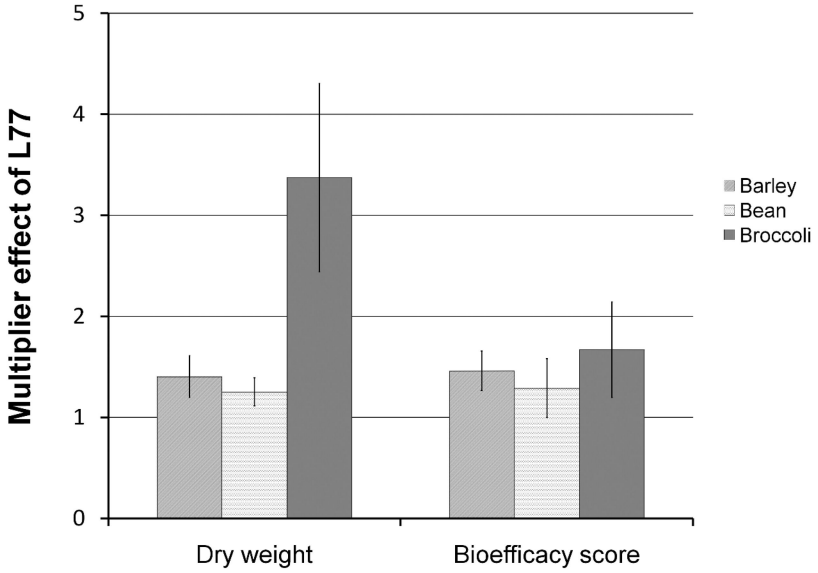

Figure 3. Multiplier effect of L-77 at 0.1% concentration on bioefficacy. Error bars show standard errors.

The Relative Influence of Uptake, Retention, and Translocation on Bioefficacy

Retention, uptake and translocation were all highly correlated (Table 8). Because of this, it was not straightforward to distinguish the relative influence of these three variables on bioefficacy. In general, all three variables increased with increasing glyphosate concentration, but because L-77 influenced retention differently to uptake or translocation (Figure 2), it was possible to compare the effects of retention with the other two variables (Model 10). However, uptake and translocation were very closely related with correlations greater than 0.98 for all species (Table 8). Essentially, a constant proportion of the glyphosate taken up (about 40-45% for all species) was translocated. This made it impossible to distinguish the relative effects on bioefficacy of uptake and translocation.

The percentage variance explained using models relating plant dry weight and bioefficacy score, as functions of glyphosate and L-77 concentration, and retention, uptake, and translocation are shown in Table 9. These show strong relationships with both measures of bioefficacy. For models including glyphosate and L-77 concentration, regressions for dry weight explained slightly higher

percentages of variance than those for bioefficacy score. However, bioefficacy score was slightly better related to retention, uptake, and translocation than dry weight.

Table 8. Mean Retention, Uptake and Translocation (μg cm^{-2}) and Spearman Correlation Coefficients between These Variables for Each Species. These Results Are for Treatment Combinations Where All Three Variables Were Obtained.

Variable	*Barley*	*Bean*	*Broccoli*
Mean retention (μg cm^{-2})	0.695	1.690	1.738
Mean uptake (μg cm^{-2})	0.176	0.795	0.594
Mean translocation (μg cm^{-2})	0.080	0.345	0.243
Correlation between retention & uptake	0.924	0.903	0.892
Correlation between retn. & translocation	0.899	0.879	0.932
Correlation between uptake & transloc.	0.983	0.983	0.997

It is interesting that using retention alone, or uptake alone, would give us reasonable trends in the predictions for the bioefficacy of glyphosate. Although it is well known that droplet size (dose) can affect uptake, and that the uptake of glyphosate increases with increasing droplet size (*60*), it is encouraging that laboratory-based screening methods to determine the relative performance in uptake among formulations, as used in the current study using 0.24 μl droplets, do relate well to the bioefficacy obtained by spraying plants (using a nozzle producing a much smaller droplet VMD). If the retention model was used, the predictions for the bioefficacy of glyphosate on bean would not relate as well to reality as the other species, while if the model for uptake was used, bioefficacy in terms of dry weight of broccoli would not relate as well to reality. The use of both retention and uptake in the bioefficacy model significantly improved predictions in these cases, and is overall the best model to use. This is in agreement with Policello *et al.* (*46*), who found that uptake alone was not an accurate indicator of glyphosate activity, and, that while there was no direct correlation between retention and glyphosate efficacy on barnyardgrass, the best model of spray efficacy included both variables. It is reasonable to expect that experimental results from retention and uptake studies (or predictions of each from models) considered together will give a good indication of the relative bioefficacy of different formulations for a given AI, across a wide range of systemic active ingredient (AI) and species, without the need for translocation results. However, this may not be the case with all herbicides (*21*). Consideration should also be made to leaf phytotoxicity/injury effects that may limit translocation and confound data (*61*). There is scope for improvement in these models by increasing the number of plant species with diverse physical, physiological and biochemical characteristics.

Table 9. Percentage Variance Explained by the Various Models Describing Bioefficacy in Terms of Glyphosate and L-77 Concentrations (Models 6 & 7), and Retention, Uptake and Translocation (Models 8-11). These Results Are for Treatment Combinations Where All Retention, Uptake, and Translocation Were Obtained.

Variable	*Models*	*Barley*		*Bean*		*Broccoli*	
		Dry weight	*Bioefficacy score*	*Dry weight*	*Bioefficacy score*	*Dry weight*	*Bioefficacy score*
Glyphosate & L-77 conc.	6 & 7	96.1	95.6	87.7	75.6	91.7	89.9
Retention	8 & 9	96.4	97.9	76.4	78.7	89.3	88.8
Uptake	8 & 9	91.8	97.4	85.1	87.6	77.6	87.5
Translocation	8 & 9	90.7	96.8	81.1	87.4	74.4	82.7
Retention & uptake	10 & 11	97.1	98.9	85.2	88.8	90.9	92.0
Retention & translocation	10 & 11	96.8	98.9	84.3	87.4	90.6	90.5

Table 10. Details of the Regression Model Describing Plant Dry Weight from Retention and Uptake Using Model 10. Shown Are Parameter Estimates With Standard Errors In Parentheses And R^2, and t-tests of the Significance of Parameter c. These Results Are for Treatment Combinations Where All Retention, Uptake, and Translocation Were Obtained.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	3.95 (0.17)	3.73 (0.12)	2.43 (0.10)
a_2	3.83 (0.17)	5.33 (0.21)	2.28 (0.15)
b_1	-1.53 (0.63)	-0.142 (0.05)	-0.900 (0.221)
b_2	-4.80 (1.46)	-0.278 (0.046)	-0.275 (0.076)
c	0.408 (0.190)	0.043 (0.144)	0.480 (0.203)
R^2	97.1	85.2	90.9
Test of hypothesis: c=1	t_{13}=1.69, p=0.12	t_{17}=3.18, p=0.0055	t_{15}=1.64, p=0.12
Test of hypothesis: c=0	t_{13}=4.85, p=0.00032	t_{17}=0.32, p=0.75	t_{15}=4.70, p=0.00028

The results of using retention and uptake (fitting Models 10 & 11) to predict bioefficacy are shown in Tables 10 & 11. For bean, bioefficacy is largely determined by uptake rather than retention. (i.e., c is not significantly different to zero). In contrast, for barley and broccoli, bioefficacy appears to be affected by both retention and uptake (c is significantly greater than zero for both dry weight and bioefficacy score). Actually, based on dry weight we cannot reject the hypothesis that dry weight is solely controlled by retention (i.e. that c=1), although this was not so for bioefficacy score.

Results of using retention and translocation (fitting Models 10 & 11) to predict bioefficacy are shown in Tables 12 & 13. Generally, results were similar to those for retention and uptake, although the percentage variance explained by these models was slightly lower for all species than for models using retention and uptake. Attempts to include all three variables (retention, uptake, and translocation) together in a combined model were not successful because of the high correlation between uptake and translocation.

Table 11. Details of the Regression Model Describing *Bioefficacy Score* from Retention and Uptake Using Model 11. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2, and t-tests of the Significance of Parameter *c*. These Results Are for Treatment Combinations Where All Retention, Uptake, and Translocation Were Obtained.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	7.0 (3.1)	-2.0 (2.0)	0.2 (5.1)
a_2	-2.6 (2.2)	2.1 (2.9)	3.1 (4.5)
b_1	61.2 (7.6)	18.7 (3.8)	51.0 (7.1)
b_2	21.4 (5.9)	15.1 (4.0)	24.7 (5.4)
c	0.335 (0.116)	-0.188 (0.082)	0.283 (0.155)
d	0.548 (0.073)	0.650 (0.238)	0.344 (0.079)
R^2	98.9	88.8	92.0
Test of hypothesis: c=1	t_{12}=3.22, p=0.0067	t_{16}=3.81, p=0.0014	t_{14}=2.38, p=0.031
Test of hypothesis: c=0	t_{12}=3.93, p=0.0020	t_{16}=1.32, p=0.20	t_{14}=2.80, p=0.014

Overall, L-77 modestly increases bioefficacy for all species (Figure 3). However, the L-77 effect on glyphosate bioefficacy on barley (1.2-1.5 times higher) is much less than its effect on retention (compare Figures 2 & 3). This demonstrates that bioefficacy is not related primarily to retention for this species. Equally, it is not related to uptake only, because L-77 has, if anything, a negative effect on uptake (Figure 2). A weighting of 40% retention and 60% uptake appears to best predict bioefficacy on barley (Tables 10 & 11, parameter *c*). The multiplier effect of L-77 on bioefficacy may be greater for broccoli although experimental error makes this result uncertain (Figure 3). Tables 10 & 11 suggest that bioefficacy is 30-50% weighted to retention and 50-70% weighted to uptake. It should be noted that the two methods for determining bioefficacy (dry weight and bioefficacy score) would lead to slightly different conclusions if considered alone. For bean, bioefficacy is moderately improved by L-77 and this must clearly be due to the positive effect of L-77 on uptake. The best estimate for bean is that bioefficacy is 100% related to uptake and not directly related to retention (Tables 10 & 11). That said, retention by bean ranged from 36% to 64% (data not shown), depending on treatment, and therefore there is a need to improve application and formulation technology in order to minimise loss to the ground. In addition, glyphosate was less effective against bean compared to barley and broccoli, and although the bean plants were significantly larger overall there was substantially more glyphosate taken up and translocated within the bean plants, indicating that not only size of the target plant, but also the natural toxicity of the a.i. towards the target plant needs to be considered.

Table 12. Details of the Regression Model Describing *Plant Dry Weight* from Retention and Translocation Using Model 10. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2, and t-tests of the Significance of Parameter *c*. These Results Are for Treatment Combinations Where All Retention, Uptake and Translocation Were Obtained.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	3.98 (0.18)	3.72 (0.13)	2.44 (0.10)
a_2	3.82 (0.18)	5.37 (0.22)	2.28 (0.15)
b_1	-1.73 (0.93)	-0.23 (0.10)	-1.70 (0.82)
b_2	-4.83 (1.94)	-0.47 (0.11)	-0.48 (0.22)
c	0.433 (0.242)	0.114 (0.087)	0.274 (0.186)
R^2	96.8	84.3	90.6
Test of hypothesis: c=1	t_{13}=1.18, p=0.25	t_{17}=2.94, p=0.017	t_{15}=1.42, p=0.21
Test of hypothesis: c=0	t_{13}=4.98, p=0.00025	t_{17}=1.87, p=0.078	t_{15}=5.07, p=0.00014

In all cases, we cannot distinguish between uptake and translocation; the above observations regarding the importance of uptake apply equally to translocation.

Overview Discussion

It is more labour intensive to measure spray retention (along with uptake and translocation), than to directly determine the bioefficacy of a spray formulation. However, determining the optimal spray application and formulation parameters requires large factorial, and therefore costly, bioefficacy trials. Hence mathematical and computational models would be beneficial to help predict biological efficacy in terms of the complex interactions of spray deposition, retention (and coverage), uptake and translocation.

It must be stressed that the empirical models for retention, uptake, translocation and bioefficacy described in this study are specific to the species, active ingredient and adjuvant used. They aid in increasing our understanding of the underlying mechanisms involved for the observed trends, but do not aid in predictions outside the region for which the model was developed. In addition, these studies produce data that can be used to evaluate or validate process-driven (driven by physical processes and their associated physiological parameters, rather than derived from experimental measurements) models, either for the individual factors (retention, uptake or translocation) or for overall bioefficacy.

Table 13. Details of the Regression Model Describing *Bioefficacy Score* from Retention and Translocation Using Model 11. Shown Are Parameter Estimates with Standard Errors in Parentheses and R^2, and t-tests of the Significance of Parameter *c*. These Results Are for Treatment Combinations Where All Retention, Uptake, and Translocation Were Obtained.

Parameter	*Barley*	*Bean*	*Broccoli*
a_1	7.2 (3.0)	-1.8 (2.2)	-0.4 (5.5)
a_2	-2.7 (2.1)	1.4 (3.2)	2.9 (4.9)
b_1	92.8 (12.6)	26.8 (5.3)	63.3 (12.1)
b_2	28.4 (7.4)	23.5 (4.7)	30.0 (7.3)
c	0.165 (0.067)	-0.001 (0.050)	0.186 (0.151)
d	0.556 (0.072)	0.671 (0.300)	0.332 (0.080)
R^2	98.9	87.4	90.5
Test of hypothesis: c=1	t_{12}=3.38, p=0.0007	t_{16}=3.27, p=0.0009	t_{14}=1.60, p=0.11
Test of hypothesis: c=0	t_{12}=4.85, p=0.0004	t_{16}=0.00, p=1.0	t_{14}=3.82, p=0.0045

Process-driven models may not have such limitations as empirical models provided the underlying mechanisms that affect the physical and physiological parameters are well understood. Good progress is being made in this area in terms of spray canopy retention (*14*, *15*), although further improvement is still required. The retention results produced in the current study were used in part to evaluate a spray canopy retention model, with many other plant species included (*16*). Although there was an almost certain correlation (Pearson's correlation coefficient=0.7615 and P-value =0.0000) across the entire dataset studied, between predicted and actual retention, for the plant species used within the current study there was only a possible correlation (while predicted versus actual retention was good for broccoli, retention to barley was significantly under-predicted for the lowest surface tension solution, 0.1% L-77, and significantly over-predicted for all solutions applied to bean). However subsequent inclusion of the effect of leaf angle on droplet shatter has improved (unpublished) the model, with it now giving an almost certain correlation between predicted and actual retention across the 3 species used within the current study (although retention to bean was still significantly over-predicted). Improvements are ongoing.

The use of a reliable process-driven spray canopy retention model would allow retention of any formulation to any species to be predicted, cutting out the need to spray plants or measure spray retention (a physical process on plants). However, once a herbicide penetrates the plant, its effectiveness is determined by complex interactions of physico-chemical and physiological processes within plants. Further details regarding these factors can be found within other chapters of this book.

The current work has successfully demonstrated the effectiveness of empirical models to provide an understanding of the relative influence of retention, uptake and translocation of a single herbicide on three different plant species. The current gaps in knowledge will reduce in time as more experimental data becomes available. In the absence of robust models for bioefficacy, using available process-driven spray retention models and measuring the uptake of an active ingredient on the species of interest, appear to be the most suitable options to indicate bioefficacy trends.

Conclusions

Uptake (and possibly translocation) of glyphosate is at least as important for biological efficacy as retention, and probably more so. A surfactant that enhances retention does not necessarily also enhance uptake and may in fact inhibit it, and vice versa. Therefore, although retention or uptake alone can explain a high percentage of the variance in bioefficacy, both retention and uptake should be studied in order to adequately understand and explain the bioefficacy of spray formulations across plant species. Translocation, at least of glyphosate, is highly positively correlated with uptake. Although that may not be the case with other active ingredients, it is reasonable to expect that experimental results from retention and uptake studies (or predictions of each from models) considered together will give a good indication of the relative bioefficacy of different formulations for a given AI, across a wide range of systemic active ingredient (AI) and species, minimising the need for translocation results.

However leaf phytotoxicity, which may limit translocation, should also be considered.

Acknowledgments

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Chapter 7

Differences in Herbicide Uptake, Translocation, and Distribution as Sources of Herbicide Resistance in Weeds

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The selection of one mechanism of herbicide resistance over another involves a delicate balance between resistance effectiveness, mutation frequency and fitness penalty. In most cases, this balance appears to be shifted towards mechanisms based on target site modification and herbicide detoxification because these mechanisms provide high levels of herbicide resistance while carrying small fitness penalties. This has led the scientific community to underestimate the importance of restricted herbicide movement as source of herbicide resistance when detected. However, when these mechanisms fail to confer an acceptable level of resistance, the alteration of herbicide movement throughout the plant arises as an alternative mechanism. This paper briefly reviews how herbicides move across the plant and how the physiology described addresses the restriction of herbicide movement as a source of herbicide resistance. Mechanisms are discussed in depth for paraquat and glyphosate, the only two herbicides for which the restriction of herbicide movement has been extensively studied, with scientific evidence related to herbicide-resistant weed biotypes supporting the different hypotheses. These few cases studied

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have shown us that, as with other resistance mechanisms, resistant biotypes share common features, such as mutated protein carriers, that affect herbicide transport through the tonoplast.

Introduction

The development of resistance to xenobiotics in nature has been constant during humankind's struggle against the biosphere. People have used all types of chemical compounds to kill harmful or unwanted organisms. Through this human-driven natural selection, these organisms developed skills to avoid being killed in a never-ending process called "resistance" that still occurs today.

While issues of resistance that affect human beings, such as antibiotic resistance in bacteria, are covered by the mass media, pesticide resistance in agriculture is a comparatively less well-known problem. Considering that there are three main families of pesticides used in agriculture (insecticides, fungicides and herbicides), herbicide resistance was not regarded to be a potential problem until the early 1970s (*1*). However, what does "herbicide resistance" mean? The Weed Science Society of America distinguishes between the terms tolerance and resistance depending on whether the resistance traits are due to genetic selection or manipulations. Therefore, while herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment (implying that neither selection pressure nor genetic manipulation occurred), herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide that is normally lethal to the wild-type plant. In an individual plant, resistance may be naturally occurring and selected via selection pressure or induced by scientific techniques, including genetic engineering or selection of variants via tissue culture or mutagenesis (*2*). The development of herbicide resistance in weeds has increasingly become a major problem in agriculture. The problem appeared to be bearable at first compared with other pesticide resistances, due to the specific biological and physiological characteristics of weeds (*3*). But this has become a worldwide issue today, affecting the most commonly used pesticides in agriculture. Far from the opinion of the 1980s, where herbicide resistance was considered "more a blessing than a curse" (*4*), there has been an exponential increase to the 429 herbicide-resistant weed biotypes belonging to 234 different species found today (*5*).

Herbicide resistance in weeds can be due to one or more of three different mechanisms. Herbicide target sites are generally physiological locations where the active ingredient in an herbicide binds and interferes with physiological processes (usually a metabolically relevant organic molecule or enzyme). Therefore, herbicide resistance can be conferred by genetic mutations in these target sites, which prevent the herbicide from inhibiting these processes (*6*). Likewise, resistant plants may metabolically detoxify the active ingredient via changes to or the overexpression of metabolic enzymes (*7*). Alternatively, the plant can physically prevent the herbicide active ingredient from reaching its target site (*8*). While the first mechanism of herbicide resistance is called

"target-site resistance" (TSR), the detoxification and restriction of herbicide movement mechanisms are grouped as "non-target-site resistance" (NTSR) (*9*). Neither NTSR nor TSR are cost-free issues. Herbicide resistance usually penalizes population fitness, where "fitness" in this case is defined as the ability of a genotype to produce viable offspring relative to all other genotypes in the population (*10*). This means that alleles carrying larger fitness penalties are less frequent than those carrying smaller ones, unless the selection pressure changes (*11*). In these terms, the prevalence of TSR over NTSR alleles, or vice versa, is not only a matter of herbicide resistance but also of fitness penalty.

Restriction of Herbicide Movement: The Ugly Duckling of Herbicide Resistance

TSR has been considered the major component of herbicide resistance in weeds, even though NTSR has been reported to be a major component of herbicide resistance in very important herbicide families, such as acetyl-CoA carboxylase (ACCase) and acetolactate synthase (ALS) inhibitors (*12*). Additionally, as we will discuss later, NTSR has been described as the most widespread type of resistance to glyphosate, the most sold herbicide worldwide (*9*). In addition, NTSR may be unpredictable (a very unwanted characteristic in agriculture) because the physiological mechanisms involved in the resistance response can confer resistance to herbicides with various modes of action, including herbicides that have never been in contact with the mutant biotype or that have not yet been marketed (*13*). Why, then, is TSR considered the most important resistance mechanism? First, alterations in the herbicide target protein usually confer a high degree of resistance to one herbicide (or multiple herbicides belonging to the same chemical family) – this leads to promotion of such traits following high dose applications of herbicides (*14*). TSR can also confer resistance that is orders of magnitude higher than that of the wild type. Additionally, TSR is important because it is easy to study and has been published about extensively. Once the herbicide target site is known and its gene is sequenced, detecting nucleotide changes is easy and provides direct evidence of physiological, biochemical, and genetic differences between herbicide-resistant and herbicide–susceptible biotypes (*8*).

On the other hand, herbicide selective pressure exerted on NTSR plants tends to cause the accumulation of NTSR alleles (especially in outcrossing species), which results in a pool of genes conferring increasingly high resistance to an increasingly broad range of herbicides over time (*12*). From the agricultural viewpoint, this is a serious issue. How many NTSR alleles can be pooled in a single biotype? Is this pool of alleles as efficient as a single TSR allele, in terms of herbicide resistance? The answer to this question is apparently not, as there appears to be a direct relationship between the number of segregating NTSR alleles and fitness penalty (*12*).

However, there are social classes even within NTSR. Herbicide resistance due to metabolic alteration of the active ingredient (herbicide detoxification) has most likely been studied to the same extent as target site resistance and has been

reviewed in detail elsewhere (*8*, *15*, *16*). Herbicide detoxification accounts for most of the cases of ACCase and ALS resistance, including tolerance to these herbicides in some major crops. This likely explains why most research has focused on herbicide detoxification within NTSR (*17*). In addition, though not comparable to TSR in terms of the resistance ratio (a ratio based on the GR50 values of the resistant biotype compared with the susceptible biotype), herbicide detoxification can confer a fair degree of resistance to the resistant biotypes.

So, where does our ugly duckling fit into the story of herbicide-resistant weed biotypes? The literature is clear: studies of the restriction of herbicide movement were usually carried out only to ensure that herbicide uptake/translocation was not affecting the "real" mechanisms of resistance. In most cases, studies of herbicide penetration and translocation just monitor radioactivity in plants as radiolabeled herbicides penetrate and move across the tissues (*17*). Therefore, not much is known about the nature of the translocated compounds. Differences in herbicide absorption and/or translocation have been described as minor components of the resistant behavior, unless they were the only components of the resistance. This means that, in contrast with TSR or herbicide detoxification, there is not a wealth of data regarding WHY some mutants restrict herbicide movement when developing herbicide resistance.

Step-by-Step: The Long and Winding Road of Herbicides from Uptake to Target Site

Herbicides can be taken up both by foliar and root absorption, depending on the application method, and then can exert their phytotoxic effect near the point of entry or can be translocated throughout the plant. Foliar uptake is typical for foliar herbicide (post-emergence) applications, whereas root absorption is mostly observed in soil-applied herbicides. A mixture of these mechanisms is also observed in multiple herbicides.

Foliar and Root Herbicide Absorption

The achievement of foliar herbicide absorption is not an easy task. This is because, in contrast to root absorption, foliar uptake involves the absorption of the herbicide by plant organs not specifically designed to do so. Once deposited on the leaf surface, the active ingredients should migrate across multiple barriers, such as the epicuticular waxes and leaf cuticle at the leaf surface, until they reach the apoplast and finally penetrate the plant cells (*17*). Alternatively, the herbicide should penetrate through the stomata until reaching the mesophyll cells (*18*). These events are ruled by external parameters, such as the chemical and physical properties of the herbicide or the external environment, as well as by internal parameters such as the physiological nature of the leaf surface. While external parameters are not a source of (heritable) herbicide resistance, internal parameters are. The leaf cuticle is a thin biological layer covering aerial parts of most plants, and its function is to prevent uncontrolled water loss from plant transpiration (*19*). Plant cuticles are complex structures, composed of an insoluble cutin

framework and soluble waxes. Some cuticles contain suberin, a shikimate-derived combination of cutin-like aliphatic polymers and aromatic moieties. Interestingly, while cutin and suberin are partly hydrophilic, the epicuticular waxes that form the outer surface of the cuticle are hydrophobic. This means that the plant cuticle does not act as a homogeneous layer in terms of hydrophobicity because it becomes more "water-friendly" deeper into the layer (*17*). Despite this lipophilic gradient, cuticles are considered to be lipophilic membranes (*20*), so the resistance of the cuticle against the penetration of polar solutes is high. Thus, non-polar herbicide penetration is usually described following a solution-diffusion model (*21*). Therefore, the ability of an herbicide to penetrate plant cuticles is proportional to the solubility and mobility of the herbicide through the cuticle (*22*). This means that changes in cuticle composition may change herbicide susceptibility. To add more fun, together with this lipophilic cuticular penetration pathway, a hypothetical hydrophilic pathway has also been proposed.

Because the penetration rates of some hydrophilic solutes are too high to be explained by the solution-diffusion model, some authors suggest that hydrophilic solutes penetrate cuticles via a pathway that is physically distinct from the lipophilic route. This additional pathway, generally called "aqueous pores" (*23*), is supposed to be generated by the adsorption of water molecules by polar moieties in the cuticular membrane and pectic cell wall (*24*). The aqueous pores pathway may involve lower size-selectivity compared with the lipophilic path. Aqueous pores should provide an additional penetration pathway exclusively available for water soluble, polar herbicides, whereas lipophilic herbicides should exclusively penetrate via the lipophilic pathway, i.e., by solution and diffusion into the cuticular matrix (*23*). Of course, the existence of these hydrophilic pores in the cuticle is a matter of controversy, as they have never been observed.

But, what happens with the pores that do connect the inner and outer parts of the leaf? Stomata have their own pathway of leaf penetration (the "stomatal penetration pathway"). Opened stomata provide herbicides a fast way to reach leaf mesophyll ceIls, provided the spraying solution surface tension is below 30 mN m-2 of surface energy (*17*). Stomata cuticles located in guard cells may be penetrated as well. In this case, the promoting effect of the stomata on solute penetration is attributed to a higher permeability of the cuticle covering the guard cells (*25*). Surprisingly, only approximately 10% of all stomata in contact with the spraying solution are penetrable. The active stomata differ from the inactive ones in terms of the wettability of the cuticle surrounding the guard cells (*18*). This wettability depends on the presence or absence of, as well as the nature of, the hygroscopic substances present at the surface of the stomatal pores (*26*).

It is often surprising to non-plant physiologists that roots are quite impermeable to water. In fact, roots account for two watertight barriers for the avoidance of water and solutes running down and out the plant once absorbed. One is an external suberized layer (the exodermis) that covers all but the active growing meristematic tissues. The other is the Casparian strip, another suberized layer located at the root endodermis. Herbicide absorption in roots mostly occurs in the root tips and root hairs (*17*), the same locations where solute absorption occurs, where both the outer exodermis and the inner Casparian strip are not fully developed. However, the role of the later layer is not clear, as it appears to be a

watertight but not "solute-tight" barrier (*17*). Herbicide uptake into the roots is a result of the combination of a dual-step process: a rapid (apoplastic?) initial entry, mostly due to non-metabolic processes such as bulk water flow and herbicide diffusion along concentration gradients, is followed by a slower phase of entry and accumulation associated with metabolic activity.

Whether water transpiration (together with water mass flow) is related to herbicide absorption is another controversial matter, with several studies either supporting (*27*) or opposing (*28*) the hypothesis.

Herbicide Translocation

While herbicide absorption can be considered short-range transport, herbicide translocation (i.e., the movement of the herbicide from the point of entry to a more or less distant target site) is considered long-range delivery that requires the two transport systems to be present in plants: the phloem and xylem vascular systems. Translocation is a desirable attribute because it allows the herbicide to reach both treated and untreated parts of the plant. Herbicides with this attribute are called "systemic herbicides."

The movement of water, sugars and other compounds such as amino acids and inorganic ions through the phloem is due to the active loading of sugars into sieve elements of the phloem by companion cells (specialized parenchyma cells that act as storage tissues for organic solutes prior to entry into the phloem proper). The high concentration of sugars in the phloem causes the water potential in the tissue to drop, so water enters from the xylem and creates a pressure gradient that pushes the water from the source leaves to the sink organs in a two-osmometers-connected model known as the Münch pressure-flow hypothesis (*29*). Solutes enter the sieve elements via both the apoplast and symplast. The symplastic system implies the passage of solutes from one cell cytoplasm to another through cell channels (the plasmodesmata). Once sugars reach the companion cells, they are accumulated in the form of oligosaccharides, which cannot diffuse back through the plasmodesmata because of their size, and are loaded into the sieve elements (*30*). The apoplastic system is constituted by the continuum of cell walls of adjacent cells, as well as by the extracellular spaces out of the plasmalemma. Thus, the apoplastic system involves the active pumping of sugars first out the mesophyll photosynthetic cells and then into the companion cells through a H+-sucrose co-transport mechanism that uses highly specific channels and is driven by the proton motive force, generated by an H^+-ATPase pump (*30*). Plants can use the apoplastic system, the symplastic system or both, depending on the species. Herbicides are believed to travel passively through the phloem system, following the solute flow direction along with many other solutes (*17*). However, how herbicides manage to enter the phloem, whether they follow the apoplastic or symplastic way, and whether there are specific sucrose-like channels to pump herbicides in and out of cell plasmalemmas are questions that remain to be addressed.

On the other hand, the xylem is an "all apoplast" system, with no functional cytoplasm. Therefore, the xylem is considered to be an open vascular tissue able to transport water, inorganic ions, amino acids, and other solutes via a high-to-low

water potential gradient. In general, xylem-transported herbicides reflect the water flux through the plant, so active ingredients accumulate in active leaves, mostly at the leaf tip and margins, coinciding with the hydathode positions that lie at the end of this vascular system. Although herbicide transport through the xylem may appear to be a simple, unspecific, all-up system, it is worth noting that xylem solutes following mass flux must leave the apoplast to overcome the Casparian strip. Therefore, these solutes must be transported through two cell membranes before reaching the xylem elements, maybe implying the existence of some type of transport selectivity.

Regardless of the way herbicides move into and along the plant, the active ingredients must eventually reach their target sites, usually located within the cytoplasm. Herbicide cell absorption involves crossing the cell wall and plasmalemma. From the perspective of herbicide transport, cell walls are very hydrophilic and relatively porous barriers that offer little resistance to the passage of herbicides into cells, but there are cases where the active ingredients end up bound to cell wall constituents, such as dichlobenil, paraquat or trifluralin (*reviewed in* (*17*)). The most accepted model reflecting the complexity of the cell membrane is the fluid mosaic model from Singer, which describes the membrane as a phospholipid bi-layer with proteins located on both surfaces as well as spanning the entire membrane (*31*). Homeostasis within the cell is maintained by cell membranes, so much of the internal regulation can be attributed to both the permeability of the phospholipidic layers and these membrane-bound proteins acting as channels, carriers, and pumps. In these terms, herbicides can move through the plasmalemma either by simple diffusion due to a concentration gradient, or by a more specific transport mechanism, using the suitable membrane channels and carriers. Regarding the putative existence of carrier-mediated, energy-requiring herbicide transport through the cell membrane, facts such as the positive effect of metabolic inhibitors on transport inhibition or the existence of saturable uptake kinetics in some herbicides support this hypothesis (see the paraquat and glyphosate sections). However, herbicide diffusion appears to be the most important mechanism of transport, at least in lipophilic fast-penetrant herbicides (*32*).

It's the Mechanism, Stupid! Paraquat and Glyphosate: The Two Herbicides with Studied Mechanisms of Resistance Based on Restriction of Herbicide Movement

Resistance based on the restriction of herbicide movement has been only deeply studied in two products: paraquat and glyphosate. This is possibly because most biotypes displaying this mechanism showed higher levels of herbicide resistance than any other biotype with a different mechanism of resistance. As successful resistance to paraquat and glyphosate could only be explained in terms of impaired herbicide movement, the mechanism deserved a second look.

Herbicide Resistance to Paraquat: Several Options but One Mechanism of Resistance

The herbicide paraquat is a strong electron acceptor, capable of auto-oxidation, in the photosystem I (PSI) that catalyzes the transfer of electrons from the PSI of chloroplast membranes to molecular O_2 radicals, producing superoxide and depleting the NADPH available for CO_2 fixation. These toxic oxygen species cause lipid peroxidation and chloroplast membrane damage (*33*). Although banned in Europe due to environmental concerns, paraquat is still widely used worldwide for total weed control in agriculture and industry. Resistance to paraquat has been evolving over decades, which is a slow pace compared with the resistance evolution observed in other herbicide families: only 55 biotypes belonging to 30 weed species show resistance to paraquat today (*5*). This may be explained by the total absence of metabolic detoxification in plants, as well as by the redox reaction that occurs between paraquat and its target site (*34*). Two mechanisms of resistance have been proposed for paraquat: the enhanced detoxification of oxygen radicals and the sequestration models (*reviewed in* (*35*)). However, the sequestration model attracts a greater consensus, as there are data that directly contradict the first hypothesis that are difficult to reconcile.

The original sequestration hypothesis postulates that paraquat is somehow compartmentalized somewhere in the resistant biotypes, preventing the herbicide from interacting with its active target site in the chloroplast (*35*). New data show that this putative mechanism is not so evident. The first question to address is whether paraquat is sequestered prior to reaching its target site at the thylakoid, therefore preventing the herbicide from binding the PSI, or if this process starts either after or in parallel to the penetration of paraquat into the chloroplast. Early chlorophyll fluorescence studies carried out in resistant *Conyza* spp. indicated that paraquat was not present at photosystem I, as the resistant biotype required a nearly 100-fold greater concentration of paraquat than the susceptible biotype to exhibit similar fluorescence quenching four hours after herbicide application (*36*). However, subsequent fluorescence studies, using shorter incubation times in paraquat-resistant biotypes of *Erigeron canadensis,* demonstrated that photosynthetic electron transport was affected in both the resistant and susceptible biotypes, the former recovering photosynthetic activity within a few hours, confirming the hypothesis that paraquat can reach the site of action in the chloroplast immediately in both the resistant and susceptible biotypes (*33*). In addition, these studies showed that light played a basic role not only in the effective initial uptake of paraquat by the chloroplast but also in the mechanism of resistance to this herbicide, as resistant plants only recovered the photosynthetic function in the light, and an increase in light intensity had a pronounced enhancing effect on the recovery of the photosynthetic activity (*33*, *37*, *38*). In fact, light appears to play a major role in another problem: the relationship between paraquat resistance and translocation. Effectively, different resistant biotypes displaying strong evidence of paraquat sequestration showed either reduced paraquat translocation rates in excised leaves (*39*) and whole plants (*40*, *41*) or no differences at all (*42*, *43*). As a leaf-applied, xylem-translocated

herbicide, paraquat translocation is supposed to be low, so differences in paraquat translocation could be a source of herbicide resistance. However, the presence or absence of reduced paraquat translocation in herbicide-resistant biotypes appears to be more a consequence of damage in the vascular tissues (phloem and/or xylem), rather than a primary mechanism (*44*). Light could modulate the disrupting effect of paraquat in cell membranes; this effect would be more intense in photosynthetically active tissues (shoots) than in non-photosynthetically active ones (roots), as well as under light conditions than in darkness.

If paraquat reaches both resistant and susceptible cells and effectively blocks photosynthesis, it must be pumped out the chloroplast somehow to be expelled out of or sequestered within the plant cell. In fact, paraquat sequestration from its active site via an alteration in membrane transport has been previously described in paraquat-resistant *Escherichia coli* mutants (*45*). But, where does paraquat accumulate in resistant plants and why is it more efficiently accumulated in these plants? A first candidate acting as a paraquat dump would be the cell wall, with its numerous negatively charged binding sites suitable for use by a divalent cation like paraquat. Things become controversial here because some authors claim that most of the paraquat bound to both resistant and susceptible cell walls is desorbed into the external solution in a short period of time (*46*) or that there is no difference in the binding of paraquat to the cell walls of resistant and susceptible biotypes (*36*). However, other authors postulate that difenzoquat (an herbicide closely related to paraquat) resistance in difenzoquat-resistant *Avena fatua* is due to the binding of a significant amount of this herbicide to the cell wall material in resistant cells, with this fraction exchanging very slowly with the external medium (*47*). However, how difenzoquat could penetrate the cell wall on the way in but become bound to the negative charges of the wall on the way out remains unclear.

The other candidate is a cytoplasmic compartment, most likely the vacuole. In this case, some type of mechanism of paraquat transport through the cell membranes, independent of herbicide concentration gradient, is suggested. This hypothesis is based on variable fluorescence kinetics, which show almost no concentration dependence at paraquat concentrations ranging from the normal agricultural concentration up to concentrations 10 times greater. Only a concentration-dependent and irreversible breakdown is observed at the highest concentrations (*33*). If paraquat transport through membranes is mediated via a membrane protein carrier, then an alteration of one or several of these proteins could confer resistance by inhibiting the net movement of paraquat, ultimately sequestering it from the thylakoids. This is logical, as the existence of a mutated membrane protein as a putative cause of paraquat resistance in *E. coli* has demonstrated (*48*).

Therefore, three different cell membranes may interact with paraquat: the plasmalemma, the tonoplast and chloroplastic membranes. As the outer barrier, the plasmalemma in paraquat-resistant plants may either impede the access of paraquat to the cytoplasm or enhance its expulsion out the cytoplasm. In these processes, a protein carrier is suspected, due to the saturable nature of paraquat transport across the membrane (*49*, *50*). The first hypothesis is not consistently supported by the facts: several fluorescence studies reveal that paraquat does reach its target site at a concentration high enough to inhibit photosynthetic transport in both resistant and

susceptible plants, with the resistant response being detected after PSI blockage. Therefore, if the plasmalemma is related to paraquat resistance, it should be due to a differential ability to pump the herbicide out of the cytoplasm, as paraquat efflux out of the cytoplasm has been already detected (*50*, *51*). Unfortunately, the data do not support this hypothesis, as the total amount of paraquat present in both cells and whole plants does not seem to differ between paraquat-resistant and –susceptible biotypes (*41*, *44*, *52*, *53*).

Following the same arguments as above, and with the same fluorescence evidence, the chloroplastic envelope in both paraquat-resistant and –susceptible plants appears to be as permeable to the herbicide as the plasmalemma, so the question remains whether there are differences between resistant and susceptible biotypes in paraquat efflux out of the chloroplast. However, studies using isolated chloroplasts have not shown any differences in terms of chlorophyll fluorescence quenching (*36*) or Hill reaction kinetics (*52*) between paraquat-resistant and –susceptible weed biotypes, so an active paraquat efflux from the chloroplast to the cytosol, if it exists, is not efficient enough to make a difference.

Confined by the vacuolar membrane or tonoplast, the water-filled vacuole can occupy 80 to 90% of the total volume of the plant cell. Together with its key role in plant cell enlargement, vacuoles act as both reservoirs and dumps. They contain water and dissolved inorganic ions, organic acids, sugars, enzymes, and a variety of secondary metabolites. The passage of these compounds through the tonoplast is closely regulated, as the presence of carriers for several inorganic ions and organic molecules plus adenosine triphosphatases and pyrophosphatases suggests (*reviewed in* (*35*)). Several studies have shown some evidence that paraquat may be transported in both directions across the tonoplast via a polyamine transporter. It has been demonstrated that paraquat accumulates in the vacuoles of maize root cells (*54*) and is transported into barley vacuoles (*55*). If resistant cells have either a greater paraquat influx or a lower paraquat efflux into and out of the vacuole, that may represent a veritable cause of paraquat resistance in resistant biotypes. Supporting this hypothesis, there is strong evidence of a system for the transport of polyamine/paraquat through membrane system. Two polyamines, spermidine and cadaverine, applied prior to paraquat treatments inhibited paraquat activity in paraquat-susceptible but not–resistant biotypes of *Arctotheca calendula* (*53*). This inhibitory effect suggests a role for these polyamines in the resistance response, although this role remains unclear. There are several, non-exclusive, possible explanations. Some authors postulate that polyamines such as cadaverine and putrescine compete with paraquat for membrane transport in paraquat-resistant weed biotypes (*49*, *53*) and paraquat-resistant *Arabidopsis thaliana* mutants (*56*). These results strongly suggest that paraquat either enters maize root cells via a carrier system that normally functions in the transport of diamines with a charge distribution similar to that of paraquat, or that paraquat is taken up by plant cells via a paraquat transporter whose function is under stringent negative regulation by these diamines (*56*). Because polyamines applied prior to paraquat treatment inhibited paraquat activity only in susceptible plants, the inhibition may be caused by polyamines competing with paraquat for absorption across the plasmalemma (*53*). However, that hypothesis is not supported by fluorescence data. Alternatively, the inhibition may be due to polyamines competing with

paraquat for tonoplast efflux. Either way, this differential response to polyamines between resistant and susceptible cells may be evidence of a lack of protein transport in the resistant biotypes that results in paraquat resistance.

In addition, polyamines are well-known protective agents against biotic and abiotic stresses in plants (*reviewed in* (*56*)). Few studies have been published about the role of polyamines in the resistance response to paraquat. Soar et al. (*53*) found, in *Arctotheca calendula,* that paraquat-resistant plants contained more free putrescine than susceptible plants. In addition, the putrescine content in the resistant biotype decreased rapidly over time after paraquat treatment, while no such behavior was observed in the susceptible biotype. This dissimilar polyamine consumption between susceptible and resistant cells may be related to either direct oxygen radical scavenging (*57*), direct competition with paraquat for membrane carriers (*49*, *53*, *56*), or membrane stabilization (*58*).

Herbicide Resistance to Glyphosate: When Movement Does Matter

Glyphosate has become the most used and sold herbicide in the world (*59*), possibly due to the release of glyphosate-resistant (GR) crops in the late 90s, including soybean, corn, canola, cotton and sugar beet plants (*60*). The target site for glyphosate action is the enzyme 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS) (EC 2.5.1.19) (*61*), a key enzyme in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Inhibition of EPSPS results in the accumulation of shikimic acid, and the reduction of biosynthesis of these amino acids, leading to plant death. Prior to the development of GR crops, glyphosate was a popular herbicide because of its unique mode of action, good leaf uptake and translocation of its salt and ester forms, low toxicity, and benign environmental profile (*62*). Glyphosate was a good choice when used in pre-seeding control or in established woody crops, but a lack of physiological selectivity was a handicap. Its popularity boosted as zero tillage systems and GR crops appeared in the market and glyphosate became an easy, cheap, stand-alone, and selective way to keep weeds out of the fields. In addition, the natural occurrence of glyphosate-resistant weed biotypes was thought to be a slow or non-occurring event because (A) there were no known mechanisms for metabolizing glyphosate in plants and (B) the enzyme kinetics of lab-created mutants with GR EPSPS were poor (*63–65*). Of course, a third mechanism was not considered.

As zero tillage systems became popular and GR crops appeared on the market, glyphosate became the sole method for weed control on millions of hectares of arable land. This enormous selection pressure exerted on weed populations resulted in a flowering of glyphosate-resistant weed biotypes. To date, there are 210 weed biotypes, belonging to 27 different weed species and affecting 24 countries (*5*).

Glyphosate resistance in weeds has been described as both TSR and NTSR, depending on the weed biotype. Unlike GR crops, where a prokaryotic enzyme-efficient, glyphosate-resistant EPSPS gene has been introduced, EPSPS mutations resulting in glyphosate-resistant weed biotypes reduce the affinity for substrates and the enzymatic efficiency (*66*). Thus, proline-to-serine and proline-to-threonine substitutions at amino acid Pro101 (P101S and P101T, respectively) only confer

a moderate glyphosate resistance in weed biotypes. However, these mutations additionally reduce the affinity of EPSPS for PEP binding (*67*), decreasing fitness. Therefore, where compared, NTSR (i.e., translocation) always provided a similar or better degree of resistance than TSR (*66*).

As glyphosate is a foliar-applied, phloem-translocated herbicide (*68*, *69*), glyphosate translocation follows a source-to sink pattern. This pathway starts in the leaf surface, with glyphosate penetrating the epicuticular wax and cuticle, moving through the mesophyll cells to the phloem elements and then, translocating following the flow of sucrose.

Due to its zwitter-ionic nature, glyphosate acid penetration through the non-polar cuticle is quite poor and follows a characteristic concentration-dependent uptake pattern (*70*). Glyphosate uptake often occurs in two stages, the first being relatively rapid and most likely contact area-sensitive, followed by a second much slower one, dependent on the concentration gradient (*71*). In addition, the role of the previously described cuticular aqueous pores in glyphosate leaf penetration remains controversial (*72*). Therefore, glyphosate leaf penetration depends both on the nature of the adjuvants present in the herbicide formulation and on the composition and structure of the leaf cuticle and epicuticular waxes. Therefore, glyphosate resistance due to either altered herbicide retention or uptake by the leaves is a very uncommon event, as it has been only described in a couple of *Lolium multiflorum* weed biotypes (*73*, *74*), where other NTSR mechanisms (altered translocation) were also present. The nature of these differences in glyphosate leaf retention and penetration remains unknown.

Once inside the plant, it appears there are two different mechanisms of glyphosate uptake by plant cells. First, there is an active system operating at low concentrations that pumps the herbicide into plant cells, possibly via a phosphate transporter. This transport works against a concentration gradient, exhibiting a saturation phase and inhibition by competitive inhibitors of phosphate uptake in plant cells (*reviewed in* (*75*)). In addition, there is a passive, gradient-dependent mass flow system. Inside susceptible plant cells, glyphosate appears to be restricted to the cytoplasm, with little accumulation in the vacuole (*68*, *76*).

Although glyphosate has both phloem and xylem mobility, the fact that it tends to accumulate in sink organs points towards the phloem system as the main transport route (*77*). Movement of glyphosate from mesophyll cells to the phloem lumen presumably occurs via the symplastic mechanism, similar to the previously described for cell uptake. For this, the putative glyphosate transporters may be the low-affinity phosphate transporters Pht2;1 and Pht1;6, responsible for phosphate efflux out of source leaves (*reviewed in* (*75*) and (*78*)). In any case, once inside the phloem sieves, glyphosate is trapped by its own hydrophilicity and is mass-transported to sink tissues. The amount of glyphosate translocated from sources to sinks is self-limiting, as the toxicity experienced by the susceptible plants as a result of herbicide action decreases the translocation efficiency of the compound (*79*). Interestingly, glyphosate translocation in highly resistant GR crops containing the *Agrobacterium* spp. CP-4 EPSP gene is greater than in parental susceptible controls, possibly due to glyphosate toxicity occurring at the source thereby limiting mobilization (*80*). How glyphosate is discharged from the phloem to sinks has not been studied.

As a highly systemic herbicide, one of the strengths of glyphosate is its ability to reach vital areas such as the roots and shoot meristems. This rapid and widespread translocation has been postulated as the source of herbicide efficacy (*81*). It is, then, fairly evident that a reduction of glyphosate translocation or a change in glyphosate distribution could confer resistance to this herbicide. Glyphosate-resistant weed biotypes tend to show similar patterns in terms of herbicide distribution. Therefore, while differences between resistant and susceptible biotypes in glyphosate absorption are small at best, herbicide translocation and accumulation differ greatly between resistant and susceptible plants. Following the usual source-to-sink phloematic transport, glyphosate applied to susceptible biotypes tends to accumulate in sink tissues, whereas in resistant biotypes is usually only present in the tip of treated leaves, with little downward translocation (*reviewed in* (*77*) *and* (*82*)). Whether this response in resistant biotypes is a phloem-independent, xylem, all-apoplast translocation mechanism remains unclear.

Shaner (*75*) describes up to four potential mechanisms explaining how cellular absorption and subsequent translocation of glyphosate could be reduced: (1) alteration in a putative phosphate transporter responsible for the active cellular uptake of glyphosate, thus rendering lower herbicide influx; (2) evolution of a new transporter that pumps glyphosate into the vacuole, thus sequestering the herbicide and preventing it from reaching either the chloroplast or the phloem; (3) evolution of a new transporter that actively pumps glyphosate out of the cell and into the apoplast; or (4) evolution of a transporter at the chloroplast envelope that pumps glyphosate out of the chloroplast, preventing the herbicide from reaching its target site.

Although none of the four hypotheses can be discarded yet, recent evidence points to vacuolar glyphosate sequestration as the primary mechanism of resistance. ^{31}P NMR studies carried out in glyphosate-resistant and -susceptible biotypes of *Conyza canadensis* have revealed that the rate of vacuole accumulation of this herbicide is more rapid and that accumulation occurs to a greater extent in the resistant tissue than in the susceptible one. This ability was shown to be present in both source and sink tissues (*83*). Low temperatures decreased both glyphosate vacuolar sequestration and glyphosate resistance at the whole plant level in the resistant biotypes, rendering results similar to those observed in the susceptible ones, and showing a direct link between glyphosate sequestration and glyphosate resistance (*84*). In addition, while glyphosate influx and efflux from the cytoplasm appear to be reversible and diffusion-controlled, once glyphosate enters the vacuole in resistant cells, it is effectively trapped, suggesting the presence of a specific tonoplast transporter for glyphosate. These results have been confirmed in different glyphosate-resistant *Lolium* spp biotypes collected on three different continents, all of which have shown that the extent of vacuole sequestration correlates to the level of glyphosate resistance (*85*).

What is the nature of these tonoplast-localized carriers responsible for glyphosate sequestration inside plant cell vacuoles? Genomic analysis of glyphosate-resistant horseweed plants exposed to this herbicide has shown the up-regulation of more than 20 genes, including four ABC transporters and several tonoplast intrinsic proteins (TIPs) (*86*). TIPs are part of the major-intrinsic-protein

family and are localized to the tonoplast membrane in the cell and expected to be involved in the transport of water, glyphosate, or glyphosate breakdown products (*86*). However, ABC transporters seem to be the most promising candidates to explain NTSR in glyphosate-resistant weed biotypes. ABC transporters are membrane-associated active transport proteins that move both plant metabolites and xenobiotics across membranes using adenosine triphosphate hydrolysis (*87*). ABC transporters are of special interest because they are responsible for exporting toxins, sequestering plant secondary metabolites in the vacuole, and translocating phospholipids (*87*). Although ABC transporters can be targeted to any internal cell membrane, the idea of tonoplast targeting is in agreement with the glyphosate vacuole sequestration theory. Mutated ABC transporters pumping glyphosate into the vacuole (or not pumping the herbicide out) would correspond with and justify the observed experimental data. Although there is no direct evidence of ABC transporters showing affinity for glyphosate, there are physiological studies showing affinity of these proteins for some other herbicides and herbicide metabolites (*88*).

Conclusion

The selection of one mechanism of herbicide resistance over another is a delicate balance between resistance effectiveness, mutation frequency and fitness penalty. In most cases, this balance appears shifted towards TSR and herbicide detoxification because these provide high levels of herbicide resistance while carrying only a small fitness penalty. This has caused the scientific community to underestimate the importance of the restriction of herbicide movement as a source of herbicide resistance, when detected. Therefore, most cases of herbicide resistance due to a lack of herbicide absorption and translocation have not been deeply studied because they were present together with another "major" mechanism. However, when these mechanisms fail to confer an acceptable level of resistance, the alteration of herbicide movement throughout the plant has proven to be as capable a mechanism as any other. These few studied cases have shown us that, as is the case with other resistance mechanisms, resistant biotypes share common features, such as mutated protein carriers that affect herbicide transport through the tonoplast. However, many questions remain to be addressed: What is the nature of these mutated carriers? Are they increasing influx or decreasing efflux of the herbicide to or from the vacuole? Is the herbicide translocated as the parent herbicide or in the form of its metabolites? Much work remains to be done.

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Chapter 8

Absorption and Penetration of Herbicides Viewed in Metabolism Studies: Case of Glufosinate and Imazamox in Wheat

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The search for new alternatives for weed control in wheat has led to the creation of new resistant lines of wheat to herbicides that are not apt for this crop. This is the case of Clearfield wheat for the family of imidazolinone herbicides and genetically modified wheat to glufosinate. The behavior of these herbicides in contact with these lines has been studied, resulting in some very interesting ideas about the transformation of these herbicides within the plant as well as the way they take through them.

Wheat and Weeds

The importance of the effect that each weed produces on the crop will depend on the type of weed management that is performed and the planting season of the wheat.

In the case of wheat, there are three herbicide application times depending on the state of the development of wheat plants and weeds: before planting, in preemergence and in postemergence. Before sowing wheat the herbicides glyphosate, sulphosate, paraquat and / or diquat, and even mixtures with other non residual herbicides that increase their effectiveness are commonly used. In preemergence, i.e. before crop and weed emergence, it is common to use

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

metsulfuron methyl and flurochloridone. Finally, in the case of postemergence, when the wheat and weeds have emerged, the herbicides used are clodinafop propargyl, chlortoluron, diclofop, isoproturon, pinoxaden, tralkoxydim, fenoxaprop-p-ethyl, bromoxynil and many others.

However, despite the wide range of herbicides, some weeds are not controlled by their use and a search for new alternatives is therefore necessary. Some of these alternatives entails the "design" of crops resistant to non-selective herbicides, either by crossbreeding and biotechnology application or by transgenesis (integration of genes into the genome without crossbreedings). In the first case we refer to Clearfield® crops specific to imidazolinone (*1*), and in the second case to genetically modified organisms such as wheat resistant to glufosinate (*2*).

Clearfield technology is considered as an integrated weed control (*3*) based on the development of varieties tolerant to imidazolinones using traditional induction mutations and conventional breeding. They are non-genetically modified seeds, and their performance has been widely assessed (*4*, *5*). The advances in genetic engineering have promoted the application of recombinant DNA technology to obtain and commercialize new varieties of genetically modified crops in which two glufosinate resistance genes (designed as *pat* and *bar*) were introduced, encoding one of the phosphinothricin acetyltransferases (PAT) (*6*) widely applied in plant genetic engineering. This permits the following-up of glutamine synthetase (GS) activity in the presence of glufosinate. In both cases, the metabolism to its respective herbicides was also found (*7*, *8*).

Importance of Absorption and Translocation Imazamox and Glufosinate in the Resistant Wheat Lines

A necessary condition for achieving the effectiveness of a herbicide is that it reaches its site of action in a sufficient concentration to be lethal. The lack of movement of a herbicide will reduce its concentration at the site of action. These low concentrations can be occurred due to a reduction in the penetration, absorption or translocation or the existence of sequestration phenomena in metabolically inactive cell organelles as shown in Figure 1 as occurs in the glyphosate. Those mechanisms are difficult to study separately, and when it is done it is hard to tell them apart, because a differential absorption implies a differential translocation, and the latter may derive from the different herbicide degradation in the absorption site, resulting in more or fewer metabolites which can be translocated.

The resistance/sensitivity to herbicides due to their lack of absorption in preemergence treatment can be associated with morphological factors (*9*) such as differences in depth or anatomical structure of the root system, or physiological factors such as the limited absorption of the herbicide active in susceptible species. Regarding the foliar path, the amount of herbicide penetrated in the plant tissue in postemergence applications is subject to the amount of it adhered to the plant. This depends on factors such as: weather conditions during the treatment, the surface tension of the herbicide solution, the volume of treatment, and foliar features such as area and leaf orientation and the amount of waxes present in

the leaves. Resistance to post-emergence treatments is often associated with the presence of differences in the leaf cuticle such as composition and epicuticular wax content (*10*).

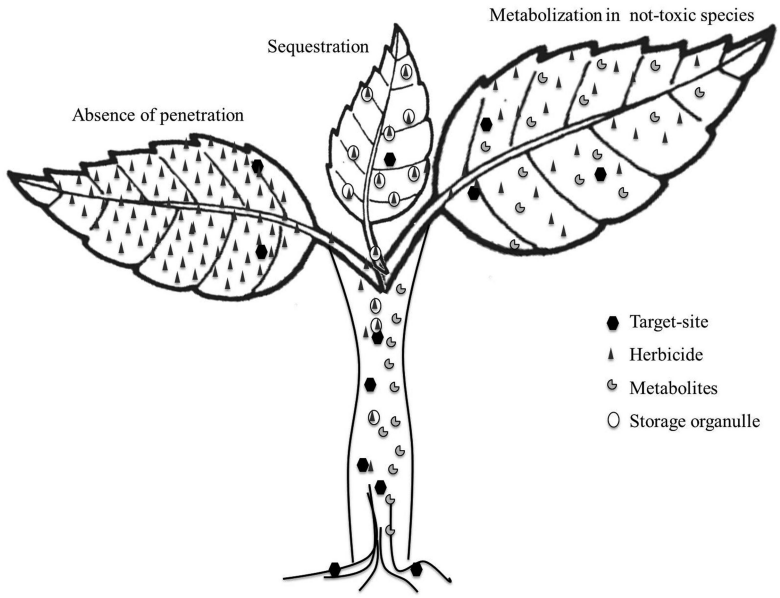

Figure 1. Mechanisms known in which the concentration of herbicide does not reach a lethal dose in the target-site and by some of which can occur resistance to imazamox and glufosinate.

The same as in the absorption/penetration processes, those of herbicide translocation in plants can be classified according to the type of herbicide treatment that has been applied (*11*). In the case of absorption via root, the movement of the herbicide will depend on its chemical nature. This is due to three causes, namely: 1) the accumulation of the unmetabolized herbicide at the root, resulting in a lack of translocation of the active ingredient to the shoots; 2) the herbicide metabolism in the root and subsequent production of immobile forms which are generally polar conjugates; 3) the restriction of herbicide movement in the vascular system (primary and secondary vessels) making it impossible to reach its primary site of action. In the case of the foliar herbicide movement, after penetration, this can be classified according to whether its transport takes place in the xylem or the phloem. While the transport of the herbicides via the xylem path freely follows the water flow in this system to the leaf margins and to the inter-vascular spaces, the transport via phloem will depend on two distinctive processes: the concentration gradient of the herbicide from the phloem cells and the mesophilic, and the ability of the herbicide to be retained by the phloem cells during transport (*11*).

Imazamox is a systemic herbicide and, hence, translocatable, but, glufosinate is mostly a contact herbicide but with a partial systemic action, so that it rarely translocates. In the case of glufosinate it has not been proved that, although the herbicide cannot be translocated, metabolites can do so.

Herbicides Metabolism: Transport of the Metabolites

Most techniques used for the study of absorption and translocation are based on using radiolabeled herbicide. This is a help when visualizing the movement of the herbicide through the plant as long as there is a radioactivity detector type phophoimager, although the latter does not give any information about metabolite transport only about the radioactivity displaced as we can see in the studies realized in the references (*10*) and (*11*).

In the case of glufosinate, the study does not have any meaning, as it hardly translocates, and, if it does, it takes a small proportion. In the case of imazamox, this makes more sense, because it is a systemic herbicide with a high translocation speed.

Studies on *Echinochloa crus-galli* (*12*) or those made in red lentil and dry bean (*13*) demonstrate imazamox translocation through the plant and the speed at which it happens. The absorption and translocation of imazamox can also be studied by sampling the foliar part (leaves) and the root. Working in this way, it was observed in works made in two cultivars of *Triticum aestivum* with Clearfield technology (*8*) that the susceptible (S) cultivar, which is unable to metabolize the herbicide, translocates imazamox from the leaves to the root throughout the experiment; therefore, translocation of imazamox through the plant occurs. On the contrary, in the resistant (R) cultivar, the metabolites are the compounds translocated to the root, thus translocation took place proportionally to dose and time. The difference between the two cultivars is the compound translocated to the root, consisting of toxic or non-toxic forms determined by the metabolism. In both cultivars, and for the same amount of imazamox applied, the amount of herbicide penetrated is the same, but it appears as such or is metabolized, depending on the cultivar. In the resistant cultivar imazamox was metabolized about 67.74 % at 120 h after treatment with 200 g ai of herbicide ha–1 and about 7.70 % corresponding of the metabolized amount was translocated. Respect to the rest of imazamox unmetabolized, only the 9.32 % was translocated. While in the susceptible cultivar about 0.98 % was metabolized and 99.35 % of unmetabolized imazamox was translocated. The results obtained in this work indicated that the low translocation of imazamox to the root could be due to the metabolization to nontoxic forms of herbicide which are translocated, occurring when the enzyme is saturated with the herbicide, which reduces the amount of intracellular herbicide that can catalyze the ALS enzyme, thus increasing the tolerance to the herbicide as compared to other populations with a single resistance mechanism and (*10*). Figure 2 shows as the herbicides and the metabolites can be transported through the plant.

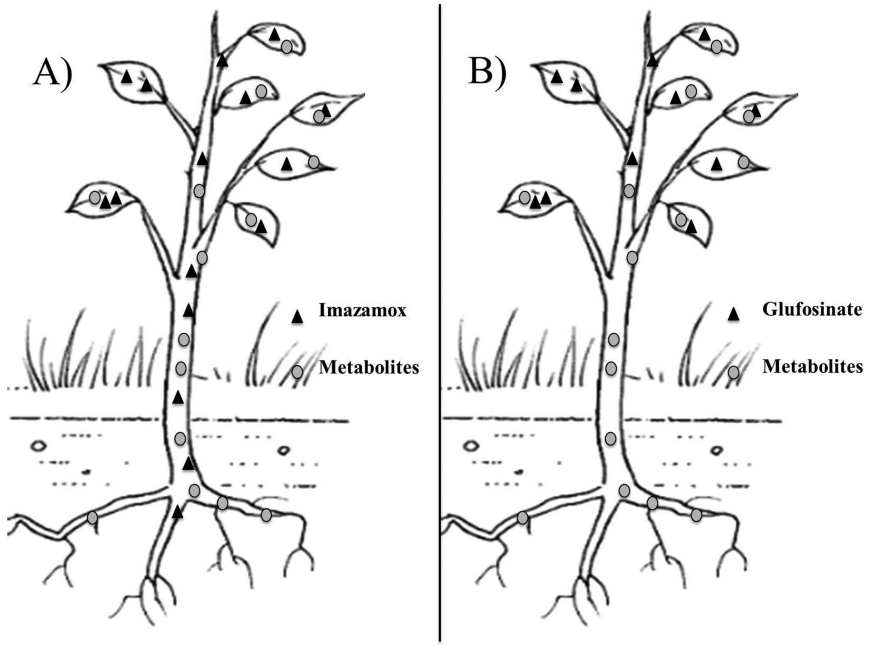

Figure 2. Illustration of distribution of imazamox and its metabolites (A) glufosinate and its metabolites (B) in plant as a result of foliar uptake.

Techniques Used To Study the Metabolism

In the case of imazamox, some authors have studied the presence of metabolites by the measure of radioactivity in extracts of plants treated with ^{14}C-imazamox (*13*, *14*) as target metabolites have not been commercialized. The absence of commercial metabolites has delayed research on degradation of the herbicide, which has been restricted to studies involving radioactivity-based methods (*13*, *14*) has required the synthesis of the target metabolites (*15*). Harir and his collegues (*16*), working with radioactivity-based methods, have measured the presence of metabolites through the amount of measured total radioactivity, which is not reliable information because the radioactivity can stem from a non-metabolic degradation occurring outside the plant by photolysis. The main problems in dealing with metabolite synthesis are its cost (both in reagents and time) and purity. The search for fast and effective procedures for the identification of metabolites without standards and without the use of radioactive compounds is a challenge in the agronomical field due to the need to find out the behavior of a plant against a given herbicide. In the case of resistant weeds, finding such a procedure would constitute a useful tool for developing new attack strategies.

Capillary electrophoresis (CE) in its different modes has been the most widely used separation technique prior to determination of imidazolinones (*17*). Thus, Ohba, Minoura, Safarpour M., Picard, and Safarpour H. (*15*) described

a method using reverse micellar electrokinetic chromatography (MEKC) with UV detection, which permitted the determination of imazamox and its hydroxy and glucoside metabolites after synthesis of both compounds. The capability of CE can be improved by coupling to a mass spectrometry (MS) detector, which has been the equipment used for several studies dealing with trace analysis of herbicides, including imazamox (*18*–*21*). In addition to the use of CE, separation of imazamox from mixtures with other herbicides and pesticides has involved gas chromatography (GC) (*22*) and, particularly, liquid chromatography (LC) (*13*, *14*, *23*) with MS detection in all instances. Despite the potential of MS in the identification of degradation products from herbicides (*16*), no MS-based studies have been reported to confirm the presence of imazamox metabolites in plants.

In the case of glufosinate, the determination of the analytes has frequently been carried out after separation by liquid chromatography (LC) (*24*–*26*), gas chromatography (GC) (*27*, *28*) or capillary electrophoresis (CE) (*29*, *30*) by different detection techniques. In metabolism studies, the preferred methods for analysis of glufosinate and metabolites in plants are based on LC separation and radioactivity detection (*31*, *32*), which require simple sample preparation protocols (usually precipitation of polysaccharides, protein and peptides and subsequent filtration or centrifugation), thus preventing or minimizing potential losses of the target analytes (particularly low concentrated metabolites) (*32*). Nevertheless, radioactivity-based methods have as major limitations the high cost of reagents, loss of radioactivity by quenching, difficulties in waste management and their inability to identify chemical structures of the metabolites. Apart from radioactivity-based methods, photometry by a diode array detector (DAD) (*29*, *30*, *33*), fluorescence detection (*24*, *25*, *29*) and mass spectrometry (MS) (*27*, *28*) have also been used, but only for glufosinate determination. Derivatization is required prior to fluorescence detection, mostly using fluorenylmethoxycarbonyl chloride (FMOC–Cl) as a fluorogenic reagent (*24*, *25*, *28*) that reacts with the amino group. In short, studies on the metabolism of glufosinate have involved only some of its metabolites (mainly its major metabolite, 3-(methylphosphinic) propionic acid, also known as MPPA), developed in aqueous media.

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Chapter 9

Uptake, Translocation, and Accumulation of Pharmaceutical and Hormone Contaminants in Vegetables

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The widespread occurrence of pharmaceuticals and personal care products (PPCPs) and natural hormones in watersheds has been recognized as an emerging environmental issue. Potential uptake and internalization of these emerging contaminants by food plants that are irrigated with contaminated water is becoming a food safety issue. In the present study, uptake, translocation, and accumulation of seven PPCPs and three steroid hormones in lettuce and tomato plants grown under hydroponic conditions were investigated. An isotopic dilution method was developed for the analysis of trace levels of PPCPs and hormones in vegetables using liquid chromatography-tandem mass spectrometry (LC-MS/MS), combined with ultrasonication-shaking extraction and solid phase extraction (SPE) cleanup for sample preparation. All target PPCPs and hormones were detected in the lettuce and tomato roots with concentrations ranging from 12.5 μg kg^{-1} up to 20.9 mg kg^{-1} when the plants were grown in hydroponic solutions containing each compound at 50 μg L^{-1}. This result indicates that these contaminants can be bound to or taken up by the plant roots. Interestingly, the concentrations of caffeine (CAF) and carbamazepine (CBZ) in lettuce and tomato leaves

In Retention, Uptake, and Translocation of Agrochemicals in Plants; Satchivi, et al.;

were much higher than those in roots, suggesting that these two PPCPs can easily translocate from plant roots to leaves via water transpiration and thereby accumulate in plant leaves. For other target PPCPs and hormones, the translocation factor (TF) values were very small in lettuce and tomato plants, implying poor translocation of these chemicals from roots to above-ground plant parts following uptake. The bioaccumulation factors (BAFs) in tomato fruits for all target compounds except CBZ were much less than 1. By contrast, three PPCPs had BAFs >15 in lettuce leaves, indicating there may be potential risk of exposure to these contaminants through consumption of this leafy vegetable.

Introduction

The widespread occurrence of emerging chemical contaminants, including pharmaceutical and personal care products (PPCPs) and natural hormones, in watersheds has been recognized as a critical environmental issue. It has been reported that over 80 pharmaceutically active compounds have been detected in the aquatic environment (*1*). In the U.S., a nationwide survey across 30 states found that approximately 90% and 48% of the 139 sampled streams contained detectable levels of PPCPs and steroid hormones, respectively (*2*).

Sewage treatment plants (STPs) and concentrated animal feeding operations (CAFOs) have been identified as major sources discharging emerging contaminants to surrounding water supplies via wastewater effluents. Due to incomplete removal in most conventional biological STPs, PPCP and hormone residues have frequently been detected in effluents and receiving waters at concentrations ranging from <0.1 to 10 ng/L (*1*–*6*), and in some STP effluents even up to μg/L levels (*7*). CAFOs such as dairy and swine facilities are another major source introducing veterinary pharmaceuticals and animal hormones to the environment through the land application of manure and manure-containing wastewater (*8*, *9*). Furthermore, reclaimed effluents are becoming an important source to supplement the increasingly scarce fresh water supply available for agricultural irrigation, especially in semiarid regions or during periods of drought. Agricultural irrigation using PPCP or hormone-containing water may introduce these emerging contaminants to crop fields, which may subsequently be taken up by plants and thereby enter food chains.

Previous studies have indicated that PPCPs and steroid hormones could be taken up, accumulated in, or metabolized in beans, wetland macrophytes, and algae (*10*–*12*). For example, some veterinary pharmaceuticals derived from animal manures were found to accumulate in alfalfa, corn, lettuce, potato, and soybean (*13*, *14*), indicating that these emerging contaminants may enter terrestrial food chains through soil via the land application of wastewater and biosolids. Recently, uptake of human pharmaceuticals in leafy vegetables grown hydroponically or in nutrient solution has also been reported (*15*–*17*). These

studies have clearly shown PPCP uptake by food plants, but predicted that the potential risk to humans through dietary uptake was negligible due to very minor exposure (*17*). However, this simple estimation based on a few compounds and plant types may not encompass all possible human health effects (*16*). Moreover, the potential accumulation of PPCPs or hormones in plants over time always raises public concern when reclaimed water is used for irrigation.

The potential for chemical uptake, internal transfer, and accumulation in plants is generally associated with chemical properties, plant species and cultivars, growth substrates, contaminant concentrations, and plant development stages. Presently, very limited information is available concerning the uptake of PPCPs and hormones by food plants that are irrigated with water containing environmental-level contaminants. Since these emerging contaminant residues have been frequently detected in the aquatic environment, it is essential to investigate possible contamination of food plants grown in PPCP or hormone-containing water. The objectives of this study were to: (i) develop a sensitive method for analyzing trace levels of PPCPs and steroid hormones in vegetables; (ii) investigate uptake, translocation, and accumulation of target contaminants within plants grown hydroponically with a nutrient solution containing PPCPs and hormones at environmentally relevant levels; and (iii) determine the accumulation magnitude of these contaminants in food plants and thereby assess the potential risk of this exposure route on human health.

Materials and Methods

Chemicals and Materials

Seven PPCPs and three steroid hormones were selected in this study on the basis of their high occurrence in aquatic environments and their wide range of physicochemical properties (e.g., pK_a and log K_{ow}) (Table 1). PPCP standards caffeine (CAF), carbamazepine (CBZ), gemfibrozil (GEM), ibuprofen (IBU), naproxen (NAP), triclosan (TCS), and sulfamethoxazole (SMO), internal standard florfenicol, and hormone standards 17β-estradiol (βE2), estrone (E1) and 17α-ethinylestradiol (EE2) were obtained from Restek (Bellefonte, PA, USA). Isotope standards including $^{13}C_3$-caffeine, D_{10}-carbamazepine, D_6-gemfibrozil, $^{13}C_3$-ibuprofen, $^{13}C_4$-naproxen, $^{13}C_{12}$-triclosan, $^{13}C_6$-sulfamethoxazole, and $^{13}C_6$-estrone were purchased from Cambridge Isotope (Andover, MA, USA). Solvents used in the study, including methanol, acetone, and acetonitrile, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). An individual stock solution of each PPCP compound was prepared in methanol and stored in an amber glass vial at -20°C.

The seeds of lettuce (*Lactuca sativa 'Red Lollo'*) and tomato (*Solanum lycopersicum 'Cherry Cascade'*) were provided by a local nursery. Lettuce and tomato were selected for this study because they are representative of edible leaf and fruit vegetables, respectively. Two commercial hydroponic systems, AeroFlo2 Model 20 and Turbogarden Aero, were purchased from General Hydroponics (Sebastopol, CA) and Botanicare (Chandler, AZ), respectively.

Table 1. Selected Physico-Chemical Properties of Target Compounds and Their Optimized MRM Parameters for LC-MS/MS Analysis

Compound	*Abbreviation*	*pK_a (18)*	*Log K_{ow} (18)*	*Retention time (min)*	*ESI mode*	*MRM ions*	*MRM ions for isotope*	*Cone (V)*	*Collision (V)*
Caffeine	CAF	14.0	-0.07	9.9	+	195.2>137.9	198.2>140.0	35	20
Carbamazepine	CBZ	13.9	2.45	16.3	+	237.4>194.2	247.4>204.2	35	16
Naproxen	NAP	4.15	3.18	17.7	–	229.2>170.1	233.2>170.1	20	15
Ibuprofen	IBU	4.91	3.97	19.9	–	205.1>161.1	208.2>163.1	20	10
Gemfibrozil	GEM	4.5	4.77	21.0	–	249.0>121.0	255.0>121.0	26	12
Triclosan	TCS	7.9	4.76	21.1	–	286.8>235.0	298.8>235.0	22	10
Sulfamethoxazole	SMO	5.7	0.89	13.1	+	254.0>156.0	260.2>162.0	35	16
Estrone	E1	10.8	3.13	16.8	–	269.3>145.0	275.2>145.0	50	40
17β-Estradiol	βE2	10.7	4.01	15.8	–	271.3>145.0	N/A	50	40
17α-Ethinylestradiol	EE2	10.2	3.67	16.1	–	295.3>145.0	N/A	50	40

Hydroponic Experiment

Plant uptake experiments were performed in a temperature-controlled greenhouse (20~25°C) operated by the Plant Care Facility at the University of Illinois at Urbana-Champaign. The lettuce seeds were germinated in Fafard superfine-germination mix (Agawam, MA) for two weeks. Ten seedlings of uniform size were transferred into the AeroFlo2 Model 20 hydroponic tanks, where they were maintained in a continuously aerated nutrient solution under a 16/8-h day/night photoperiod at 25 ± 1 °C and 20 ± 1 °C day/night temperature. Prior to transfer, the seedlings were thoroughly washed to remove any substrate particles attached to the plants. After one-week acclimation of the lettuce plants in nutrient solutions, seven target PPCPs and three steroid hormones were spiked into the hydroponic tanks, resulting in initial concentrations of each compound at 50 μg L^{-1}. The chemical-spiked nutrient solutions were replaced twice per week to avoid nutrient depletion and restrict bacterial growth. Lettuce plants were also grown in nutrient solutions without chemical spiking as a control. After three weeks, the whole lettuce plants were harvested from each hydroponic tank for further analysis.

Similarly, the tomato seeds were germinated in soil in the greenhouse (20~25°C). After three weeks, the seedlings were transferred to a continuously aerated nutrient solution under a 16/8-h day/night photoperiod in the Turbogarden Aero tanks. The plants were acclimated in nutrient solutions for four weeks until the first tomato fruit appeared. Six tomato seedlings were grown in the hydroponic tanks and thinned to three before spiking chemicals to allow for adequate space in the hydroponic system for full growth of tomato plants. Seven target PPCPs and three steroid hormones were fortified in the hydroponic solutions, resulting in initial concentrations of each compound at 50 μg L^{-1}. The chemical-spiked nutrient solutions for this study were changed once per week. Additionally, the tomato plants were grown in only nutrient solutions as a control. After five weeks, the whole tomato plants were harvested by removing them from the hydroponic tanks.

Sample Preparation and Extraction

After harvesting, all plants were rinsed under a stream of deionized water for 5 min, left to drain, and then blotted dry. The lettuce plants were separated into roots and leaves. The whole tomato plants were separated into roots, stems, leaves, and fruits. All plant components (roots, stems, leaves, and fruits) were weighed individually. Plant leaves (lettuce and tomato) and fruits (tomato) were homogenized using a food processor. All plant roots and stems were cut into small pieces and then freeze-dried using a freeze dry system (Labconco, Kansas City, MO). The dried samples were ground to powder using a mill (Glen Mills, Maywood, NJ). After measuring their moisture contents, all plant components were stored at -20°C until extraction.

PPCPs and hormones in plant samples were extracted using ultrasonication-shaking methods and cleaned up by solid phase extraction (SPE) cartridges based on EPA Method 1694 with some modification (*19*, *20*). First, three dual-solvent

(solvent A/solvent B) systems were evaluated to optimize the extraction method: (i) methyl tert-butyl ether (MTBE)/acetonitrile; (ii) acetonitrile/phosphate buffer; and (iii) acetonitrile/water. Briefly, the uncontaminated tomato fruits were weighed into centrifuge tubes and spiked with 100 ng of each PPCP or hormone standard. After premixing, 20 mL of extraction solvent A was added to the test sample for extraction. The sample was vortexed for 1 min, sonicated for 30 min, shaken for 30 min, and then centrifuged at 10,000 RPM for 15 min. The supernatant was poured out into a turbovap tube. The solid phase was further extracted by adding 15 mL of solvent B, followed by vortexing, sonicating, shaking, and centrifuging. The aqueous layer was poured out into the same turbovap tube. The solid sample was extracted one more time using 20 mL of solvent A according to the above extraction procedure. Extracts from each sample were combined and then mixed thoroughly. The extracts were concentrated to 1.0 mL using a closed cell concentrator (Turbo Vap@500, Hopkinton, MA) at 40°C, followed by addition of ultrapure water (49 mL at pH 2) to each sample.

Oasis HLB cartridges as SPE columns were used to clean up the samples. Before loading the sample extracts, the SPE cartridges were preconditioned with 10 mL methanol, 10 mL water, and 10 mL pH=2.0 water in series by gravity. Sample extracts were passed through the SPE cartridges, using a vacuum to control the flow rate at 3-5 mL/min. For PPCP extraction, the cartridges were washed with 10 mL water after loading the sample and dried under vacuum for about 30 min. Each sample was eluted with 10 mL methanol and 6 mL acetone:methanol (1:1) by gravity. The combined sample extracts were blown down to dryness under gentle nitrogen gas and reconstituted with 1.0 mL acetonitrile:water (1:1). For hormone extraction, Oasis HLB cartridges were washed with 5 mL methanol:water (5:95) and dried under vacuum for about 30 min after loading the samples. The samples were then eluted with 6 mL of ethyl acetate:methanol (9:1). The extracts were blown down to dryness under gentle nitrogen gas and reconstituted with 1.0 mL acetonitrile:water (1:1).

To reduce interference from the plant matrix on LC-MS/MS analysis, an isotope dilution method was utilized to analyze all harvested plant samples. In brief, each plant sample was weighed into centrifuge tubes and spiked with PPCP or hormone isotope standards as surrogates. All samples were extracted and cleaned up according to the procedures described above. Analysis of each plant sample was carried out in triplicate.

Instrumental Analysis and Optimization

Concentrations of PPCPs were determined by LC-MS/MS (Waters, Quattro Macro, QA1140, Milford, MA). All target PPCPs were separated on a Symmetry C18 column (3.5 µm particle size, 2.1x150mm, Waters) by HPLC (2695 module, Waters). A gradient separation was achieved using two mobile phases: solvent A, 0.1% ammonium acetate and 0.1% acetic acid in water; and solvent B, 1:1 methanol:acetonitrile. The gradient began with 90% solvent A and 10% solvent B and was maintained for 2 min. The gradient was then ramped up to 5% solvent A and 95% solvent B linearly in 13 min and maintained for 8 min. The

gradient changed back to 90% solvent A and 10% solvent B in 0.5 min and was re-equilibrated for 5.5 min. Sample extracts were spiked with 100 ng internal standard florfenicol, and 30 μL of each sample was injected.

Hormones were analyzed by the same LC-MS/MS system. Two mobile phases were applied for separation: solvent C, water with 10mM ammonium hydroxide; and solvent D, acetonitrile with 10mM ammonium hydroxide. The gradient began with 90% solvent C and 10% solvent D and was maintained for 2 min. The gradient was then ramped up to 5% solvent C and 95% solvent D linearly in 13 min and maintained for 8 min. The gradient changed back to 90% solvent C and 10% solvent D in 0.5 min and was re-equilibrated for 5.5 min. Sample extracts were spiked with 100 ng internal standard florfenicol, and 30 μL of each sample was injected.

An LC system was coupled with a Quattro Macro mass spectrometer (QA1140, Waters, Milford, MA) equipped with an electrospray ionization (ESI) source. For PPCP analysis, the mass spectrometer was operated in positive and negative ESI mode simultaneously with optimized instrument conditions: desolvation gas flow rate 650 L/min; capillary voltage 3.0 kV for positive and 3.5 kV for negative mode. For hormone analysis, negative ESI mode was applied with the same desolvation gas flow and capillary voltage. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode and optimized parameters including collision energy and cone voltage for each target analyte are listed in Table 1. Confirmation of the analytes in plant sample extracts was based on the MRM ion transitions as well as comparing the retention time of each peak to its corresponding isotopic standard.

Results and Discussion

Optimization of Extraction Method

A series of preliminary experiments were performed to investigate the effects of extraction conditions on recoveries of PPCPs and hormones in plant samples. It has been reported that ultrasonic extraction showed better recoveries for most target PPCPs than accelerated solvent extraction (ASE) (*16*, *19*). One of the studies also showed that sonication extraction using acetonitrile or MTBE resulted in a higher extraction efficiency compared to other solvents such as methanol, acetone, and ethyl acetate (*16*). In accordance with EPA Method 1694, PPCPs in solid samples were extracted using ultrasonic extraction with acetonitrile (*19*).

To further improve extraction efficiency of PPCPs and hormones from plant samples, different solvent-mixture systems were examined under a two-step extraction procedure involving ultrasonication and shaking. The absolute recoveries of all of the target analytes in the uncontaminated tomato fruits under three solvent extraction systems are displayed in Figure 1. The absolute recovery for each analyte was calculated as the amount detected over that spiked. The recoveries ranged from 42%~115% for acetonitrile/MTBE, 52%~121% for acetonitrile/phosphate buffer solution, and 9%~127% for acetonitrile/water, respectively. Using acetonitrile/phosphate buffer solution as the extraction solvent led to better recoveries for most target compounds. Compared to the

acetonitrile/MTBE solvent system used in a previous study for PPCP extraction (*16*), the acetonitrile/phosphate buffer system appeared to have less variation in recovery for most target compounds in this study (Figure 1). Therefore, the acetonitrile/phosphate buffer solution extraction was selected for use in this study.

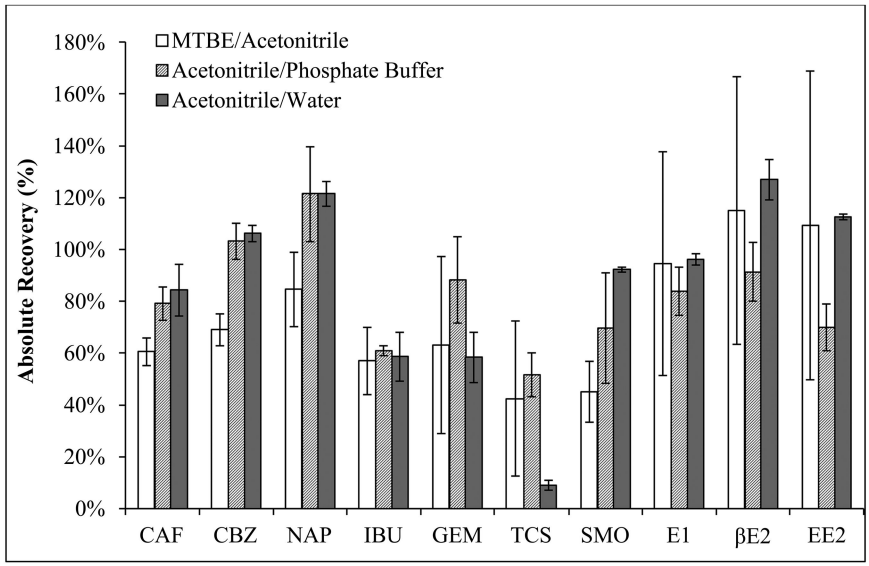

Figure 1. Effect of extraction solvents on the recoveries of PPCPs and hormones. Standard deviation of triplicate samples is shown as error bars.

Method Validation

The entire procedure used to detect and quantify the residues of PPCPs and hormones in plant samples included isotope standard addition, ultrasonication-shaking extraction, SPE cartridge cleanup, and quantification by LC-MS/MS. The performance of the developed method was evaluated by considering response linearity, recoveries, and limits of detection (LODs) of target PPCPs and hormones in the plant samples. For analyte quantification, six-point calibration curves (1~500 μg L^{-1}) were performed for each PPCP and hormone. Calibration curves of each PPCP were estimated as the relative response to the corresponding stable isotope surrogate standards. For the three steroid hormones, calibration curves were estimated as response of each hormone relative to the $^{13}C_6$-estrone surrogate. Good linearity was achieved for all the compounds and the squares of correlation coefficients (r^2) were all higher than 0.998.

The absolute recovery and corrected recovery for each analyte based on the developed method are shown in Table 2. The absolute recoveries of most target compounds in plant samples were relatively low and variable compared to the corrected recoveries. The corrected recovery was calculated as the amount detected, after correction with the corresponding isotopic standard surrogate

(*16*). The use of isotopic standards as recovery surrogates can offset the matrix effects in ionization, analyte loss during sample preparation, and variations in the instrumental response from injection to injection. The corrected recoveries of target PPCPs and hormones in the test plant samples were calculated to be in the range of 94~107%, indicating that the isotopic dilution method was able to provide good quality control in the simultaneous analysis of a broad range of compounds in a complicated matrix.

Table 2. Recoveries and Method LODs of the Target Compounds

Compound	*Absolute Recovery (%)*	*Corrected Recovery (%)*	*LOD (μg kg^{-1}, dw)*	*LOQ (μg kg^{-1}, dw)*
Caffeine	79 ± 6	102 ± 6	1.4	4.2
Carbamazepine	103 ± 7	106 ± 4	0.4	1.2
Naproxen	121 ± 18	102 ± 6	1.0	3.0
Ibuprofen	61 ± 2	99 ± 8	0.6	1.8
Gemfibrozil	88 ±17	99 ± 9	0.04	0.12
Triclosan	52 ± 8	93 ± 8	0.8	2.4
Sulfamethoxazole	70 ± 21	102 ± 3	0.08	0.24
Estrone	84 ± 9	98 ± 3	1.5	4.5
17β-Estradiol	91 ± 11	94 ± 7	1.9	5.7
17α-Ethinylestradiol	70 ± 9	101 ± 10	2.6	7.8

The study of the method limits of detection (LOD) was performed according to a previous report (*21*). Table 2 shows that the LODs for all target compounds were in the range of 0.04~2.6 μg kg^{-1} dry weight (dw) for the plant samples, which were similar to a previous study (*16*). The low LOD values suggest that this method can be used to detect the residues of target PPCPs and hormones in vegetables that may be impacted by the use of reclaimed water or contaminated water. Limits of quantification (LOQs) were defined as 3-fold LODs (Table 2). Reporting limits in the following study were chosen to be greater than or equal to LOQs.

PPCP and Hormone Uptake and Accumulation in Lettuce

Concentrations of PPCPs and steroid hormones in both leaves and roots of the lettuce are shown in Figure 2. All target compounds were detected in the lettuce roots that were grown in the nutrient solution enriched at an initial concentration of each compound at 50 μg L^{-1}. None of the target compounds were detected in the control lettuce plants that were only grown in the nutrient solution (data not shown). The concentrations of all compounds in the lettuce roots ranged from

215 to 28,985 μg kg^{-1} based on plant dry weight (dw). This result suggests that these emerging contaminants can be bound or taken up by lettuce roots. The pharmaceutical SMO was detected at the highest residual concentration in lettuce roots, which is similar to its uptake in cabbage plants (*15*).

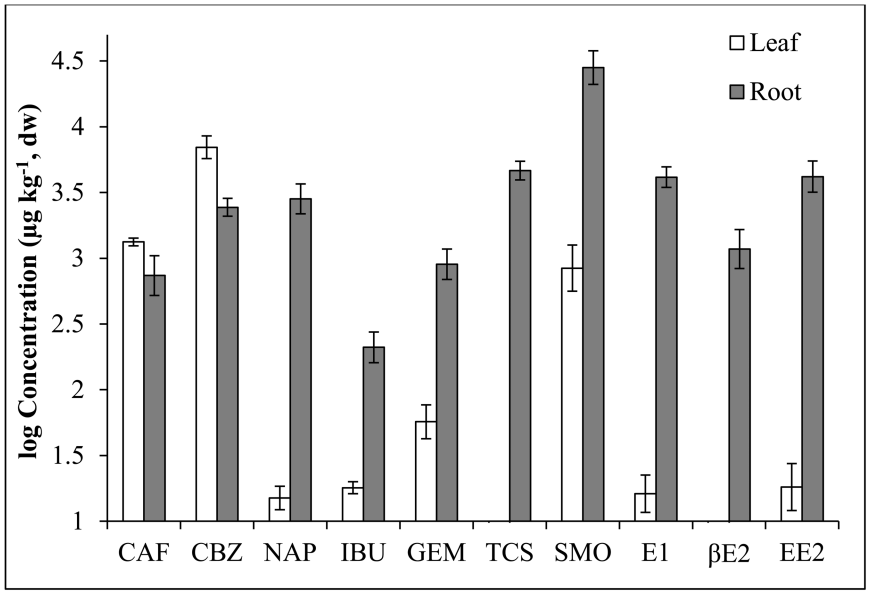

Figure 2. Concentrations of PPCP and hormone contaminants in leaves and roots of the lettuce grown hydroponically in nutrient solution containing each compound at 50 μg L^{-1}. Standard deviation of triplicate samples is shown as error bars.

Eight out of the ten compounds were detected in the lettuce leaves at concentrations ranging from 15.3 to 7,078 μg kg^{-1} dw (Figure 2). One PPCP (TCS) and one hormone (βE2) were not found in the lettuce leaves, indicating that these two compounds would be unlikely to accumulate in this leafy vegetable. Three PPCPs (CAF, CBZ, and SMO) had the highest concentrations accumulated in the lettuce leaves among all target PPCPs and hormones tested (Figure 2). Previous studies have shown that many factors impact the uptake and transfer of organic compounds within plants including chemical hydrophobicity and ionization (*15*, *17*, *22*). It has been reported that polar organic compounds have a great potential for root uptake and translocation (*23*, *24*). The high concentrations of these three PPCPs observed in the lettuce leaves may be attributed to their low log KOW values (< 3). More interestingly, two PPCPs (CAF and CBZ) were found at significantly higher concentrations in lettuce leaves than in roots (Figure 2). By contrast, the levels of the other target PPCPs and hormones in lettuce leaves were 1~2 orders of magnitude lower than those in roots (Figure 2). These results imply that CAF and CBZ can readily transfer from lettuce roots to leaves and accumulate in the edible portion of the vegetable. Previous studies reported

that significantly greater accumulation was observed for neutral compounds than anionic compounds in collard plants (*17*), since molecular ionization of organic compounds may reduce their ability to permeate cell membranes and thereby result in a reduced internal transfer potential (*22*). The high pK_a values of CAF and CBZ (Table 1) reflect the fact that these two PPCPs were less ionized in the nutrient solutions compared to other compounds, thereby facilitating their transfer from lettuce roots to leaves. Accordingly, CAF and CBZ accumulated to a greater extent in lettuce leaves rather than in roots.

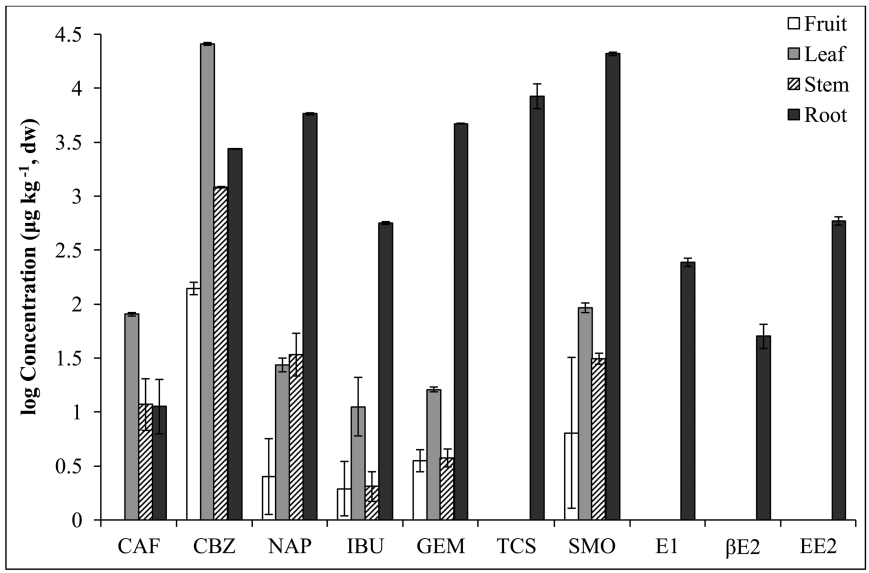

Figure 3. Concentrations of PPCP and hormone contaminants in roots, stems, leaves, and fruits of the tomatoes grown hydroponically in nutrient solution containing each compound at 50 µg L^{-1}. Standard deviation of triplicate samples is shown as error bars.

PPCP and Hormone Uptake and Accumulation in Tomatoes

The roots, stems, leaves, and fruits from tomato plants were analyzed separately to understand differential uptake, translocation, and accumulation of PPCPs and hormones in different parts of a plant. Figure 3 shows the concentrations (µg kg^{-1}, dw) of all PPCP and hormone compounds in the roots, stems, leaves, and fruits of tomatoes that were grown in the nutrient solution with each target compound at 50 µg L^{-1}. All target PPCPs were found in tomato roots. Six out of the seven PPCPs were detected in tomato leaves and stems. SMO (20,909 µg kg^{-1}, dw) was the PPCP detected at the highest concentration in the roots whereas CBZ (25,752 µg kg^{-1}, dw) had the highest concentration in the leaves. Similar to the results from lettuce experiments, the concentrations of CAF and CBZ in tomato leaves were higher than those in tomato roots and stems.

A previous report showed that CAF concentrations in macrophyte shoots were much higher than in its roots, suggesting this compound is likely to be taken up by plant roots and translocated to shoot tissues (*23*). Five target PPCPs (CBZ, NAP, IBU, SMO, and GEM) were detected in tomato fruits with concentrations ranging from 1.8 to 140 μg kg^{-1} (dw). Except for CBZ, the pKa values of the four other PPCPs detected in tomato fruits were between 4 and 6 (Table 1), which are favorable for phloem trapping and translocation of organic compounds in plants.

All three target hormones were detected in the tomato roots (Figure 3). Their concentrations in tomato roots ranged from 21.5 to 589 μg kg^{-1} (dw), much less than most of the PPCP concentrations in roots. No hormones were found in the stems, leaves, and fruits of the tomatoes. This may be attributed to a lack of systemicity for these hormone compounds in tomato plants, which may result in them only being bound to the surface of roots and not readily taken up into the plants. Thus, the accumulation of steroid hormones in tomato plants irrigated with hormone-containing water is unlikely and therefore would cause negligible contamination in tomato fruits. This finding is consistent with previous reports that hydrophobic chemicals (log K_{OW} > 3) cannot easily translocate within the plant (*23*, *24*).

Translocation and Bioaccumulation of PPCPs and Hormones in Vegetables

Translocation Factors (TFs) were calculated based on total PPCPs and hormones in above ground plant parts relative to their amounts in roots. Table 3 summarizes the TF values of all target compounds in the lettuce and tomatoes. TF values were not calculated for TCS and βE2 in lettuce and for TCS and all hormones in tomatoes because those compounds were not detected in any of the above ground plant parts. Except for CAF and CBZ, the calculated TF values of the other PPCPs and hormones were very small (<<1) in both test vegetables, suggesting poor translocation of these chemicals from roots to upper plant parts after uptake. Considering that these chemicals preferentially accumulate in plant roots as compared to above-ground parts, the potential risk for human consumption from those contaminants may be significantly greater for root vegetables such as radishes and carrots. All TF values for CAF and CBZ from lettuce and tomato plants were greater than 1 (Table 3), which further confirms that these two PPCPs can easily translocate from plant roots to leaves via water transpiration. A previous study illustrated that the translocation of non-ionized chemicals from plant roots into shoots is a passive process that occurs in proportion to the amount of water transpired (*25*).

Bioaccumulation Factors (BAFs) are defined as the ratio of detected concentrations (dw) of target compounds in the plant tissues to their initial concentrations in the nutrient solutions. Table 4 shows BAF values for the target PPCPs and hormones in each plant part. All BAF values for both lettuce and tomato roots were more than 1 with the exception of CAF in tomato roots, indicating that most of the target compounds have the potential to accumulate in plant roots. SMO had the highest BAF values for both lettuce roots (BAF=580) and tomato roots (BAF=418).

Table 3. TF Values of PPCPs and Hormones from Plant Roots to above Ground Parts

Compound	*Lettuce*	*Tomato*
Caffeine	1.73	7.46
Carbamazepine	2.88	9.85
Naproxen	0.0053	0.011
Ibuprofen	0.084	0.029
Gemfibrozil	0.064	0.0050
Triclosan	N/A	N/A
Sulfamethoxazole	0.030	0.0065
Estrone	0.0040	N/A
17β-Estradiol	N/A	N/A
17α-Ethinylestradiol	0.0045	N/A

Table 4. BAF Values of PPCPs and Hormones in Lettuce and Tomatoes

Compound	*Lettuce*		*Tomato*			
	Root	*Leaf*	*Root*	*Stem*	*Leaf*	*Fruit*
Caffeine	15.4	26.7	0.25	0.26	1.61	N/A
Carbamazepine	49.2	142	55.0	24.2	515	2.80
Naproxen	57.8	0.30	116	0.74	0.55	0.054
Ibuprofen	4.31	0.36	11.3	0.042	0.25	0.036
Gemfibrozil	18.5	1.18	93.7	0.076	0.32	0.072
Triclosan	93.7	N/A	173	N/A	N/A	N/A
Sulfamethoxazole	580	17.8	418	0.63	1.86	0.22
Estrone	83.6	0.34	4.89	N/A	N/A	N/A
17β-Estradiol	24.4	N/A	1.03	N/A	N/A	N/A
17α-Ethinylestradiol	85.6	0.39	11.8	N/A	N/A	N/A

The accumulation of PPCPs and hormones in the edible parts of the two vegetables varied. The BAF values of all compounds except CBZ (BAF=2.8) in tomato fruits were much less than 1. By contrast, three PPCPs had high BAF values for lettuce leaves. CBZ (BAF=142) showed the highest accumulation potential in the lettuce leaves, followed by CAF and SMO with BAFs of 26.7 and 17.8, respectively. This indicates that these three PPCPs are readily accumulated in lettuce leaves and may pose a potential risk to public health

through consumption of this leafy vegetable. Therefore, the potential occurrence of these PPCPs in leafy vegetables including lettuces, cabbages, and spinaches irrigated with reclaimed water needs to be preferentially investigated.

Currently, hydroponics is a growing area of commercial food production. Compared to soil cultivation systems irrigated with reclaimed water, the use of hydroponics utilizing reclaimed water may result in greater accumulations of PPCPs and hormones in vegetables, because contaminant sorption on soils can reduce their availability for plant uptake. However, the concentrations of emerging contaminants in bio-solids such as manure and STP sludge are usually much higher than those in reclaimed water. Land application of these bio-solids on food crops may result in an enhanced uptake and accumulation of PPCP and hormone contaminants in vegetables compared to irrigation with reclaimed water. The effects of different cultivation systems and management practices on accumulation of emerging contaminants in food crops need to be further evaluated.

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Chapter 10

The Use of Systemic Anthranilic Diamide and Neonicotinoid Seed Treatments in Rice Pest Management

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The rice water weevil, *Lissorhoptrus oryzophilus*, is the key insect pest of rice in the United States. Over the last five years, anthranilic diamide and neonicotinoid seed treatments have been introduced into the U.S. rice market for management of rice water weevils and other early and mid-season pests. This chapter reviews several aspects of the use of seed treatments in rice. In a four-year field study involving over 40 commercial fields throughout Louisiana, threshold densities of rice water weevil larvae were exceeded in over 80% of untreated fields. Seed treatments of thiamethoxam, a neonicotinoid, were about as effective as foliar applications of pyrethroids at reducing densities of rice water weevil larvae in commercial fields, while seed treatments containing chlorantraniliprole were significantly more effective than pyrethroids or thiamethoxam. A series of greenhouse studies demonstrated that seed treatments of chlorantraniliprole and thiamethoxam have differential effects on life stages of the rice water weevil. Seed treatment with thiamethoxam reduced survival of adults feeding on leaves of treated plants, and had pronounced effects on egg-laying and early instar survival; chlorantraniliprole seed treatment, in contrast, had no effects on adults and exerted its effects primarily on root-feeding larvae. These differences in biological activities were consistent with patterns of distribution of insecticides in tissues of treated plants: high concentrations of chlorantraniliprole were found

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in roots of both greenhouse and field-grown plants, while thiamethoxam concentrations were higher in above-ground portions of plants. The greater efficacy of chlorantraniliprole seed treatment in field experiments is probably attributable to the greater persistence of this chemical in rice plants and to the tendency of this chemical to accumulate in root tissues. Standard and reduced rates of chlorantraniliprole seed treatment were compatible with two alternative management practices, shallow flooding and plant resistance, in a small-plot field study. Chlorantraniliprole and neonicotinoid seed treatments had differential effects on other pests of rice, including the fall armyworm, *Spodoptera frugiperda* and sugarcane borer, *Diatrea saccharalis*. The ubiquity and severity of the rice water weevil as a pest, the effectiveness of seed treatments against both the rice water weevil and sporadic pests, and the lower impact of seed treatments on key non-target organisms provide solid justification for the use of seed treatments in rice.

Insect pests are major limiting factors in rice production world-wide (*1*). In the United States, the rice water weevil (RWW), *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), is the most destructive insect pest of rice (*2*) and is a key early season driver of pest management decisions. Adult RWWs feed on leaves of young rice plants, causing characteristic feeding scars parallel to the venation of leaves. Oviposition is triggered by the presence of standing water, and females deposit eggs primarily in submerged leaf sheaths (*3*, *4*). The incubation period of eggs is 5–9 days (*5*). Neonates may mine leaf sheaths or stems for a short period of time, but quickly move down and establish feeding sites on or in rice roots (*4*, *6*).The insect passes through four instars and pupates on roots in 27–30 days (*7*). Severe root pruning can result in poor crop stand and reduced tillering at vegetative stage of crop growth and reduced panicle size and grain weight at crop maturity, thus causing economic losses to the crop (*8*). Over the past several years, the RWW has invaded other important rice-producing regions of the world, including Asia and Europe, and now poses a global threat to rice production (*9*).

Historically, the RWW has been managed primarily through the use of insecticides. In the 1960s, seed treatments of the organochlorine aldrin were widely used against RWW, but weevils developed resistance in the latter half of the decade (*10*). Beginning in the 1970s, the only insecticide available for RWW management was a granular insecticide, Furadan (AI: carbofuran, a carbamate insecticide), that was applied to flooded soils several weeks after flooding of rice fields to kill root-feeding RWW larvae. However, carbofuran is highly toxic to birds and its use in rice in the U.S. was disallowed by the Environmental Protection Agency in the mid-1990s. Following this regulatory action, several new insecticides were registered for use against the RWW, including an insecticidal seed treatment, Icon (AI: fipronil, a phenyl pyrazole insecticide), which targeted RWW larvae, and several foliar pyrethroids, including Karate

Z (lambda-cyhalothrin) and Mustang Max (zeta-cypermethrin), which targeted adult weevils. These new insecticides were quickly adopted by rice farmers and gave control equal to or better than that formerly given by Furadan. However, the introduction of Icon coincided in 1999 and 2000 with sharp declines in crawfish production in southwest Louisiana and a class action lawsuit was brought against the manufacturer of Icon alleging that Icon had contributed to crawfish kills. A $45 million settlement was reached in 2004 with crawfish producers, and Icon was withdrawn from the U.S. rice market in the same year. This lawsuit has heightened awareness of the need to integrate practices for RWW management with crawfish production.

For most of the decade following the removal of Icon from the market, RWW and other pests were managed through foliar applications of pyrethroids. The use of pyrethroids for RWW management in rice, however, has several limitations. Widespread use of pyrethroids raises concerns about persistence in soils and non-target effects on invertebrates, particularly crayfish (*Procambarus clarkii*), and fish (*11–14*). In addition to these environmental concerns, foliar applications of pyrethroids are ineffective on root- feeding larvae. Moreover, the short residual activity of pyrethroids may necessitate multiple applications to achieve adequate control of this insect (*15*). Finally, heavy use of a single class of insecticide is an unwise strategy due to insecticide resistance issues. Therefore, alternatives to pyrethroids were sought for rice pest management in the southern United States.

Since 2008, three insecticidal seed treatments, Dermacor X-100™, which contains the anthranilic diamide insecticide, chlorantraniliprole (CAP), CruiserMaxx™, which contains the neonicotinoid, thiamethoxam (TMX), and NipsitInside™, which contains another neonicotinoid, clothianidin (CLO), have been introduced for use in rice against RWW and other pests. The binding of CAP at ryanodine receptors causes uncontrolled release of calcium ions from sarcoplasmic reticulum of muscle cells resulting in paralysis in insects (*16*). The high selectivity of CAP toward insects has been attributed to its high affinity (ranging from ~300-fold to >2000-fold) for ryanodine receptors of insects relative to those of mammals (*17*, *18*). This insecticide has been developed world-wide in several crops to control a range of pests belonging to the Order Lepidoptera and some species of Coleoptera and Diptera (*19*). Neonicotinoids are selective insecticides that interfere with nervous transmission in insect nervous systems, and exhibit stronger affinity for insect than mammalian nicotinic acetylcholine receptors (*20*, *21*). Neonicotinoids provide excellent control of a wide range of sucking and chewing insects belonging to orders Hemiptera, Thysanoptera, Coleoptera, Lepidoptera and Diptera (*22*, *23*).

In addition to the RWW, a number of other pests can attack rice in the United States during the seedling, vegetative and early reproductive phases of rice development. The importance of these pests varies regionally. In Arkansas, for example, *Colaspis* sp. can be important pests of rice seedlings and can severely reduce early season stands (*24*). In Louisiana and Texas, several Lepidopterans are important, including the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), and the sugarcane borer, *Diatraea saccharalis* (Lepidoptera: Crambidae). While these pests are not consistently important constraints on yield,

the threat of these sporadic pests can influence pest management decisions, and in particular the choice of insecticides.

Insecticidal seed treatments have been widely adopted since their introduction into the rice market in 2008. A recent survey of grower practices for RWW management in rice in Louisiana and Texas revealed that over 65% of producers and consultants used seed treatments on one or more of the fields for which they were responsible (*25*), and adoption rates are similarly high in Mississippi and Arkansas (*26*, *27*). However, indiscriminate and widespread use of seed treatments create concerns about environmental and resistance management issues.

This chapter describes a series of field and greenhouse studies investigating the use of prophylactic seed treatments to manage the RWW and other early and mid-season insect pests in U.S. rice. The initial section of the chapter summarizes a study conducted on commercial rice farms throughout Louisiana to compare the efficacies of registered seed treatments and foliar pyrethroids against the RWW. Next, a set of greenhouse studies is described in which the biological activities of TMX and CAP seed treatments on various life stages of the RWW were characterized, and these biological activities were compared to the patterns of insecticide distribution in plants. The final section describes small-plot field and greenhouse experiments designed to investigate the potential role of seed treatments as a component of an integrated management program in U.S. rice.

Is the Use of Insecticidal Seed Treatments a Rational Component of an Integrated Pest Management Program in Rice?

Seed treatment insecticides offer many advantages, most notably, from a grower's perspective, ease of use. Small-plot evaluations of seed treatments conducted before their registration indicated that they were as or more effective than pyrethroids against the RWW (*28*). However, seed treatments are generally more expensive than foliar insecticides, and prophylactic applications of insecticides may not be warranted when pest densities are low. Moreover, widespread use of seed treatments raises issues related to environmental impact. Thus, the question of whether the use of seed treatments justifies their environmental and economic costs is an important one.

To partially address this question, a four-year study involving 41 commercial field sites in 15 parishes across Louisiana was conducted to compare the effectiveness of seed treatments and foliar pyrethroid treatments against RWW (*29*). All field sites included untreated areas that allowed an assessment of the extent and severity of RWW infestations. Larvae were sampled by taking multiple root/soil core samples from each field site three or four weeks after establishment of permanent flood and counting the larvae and pupae associated with roots in cores. Data from these samples are shown in Table 1. In untreated areas, mean weevil densities averaged over 11 larvae per core sample across all fields and years of the study, a density well above the threshold used previously to trigger Furadan applications in flooded fields (three larvae per core sample).

Furthermore, the threshold value of three larvae per core was exceeded in over 80% of untreated areas. Although all insecticide treatments suppressed weevil populations, the effectiveness of insecticide treatments differed (Table 1). In particular, CAP seed treatments were consistently more effective than TMX seed treatments and pyrethroid sprays. In 2010, for example, when CAP, TMX and pyrethroid treatments were evaluated simultaneously, weevil suppression was greatest in CAP-treated plots (84%), followed by pyrethroid (62%) and TMX treatments (50%). These data indicate that seed treatments are generally justified in Louisiana by the severity of the weevil problem and the effectiveness of the tactic.

Table 1. Densities of RWW Larvae and Pupae in Commercial Rice Fields Treated with Insecticides or Left Untreated

Treatments[a]	*Mean number ± SEM of immature weevils*			
	2008	*2009*	*2010*	*2011*[b]
Untreated	11.7 ± 1.1	11.4 ± 0.9	8.4 ± 1.5	12.9 ± 2.9
Pyrethroid	5.1 ± 2.1	2.6 ± 0.9	3.1 ± 1.8	-
CAP	1.7 ± 1.1	0.6 ± 0.9	1.3 ± 1.4	2.6 ± 2.9
TMX			4.2 ± 1.4	7.9 ± 2.9
CLO				7.9 ± 2.9

[a] Pyrethroid treatment consisted of early post-flood foliar applications of lambda-cyhalothrin; the remaining insecticides were applied as seed treatments. [b] Only seed treatments were evaluated in 2011. (Reproduced with permission from reference (*29*).) (Copyright 2014 xx.)

Biological Activities of Seed Treatments on RWW Life Stages and Their Relation to Patterns of Distribution of Active Ingredients in Rice Plants

All life stages of the RWW feed on, or are otherwise associated with, rice plants. Thus, systemic insecticides applied to rice as seed treatments could affect multiple life stages. The differences in efficacies of CAP and TMX observed in small-plot experiments and commercial rice fields could be due to differences in the persistence or potency of active ingredients against multiple life stages of this insect. These differences could arise, in turn, from the distinct physical and chemical properties of the active ingredients and the resultant differences in their systemic distributions in rice plants. A series of greenhouse and laboratory experiments were conducted to investigate the impact of CAP and TMX seed treatments on survival, feeding and egg-laying by adults, and on survival of first and late instars (*30*). Biological impacts on weevils were also related to the distribution of active ingredients in plants.

Impacts on Adult Survival and Foliar Feeding

As adult weevils use rice foliage as a food source, the impact of feeding on adults was investigated by using foliage from plants treated as seeds with insecticides at different rates (CAP: 0-100 µg AI/seed; TMX: 0-35 µg AI/seed) (*31*). Plants at the 5-6 leaf stage (approximately four weeks after planting) were used for foliar assays. The two top leaves of plants were excised and basal (cut) portions of leaves were inserted in Petri dishes lined with agar (1.5%). Field-collected adult RWWs were released in Petri dishes and allowed to feed for 72 h. Leaves were replaced daily with leaves from fresh plants, and adult mortality and feeding activity (scarring) were determined daily. Cumulative adult mortalities and rates of foliar consumption in CAP and TMX treatments are shown in Table 2.

Table 2. Comparison of Adult Rice Water Weevil Mortality ± SE and Feeding Activity ± SE on Foliage of Plants Treated as Seeds with CAP or TMX

Seed treatment	*Rate(µg AI/seed)*	*% mortality*[a]	*Leaf consumption rate ($mm^2 weevil^{-1}\ day^{-1}$)*
CAP	0	15.0 ± 2.9	8.3 ± 0.7
	10	10.0 ± 5.8	8.3 ± 0.6
	25	20.0 ± 4.1	6.7 ± 0.9
	50	12.5 ± 6.3	7.3 ± 0.9
	100	16.3 ± 4.9	9.4 ± 0.7
TMX	0	3.8 ± 1.8 a	8.3 ± 0.5 a
	7	22.5 ± 4.5 b	6.9 ± 0.3 a
	14	21.3 ± 3.5 a	6.6 ± 0.6 b
	21	28.8 ± 3.0 b	5.5 ± 0.8 b
	28	42.5 ± 6.4 b	5.2 ± 0.3 b
	35	45.0 ±7.7 b	6.4 ± 0.5 b

[a] Mortality was assessed after 72 h of feeding and was defined as a lack of mobility for 10 min after being prodded with a camel hair brush (*31*).

Adult feeding on foliage from plants treated as seeds with CAP affected neither adult survival nor foliar consumption rates; in contrast, seed treatment with TMX resulted in mortality of adult weevils and affected foliar consumption rates. Adult mortalities generally increased with the rate of TMX applied to seeds.

Lethal Doses of TMX Based on Consumption of Active Ingredients in Foliage

Next, the relationship between actual levels of TMX consumed by adult weevils (oral doses) and adult mortalities was quantified. Oral doses of insecticides were estimated by combining estimates of leaf biomass removed by adult weevils during feeding with estimates of foliar concentrations of TMX as determined by LC/MS/MS analyses. The rates of TMX used in these experiments ranged from 0 to 63 μg AI/seed, and the experiment was conducted with greenhouse-grown plants at both the 2-3 leaf stage and the 3-4 leaf stage. For bioassays, excised leaves were inserted in a petri dish and 40 weevils were released per dish and allowed to feed for one hour, at which time mortality was assessed. The criterion used to score mortality in these experiments was the inability of weevils to right themselves in ten minutes after placing them on their backs. Leaf samples from separate plants of the same age treated at the same rates were collected and concentrations of TMX and CLO (a metabolic product of TMX) in leaves were determined using an LC/MS/MS analytical method as described previously (*31*). After mortality assessment, damaged leaves were scanned and areas of feeding scars were measured by an image analysis software. Oral doses were estimated by using the values for insecticide residues in leaves and estimates of ingested leaf biomass. The relationship between area of leaf scars and mass of leaf tissue consumed was determined earlier by conducting feeding assays on leaves of untreated plants as described previously (*31*). The relationship between the dose and mean weevil mortality was determined by using probit analysis as described by Finney (*32*). Thus the bioassay method described here used biologically relevant mode of exposure to characterize acute toxicity of systemic insecticide in foliage.

Weevil mortality was dose dependent at both 2-3 and 3-4 leaf stages of rice plants (Figure 1). The LD_{50} for weevils feeding on 2-3 leaf stage plants was 447 pg TMX+ CLO/weevil (95% fiducial limits = 25-830 pg/weevil; slope = 1.5 ± 0.5) but was lower (142 pg/weevil; 95% fiducial limits: 102-180; slope = 1.86 ± 0.23) in experiments with 3-4 leaf stage plants. To our knowledge, these LD_{50} s are the first LD_{50} s for leaf feeding insects on foliage of plants treated as seeds with TMX. Leaf consumption (Figure 1, inset) in these bioassays decreased with increasing foliar concentration of TMX for both 2-3 leaf ($F_{1,4} = 14.9$, $P = 0.02$; slope = -24.3 ± 6.3) and 3-4 leaf stage plants ($F_{1,5} = 7.1$, $P = 0.04$; slope = -26.2 ± 9.8).

The overlapping confidence intervals of the LD_{50} estimates from these assays preclude definitive statements about the relative potencies of TMX at 2-3 and 3-4 leaf stages. Variability in estimates of acute toxicity is not surprising based on factors such as variation in location of adult weevil feeding sites, direct effects of TMX on feeding behavior, and non-uniform distribution of insecticides in leaves. With respect to the latter factor, higher accumulation of TMX in the tips of rice leaves than at basal regions of leaves after foliar treatment has been reported (*22*). Similarly, in cotton treated as seeds with imidacloprid, insecticide concentrations were higher at apical portions of leaf blades than in central portions (*33*).

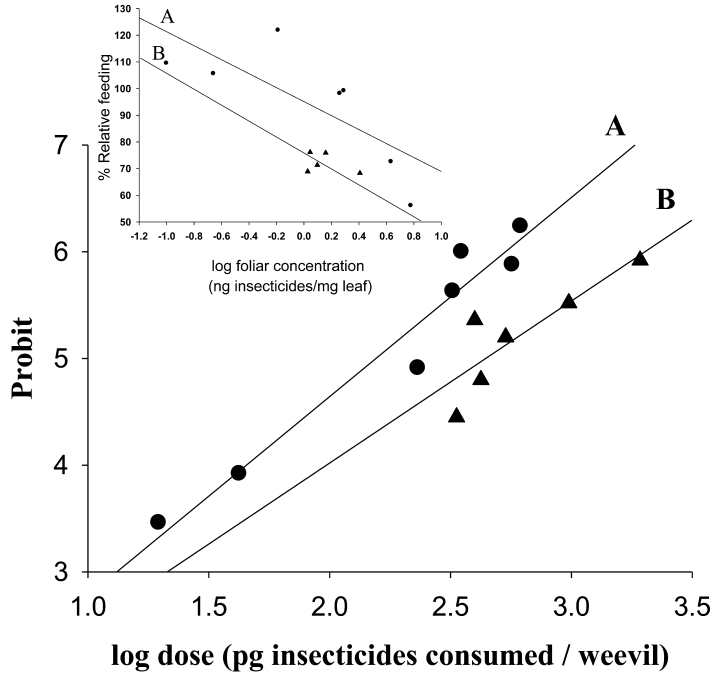

Figure 1. Dose responses of mortality and feeding activity (inset) in adult RWWs after seed treatments with TMX in rice as measured in 1h feeding assays conducted with plants at the 3-4 (A) or 2-3 (B) leaf stage. Doses of TMX + CLO per weevil (pg) were estimated based on quantification of leaf mass consumed (mg) and on LC/MS/MS analysis of residues (ng insecticides / mg leaf). Feeding damage was assessed by digital scanning and image analysis and is expressed as percent relative feeding = [(feeding in treatment/feeding on control) X 100]. (Reproduced with permission from reference (31).) (Copyright 2012 Society of Chemical Industry.)

Plant Growth-Related Dilution of Adulticidal Activity of TMX

Because thiamethoxam is highly systemic in plant leaves [22], dilution in adulticidal activity is expected as plant above-ground biomass increases. The effects of plant stage on the adulticidal activity of TMX were investigated by comparing mortalities of adult weevils fed on leaves from greenhouse-grown plants at different growth stages (5-6 leaf, approximately 4 w old, and 4-5 tiller stages, approximately 8 w old). Four-day feeding assays were conducted using the youngest (top two) leaves of rice plants treated as seeds with rates of TMX ranging from 0 to 63 µg AI/seed. Three replicate assays were conducted over consecutive days using foliage from different plants and using separate collections

of weevils. For each treatment rate, forty weevils were released per dish on each day. Levels of TMX in rice leaves (fresh leaves from separate plants at the same stages and treated with the same rates) were analyzed using a competitive ELISA adapted from that described earlier for the quantification of imidacloprid in avocado leaves (*34*, *35*).

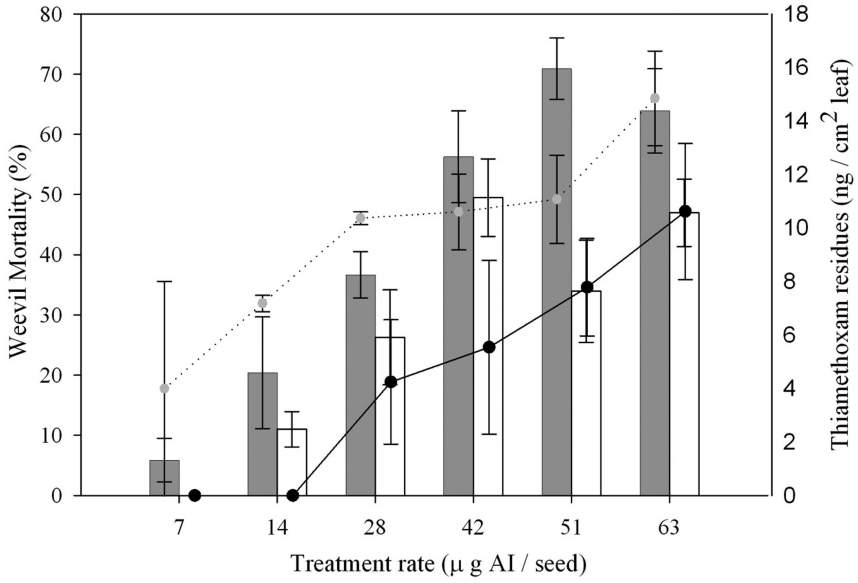

Figure 2. Effects of plant stage on adult mortality from TMX seed treatment. Mortalities were recorded 96-h after weevil release on excised foliage from plants at the 5-6 leaf stage or tillering stage . Leaves were analyzed for residues of TMX by ELISA. TMX was measured in 5-6 leaf and tillering stage plants. (Reproduced with permission from reference (31).) (Copyright 2012 Society of Chemical Industry.)

Adult mortalities following leaf feeding were higher in younger than older rice plants, suggesting a plant age-related decline in adulticidal activity of TMX. Adult mortalities resulting from feeding on foliage generally increased with increasing seed treatment rate (Figure 2). Mortalities on leaves from 5-6 leaf stage plants ranged from 6-64%, depending on seed treatment rate, whereas mortalities on leaves from tillering stage plants ranged from 0-47%. For weevils on leaves from 5-6 leaf stage plants, mortality increased with seed treatment rate between 7 and 51 µg ai/seed, although no marked increase in mortalities were measured at rates > 28 µg ai/seed. On the other hand, for weevils on leaves from tillering plants, no mortality was observed on treatments at 7 µg ai/seed, and mortalities on leaves from plants treated at 42, 51 and 63 µg ai/seed differed from mortalites on leaves from the 14 µg ai/seed treatment. Consistent with differences in weevil mortalities

at different treatment rates, TMX residues measured by ELISA increased with seed treatment rate and differed between plant stages. No residues were measured at the rates of 7 and 14μg ai/seed in the foliage of tillering stage plants. Similar age-related declines in TMX activity have also been found in snapbeans against *Empoasca fabae* immatures (*36*), in avocado trees against *Scirtothrips perseae* (*35*), and in soybeans against *Aphis glycines* (*37*). Plant growth-related dilution in TMX levels and activities may be one explanation for lower field efficacies of TMX-treated plants in commercial fields.

Impact of Seed Treatments on the Egg-Laying and First Instar Survival of RWW

As weevils feed on leaves and uses leaf sheaths as substrates for oviposition and early instar feeding, exposure of adults to CAP- or TMX-treated plants could have various consequences for egg-laying and first instar mortailty. To determine the impact of CAP and TMX treatments on abundance of eggs and first instars, plants treated as seeds with different rates of CAP (0, 10 and 25 μg AI/seed) and TMX (0, 21 and 28 μg AI/seed) were infested with mating pairs of adult RWWs for 4 days following flooding of the 5-6 leaf-stage plants in a greenhouse. Pots with plants treated at single treatment rate for each chemical were placed in cages. Mating pairs of weevils were released in cages at a density of one pair per plant and allowed to feed, mate and oviposit for four days under flooded conditions in basins lined with plastic. Water was drained from basins and adult weevils were removed from plants. The impacts of seed treatment with CAP and TMX on egg numbers and first instars were determined by sampling half of the plants from each cage for eggs and the other half for first instars. For egg counts, plants were bleached in alcohol and eggs in leaf sheaths were counted under dissection microscope. For first instars, larval emergence was monitored from plants incubated in a growth chamber in test tubes containing water. To do this, entire plants were removed from soil and placed in a growth chamber in test tubes filled with water. First instars emerging from these plants sink to the bottom of tubes and can be counted until no futher emergence was found for three consecutive days.

Both egg numbers and first instar emergence were reduced on plants treated with CAP and TMX (Figure 3). The reduction in egg densities on CAP-treated plants relative to untreated plants was 40-50% and a similar reduction was seen in first instar densities. On TMX-treated plants, the magnitude of suppression of egg and larval densities was up to 90%.

Reductions in numbers of eggs on treated plants could be the consequence of three mechanisms. First, reductions in egg numbers could have resulted from mortality of adults before they oviposited. This mechanism likely provides a partial explanation for reduced egg numbers on TMX-treated but not on CAP-treated plants (Table 2). Second, oviposition may have been directly deterred by the presence of insecticides in above-ground tissues. Finally, ingestion of sub-lethal amounts of insecticide by adults while feeding on leaf tissue of treated plants may have resulted in a “toxicant-induced malaise”. These insecticides have been reported previously to induce oviposition dysfunction in insects (*39*, *40*).

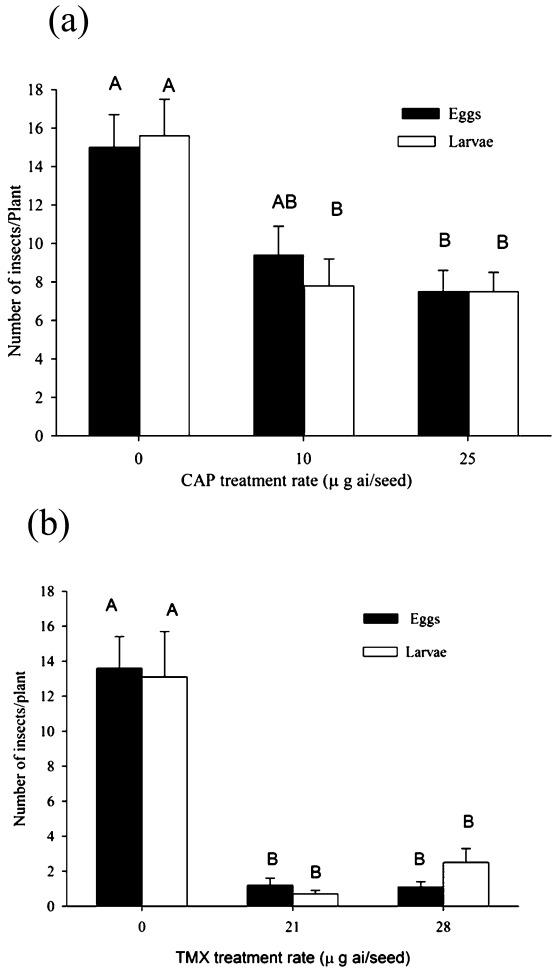

Figure 3. Densities of eggs (closed bars; eggs/plant ± SE) and first instars (open bars; larvae/plant ± SE) on plants treated as seeds with chlorantraniliprole (CAP; Figure A) and thiamethoxam (TMX; Figure B). Bars accompanied by the same letter indicate means not significantly different from one another. (Reproduced with permission from reference (38).) (Copyright 1976 Entomological Society of America.)

To more directly test the latter hypothesis - that ingestion of sub-lethal levels of insecticide by foliage-feeding adults reduces subsequent egg-laying on untreated plants - a two-stage protocol was adopted. In the first stage, adult weevils were allowed to feed for four days in Petri plates on excised foliage from plants treated or untreated as seeds with CAP or TMX. In the second stage, apparently healthy weevils from the first stage (i.e., weevils displaying coordinated movement of legs within five min after being placed on their dorsum on a flat surface) were released in infestation cages on plants untreated or treated as seeds with the two insecticides.

The rates used for foliar exposure to CAP were 0 (untreated), 25 and 50 µg [AI]/seed (i.e., approximately 1 and 2 times the field rate, respectively), and for TMX the rates used were 0 and 7 µg [AI]/seed (approximately 4 times lower than the field rate). At this rate of TMX treatment, adult mortality was minimal (Table 2). Regimes of exposure to CAP and TMX are summarized in Table 3.

Table 3. Exposure Regimes Used to Test the 'Toxicant-Induced Malaise' Hypothesis. Adult Weevils Were Initially Exposed for Four Days to Excised Foliage from Plants Treated as Seeds with Various Rates of CAP or TMX, Then Released on Whole Plants Treated as Seeds with Varying Rates of CAP or TMX. The Impact of Exposure Regime on Egg Numbers and Survival of First Instars in Plants Was Then Assessed.

Chemical	*Foliar Feeding (µg AI/seed)*	*Whole-plant infestation (µg AI/seed)*	*Treatment designation*[a]	*Regime of Exposure*[b]
CAP	25	0	25-0	Foliar feeding
	50	0	50-0	
	0	25	0-25	Whole plant
	0	50	0-50	
	0	0	0-0[c]	No Exposure
TMX	7	0	0-7	Foliar feeding
	0	7	7-0	Whole plant
	0	0	0-0	No exposure[b]

[a] The pre-hyphenated term indicates the seed treatment rate used in foliar feeding period and the post-hyphenated term is the seed treatment rate used for infestation. [b] The "foliar feeding" regime consisted of initial exposure of weevil adults to excised foliage from treated plants and subsequent release of weevils on untreated plants; the "whole plant" exposure regime consisted of initial exposure of weevil to excised foliage from untreated plants and subsequent release on treated plants. (Reproduced with permission from reference (*38*).) (Copyright 1976 Entomological Society of America.)

The exposure regime in which weevil adults were exposed to excised foliage from CAP- or TMX-treated plants before release on untreated plants ("foliar feeding" regime in Table 3) was intended to determine the impact of sub-lethal feeding exposure to insecticides in adults on the number of eggs laid and resulting first instars. Numbers of eggs and first instar weevils resulting from 'foliar feeding exposure' regimes were contrasted with those resulting from the exposure regime termed 'whole plant exposure', in which weevil adults were initially exposed to foliage from untreated plants, then released on plants treated as seeds with CAP or TMX (see Table 3). This contrast between the regimes of exposure was done

to distinguish toxicant-induced malaise from direct effects of CAP and TMX on oviposition and first instar survival. An overall control regime was included in experiments in which weevils were both fed on foliage from untreated plants and then released on untreated plants.

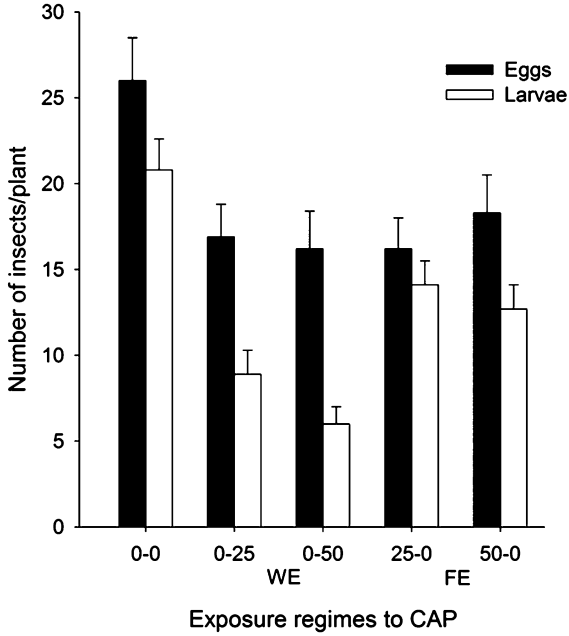

Figure 4. Impact of different CAP exposure regimes (Table 3) on densities of eggs (closed bars; eggs/plant ± SE) and first instars (open bars; larvae/plant ± SE). For "Whole-plant exposure" regimes (WE), weevil adults were fed foliage from plants not treated as seeds (0 µg AI/seed) and then used to infest plants treated as seeds with 25 or 50 µg AI/seed (0-25 or 0-50). For "foliar feeding exposure" regimes (FE), weevil adults were fed on foliage from plants treated as seeds (25 or 50 µg AI/seed) and then provided with untreated plants (0). (Reproduced with permission from reference (38).) (Copyright 1976 Entomological Society of America.)

In the foliage-feeding pre-exposure period, adult feeding on leaf material from CAP-treated plants did not impact mortality or foliar consumption. Interestingly, however, ingestion of foliage from CAP-treated plants by adults significantly reduced subsequent egg-laying and first instar emergence when untreated plants were infested with these adults (compare treatments 25-0 and 50-0 with treatment 0-0 in Figure 4). The magnitude of reduction in egg numbers in these latter treatments was similar to the reduction observed in weevils exposed directly to CAP-treated plants (compare treatments 25-0 and 50-0 and treatments 0-25 and 0-50). However, significantly fewer larvae emerged from plants when

weevils were directly exposed to CAP-treated plants without prior exposure to CAP than when weevils were pre-exposed to CAP, then exposed to untreated plants (treatments 0-25 and 0-50 versus treatments 25-0 and 50-0), suggesting larvicidal activity in CAP-treated plants.

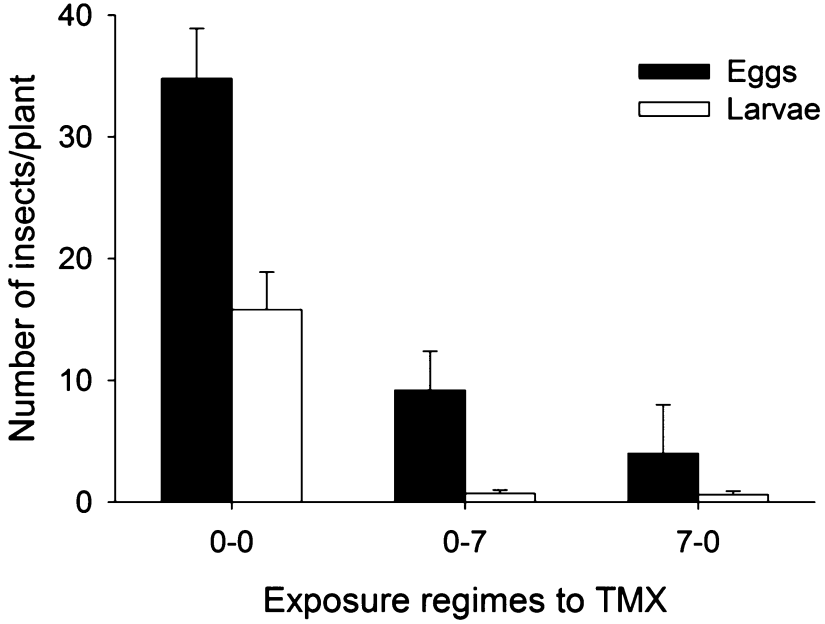

Figure 5. Impact of exposure regimes to TMX (Table 3) on densities of eggs (closed bars; eggs/plant ± SE) and first instars (open bars; larvae/plant ± SE). For "Whole-plant exposure"regime (WE), weevil adults were fed foliage from plants not treated as seeds (0 µg AI/seed) and then used to infest plants treated as seeds with 7 µg AI/seed (0-7). For "foliar feeding exposure" regime (FE), weevil adults were fed on foliage from plants treated as seeds (7 µg AI/seed) and then provided with untreated plants (0). (Reproduced with permission from reference (38).) (Copyright 1976 Entomological Society of America.)

In experiments with different TMX exposure regimes (Table 3), exposure of adult weevils to foliage from TMX-treated plants reduced survival of adults by 17% but did not impact foliar consumption (data not shown). In addition, prior ingestion of foliage containing TMX reduced egg numbers and first instar emergence when adult weevils were used to infest untreated plants (Figure 5). Ingestion of thiamethoxam by adults during the pre-exposure period reduced egg-laying and first instar emergence on untreated plants by 89% and 96%, respectively, when compared to weevils not exposed to thiamethoxam (treatment 7-0 versus treatment 0-0). In addition, as previously observed, adult weevils

without prior exposure to TMX laid fewer eggs on TMX-treated plants than on untreated plants (treatment 0-7 versus 0-0). Significantly fewer first instars emerged from TMX-treated plants than from untreated plants when infested with weevils without prior exposure to TMX.

There was no difference in egg deposition between unexposed weevils placed directly on TMX-treated plants and weevils pre-exposed to TMX placed on untreated plants. Egg deposition in the treatment 0-7 was reduced by 74% and the reduction was 89% in the 7-0 treatment). First instar emergence was reduced by 96% in both regimes. Thus in experiments with TMX, evidence for ovicidal or larvicidal effects was equivocal due to low egg numbers.

The fact that exposure of weevils to insecticides through foliar feeding resulted in reduced egg numbers is consistent with the toxicant-induced malaise hypothesis. However, this result does not exclude the possibility of direct deterrence of weevil oviposition on treated plants due to the presence of insecticides in leaf tissue. Further studies on feeding behavior and other behaviors on whole plants are required to disentangle the relative roles of oviposition deterrence and toxicant-induced malaise. This study is the first study to show an impact of adult exposure to CAP and TMX seed treatments on egg numbers in Coleoptera. Disruption of mating behavior and reduced fertility in adult insects following exposure to CAP has been previously reported in Lepidoptera (*41*) and Diptera (*39*). In addition to demonstrating reduction in egg numbers following ingestion of TMX and CAP by adults, the pre-exposure experiment provides evidence for egg and/or early-instar mortality of rice water weevils in CAP-treated plants. Effective control of annual bluegrass weevil, *Listronotus maculicollis* Kirby (Coleoptera: Curculionidae) is thought to depend on systemic effects of insecticide on young larvae as they begin to chew their way into the stem of annual bluegrass plant, *Poa annua* L. treated with Acelepryn® (chlorantraniliprole: 20%) (*42*).

Activity of TMX and CAP on RWW Life Stages As Affected by Insecticide Distribution

In the greenhouse experiments described above, the impacts of TMX seed treatment on adult RWW survival, egg-laying, and first instar emergence were similar to or greater than the impacts of CAP seed treatment. This contrasts sharply with results of field trials showing CAP to be more effective at reducing population densities of RWW. The inconsistencies between greenhouse and field results suggested that insecticide activity against later instars was an important factor in determining field efficacy. To further investigate the larvicidal activity of seed treatments, plants treated as seeds at different rates of TMX and CAP were infested with adult weevils and different life stages of RWW (i.e., adults, eggs, first and late instars) were sequentially sampled. The impacts of seed treatments were investigated at different seed treatment rates (CAP: 0, 5 and 50 µg AI/seed; TMX: 0, 14 and 28 µg AI/seed). In addition, the activities of each seed treatment on life stages of RWW were related to insecticide concentrations in different parts of rice plants.

Adult weevils were caged on individual pots containing 6 plants each for four days after which time numbers of surviving adults on plants were counted and weevils removed. Plants were then sampled at appropriate time points to monitor for numbers of eggs laid in leaf sheaths, first instars emerging from plants, and late instars on roots. To enable correlations of biological activities with insecticide distributions, separate plants treated with TMX or CAP at the same rates were used for chemical analyses. Plant material from each pot was separated into three portions - foliage (leaf blades), shoots (leaf sheaths and stems) and roots- and chemical analysis of TMX and CAP residues was conducted for each portion as explained previously (*43*).

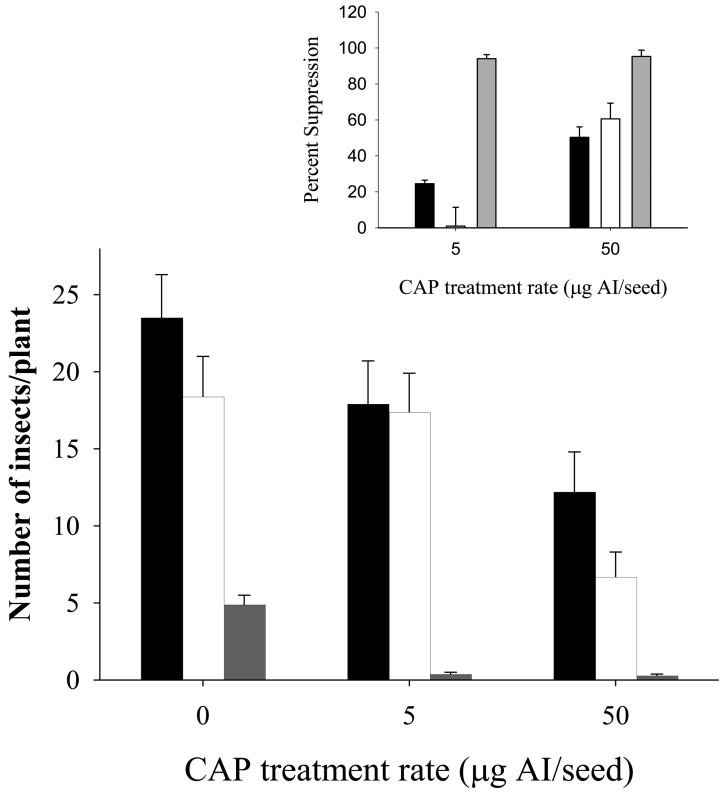

*Figure 6. Impact of seed treatment with CAP on life stages of RWW. Egg densities (closed bars; eggs per plant), first instar emergence (open bars; larvae per plant) and late larval abundance (gray bars; larvae per plant) are shown following infestation of whole plants in a greenhouse. The inset shows the impact on life stages as expressed by percent reduction in the density of each life stage relative to controls [(density in control-density in treatment/density in control)*100]. (Reproduced with permission from reference (43).) (Copyright 2012 Society of Chemical Industry.)*

In this experiment, the natural decline in densities of different weevil life stages on untreated plants from the egg stage to the first instar stage was approximately 22%, and the decline from first to late instars was approximately 74%. This decline was differentially altered by the two seed treatments. Seed treatment with CAP had no effect on adult survival (data not shown), but numbers of eggs laid by weevils on CAP-treated plants were reduced (Figure 6). Fewer first instars emerged from plants treated at 50 µg AI/seed (61% reduction; Figure 6 inset) than from untreated or plants treated with 5 µg AI/seed. At 5 µg AI/seed, the percent relative reduction in first instar emergence was 2.4%. Significantly, at both treatment rates, densities of late instars were reduced by 90%.

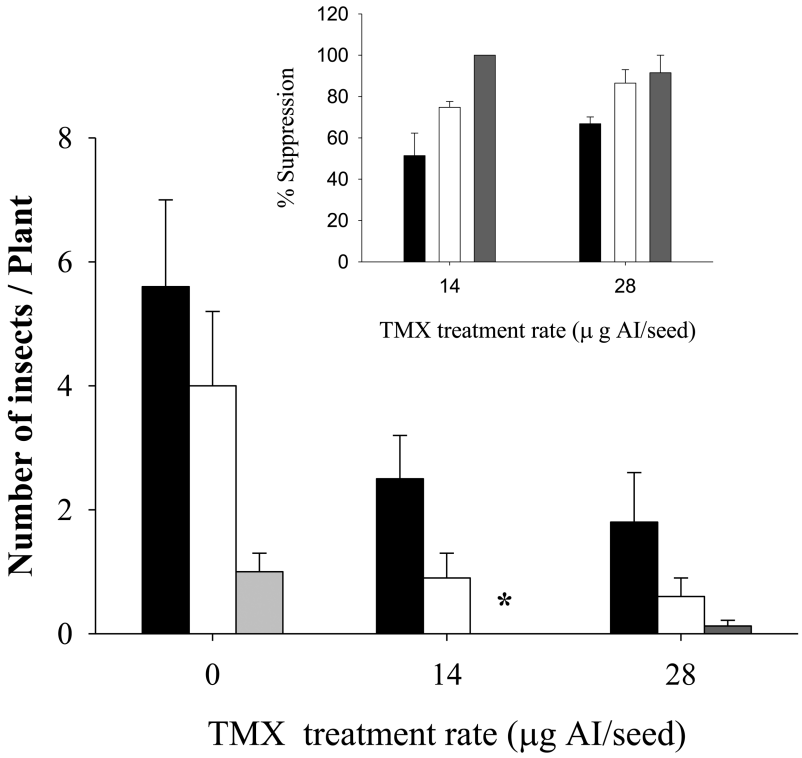

Figure 7. The impact of seed treatment with TMX on life stages of rice water weevil. Egg densities (closed bars; eggs per plant), first instar emergence (open bars; larvae per plant) and late larval abundance (gray bars; larvae per plant) following infestation of whole plants in a greenhouse. The inset shows the impact on life stages as expressed by percent reduction in the density of each life stage relative to controls [(density in control-density in treatment/density in control) X 100]. indicates no larval recovery at 14 µg AI/seed. (Reproduced with permission from (43).) (Copyright 2012 Society of Chemical Industry.)*

Seed treatment with TMX, in contrast, significantly reduced adult survival as evidenced by the lower recovery of adult weevils after four days of infestation in cages (data not shown). Numbers of eggs laid by weevils was also reduced, with reductions of 52 and 67% at treatment rates of 14 and 28 µg AI/seed, respectively (Figure 7 inset). First-instar emergence from plants was also adversely affected, with reductions of 75 and 80% at 14 and 28 µg AI/seed respectively. Finally, the mean number of late instars recovered from untreated plants was very low (1.0±0.3) (Figure 7); only three larvae were recovered from 28 µg AI/seed, and none from 14 µg AI/seed.

Thus, for CAP, the greatest reduction in weevil densities occurred in late instars feeding on roots. For TMX, in contrast, impacts on weevil populations were largely due to adult mortality and disruption in egg-laying; effects on late instars were less clear because of poor egg-laying on TMX-treated plants.

Table 4. Residues in Tissues of Rice Plants at 6-7 Leaf Stage Rice Plants after Seed Treatments

Insecticide	*Rate (µg AI/seed)*	*Tissue concentrations (ng/g)*[a]		
		Leaves	*Shoots*	*Roots*
CAP [a]	5	73.0 ± 13.0	7.0 ± 0.9	43.0 ± 27.0
	50	161.0 ± 17.0	24.0 ± 2.0	1868.0 ± 730.0
TMX [b]	5	116.0 ± 20.0	64.0 ± 11.0	52.0 ± 14.9
	50	478.0 ± 99.0	228.0 ± 30.0	156.0 ± 38.0

[a] Limit of Detection (LOD) in ppb (ng/g fresh weight): Shoots: 1.0; Roots: 20; Leaf: 2.0. [b] LOD (ng/g fresh weight): 20 ng/g (foliage, shoots and roots).

These differences in patterns of activity against weevil life stages were strongly correlated with distributions of insecticides in plant tissues. Concentrations of CAP were influenced by treatment rate and plant tissue (Table 4). Overall, higher levels of CAP were found in roots than in shoots or leaves, although at the low CAP rate, concentrations in roots and leaves were similar. Further studies are required to examine characteristics of CAP uptake by roots and distribution of CAP in various plant tissues. The residues of TMX in treated rice plants, as in CAP-treated plants, were influenced by treatment rate and the type of tissue. Higher levels of TMX were found in leaves than in roots or shoots. At both seed treatment rates, TMX concentrations were highest in leaves. The four-fold higher concentrations of TMX in above-ground than in below-ground tissues in rice plants (Table 4) is consistent with the well documented, systemic properties and high acropetal translocation of TMX in apical portions of leaves when

applied at basal regions (*22*, *33*). Thus the divergent activity of CAP and TMX on adult weevils (i.e., greater disruption in egg-laying and first instar emergence in TMX-treated plants than in CAP- treated plants) was consistent with higher above ground insecticide concentrations in TMX- than in CAP-treated plants. In contrast, the below-ground concentrations of CAP were 10-fold higher than the above-ground concentrations at 50 µg AI/seed (Table 4). Thus, failure of CAP to affect adult survivorship may be due to low systemicity of CAP. Alternatively, CAP may have lower intrinsic potency as an adulticide than TMX. The latter hypothesis is supported by the fact that foliar concentrations of TMX lower than the foliar CAP concentrations observed at 50 µg AI/seed caused significant adult mortality in a prior study (15% mortality when foliar concentrations of TMX were as low as 100 ng/g) (*31*).

(Adapted from reference (*43*)) (Copyright 2012 Society of Chemical Industry)

Foliar concentrations of CAP insufficient to cause adult mortality were apparently sufficient to reduce egg-laying. The reduction in the emergence of first instars from CAP-treated plants suggests an ovicidal or larvicidal activity of CAP. The higher percent reduction in weevil densities from egg to first instar stages in plants treated with 50 µg AI/seed (45%) than 5 µg AI/seed (2.8%) was consistent with high CAP residues in shoots from the 50 µg AI/seed treatment compared to the 5 µg AI/seed treatment. The apparently small effect on first instars on plants at the 5 µg AI/seed treatment rate contrasts with the fact that egg-laying was reduced by 24% compared to untreated plants at this rate. It may be possible that egg-laying by weevils is more sensitive to CAP than are first instars, or that CAP concentrations in shoots were too low to cause mortality when ingested by first instars. Nonetheless, the disruptive influence on egg and early instar numbers was more intense on TMX-treated plants; this again was consistent with results of a study presented previously (Figure 3) that showed greater than 90% reduction in egg-laying and first instar emergence in plants treated with TMX at 21 and 28 µg AI/seed. Future studies with TMX might examine the larvicidal effects of this seed treatment by directly releasing first instars on shoots of treated plants.

Use of Seed Treatments as Components of an Integrated Management Program

In the greenhouse studies described above, both CAP and TMX seed treatments provided excellent suppression of RWWs, although they did so by targeting different weevil life stages. These results contrast somewhat with results of field trials (e.g., Table 1), which generally have shown superior control of weevil larvae by CAP than by TMX. In addition, the greenhouse experiments showed significant impacts on weevil biology at rates lower than those labeled for use in the field. To further investigate these and other issues, small-plot field studies were conducted in which TMX and CAP were used alone and in combination with other management rates at standard (label) rates and at rates lower than currently specified on their labels.

Integrated Use of Reduced Rates of CAP, Shallow Flooding, and Plant Resistance in a Weevil Management Program

An initial field study addressed the use of reduced and label rates of CAP in combination with additional management practices. Simultaneous use of multiple management practices is desirable not only because it reduces selection pressure on populations of target pest for insecticide resistance, thereby increasing the sustainability of the program, but also because it potentially increases the effectiveness of the program. Ensuring the compatibility of different tactics is therefore an essential step in designing sustainable integrated management programs (*44*). Furthermore, if the alternative tactics used in an integrated program provide effective control, it may also be possible to reduce seed treatment rates, thereby reducing potential environmental impacts of the management program (*45*).

The alternative tactics explored in the field study were shallow flooding and use of a cultivar, 'Jefferson', possessing moderate levels of RWW resistance. Both tactics had been shown previously to reduce weevil populations in small-plot field trials (*46–48*). To evaluate integrated use of these tactics, a split-plot experiment was conducted over two years, with three replications during both the years. In each year, a six leveed area was divided into three blocks, with one leveed area in each block assigned to a "shallow flood" (5 cm flood) treatment and one to a "deep flood" (12.5 cm flood) treatment. Flooding depth was thus the main-plot factor in the split-plot design. Nine subplots in each main plot consisted of factorial combinations of three varieties and three rates of insecticidal seed treatment. In both years, 'Jefferson' was included as the resistant cultivar and 'Cocodrie' as the susceptible cultivar. As the third cultivar, CL 131 in 2009 and Neptune in 2011 were used. The treatment rates of CAP used for 2009 were 0, 10 and 25 µg AI/seed (commercial rate). The treatment rates used in 2011 were 0, 5 and 50 µg AI/seed. Densities of RWW immatures (larvae and pupae) in subplots were determined at different time points using a soil-root core sampler with diameter of 9.2 cm and a depth of 7.6 cm. Treatment effects on mean larval density in each subplot were analyzed using a repeated-measure split plot by analysis of variance (ANOVA) using general linearized mixed model using PROC GLIMMIX in SAS (*49*). To estimate appropriate degrees of freedom, the Kenward-Roger adjustment of degrees of freedom was used in the model statement.

The use of CAP seed treatment strongly reduced population densities of rice water weevil larvae, even at a treatment rate of 5 µg AI/seed, a rate five times lower than the lowest labeled rate. Furthermore, there were consistent trends over both years for shallow flood depth and the use of the resistant variety 'Jefferson' to reduce densities of the rice water weevil (Figure. 8a & b).

In the statistical analysis of these experiments, the main effects (flood depth, rice variety, and insecticide treatment) were significant in both years (insecticide treatment) or in one of the two years (flood depth, rice variety). Importantly, no antagonisms or incompatibilities were found among flood depth, cultivar and seed treatment; the only significant interactions in the statistical analyses of the two experiments arose from the strong and temporally consistent suppression of weevil larvae by CAP treatments. Thus, use of shallow flooding and resistant varieties did

not antagonize or compromise the effects of CAP seed treatment, even at reduced rates, although the use of CAP tended to mask the effects of the other two tactics. These results suggest that seed treatments can function as a component of a multi-tactic management program, and that it may be possible to reduce rates of CAP seed treatments and still achieve superior control of this pest.

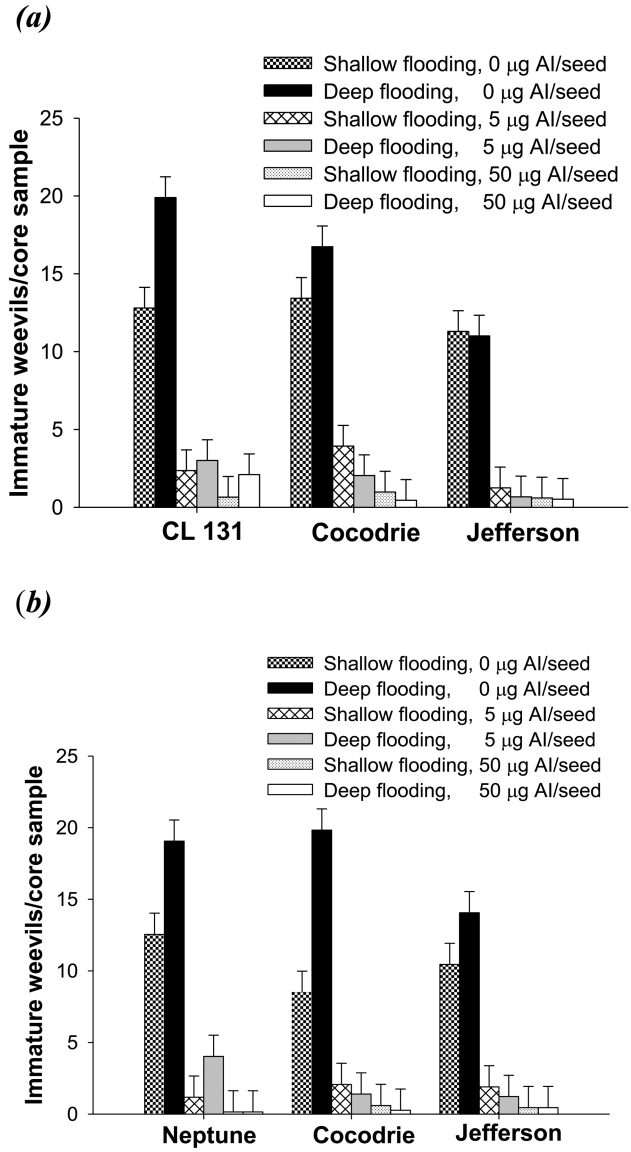

*Figure 8. Densities of immature weevils on different cultivars treated as seeds with CAP at different regimes of flood depth. No three-way interactions between flood depth, CAP treatment rate and rice cultivar were found in the year 2009**(a)** and 2011**(b)**. Plots were flooded 4-5 weeks after sowing to depths of 5 cm or 12.5 cm.*

Persistence of CAP and TMX in Roots and Shoots of Rice and Its Relationship to Field Efficacy

The superior efficacy of CAP relative to TMX in field trials (Table 1) as opposed to greenhouse experiments (Figure 7, 8) may be partly explained by the greater persistence of CAP and by the tendency of CAP to accumulate in roots rather than shoots. To further investigate this possibility, both insecticides were evaluated at four application rates in small experimental plots during the summer of 2013. The rates used included commercial rates (CAP: 32 µg AI/seed; TMX: 33 µg AI/seed) and rates higher and lower than commercial rates (Table 5). The 10 treatments were included in the same experimental field and each rate was replicated four times. Weevil larvae were sampled from all plots by taking root/soil core samples three and four weeks after flooding. For above and below-ground analyses of insecticide residues in rice plants, rice plants were sampled at two time points: one at permanent flood and another at 4 weeks after permanent flood. Three plants were randomly sampled from inner rows of rice plots. The above-ground portions of plants (foliage and shoots) and below-ground portions (i.e., roots) were separated and analyzed for tissue concentrations of CAP and TMX.

Sampling of immature weevils from plots revealed, in contrast to greenhouse experiments but consistent with prior field experiments, consistent suppression of RWW weevils in plots treated with CAP but not TMX (Table 5). The relative suppression of RWW larval densities in CAP-treated plots ranged from 74% at 3 µg AI/seed to 97% at 49 micrograms active ingredient per seed, whereas, for TMX-treated plots, suppression ranged from 11% at 5 µg AI/seed to 80% at 50 µg AI/seed (Table 5).

The chemical analyses of insecticide residues in above and below- ground tissues of rice plants at permanent flood (4 weeks after planting) and four weeks later (8 weeks after planting) revealed differences in both the distribution and persistence of the two active ingredients in rice (Table 6). At four weeks after planting, roots showed measurable concentrations of CAP at all treatment rates. CAP concentrations declined in below-ground tissues with age; however, high concentrations of this insecticide (100-fold higher than LOD) at commercial and high application rates demonstrated the high persistence of this insecticide in roots (Table 6). In contrast to high concentrations of CAP in roots, TMX levels in roots were below the level of detection (30 ppb) at both sampling times (Table 6). The above-ground portions of plants showed measurable concentrations of TMX only at commercial and high application rates at 4 weeks after planting. At 7 weeks after planting, no above ground tissues had TMX levels above the level of detection. The fact that larval densities were reduced at time points when TMX was not detectible in below-ground portions suggests that activity on adults and first instars is an important component of the field efficacy of TMX.

As noted above, the distinct patterns of distribution of CAP and TMX in rice plants may be due to differences in the physicochemical properties of the insecticides such as water solubility (CAP: 0.001 g l^{-1}; TMX: 4.1 g l^{-1}) and biphasic constant, i.e., log p (CAP: 2.86; TMX: -0.013). The suppression of weevil larvae in TMX-treated plants despite the low concentrations of active ingredient in tissues at

4 weeks after planting suggests the potential role of sublethal effects on egg-laying and early instars. The concentrations of TMX in the above-ground tissues of plants at 4 weeks after sowing were two or three-fold lower than the foliar concentrations that caused 15% adult RWW mortality in previous experiments (100 ppb) (*31*).

Table 5. Larval Densities of RWW on Roots of Rice Plants Sampled at Two Time Points, 3 Weeks after Permanent Flooding (WPF) and 4 WPF of Rice That Received Different Rates of Active Ingredient Per Seed

Insecticide	*Treatment rate (µg AI/seed)*	*Immature weevil densities per core sample*	
		3 WPF	*4 WPF*
CAP	0	11.4 ± 1.5	13.5 ± 2.1
	3	2.8 ± 0.6	3.5 ± 0.9
	8	1.1 ± 0.3	2.8 ± 0.8
	32	0.8 ± 0.2	1.1 ± 0.7
	49	0.6 ± 0.1	0.4 ± 0.2
TMX	0	8.0 ± 1.0	11.6 ± 0.6
	5	7.1 ± 1.1	6.4 ± 1.4
	13	4.8 ± 1.0	7.1 ± 0.9
	33	1.9 ± 0.7	5.9 ± 1.2
	50	1.7 ± 0.4	2.1 ± 0.6

Although reducing the use rates of CAP appears to be feasible from an efficacy stand point, there is a debate whether reducing insecticide rates is compatible with insecticide resistance management (*50*). Field studies demonstrate that the development of target-site resistance is slower at low use rates and did not not engender evolution of insecticide resistance as quickly as under high use rate or high selection pressure conditions (*50*). Where polygenic inheritance is involved, it has been repeatedly shown that the initial use of low dose-rates facilitates rapid evolution of resistance (*51*). Effective suppression of weevil larvae at low use rates in the present study suggests that further research is needed to characterize rate-dependent responses of weevil larvae to CAP and effects on larval survival and ability to complete the stage.

Table 6. Chlorantraniliprole Concentrations in the above- and below-Ground Tissues of Rice Plants Collected from Experimental Plots at Two Time Points, 0 Weeks after Permanent Flood (WPF) and 4 WPF of Rice That Received Different Rates of Active Ingredient Per Seed[a]

Insecticide/tissue	*Rate (μg AI/seed)*	*Concentration in ppb (ng/g fresh weight) at*	
		0 WPF	*4 WPF*
CAP/above-ground	3	12.5 ± 12.5[b]	0
	8	95 ± 65	0
	32	123 ± 55[c]	27.5 ± 32.0[d]
	49	269 ± 62	65.8 ± 12.2
CAP/below-ground	3	148 ± 46	0
	8	2000 ± 680	0
	32	8001 ± 4200	4740 ± 2430
	49	18850 ± 3400	3655 ± 2676[c]
TMX/above-ground	5	0	0
	13	0	0
	33	45 ± 18[c]	0
	50	38 ± 15[c]	0
TMX/below-ground	5	0	0
	13	0	0
	33	0	0
	50	0	0

[a] The 0 ppb values indicate concentrations below the limit of detection (30 ppb). The mean concentration of CAP was estimated by averaging four values obtained from four blocks. [b] three samples ND. [c] one sample ND. [d] two samples ND.

Effects of CAP and TMX on Other Early and Mid-Season Pests of Rice

As noted above, there are a number of insect pests of sporadic or regional importance in U.S. rice production, and the threat of these pests can influence choices of management tactics. Importantly, CAP and neonicotinoid seed treatments have different spectra of activity against these sporadic rice pests. These differences can be an important consideration in the choice of seed treatments. In Arkansas, for example, *Colaspis* sp. and thrips can be important pests of seedling rice. These insects are more effectively suppressed by

neonicotinoids than by Dermacor X-100, and farmers in Arkansas often choose to use CruiserMaxx or NipsitInside for this reason. In south Louisiana and Texas, in contrast, the South American rice miner, *Hydrellia wirthi*, is a sporadic pest against which Dermacor has been shown to have activity (*52*).

Another sporadic pest of concern in Louisiana is the fall armyworm (FAW). The FAW is a polyphagous insect, although cereals and grasses are the preferred hosts (*53*). When infesting rice, FAW larvae rapidly defoliate seedlings. Larvae typically develop fully in two to three weeks. Larvae prefer to feed on young rice plants or new growth of grasses (*54*). Most larvae that develop on flooded rice never pupate, as they normally pupate in the soil; because of this, FAW is considered a sporadic pest of rice in the southern United States (*55*, *56*). In other countries, however, this insect has been reported to cause severe damage to rice at the seedling stage (*57–59*).

The differential activities of CAP, TMX and CLO seed treatments on FAW were evaluated by foliar feeding assays using greenhouse-grown rice seedlings at the three-four leaf stage (Lanka and Stout, Unpublished data). Field rates of CAP (25 µg AI/seed), TMX (33 µg AI/seed) and CLO (17 µg AI/seed) were assessed for effects on FAW. CAP and the neonictonoids markedly differed in their activity against FAW larvae. Seed treatments of CAP were lethal both to neonates and third instars. TMX and CLO differentially affected FAW: TMX showed no effect either on survival or on larval growth while CLO affected FAW in a rate-dependant manner. At the label rate, seed treatments of CLO had no lethal effects FAW, but inhibited growth of neonates and decreased relative growth rates of third instars. At a seed treatment rate two-fold higher than the label rate, CLO caused 60% mortality on neonate FAW, while a comparable increase in seed treatment rate of TMX did not impact survival of neonates or growth. The superior activity of CLO relative to TMX seed treatment in this study is consistent with high efficacy of CLO reported earlier against FAW by Nauen et al. (*60*). In leaf dip bioasays using FAW larvae on cotton, these latter authors found the concentrations required for TMX were 5-fold higher than CLO to accomplish mortality of 100% .

Another sporadic pest of concern in Louisiana is the sugarcane borer (SCB). The SCB is an important pest of graminaceous crops in the southern U.S. Female adult moths of *D. saccharalis* lay cream-colored, flattened, oval-shaped eggs in groups containing 2-100 eggs. Eggs within a cluster hatch about the same time and upon hatching larvae move toward the space between leaf sheaths and plant stems. Larvae mine inside the leaf sheaths and after the second or third molt bore into the stems. Feeding on plant tissue in the stalks can lead to lodging, deadhearts and whiteheads (*61*, *62*). At the vegetative stage of rice plant growth, feeding by stem borer larvae results in “deadhearts”, in which the young tillers and the leaves of the tillers die. Larval feeding at reproductive stage of rice can result in “whiteheads” (discolored panicles with empty or partially filled grains). Normally larvae pass through three to 10 stadia (*63*). Larval development time is usually 25 to 30 days during warm weather.

Experiments in which SCB larvae were fed excised leaves and stems from 60-d old rice plants treated as seeds with label rates of Dermacor X-100 resulted in moderate rates of stem borer mortality, with about twice as many larvae dying on treated tissues as on control tissues (*64*). Larval mortality on intact

Dermacor-treated plants following the release of larvae on plants was higher, up to70% on treated plants versus less than 20% on controls. Results of a field study were consistent with these laboratory and greenhouse results: borer damage was significant reduced in CAP-treated plots but not in TMX-treated plots (*64*). Related experiments on rice in Asia have shown high activity of CAP as soil and foliar applications on several late season pests such as rice leaf folder and Noctuid and Pyralid borers (*65*, *66*). A soil granular formulation of CAP (Ferterra™) in irrigated rice applied at rates (40 g AI/ha) lower than commercial recommended rates (50 g AI/ha) resulted in significant reduction of white heads and dead hearts of yellow stemborer, *Sciropophaga incertulas* (*66*). Thus, the use of CAP as a seed treatment in the US rice market has the additional benefit of providing long-lasting suppression of stem-borers in mid-season rice.

Conclusions

Since the introduction of insecticidal seed treatments against the RWW into the U.S. rice market in 2008, the seed treatment method has been widely adopted (*29*). Replacement of pyrethroid insecticides by newer classes of insecticides offers several benefits to rice growers, including ease of use, greater effectiveness against the primary target pest, and activity against sporadic pests. In addition, the active ingredients in these seed treatments are less harmful than the pyrethroids to the red swamp crayfish, *Procambarus clarkii* (*14*, *67*), which are produced in close proximity to, and often in association with, rice in southwest Louisiana. However, prophylactic use of insecticides raises some concerns about cost-effectiveness, sustainability (development of resistance) and environmental impact of potentially unnecessary insecticide (seed treatment) applications. This review addressed some of these concerns related to the use of seed treatments.

A study involving over 40 commercial rice fields across Louisiana confirmed the ubiquity and severity of the RWW as an early season pest of rice; over 80% of untreated rice fields sampled showed population densities above treatment threshold. The field study also confirmed the results of prior small plot studies by showing that seed treatments were as or more effective than pyrethroid applications, and furthermore that CAP seed treatments were generally more effective than neonicotinoid seed treatments. The effectiveness of seed treatments against this major widespread pest, coupled with activities against sporadic pests and reduced effects on non-target crayfish, provide a solid justification for the adoption of these seed treatments.

The mechanisms by which CAP and TMX seed treatments accomplish reductions in population densities of RWW, and the reasons for the superior field efficacy of CAP relative to TMX, were investigated in a series of greenhouse experiments. Seed treatment with TMX but not with CAP reduced adult RWW survival. Dose-response relationships for TMX were characterized by using estimates of foliar biomass removed by weevils in conjuction with foliar concentrations of insecticides (TMX+CLO). Weevil mortalities were dose

dependent and the LD_{50} for weevils feeding on TMX-treated rice at the 2–3-leaf stage was 447 pg insecticide/weevil but was lower (142 pg/weevil) in experiments with 3–4-leaf-stage plants. Adulticidal activity of TMX decreased with age of rice plants. A competitive ELISA method for TMX in foliage detected lower insecticide residues in plants at the tillering stage than at the 5-6 leaf stage of rice plant development. This dilution in adulticidal activity provides a partial explanation for inferior efficacy of TMX in the field.

Experiments on the impact of seed treatments on egg-laying and emergence of first instars from treated plants revealed sublethal impacts of CAP and TMX on the number of eggs laid by adult RWWs. Notably, these effects were found in CAP even at field use rates. The greater reduction in egg numbers in TMX treatments than in CAP treatments reflected severe disruption of above-ground interactions of adult weevils by TMX.

The effects of seed treatments on various weevil life stages were monitored over time in another greenhouse experiment. This experiment showed that CAP seed treatments had their greatest impact on root-feeding larvae. The higher impact of CAP on root-feeding larval stages was consistent with high concentrations of CAP found in roots. In contrast, high disruption of above-ground interactions of adult RWW with rice plants by TMX seed treatment was consistent with high above-ground concentrations of this insecticide. The excellent control of weevils under greenhouse conditions by both CAP and TMX, albeit by targeting different life stages, contrasts somewhat with results of field trials, which generally have shown superior control of weevil larvae by CAP than by TMX. In addition, the greenhouse experiments showed significant impacts on weevil biology at CAP and TMX rates rates lower than those labeled for use in the field.

Small plot field studies showed that CAP seed treatment was compatible with two other management tactics, plant resistance and shallow flooding, and furthermore showed effective control of RWW at rates much lower than label rates. An additional small-plot study in which insecticide concentrations in roots and shoots were investigated in relation to densities of immature weevils revealed differences in the distribution and persistence of CAP and TMX consistent with results of previous studies. Overall, the results of greenhouse and field studies suggest that the greater field efficacy of CAP relative to TMX is probably due to a combination of the greater persistence of CAP in plants and the tendency of CAP to accumulate in roots rather than above-ground tissues.

Effective suppression of weevil larval densities by CAP at reduced rates together with its persistence in roots suggests the potential for reducing the use rates of this insecticide and thereby reducing environmental and economic costs associated with RWW management. However, the effectivess of reduced rates on sporadic pests such as stemborers and South American rice miner requires further study, as do the implications of low use rates for resistance management. Seed treatments could have direct effects on plants by way of altering plant defenses (*68*) and plant responses to abiotic stresses (*69*). Research in these areas is required to understand the overall economic and environmental impacts of the use of seed treatments in rice.

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