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Edited by Andrew Price, Jessica Kelton and Lina Sarunaite

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Meet the editors

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Jessica Kelton earned her M.S. degree from Auburn University in agronomy and soils with a concentration in weed science. Mrs. Kelton is a program coordinator in agronomics with the Alabama Cooperative Extension System (ACES), an outreach organization of Auburn University, USA. As a member of the Agronomics Team for ACES, she works in conjunction with other extension personnel to provide farmer education about current production practices to ensure successful grower application of new and ongoing scientific research in agriculture.

Lina Sarunaite is a senior researcher at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. Dr. Sarunaite received both B.S. and M.S. degrees from Aleksandras Stulginskis University majoring in agro business and organic agriculture at the Faculty of Agronomy. The topic of her Ph.D. study was "Investigation of ecologically sustainable multifunctional legume-grass swards." After Dr. Sarunaite received a doctoral degree in 2007 at the Institute of Agriculture, LRCAAF, she extended her research to the plant intercrops of various plant combinations in crop rotation, sole crop and its alternative cultivation technologies directed toward effective N utilization, enhancement of crops' competitive power and quality improvement of product, and weed management in sustainable and organic agriculture.

Contents

Preface

Most forms of agriculture depend on integrated pest management strategies, which include herbicides. Herbicide efficacy has contributed to increased food and feed production, improved control of invasive and nonnative weed species, and numerous other benefits for agriculture production as well as consumers. As pest management challenges appear, research is required to improve upon existing herbicide formulations and application methods and to further understand the mechanisms by which herbicides function and their off-target effects in order to ensure the future benefits of herbicides.

In addition, with the increase in chemical inputs into production systems, many concerns have been raised with regard to the detrimental impact of these products on non-target species, including humans, and on the environment. In this respect, research has been necessary to reduce environmental concerns, identify potential contamination risks from herbicide use, and ensure the protection of human health and well-bei

In this book, contributing authors have provided a broad scope of topics related to recent herbicide research. Research detailed in these chapters is focused on their chemistry and physiology of action or their impact on the surrounding environment. Research topics include herbicide chemistry, transformation, and herbicides in the environment.

The information provided in this book serves as a valuable tool for describing many areas of current herbicide research affecting both agricultural use and the environment. *Herbicides, Physiology of Action, and Safety* should be particularly useful for beginning and established scientists interested in developing research projects focused on understanding herbicide functions, environmental behavior, and safety of off-target organisms. It is hoped that this book will serve the scientific community as a source of current vital research information to help shape future research and understanding of herbicides.

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Section 1

Environmental Aspects

Chapter 1

Reducing Herbicide Residues from Agricultural Runoff and Seepage Water

George F. Antonious

Additional information is available at the end of the chapter

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Abstract

Herbicide use while being of a great benefit in controlling weeds in agricultural systems can also pose a threat to environmental quality due to off-target and off-site impacts. The increasing concern about risks associated with agricultural chemicals and specifically their impact on surface and groundwater quality is a national and international concern. In Kentucky, herbicide off-site movement occurs, allowing them to enter the Kentucky River watershed and impact surface and groundwater quality. Accordingly, it is necessary to assess the distribution and degradation/dissipation of herbicides in agricultural soils and runoff water after field application and develop management practices and/or remediation techniques to mitigate environmental pollution by agrochemicals. The overall goal of the best management practices is to develop sustainable agricultural techniques that strike an acceptable balance between crop production benefits and ecological conservation by reducing herbicide impact on environmental quality to 1) protect watersheds by reducing the mobility of herbicides from soil into runoff and seepage water using binding agents; 2) enhance soil microbial activity that mineralizes herbicides in soil; and 3) enhance growers' knowledge about bioremediation techniques (soil amendments, biofilters, biochar, and soil microorganisms) that could be implemented to reduce herbicide mobility and protect natural water resources.

Keywords: Biofilter, Biochar, Soil amendments, Organic matter, GC-MSD

1. Introduction

In Kentucky agriculture, herbicides (metribuzin, bensulide, dacthal, halosulfuron, carfentrazone, trifluralin, napropamide, and pendimethalin) are applied according to crop production guidelines [1]; however, their application period typically coincides with seasonal rainfall. In intensively cultivated areas, agriculture is a significant source of herbicides associated with

runoff. According to the US Environmental Protection Agency (EPA), over 441 million kg of conventional pesticides were used in the USA in 2001 [2]. Of that total, about 340 million kg were used in agricultural applications, and 48 million kg were used for home and garden purposes. In the USA, pesticide residues caused about 1,200 water-body impairments [3]. The USEPA also reported the consumption of 3 billion kg of herbicides during 2001 [4].

Pesticides play an important role in the success of modern farming and food production [5]. A commonly quoted estimate is that farmers save $ 3 to $ 5 for every $ 1 spent on pesticides [6]. In the USA, herbicides are used on > 90 % of most crops. Prior to the adoption of herbicides, 120 hours of labor and 16 cultivation trips per acre were used to control weeds. Without herbicides, the US food and fiber production would be reduced by 13.3 billion due to less effective weed control. Pesticides play an important role in the success of modern farming and food production [7]. The total loss in production would amount to 288 billion pounds, which represents approximately 21 % of the national production. In addition, the adoption of no-till practices prevents annual erosion of 304 billion pounds of soil every year [7]. Approximately 41,511 water-body impairments across the USA are attributed to pesticides, and of that total, 1,300 water-body impairments are within the state of Kentucky [8]. Based on estimated pesticide sales data for agricultural applications in 2000, a total of 1.5 million pounds of herbicides were applied in the Kentucky Green River Basin. Five herbicides (atrazine, simazine, metolachlor, tebuthiuron, and prometon) were detected in eight karst springs water in the Green River drainage basin [9]. Runoff water and sediment are frequent in sloping areas where most of the arable lands are highly erodible. Concerns about soil erosion, nutrient runoff, loss of soil organic matter, and the impairment of environmental quality from sedimentation and pollution of natural water resources by agrochemicals, N, P, trace elements, and other environmental contaminants have stimulated interest in proper management of natural resources. Herbicides cause water pollution by running off agricultural fields and domestic gardens into nearby water sources. Although agriculture has been identified as a source of pesticides found in surface water, other sources exist. Other sources may be pesticide manufacturing industries, industries using pesticides in their processes (such as woolen goods manufacturers), and direct application of herbicides to surface waters to control aquatic plants, lawn care, and golf course care.

A wide range of active ingredients are used as pesticides, and millions of tons of so-called "inert" ingredients are added to pesticide formulations as carriers, stabilizers, emulsifiers, etc. Some of these ingredients are dangerous in their own right. Ethylene dichloride, a nerve poison, is an example of an inert ingredient linked with damage to the eyes, heart, liver, and adrenal glands [10].

Portions of the active ingredient may transport to neighboring water bodies via drift during pesticide spraying, wind erosion, and runoff. Accordingly, it is necessary to assess and monitor the distribution and degradation/dissipation of herbicide residues in soil and water after field application. Runoff from agricultural watershed is found to carry enormous amounts of pesticides [11]. Following natural rainfall events, water flow may change from < 1 to > 10,000 ft^3/s^{-1} (1 ft^3 is equal to 28.3 L) in a matter of minutes to hours, enhancing pesticide transport to receiving water [12]. Streams and their tributaries that drain into the Kentucky River add

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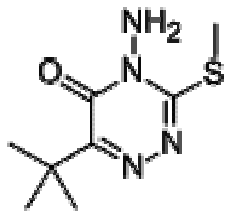
fertilizers and herbicides. In Kentucky, the watershed lies above thick layers of easily dissolved limestone that form carbonate aquifers. Groundwater flows through channels in the limestone, so caves and springs—and sinkholes—are common in regions with this karst geology [13]. Accordingly, Kentucky's citizen who uses fertilizer, herbicides, and other chemicals on his lawn, landscaping, and gardens adds to this water pollution problem.

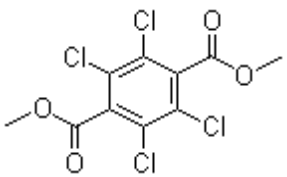
A significant association was found between the season of elevated agrichemicals in surface water in April–July and higher risks of birth defects in live births in the USA [14]. The most frequently detected herbicides in surface waters include several triazines (atrazine, cyanazine, and simazine), acetanilides (metolachlor and alachlor), and 2, 4-dichlorophenoxyacetic acid (2, 4-D) [15]. In Missouri men, high urine levels of atrazine and alachlor were associated with abnormal sperm [16]. Concentrations of the herbicide atrazine and other pesticides also were higher in the months of April–July. Chlorophenoxy herbicides are widely used in the USA for broadleaf weed control in grain farming and park maintenance. In Minnesota, Montana, North Dakota, and South Dakota, about 85 % of the spring and durum wheat acreage is treated with chlorophenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA).

Schreinemachers [17] found that infants in these four wheat-producing states conceived in April–June, the time of herbicide field application, were more likely to have circulatory/respiratory (excluding heart) malformations compared to births conceived during the other months. According to the EPA's most recent data, public water systems using groundwater as a drinking source serve about 105 million people nationwide [18]. The total number of people drinking groundwater increases when factoring in households supplied by private drinking water wells. Halosulfuron-methyl is a pyrazole sulfonylurea (Figure 1) used as a selective broadleaf postemergence herbicide. When applied preemergence or to the paddy water, intact halosulfuron-methyl was not detected or recovered in maize, sugarcane, and rice, indicating extensive metabolism of halosulfuron-methyl, probably initiated by a breakdown of the molecule in the soil or water. Chlorosulfonamide acid was the major metabolite identified in all commodities tested [19]. Halosulfuron-methyl exhibited very high to medium mobility in soil. Toxicological studies were carried out on two metabolites. The metabolite chlorosulfonamide acid (CSA) was found in plants and in groundwater at levels potentially exceeding 0.1 $\mu g\ L^{-1}$, whereas its metabolite halosulfuron-methyl showed low acute toxicity. Mitigation of herbicides, fungicides, and insecticide residues in soil and natural water resources could be achieved using modern management practices such as biofilters [20], adsorption techniques [21, 22] or biochar [23], soil microorganisms and the enzymes they produce [24], and wetland microcosms [22]. Among these management practices is the use of soil amendments.

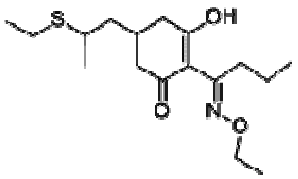
The USA produces nearly 15 million dry tons of municipal sewage sludge each year, and the tonnage is increasing due to population growth. In addition, the rapid growth in poultry industry has resulted in significant manure generation. More than 11.4 million tons of poultry litter was generated in the USA [25]. Sewage sludge and chicken manure, that must be disposed, are excellent fertilizers [26-29]. Addition of soil amendments such as chicken manure and sewage sludge to native agricultural soil increased water infiltration, lowering runoff



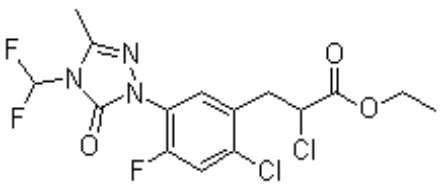
Metribuzin



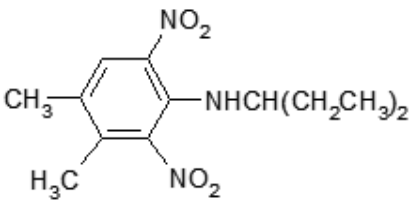
Dacthal



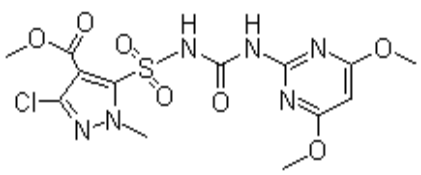
Sethoxydim



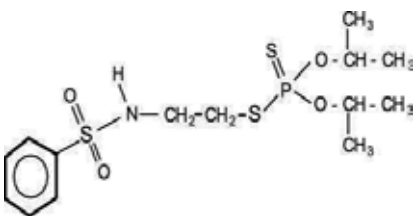
Carfentrazone



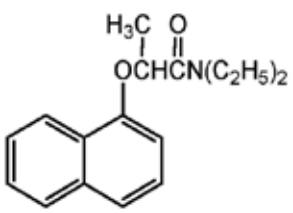
Pendimethalin



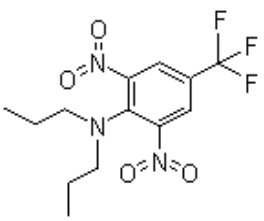
Halosulfuran-methyl



Bensulide



Trifluralin



Napropamide

Figure 1. Chemical structures of metribuzin [4-amino-6-*tert*-butyl-4, 5-dihydro-3-methylthio-1, 2, 4-triazin-5-one]; dacthal [1, 4-Benzenedicarboxylic acid, 2, 3, 5, 6-tetrachloro-, dimethyl] ester; sethoxydim [2[1(ethoxyimino) butyl]-5-[2-(ethylthio) propyl]-3-hydroxy-2-cyclohexen-1-one]; carfentazone, ethyl (RS)-2-chloro-3-{2-chloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorophenyl}propionate; pendimethalin; Prowl 3.3 E, N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine; halosulfuran methyl (Sandea 75 DF), 3-chloro-5-[(4, 6-dimethoxypyrimidin-2-yl) carbamoylsulfamoyl]-1-methyl-pyrazole-4-carboxylic acid methyl ester; bensulide [O, O-diisopropyl S-2-phenylsulfonylaminoethyl phosphorodithioate]; trifluralin [Treflan, (2,6-dinitro N,N-dipropyl-4-trifluoromethyl) benzenamine]; and napropamide [Devrinol, (N, N-diethyl-2-(1-naphthyloxy) propionamide)].

water volume and herbicide residues in runoff following natural rainfall events. Studies revealed that increasing soil organic matter by the addition of soil amendment to native soil reduced the transport of dimethyl tetrachloroterephthalate (DCPA, dacthal) herbicide down the land slope into runoff water [30]. The use of sewage sludge in land farming could become a useful technique for trapping herbicides such as trifluralin [31] and dimethazone [32, 33], metribuzin [30], and napropamide [34]. In addition to soil amendments, slot-mulch techniques (biobeds or biofilters—a cavity in the ground filled with a mixture of composted organic matter, topsoil, and a grass layer on top) provide a potential solution to herbicide contamination of surface waters arising from agricultural fields. Antonious [20] installed the first biofilter in the USA (www.biobed.org) for mitigation of herbicides in runoff water from vegetable fields. The use of biobeds and adsorption techniques are unique ways of treating contaminated soil and agricultural runoff. The filling materials of biobed systems (mixture of straw, peat moss, and native soil) have increased sorption capacity and microbial activity for degradation of pesticides [35]. The mechanism of biosorption process includes chemisorption, complexation, adsorption, diffusion through the pores, and ion exchange [36]. Biobeds were tested for their ability to retain and degrade chlorpyrifos (an insecticide), metalaxyl (a fungicide), and imazamox (an herbicide) using farm-available materials (vine branch, citrus peel, urban waste, and green compost). Degradation of the three pesticides in biobeds was found to be faster than published values for degradation of these pesticides in soil. The half-life of the three pesticides used was less than 14 days compared to literature values of 60–70 days in bare soil [37]. In addition, microorganisms in biobeds and soil are capable of degrading both sorbed and bound pesticide residues [38]. These findings indicated that biobeds or biofilters could substantially reduce pesticide concentrations in agricultural runoff. The biobed system can also be built on the farmland using locally available materials. This developed methodology to mitigate the impact of pesticides on the ecosystem is urgently needed. The risk of groundwater contamination resulting from rapid leaching of dimethazone and trifluralin herbicides was minimized through herbicide adsorption on the biobed filling materials under field conditions [20, 22]. In addition, biobeds (biofilters) have been used in northern Europe for minimizing point-source contamination of water resources by pesticides [39]. Antonious [20] provided evidence that biobeds enhanced dimethazone (a hydrophilic herbicide) and trifluralin (a lipophilic herbicide) dissipation and reduced their residues in runoff. To the best of the author's knowledge, the use of biobeds at Kentucky State University (KSU)/College of Agriculture (Frankfort, KY) represents the first field application of biobed systems in the USA for reducing runoff water loss and mitigation of off-site movement of herbicides. Installing biobeds in Kentucky farms (current project at KSU), where most of the arable lands are highly erodible, is a unique way of mitigating environmental pollution by herbicides before they enter rivers and streams.

Biochar, a product of the pyrolysis of organic material, has been credited with many desirable properties as a soil amendment, including soil conditioning [40], enhanced soil fertility [41], and sorption of pollutants [23] and soil hormones [42], and as an agent of C sequestration [43-45]. Although one study did report enhanced biological N fixation in biochar-amended soils [46], the effects of biochar on the biological activity of soil need greater investigation to evaluate the potential repercussions of wide application of such material when amended with municipal sewage sludge or chicken manure used for land farming. Currently, little informa-

tion exists in literature if biochar amendment to soil can reduce the plant uptake of pesticide residues and/or trace-element bioavailability. Such practice, if found effective, can assist in the management of contaminated agricultural and urban soils from current and past use of herbicides and other contaminants. Interest in biochar, made by pyrolysis of biomass, as a multifaceted solution to agricultural and pesticide pollution issues is growing at a rapid pace both nationally and internationally. The use of biochar in the US agricultural field is a new area of research. The infiltration of harmful quantities of nutrients and herbicides into groundwater as well as soil erosion and runoff into surface waters could be limited with the use of biochar [47] as a soil amendment. Biochar reduced the release of nitrous oxide [48]. About 40 % decrease in nitrous oxide emissions has been reported when biochar was applied to soil. This gas is approximately 310 times stronger than CO_2 in terms of global warming potential [49].

Greenhouse gas emission reductions might be 12 %–84 % greater if biochar is land applied instead of combusted for energy purposes [47, 48]. Biochar's fate as a viable component of the long-term solution to mitigate climate change by way of carbon sequestration depends upon further development by the scientific and technology transfer communities. Little is known about how biochar could successfully be implemented in the agricultural fields. Improving methods of pesticide detection and monitoring the performance and impact of soil amendments (such as sewage sludge, chicken manure, and biochar) and soil management practices on concentrations and transport of pesticides, nutrients, and trace elements into runoff and seepage water are the main focus of the current agricultural remediation methods. About $ 2.1 million was spent in 2009 on biochar research by the Agricultural Research Service (ARS) [50]. Converting biomass into biochar and bio-oil at various labs nationwide is one of ARS's priorities. The USA could use biochar to sequester 139 Tg of carbon annually if it were to harvest and pyrolyze 1.3 billion tons of biomass [50]. A series of presentations delivered at the United Nations Climate Change Conferences elevated interest in biochar as an immediate response to mitigate climate change, given its carbon sequestration ability. Biochar sequesters carbon while simultaneously enhancing the fertility of the soil. In addition, Ute et al. [51] found that some microorganisms were able to live on biochar as C source. The activity of purified N-acetyl glucosaminidase increased 50 to 75 % following biochar exposure, suggesting a chemical enhancement of enzyme function [52].

Biochar also is a potential solution to environmental contamination by herbicides. A survey of current adsorbents indicated that the large surface area of activated carbon (ranging from 500 to 2,000 $m^2\ g^{-1}$) makes it a perfect candidate for pesticides and trace-element adsorption. Adsorption on activated charcoal occurs through van der Waals forces that allow it to adsorb different types of pollutants including pesticides [53-55]. Knowledge about the environmental problems and adoption of appropriate solutions and practices to enhance and protect our national water quality from environmental pollution by herbicides require timely delivery of research and educational technology. The development of production systems and mitigation techniques that reduce the introduction of agrochemicals like herbicides into the environment presents a continuing challenge.

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2. Literature review

Soil erosion reduces crop productivity by decreasing soil depth, removing nutrients required for plant growth, and altering soil physical properties resulting in less water infiltration, poorer crop establishment, and root penetration. Intensive use of herbicides and other pesticides in many parts of the USA increases the potential for non-point source contamination of soil and receiving water. One of the major problems with the application of pesticides is targeting the chemicals to the pest. Often, less than 0.1 % of the pesticides applied to crops under field conditions reach the target pests. The remaining 99.9 % residues can have a number of adverse effects that include health risks to both humans and wildlife. The amount of herbicides reaching target weeds is generally much higher. From 0.1 to 5 % of postemergence herbicides applied reach the target weeds [56]. Occurrence of herbicides and other pesticides in runoff and infiltration water [32-34, 57-64] increases the potential impact on human health and environmental quality. The lack of pesticide field dissipation data and the increasing concern about herbicides and other agricultural chemicals and their impact on surface and groundwater quality have made environmental pollution by herbicides a national concern.

In addition, an increase of organic waste originating from different human and productive activities is a continuous concern. Waste application to soil is a solution to disposal. This practice is popular in the agricultural fields because of the value of this waste as organic fertilizer. Application of organic amendments to agricultural soils makes good use of natural resources and reduces the need of synthetic fertilizers. Recently, increased interest has been focused on assessing the influence of organic waste added to the soil on pesticide (herbicides, fungicides, and insecticides) adsorption, movement, and biodegradation. There is a continuing search for inexpensive, locally available sources of organic matter for use in growing horticultural crops. The addition of organic amendment to soil normally results in an increase in the microbiological activity [24, 65-67] due to the availability of simple organic molecules such as sugar and amino acids. Composting provides an organic amendment useful for improving soil structure and nutrient status [65] and generally stimulates soil microbial activity [24, 68, 69]. Among the major parameters of soil fertility and biological properties, special emphases are given to enzyme activity. As more sewage sludge treatment districts turn to composting as a viable means of sludge stabilization, sewage sludge will become available in increasing quantities. With the increased interest in recycling waste, there is a need to monitor the three enzymes (invertase, urease, and phosphatase) of the C, N, and P cycles, respectively, as indicators of increased soil microbial populations in the rhizosphere of growing plants [24]. Soil microorganisms (bacteria, fungi, protozoa, algae) in sewage sludge and chicken manure excrete ureases, invertases, dehydrogenases, cellulases, amylases, and phosphatases to degrade herbicides in soil and water ecosystems. Microorganisms produce sticky substances (polysaccharides) that help soil particles adhere to one another and help the soil resist erosion that could diminish agriculture productivity [70]. Emphasis has been given to soil enzymes in relation to reclamation management and the enzymatic processes that play a significant role in bioremediation of pesticides and heavy metals. Work carried out by Antonious [22, 24] has

provided detailed information on enzymatic and microbial population responses in soil in a series of ecosystems. Remediation of contaminated soils is based on the degrading activity of soil microbiota. However, sewage sludge and chicken manure contain significant amounts of heavy metals that may impact soil microorganisms and the enzymes they produce by blocking of either the enzyme or substrate when present in excessive concentrations [71]. Accumulation of trace elements in plants grown in biosolids (sewage sludge) varied among plant species and even among accessions of the same species [72]. The application rate of sewage sludge and chicken manure applied to agricultural soil is proposed according to soil nitrogen, phosphorus, and potassium requirements, with a maximum application rate of 15 t $year^{-1}$ [73]. In fact, a specific rule for animal manure application to soil does not exist, but is proposed through good agricultural practices for use as fertilizer and as herbicide binding agent due to its organic matter content. Several studies have shown that an increase in soil pH results in an increase in soil microbial biomass and enzymatic activities, favoring a rapid growth-linked degradation of phenylurea isoproturon herbicides as a result of interaction between degradative *Sphingomonas spp*. and pH [74] and biodegradation of fenamiphos [75]. Therefore, the question here is what can be done to increase soil pH? The answer is the use of either lime (CaO) or biochar mixed with native soil.

3. Overview of soil amendments and herbicide field studies

At Kentucky State University H.R. Benson Research and Demonstration Farm (Franklin County, KY), studies were conducted on a Lowell silty loam soil of 12 % clay, 75 % silt, and 13 % sand. Plots (18) of 22 × 3.7 m each were established on 10 % slope to investigate herbicide mobility in relation to soil amendments under field conditions. The plots were separated using metal borders 20 cm above ground level to prevent cross contamination between adjacent treatments (Figure 2). In the first year, three soil management practices were used: i) sewage sludge (Figure 3) obtained from Nicholasville Wastewater Treatment Plant, Versailles, KY, was treated with lime (CaO) at 1:10 (w/w) ratio and mixed with yard waste compost (made from yard and lawn trimmings and vegetable remains) at 15 t $acre^{-1}$ (on dry-weight basis) with a plowing depth of 15 cm, ii) sewage sludge was mixed with native soil at 15 t $acre^{-1}$ (on dry-weight basis) with a plowing depth of 15 cm, and iii) a no-mulch (NM) control treatment (rototilled bare soil) was used for comparison purposes to monitor the herbicide bensulide (Figure 1) and its metabolite mobility. In year 2, the three soil management practices were chicken manure, sewage sludge (Figure 3), and no-mulch native soil used for comparison purposes to monitor the impact of soil amendments on metribuzin and DCPA (dacthal) herbicide mobility under field conditions. In subsequent years, the persistence of trifluralin and napropamide herbicides (Figure 1) was also investigated. Herbicides selected in this research are commonly used in Kentucky agriculture and were applied according to Kentucky-recommended rates of application [1]. Following herbicide treatment, total runoff water loss per runoff event per each 0.02-acre plot was used to monitor mobility of herbicides tested.

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Figure 2. Kentucky State University/ College of Agriculture erosion field plots.

Figure 3. Soil amendments, chicken manure (left) and sewage sludge (right) used for mitigation of herbicide residues at Kentucky State University/College of Agriculture (Franklin Conty, KY).

3.1. Runoff water measurement

Runoff water was collected and quantified at the lower end of each plot throughout the growing season using a tipping-bucket runoff metering apparatus (Figure 4). Buckets were calibrated (one tip represents 3 L of runoff) and maintained to provide precise measure of amount of runoff per tip. Numbers of tips were counted using mechanical runoff counters (ENM Company, 5617 Northwest Highway, Chicago, IL 60646). Collection of samples in 3.79 L borosilicate glass bottles was carried out through a flow-restricted composite collection system (approximately 40 mL per tip was collected). Following rainfall events, runoff water samples were transported on ice to the laboratory, stored at 4 ^{O}C for extraction and analyses of herbicide residues. Runoff water volume (L acre^{-1}) from each soil treatment was based on total runoff water loss per runoff event per 0.02-acre plots.

Figure 4. Surface runoff water collection using tipping buckets installed down the field slope. A gutter was installed across the lower end of each plot with 5% slope to direct runoff to the tipping buckets and collection bottles for runoff water measurement and sampling

3.2. Leachate measurement

Pan-lysimeters (18) were used to monitor herbicide seepage and presence or absence of herbicides like bensulide residues and/or its metabolites in the vadose zone (the unsaturated water layer below the plant root). Pan-lysimeters (Figure 5) were 4 ft^2 each and were installed in a tunnel at 2.0 m underground, leaving the soil column above it intact. This system allowed the collection of infiltration water under normal field conditions (zero tension). Leachate water percolated into the vadose zone was collected in borosilicate amber bottles. Volumes of water collected were recorded following each rainfall or irrigation event.

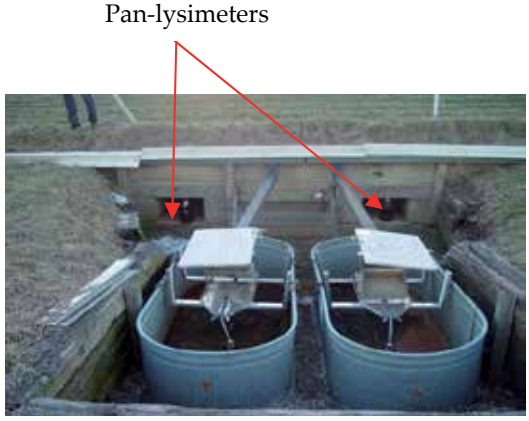

Figure 5. Pan lysimeters installed down the soil column (2 m deep) at the end of field plots for collection of infiltration water (Kentucky State University, Franklin County, KY).

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3.3. Monitoring herbicide residues in soil

Soil samples (6 replicates per treatment) were collected from the different treatments using a soil core sampler (Figure 6) equipped with a plastic liner tube (Clements Associates, Newton, IA) of 2.5 cm ID for maintenance of sample integrity. Soil samples were taken to a depth of 15 cm from the rhizosphere of growing plants within the treatments prior to and after herbicide application during the course of the study. Samples were air-dried in the dark at room temperature for 48 h and sieved to a size of 2 mm for herbicide residue analyses. At KSU College of Agriculture, nine biobed systems were installed. A low-cost biobed system (a hole filled with a mixture of chopped wheat straw, peat moss, and top soil) was developed and used in Sweden since 1993 [35] to degrade pesticides from point sources. High-quality compost made from garden residues or municipal waste contains numerous microorganisms with differing activities and has provided a good retention capacity for pesticides [37]. The soil in biobeds provides sorption capacity and degrading microorganisms, and the peat contributes to high sorption capacity and regulates the humidity of the system. The grass layer (living fescue) that covers the biobed system helps to keep the system humid. Castillo et al. [76] reported that a straw to peat to soil ratio of 50:25:25 % is a recommended biomixture composition for biobeds. This is because organic amendments that increase soil organic matter content offer enhanced pesticide sorption capacity [77, 78]. Concentration of herbicide residues adsorbed to yard waste, sewage sludge, or chicken manure-amended soils was calculated as described by Antonious et al. [21]. The adsorption coefficient (Kd) was calculated using the Freundlich equation ($q = Kd\ C\ 1/n$), where q is the amount of solute (pesticide) adsorbed per unit mass of soil, C is the equilibrium concentration of the solute, and n is the correction factor. Plotting the linear form of the above equation as log q vs (1/n log c + log Kd) provides the slope of the regression line as 1/n and the intercept with the y-axis as log Kd. Pesticide residue data from three soil treatments and from adsorption isotherm experiments were statistically analyzed by analysis of variance (ANOVA) and Duncan's multiple range test for mean comparisons. Replacement of some of the original materials in the Swedish biomixture (straw, peat, and soil) can also change the performance of the biobed system [76].

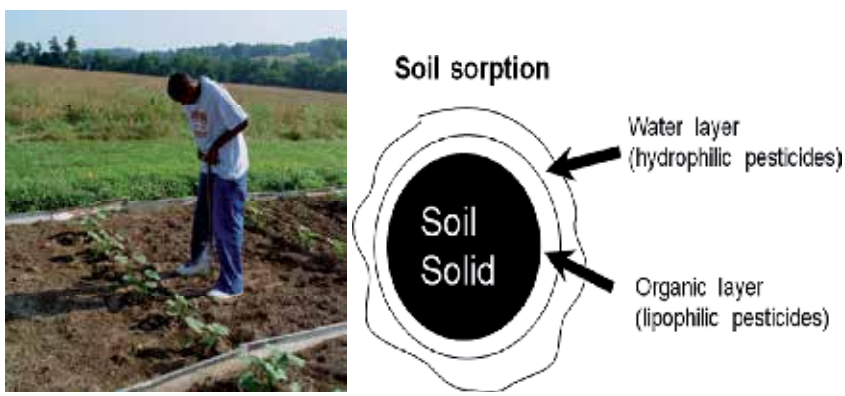

Figure 6. Collection of soil samples using a core sampler equipped with a plastic liner tube (left photo) and a schematic representation of sorption on soil particle (right sketch).

The biobed system has been modified and sometimes renamed as biomassbed in Italy, biofilter in Belgium, and Phytobac and biobac in France. The potential of using biobeds to contain and degrade pesticides has been evaluated in a series of experiments using laboratory-scale biobeds located in greenhouses in Utah, USA. The study was performed by EarthFax Development Corporation and funded by the US EPA. The study in Utah involved application of selected herbicides to the surface of the biobed system, which were prepared to assess various factors (e.g., substrate mixtures with and without fungal inoculation). In Utah, the herbicide degrading potential of the biobed substrate mixtures was determined by analyzing soil/peat/straw (or corn stover or corn cob) mixture of subsamples taken from various depths in the beds to determine residual herbicide concentrations over time. Accordingly, the degradative performance of biobeds for several of the most commonly used herbicides in the USA was exceptional, particularly for the most heavily used herbicide in the USA (atrazine). Biobeds have been used in northern Europe for minimizing point-source contamination of water resources by pesticides [39]. Biobeds were tested for their ability to retain and degrade chlorpyrifos (an insecticide), metalaxyl (a fungicide), and imazamox (an herbicide) using farm-available materials (vine branch, citrus peel, urban waste, and green compost). The filling materials (mixture of modified straw, peat moss, and native soil) of biobeds have increased sorption capacity and microbial activity for degradation of pesticides. Degradation of the pesticides in biobeds was found to be faster than published values for degradation in soil. The half-life of pesticides tested was less than 14 days compared to literature values of 60–70 days in soil [37]. Biobeds also reduced the concentration of sediment, so they might reduce the concentration of pesticides that are strongly sorbed to sediment. Little is known regarding biobed used in the USA. The installation and application of biobed system under field conditions (Figures 7 and 8) is the first application of biobed systems for reducing runoff water loss and mitigation of off-site movement of herbicides in runoff (non-point source contamination) in Kentucky agriculture, where most of the arable lands are highly erodible. In Kentucky agriculture, nine biobeds were used for testing the performance of these systems in treating residues of the two herbicides dimethazone and trifluralin (Figure 1) in runoff and seepage water arising from agricultural production under three soil management practices (municipal sewage sludge, sewage sludge mixed with yard waste, and no-mulch native soil). Methodologies to mitigate the impact of pesticides on the ecosystem are urgently needed. Since 1991, Kentucky State University (KSU) Water Quality & Environmental Toxicology Research of the Land Grant Program in Franklin County, KY (USA), has been involved in several field and laboratory projects to investigate the relationships between soil farming practices, soil erosion processes, vegetable yield, fate of pesticides and pesticide metabolites in soil, runoff, and infiltration water. Various agricultural and management practices have been used to mitigate environmental pollution by herbicides. Planting living fescue strips against the contour of the land slope reduced runoff but has the disadvantage of increasing the potential of soil infiltration by pesticides [61, 79], whereas, plastic mulch, which can cover between 50 and 70 % of a field, increased surface water runoff from both rainfall and irrigation [32]. This means that much of the herbicides applied in living fescue strips used as frequent barriers to runoff or in plastic-mulched fields may seep into groundwater or leave the field into surface runoff. In addition, bensulide (O, O-diisopropyl S-2-phenylsulfonylaminoethyl phosphorodi-

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thioate) is one of the few herbicides from the organophosphate group used for control of weeds that threaten numerous crops. Half-life ($T_{1/2}$) values of bensulide in soil were 44.3, 37.6, and 27.1 d in sewage sludge mixed with yard waste at 1:1 ratio, sewage sludge alone, and no-mulch bare soil treatments, respectively. The addition of sewage sludge mixed with yard waste and sewage sludge alone to native soil increased water infiltration, lowering runoff water volume and bensulide residues in runoff following natural rainfall events.

Figure 7. Preparation of soil cavity for biofilter installation at Kentucky State University (Franklin Connty, KY) for trapping herbicides residues in surface runoff water.

4. Herbicide residue analysis

Soil samples (100 gm) was shaken with methylene chloride: acetone (1:1 v/v) for 1 h using a Multi-wrist shaker (Lab-Line Instruments Inc., Melrose Park, IL, USA) to extract the herbicide bensulide. The solvent mixture was filtered through Whatman 934-AH glass microfiber discs (Fisher Sci., Pittsburg, PA). Extracts were passed through anhydrous Na_2SO_4 to remove any traces of water and concentrated by rotary vacuum (Buchi Rotavapor Model 461, Switzerland) and N_2 stream evaporation. Each concentrated extract was subsequently passed through a 0.45

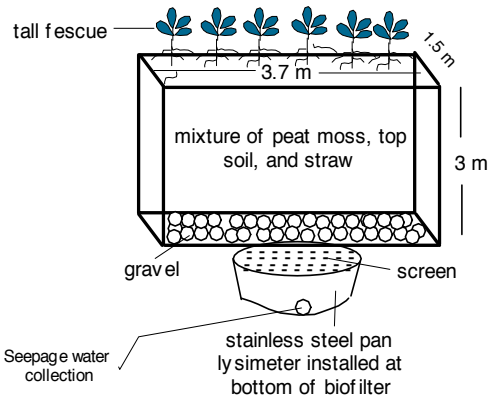

Figure 8. A biofilter system installed at Kentucky State University Research Farm for trapping herbicides and other contaminants in surface water runoff before they enter the Kentucky River.

μm GD/X disposable syringe filter (Fisher Scientific, Pittsburg, PA). One μL of the concentrated extracts was injected into a gas chromatograph (GC) equipped with an NP detector. The gas chromatograph (HP 5890, Hewlett Packard, Palo Alto, CA) was equipped with a 30 m (0.23 mm diameter, 0.33 μm film thickness) fused silica capillary column with HP-5 (5 % phenyl polysiloxane, 95 % methyl polysiloxane) liquid phase. Operating conditions were 230, 250, and 280 ^{O}C for injector, oven, and detector, respectively. Area units were obtained from 1 μL injections. Linearity over the range of concentrations was determined using regression analysis ($R^2 > 0.95$). Quantification was based on average peak areas from three consecutive injections obtained from external standards of bensulide. Peak identity was confirmed by consistent retention time and coelution with standards under the conditions described. Bensulide residues were also confirmed using a gas chromatograph/mass selected detector (GC/MSD, Hewlett Packard Model 5971a, Palo Alto, CA) operated in total ion monitoring with electron impact ionization (EI) mode and 70 eV electron energy. Under these conditions, retention times (R_t) of bensulide averaged 19.6 min. Bensulide standard material of 99 % purity was obtained from Chem Service (West Chester, PA, USA). Bensulide standard solutions in acetone ranging from 0.1 to 15 ng L^{-1} were prepared and used to spike blank soil, runoff, and infiltration water samples obtained from soil treatments for evaluating the reproducibility and efficiency of the analytical procedures used. After fortification at 30 and 45 μg g^{-1} soil and 30 and 45 μg mL^{-1} water samples, bensulide in fortified samples was extracted and determined using the same procedures described above. Bensulide residues detected in soil were used to calculate half-lives in each of the three soil treatments. Half-lives were calculated from regression lines using the equation $T_{1/2} = \ln 2/K$, where K = -2.302 × slope of the line. Quality control (QC) samples included three field blanks to detect possible contamination during sampling, processing, and analysis. Three sets of duplicate samples and three sample-matrix spikes were used to evaluate potential bias of the data collected and the ability of the analytical procedure to recover the analyte from field samples. Residues of bensulide in soil and water and volume of runoff and infiltration water were related to soil management technique and statistically analyzed using ANOVA procedure and Duncan's multiple range test for mean comparisons.

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5. Quantification of herbicide residues

A mass spectrometer is an excellent tool for identifying the chemical structure of a single herbicide when present with a mixture of compounds. Bensulide residues were confirmed using GC/MSD which showed spectral data with molecular ion peaks (M+) at m/z 170, 141, 77, and 51 (Figure 9) and at m/z 157, 141, 93, 77, and 51 that correspond to benzenesulfonamide ($C_6H_7NSO_2$), a bensulide metabolite, along with other characteristic fragment ion peaks (Figure 10). These mass spectral data are in agreement with those reported by the National Institute of Standards and Technology [80]. The organic carbon sorption coefficient (K_{OC}) of the herbicide bensulide is 3,900 mL g^{-1}, whereas azafenidin (an herbicide) K_{OC} is 298, which indicates that azafenidin does not bind strongly to soil particles [81]. Herbicides with a strong sorption rate remain near the soil surface, increasing the chances of being carried to a stream via surface runoff. On the contrary, herbicides with high persistence and a weak K_{OC} may be readily leached through the soil column and are more likely to contaminate groundwater. Herbicides having high K_{OC} value, i.e., bensulide, will bind to soil and organic matter. Accordingly, herbicides can be ranked as leachers or non-leachers to assess their potential for off-site surface or subsurface movements under field conditions. Pesticide adsorption to soil is related more to soil organic matter than to other soil chemical and physical properties [81, 82]. Therefore, addition of soil amendments having high organic matter content (such as sewage sludge and chicken manure) is a management practice that should be exploited to trap nonionic herbicides like bensulide to reduce its surface and subsurface mobility under field conditions.

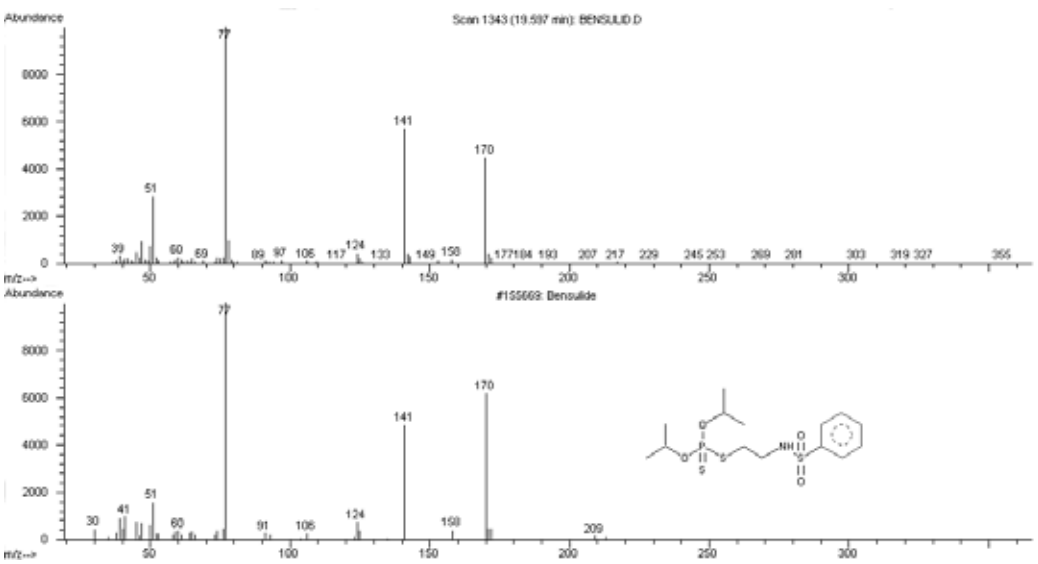

Figure 9. Electron impact mass spectrum of bensulide ($C_{14}H_{24}NO_4PS_3$) extracted from soil indicating the molecular ions of m/z 51, 77, 141, and 170, along with characteristic fragment ions.

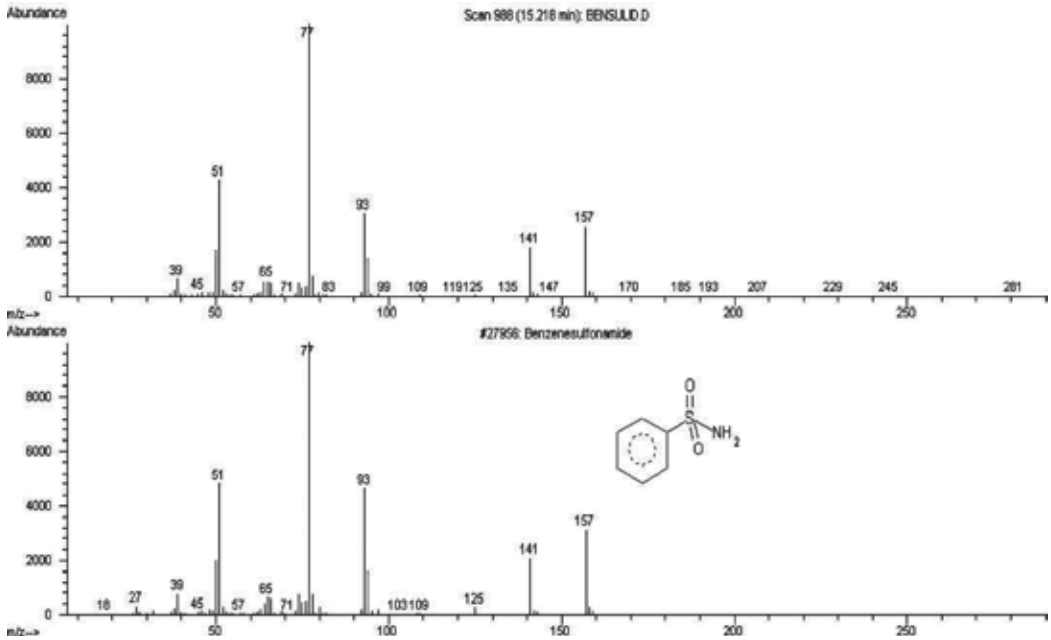

Figure 10. Electron impact mass spectrum of benzenesulphonamide ($C_6H_7NSO_2$), a bensulide metabolite, detected in soil indicating the molecular ions of m/z 157, 141, 93, 77, and 51, along with characteristic fragment ions.

Humic acids (Figure 11), the fraction of humic substances that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values, are dark brown to black in color, whereas fulvic acids, the fraction of humic substances that is soluble in water under all pH conditions, are light yellow to yellow brown in color. Humin, the fraction of humic substances that is not soluble in water at any pH value, is black in color. The presence of carboxylate and phenolate groups gives the humic acids the ability to form complexes with ions such as Mg^{2+}, Ca^{2+}, Fe^{2+}, and Fe^{3+}. Many humic acids have two or more of these groups arranged to enable the formation of chelate complexes [83-85]. The formation of chelate complexes is an important aspect of the biological role of humic acids in regulating bioavailability of metal ions [86] and binding of pesticides to soil organic matter.

The soil organic matter content and the water solubility of the pesticide are the two most important characteristics determining soil adsorption of a pesticide. Adsorption of nonionic pesticides on soil particles depends directly on the organic carbon content (K_{OC}) of the compound and the adsorbing phase. K_{OC} coefficient represents the sorption on a unit carbon basis and could be used for comparison of sorption extent on soils with different organic matter contents. The greater the K_{OC} value of a pesticide, the stronger the binding to the soil [21, 83, 87]. Walker and Welch [88] studied the degradation rates of three herbicides and their strengths to adsorption; their results showed an order of degradation rate of metribuzin > alachlor > atrazine and an order of adsorption of alachlor > atrazine > metribuzin. Metribuzin mineralization to $^{14}CO_2$ proceeded more slowly in amended soil than in un-amended soil [89].

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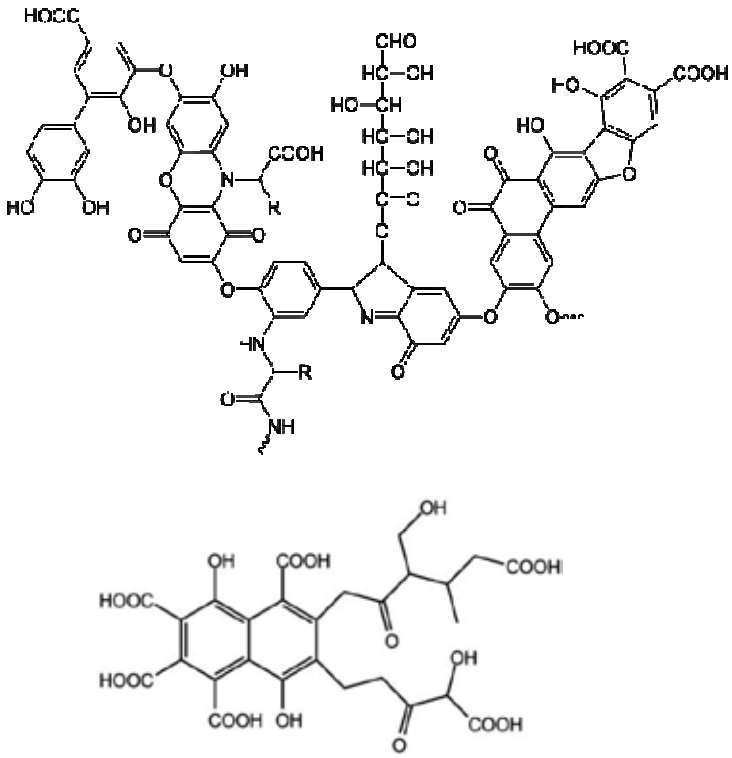

Figure 11. Chemical structure of a typical humic acid (upper structure) and fulvic acid (lower structure) having quinone, phenol, catechol and sugar moieties.

The presence of metribuzin (an herbicide) in soil was confirmed using GC/MSD which showed spectral data with molecular ion peaks (M+) at m/z 198 along with characteristic fragment ion peaks at 57, 74, 103, 144, and 171 (Figure 12, upper graph), whereas the presence of DCPA (dacthal) in soil and water was confirmed using molecular weight of 332 and spectral ions of m/z 59, 107, 142, 177, 221, 273, and 301, along with characteristic fragment ions (Figure 12, lower graph). These mass spectral data are in agreement with those reported by the National Institute of Standards and Technology [80]. DCPA has a low vapor pressure, 2.5×10^{-6} mmHg, and very low water solubility, 0.5 ppm. DCPA with its low water solubility, <0.5 ppm, high K_{OC} of 5,900 and stability to UV light has great affinity for binding to soil particles. Consequently, low residue levels of DCPA would be available on the soil surface. In soil, biodegradation of DCPA into tetrachlorophthalic acid, which is extremely mobile in the environment, is slow [90]. Work carried out by Antonious et al. [30] revealed that runoff water volume from no-mulch soil (200,000 L plot^{-1}) was significantly greater than runoff from soil amended with chicken manure and sewage sludge-amended soils (45,000 and 85,000 L plot^{-1}, respectively). This might be due to reduced bulk density and increased soil interspaces after addition of soil amendments that increased water infiltration into the soil column toward the vadose zone (the unsaturated water layer below the plant root), reducing surface water runoff from chicken

manure and sewage sludge incorporated soil. Water infiltration into the vadose zone also varied between soil treatments. Generally, substantial portions of soil-applied herbicides are leached from the site of application by percolation into the vadose zone and enter the groundwater supply. DCPA residues were detected at very low concentrations in infiltration water collected from the vadose zone. One should consider that precipitation during June and July storms resulted in increased water infiltration from chicken manure and sewage sludge compost treatments compared to no-mulch treatment. The decrease of DCPA residues in runoff and infiltration water from chicken manure and sewage sludge treatments might be due to DCPA environmental fate characteristics (i.e., high sorption capacity) that increased its adsorption to the soil particles and the greater organic matter content of chicken manure (5.37 %) and sewage sludge treatments (5.35 %) compared to 2.2 % in no-mulch treatment which might have increased DCPA binding to humic substances, reducing its mobility in the soil column into the vadose zone and down the land slope into surface water. This may be explained by the nonpolar properties of the herbicide DCPA which increases its adsorption to soil particles, thereby decreasing its availability in leachate water at lower depth.

Other reports also suggested that the mobility of metribuzin within soils is limited in organic soils [91, 92] because metribuzin is tightly bound to soils with high clay or organic matter content. In addition, Nicholls et al. [93] reported little movement (<10 cm) of metribuzin applied at the surface of sandy loam that was maintained fallow over the entire growing season. Khoury et al. [94] indicated that soil microorganisms contribute to the rapid degradation of metribuzin and the increase in soil organic matter favored microbial development and subsequently accelerated the degradation of metribuzin. They concluded that the degradation speed of metribuzin in non-sterile soils was found to be 7–12 times higher than that in sterilized soils. Benoit et al. [95] investigated metribuzin sorption and found that metribuzin is weakly sorbed in different soils and concluded that the observed relationship between organic carbon and herbicide mineralization was related to the activity of soil microorganism.

The persistence of an herbicide is defined as the time in which the molecule remains in the soil and is usually expressed as half-life ($T_{1/2}$). The leaching index (LI) of an herbicide can be calculated using the equation developed by Laskowski et al. [96]

(LI = (S) ($T_{1/2}$)/ (VP) (K_{OC})), where S is the water solubility of a pesticide in mg L^{-1} at 25 ^{O}C, $T_{1/2}$ is the half-life of a pesticide in soil in days, VP is the vapor pressure in mmHg at 25 ^{O}C, and K_{OC} is the organic carbon partition coefficient that can be calculated using the equation K_{OC} = Kd/% soil organic carbon. Using S and VP values from the pesticide manual [97], $T_{1/2}$ in no-much soil taken from Antonious et al. [30] and K_{OC} from Kim and Feagley [98], the LI value of metribuzin in soil was calculated as [LI = (1050) (12)/(5.8×10^{-7}) (96)] = 2.26×10^{8}, whereas LI value of DCPA in soil was calculated as [LI = (0.5) (26.17)/(1.57×10^{-6}) (5900)] = 1.41×10^{3}, indicating the weak soil leaching of DCPA and the high leaching of metribuzin into the soil column.

In other studies conducted in a field experiment, a silty loam soil was sprayed with a mixture of two preemergent herbicides, dimethazone and trifluralin (Figure 1) formulations. One hundred twenty-five milliliters of Command 3ME (dimethazone) formulation obtained from Platte Chemical Company (18th street, Greeley, CO) and 300 mL of Treflan (trifluralin)

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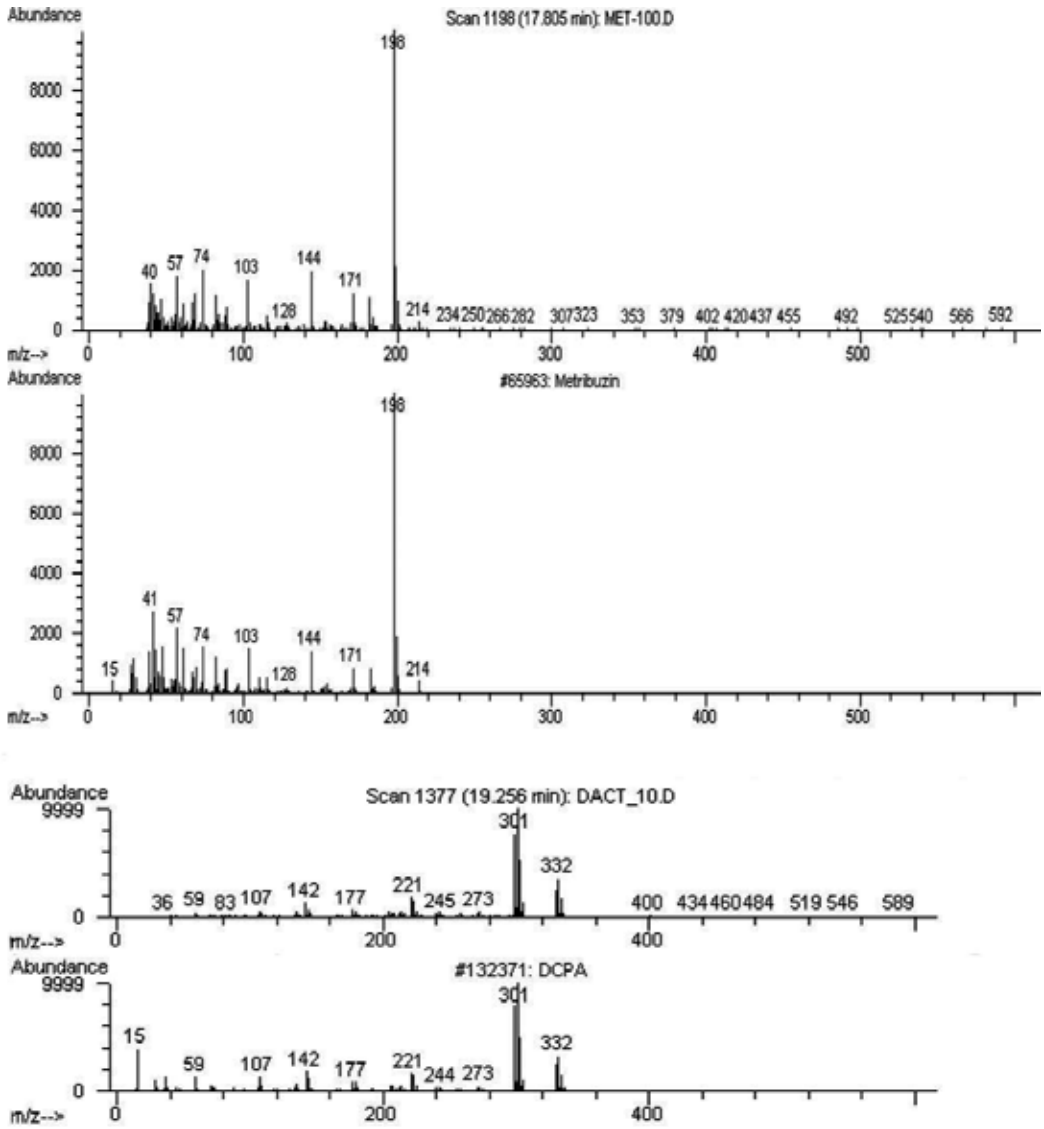

Figure 12. Electron impact mass spectrum of metribuzin extracted from soil indicating the molecular weight of 214 and molecular ions of m/z 57, 74, 103, 144, 171, and 198, along with characteristic fragment ions (upper graph) and DCPA extracted from soil indicating the molecular weight of 332 and molecular ions of m/z 59, 107, 142, 177, 221, 273, and 301, along with characteristic fragment ions (lower graph).

formulation (Dow AgroSciences) were used at the recommended rates of application in Kentucky [1]. The two herbicides were mixed in a total volume of 15 gallons of water and sprayed uniformly on the field plots (total area = 0.36 acre) using a portable backpack sprayer equipped with one conical nozzle operated at 40 psi (275 kPa). Following herbicide spraying, soil samples were collected, dried, sieved to a size of 2 mm, and extracted with 100 mL of acetonitrile: hexane: methanol mixture (45:45:10 v/v) using Soxhlet extraction apparatus

(Figure 13). The extracts were dried over anhydrous Na_2SO_4 to remove any traces of water and concentrated by rotary vacuum (Buchi Rotavapor Model 461, Switzerland) and N_2 stream evaporation.

Figure 13. Use of soxhlet extraction units and liquid-liquid partition for extraction of dimethazone and trifluralin herbicides from soil.

Trifluralin and dimethazone herbicides were extracted from runoff and infiltration water samples with a mixture of methylene chloride (CH_2Cl_2) + acetone (6:1, v/v) and sodium chloride solution by liquid-liquid partition. The solvent was filtered through a Buchner funnel containing Whatman 934-AH, of 55-mm-diameter glass microfiber filter (Fisher Scientific, Pittsburgh, PA), passed through anhydrous sodium sulfate (Na_2SO_4), and concentrated by rotary vacuum evaporator to a known volume. Concentrated extracts were injected into a gas chromatograph (GC) equipped with flame ionization detector (FID). Retention times (Rt) of trifluralin and dimethazone averaged 16.29 and 17.43 min, respectively (Figure 14). The molecular weight of trifluralin (335) is greater than that of dimethazone (239). However, a trifluralin peak appeared before dimethazone. This might be because of the greater vapor pressure of trifluralin as indicated in Table 1. The electron impact mass spectrum of trifluralin (Figure 15) showed spectral data with molecular ion peaks (M+) at m/z 306, 290, 264, and 43. Dimethazone electron mass spectrum (Figure 16) with spectral data at m/z 204, 125, 89, and 41 is consistent with those reported by the National Institute of Standards and Technology [80]. In soil, the ion m/z 204 is formed by loss of the atom of chlorine and the m/z 125 is formed by the breakage of the molecule of dimethazone at the carbon bond with nitrogen and the subsequent loss of the $–C_5H_8NO_2$ of m/z 114.

Results indicated that dimethazone residues extracted from sewage sludge and sewage sludge mixed with yard waste compost increased by 14 and 50 %, respectively, compared to no-mulch soil. Similarly, trifluralin residues increased by 17 and 75 % in sewage sludge and sewage sludge mixed with yard waste compost, respectively (data not shown), compared to no-mulch native soil. This could be explained by the adsorption properties of dimethazone on soil particles that varied with increasing percentages of soil organic matter following the addition

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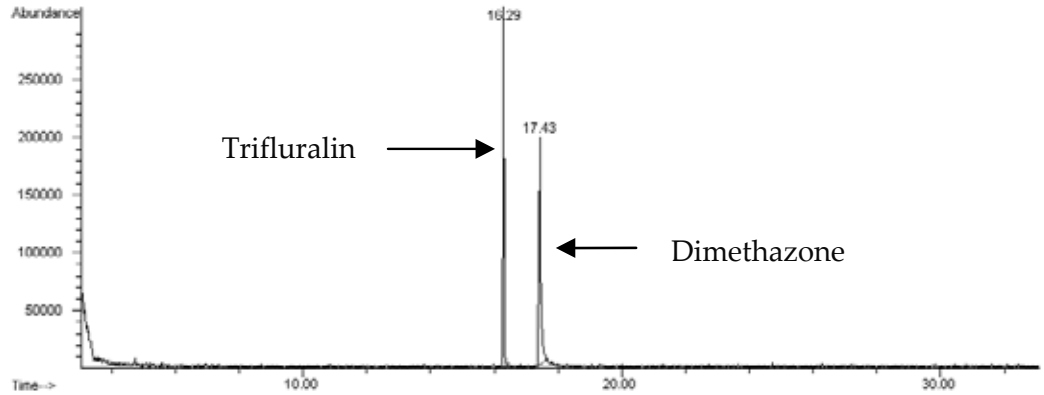

Figure 14. Gas chromatographic (GC) chromatograms of soil extracts prepared in acetonitrile: hexane: methanol (45:45:10 v/v) one hour following spraying with a mixture of dimethazone and trifluralin formulations.

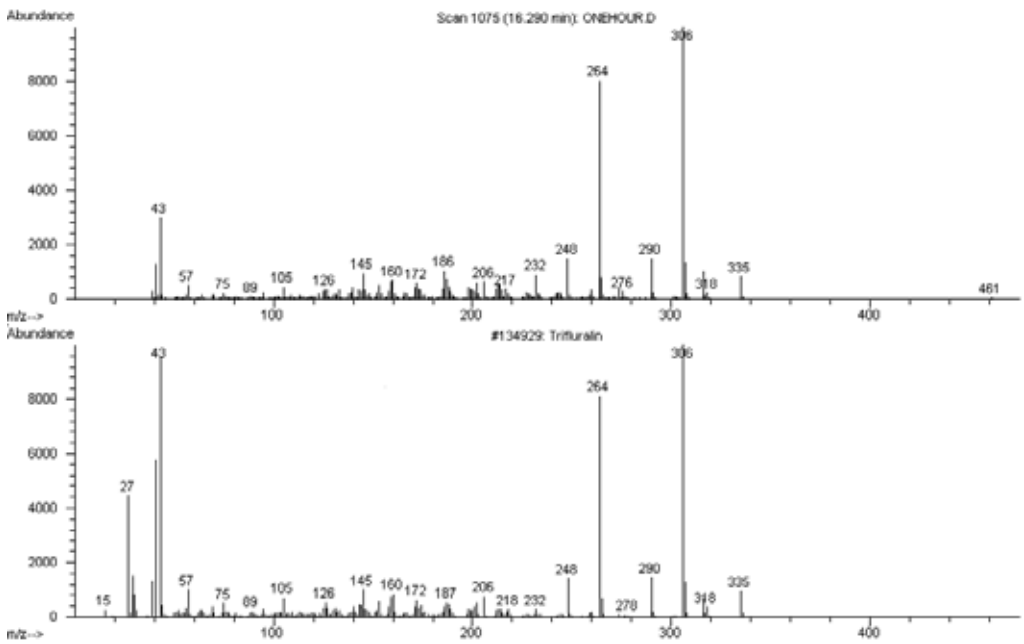

Figure 15. Electron impact mass spectrum of trifluralin ($C_{13}H_{16}N_3O_4F_3$) extracted from soil indicating the molecular ions of m/z 306, 290, 264, and 43 along with other characteristic fragment ions.

of amendments. Loux et al. [99] proposed hydrophobic bonding to organic matter to be the primary mechanism of dimethazone sorption and that bioavailability and dissipation of dimethazone in soil are determined by dimethazone adsorption properties. Soil amendments such as yard waste compost contain significant concentrations of humic acid, the main constituent of soil organic matter. Tavares and Rezende [100] indicated that functional groups in humic acid, namely, carboxylic and phenolic groups (Figure 11) appeared to be the principal

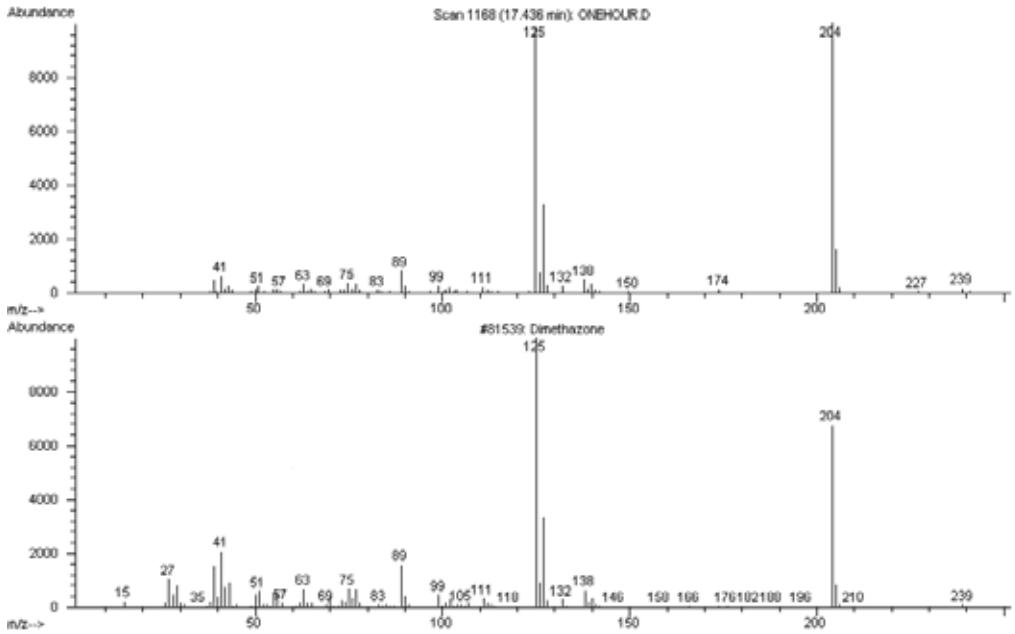

Figure 16. Electron impact mass spectrum of dimethazone ($C_{12}H_{14}$ Cl NO_2) extracted from soil indicating the molecular ions of m/z 204, 125, 89, and 41 along with other characteristic fragment ions.

sites for the adsorption and interaction with trifluralin. This might explain why trifluralin residues were higher in compost-amended soil than in no-mulch bare soil. Due to mechanical incorporation of the herbicide in the top 10–15 cm of soil, an equilibrium is usually established between the pesticide adsorbed to the soil and that in solution. This equilibrium reduces the transport and movement of strongly adsorbed pesticides such as trifluralin [101]. Accordingly, recyclable wastes have unique properties that should be thoroughly investigated in the soil/water/plant ecosystem. Binding of pesticides to humus [83] might decrease the amount of pesticides available to interact with biota, thus reducing the toxicity of the pesticide active ingredients. Binding might inhibit the mobility of xenobiotics via leaching and runoff, thus preventing the contamination of aquatic environments. This is particularly important because the extensive use of herbicides such as trifluralin has a high toxicity to fish (Table 1). Some herbicides are highly soluble in water, but because of their ionic properties, they bind tightly to the soil particles and organic matter in recycled waste and pose minimal risk for ground and surface water contamination.

Table 1 indicated that the soil binding property (Koc) of dimethazone is only 150–562 mL g^{-1}, while Koc of trifluralin is 8,000 mL g^{-1}. Greater Koc values of trifluralin indicated a tighter binding to the soil particles [102, 103]. Occurrence of trifluralin at concentrations of 50 to 130 ng g^{-1} on dry-weight basis has been reported in soils 30 months after the last application [104], and since the adsorbed herbicide becomes biologically inactive, therefore higher volume application rates are needed for soils rich in organic matter. Dimethazone is soluble in water

http://dx.doi.org/10.5772/60870

Property	Dimethazone	Trifluralin	Reference
Water Solubility (g L^{-1})	1.1	0.22	[92]
Fish LC_{50} (mg L^{-1}) Rainbow trout Bluegill sunfish	 19 34	 0.01-0.04 0.02-0.09	 [92] [92]
Log K_{OW} †	2.5	5.1	[100] [101]
K_{OC} (mL g^{-1}) ‡	150-562	8,000	[102] [82]
Vapor Pressure (mm Hg at 29 ^{O}C)	1.44×10^{-4}	1.99×10^{-4}	[92]
Concentration (μg g^{-1} Soil)	0.4	1.1	[15]
Concentration (mg $Plot^{-1}$) in infiltration water	0.08	0.009	[15]

† Partition coefficient between n-octanol and water (as log value)

‡ Organic carbon partition coefficient

Note that one plot is 0.02 of an acre

Table 1. Physical and chemical properties of dimethazone and trifluralin herbicides sprayed at Kentucky State University Research Farm, Franklin County, Kentucky, USA and their concentrations in runoff and infiltration water following natural rainfall.

and hence poorly bonds to most soils, giving it a potential for leaching into the soil column. These findings indicated that soil amendments and farm management practices play a major role in influencing herbicide residue levels in soil and their off-site mobility into natural water resources.

6. Conclusion

Herbicide washes off from agricultural fields and the Kentucky River watershed impacts surface water quality. Analysis of the herbicide metribuzin in the top soil indicated that considerable residues were detected in soil amended with chicken manure and sewage sludge compared to native soil. Similarly, soil amended with chicken manure or sewage sludge retained DCPA residues up to 99 d compared to unamended soils. Runoff water volume from no-mulch soil (200,000 L $plot^{-1}$) was significantly greater than runoff from soil amended with chicken manure and sewage sludge-amended soils (45,000 and 85,000 L $plot^{-1}$, respectively). Knowledge about the environmental problems and adoption of appropriate solutions and practices to enhance and protect our national water quality from environmental pollution by herbicides require timely delivery of research and educational technology. The development of production systems and mitigation techniques that reduce the introduction of agrochemicals like herbicides into the environment presents a continuing challenge. Soil mixed with chicken manure and sewage sludge increased water infiltration into the soil column toward the vadose

zone, reducing surface water runoff down the land slope. Agricultural runoff is the main contributor to poor water quality. Recycling waste for use as a low-cost fertilizer promoted the restoration of ecologic and economic functions of soil. Composts provide a stabilized form of organic matter that improves the physical properties of soils by increasing nutrient and water holding capacity, total pore space, aggregate stability, erosion resistance, and temperature insulation and decreasing apparent soil density [105] and binding of herbicides to soil particles and organic matter in soil amendments through herbicide sorption process that include chemisorption, complexation, adsorption, diffusion through the pores, and ion exchange [36]. Mobility of herbicides is affected by many site-specific variables, including the amount of soil organic matter, particle size distribution, porosity, rainfall, and application rates [30]. Field trials related to the mobility of trifluralin herbicide were conducted on a Lowell silty loam soil (pH 6.7, 2 % organic matter) of 10 % slope located at the Kentucky State University (KSU) H.R. Benson Research Farm (Franklin County, KY, USA). The farm is located in the Kentucky River watershed in the Bluegrass region. Results indicated that yard waste compost mixed with native soil reduced trifluralin residues in surface runoff water from June to July rainfall by 76 and 84 %, respectively. When sewage sludge and chicken manure were mixed with native soil, half-life ($T_{1/2}$) values of metribuzin were 24, 18, and 12 d in chicken manure, sewage sludge, and no-mulch treatments, respectively, whereas $T_{1/2}$ values of DCPA (dacthal) were greater in chicken manure and sewage sludge-incorporated soil (45.8 and 52.2 d, respectively) compared to NM native soil (26.2 d). A strong positive relationship was found between napropamide (an herbicide) concentrations and organic matter content in soil leachates. Sewage sludge and yard waste mixture added to native soil increased water infiltration, lowering surface runoff water volume and dimethazone (an herbicide) residues in runoff following natural rainfall events. Accordingly, soil amendments could be used to intercept herbicide-contaminated runoff from agricultural fields and might provide a potential solution to herbicide contamination of surface and seepage water from farmlands.

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Chapter 2

Potato Production near Glyphosate-resistant Crops — Injury Potential

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Additional information is available at the end of the chapter

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Abstract

The herbicide glyphosate is used in many countries because of low cost and effective weed control, but low levels of glyphosate on potato can reduce yield, marketability, and seed quality. Glyphosate is a phloem-mobile herbicide that can translocate to tubers, causing malformations that reduce the quality of current-season production. Potato plants are most susceptible to glyphosate at the hooking or tuber initiation stage. Tubers exposed at these stages often will become malformed and yield loss can occur. Seed production can be affected because glyphosate degradation is slow and it translocates to tubers. Seed potato exposed to glyphosate can store glyphosate residues until they are planted the next season. Tubers planted with glyphosate residues will have an erratic and slow emergence pattern, bending and twisting of leaves, multiple shoots from eyes, "candelabra" or "cauliflower" formation of shoots, or completely inhibited shoot growth, depending on the rate and cultivar. Glyphosate-affected seed tubers produce less tuber set and tubers with reduced weight. Tubers suspected to have glyphosate injury should be tested at a reputable laboratory to confirm glyphosate residues are present. Good management practices can help prevent potato from being exposed to glyphosate.

Keywords: "Red Norland", "Russet Burbank", elephant hide, growth cracks, graded yield

1. Introduction

Introduced in 1974 as a nonselective postemergence herbicide, glyphosate has become the most commonly applied herbicide in the world with approximately 80% of the transgenic crops worldwide as glyphosate resistant [1]. Glyphosate-resistant crops were first introduced in 1996 for soybean [*Glycine max* (L.) Merr.] and canola (*Brassica napa* L.), followed by cotton (*Gossypium hirsutum* L.) in 1997, corn (*Zea mays* L.) in 1998, alfalfa (*Medicago sativa* L.) in 2006, and sugar

beet (*Beta vulgaris* L.) in 2007. Green [2] attributed the unprecedented increase in glyphosate usage due to broad-spectrum weed control and more profitable yields with the glyphosate-resistant crop systems. However, glyphosate also is commonly applied preplant to kill existing vegetation prior to planting spring-seeded crops and preharvest on glyphosate-resistant and non-glyphosate-resistant crops such as barley (*Hordeum vulgare* L.), flax (*Linum usitatissimum* L.), lentil (*Lens culinaris* Medic.), pea (*Pisum arvense* L.), safflower (Carthamus tinctorius L.), sunflower (*Helianthus annuus* L.), and wheat (*Triticum aestivum* L.) [3].

Glyphosate is often applied multiple times in a year, using either ground or aerial equipment. Ding et al. [4] reported that the average number of glyphosate applications in glyphosate-resistant soybean and cotton was 2.6 and 3.1, respectively. Numerous studies have reported injury from simulated glyphosate drift on conventional corn [5], cotton [6], onion (*Allium cepa* L.) [7], peanut (*Arachis hypogaea* L.) [8], potato (*Solanum tuberosum* L.) [9], rice (*Oryza sativa* L.) [10], tomato (*Lycopersicon esculentum* Mill.) [11], soybean [12], and wheat [13]. Glyphosate spray drift is the physical movement of glyphosate particles onto an off-target plant and can occur during applications when weather conditions promote drift. In virtually all pesticide applications, a small fraction of the pesticide moves downwind and onto off-target surfaces. The amount of spray particles moving off-target depends on many factors such as environmental conditions, herbicide formulation, droplet size spectrum from specific nozzles, and spray release height above the target [14,15]. Bode [14] reported that between 1% and 16% of the applied rate could drift downwind from the intended target. Glyphosate drift is particularly important because the herbicide is nonselective and highly active on sensitive plant species at low doses, but also because conventional (non-glyphosate-resistant) crops are frequently planted adjacent to glyphosate-resistant crops.

2. Commercial potato production

Potato production originated in the Andean region of South America as early as 7,000 years ago [16]. The first record of potato coming to Europe from South America was approximately 1570 AD. Potato reached North America by the early 1700s and is currently grown in over 95 countries through the world. Potato ranks fourth among all food crops in total production. The United States ranks fifth behind China, Russia, Poland, and Germany in potato production. However, in North Dakota, potato is one of the most important vegetable and horticultural crops with approximately 31,566 ha harvested in 2013 and a farm-gate value over $232.9 million [17].

2.1. Potato growth stages

Potato growth and deployment can be separated into five stages. The first stage is when a whole or cut seed tuber is planted, dormancy is broken, and sprouts begin to develop from the eyes and grow toward the soil surface. The second stage begins when the shoot emerges and vegetative growth continues until tuber initiation. Stage three, or tuber initiation, is when tubers form at the end of stolons. Flowering of many potato cultivars occurs close to this time or at the end of tuber initiation. The fourth stage is tuber bulking, or when tubers enlarge. This

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is the most critical stage for tuber growth and yield. The final stage consists of leaf and stem senescence and tuber maturation [18].

2.2. Determinate and indeterminate

Potato cultivars can be classified as determinate, semi-determinate, or indeterminate. Short-season cultivars are often assumed to be determinate. They tend to have shorter vine lengths and fewer flower clusters. These plants grow leaves for a determined period, approximately 10–13 leaves, flower, and cease vine growth once tuber initiation begins. The longer-season potatoes are those of a semi-determinate or indeterminate nature. These plants will continue to grow leaves, flowers, and can initiate tubers throughout the growing season, requiring a longer season to complete their growth cycle.

3. Glyphosate injury to potato

The widespread adoption of glyphosate-resistant crops has increased glyphosate applications and widened the application window. Reddy et al. [19] reported that the number of observed herbicide drift cases onto nontarget crops increased by 60% from 2007 to 2008 in Mississippi. Glyphosate drift caused 58% of all drift cases. In general, grass species are more sensitive to glyphosate than broadleaf species; thus, glyphosate drift onto crops such as corn, rice, and wheat can cause significant yield reduction, especially if the drift occurs during a sensitive growth stage [5,13,19, 20]. However, the economic loss in these crops may not be as great as from drift to less sensitive high-value broadleaf crops that are consumed with little processing and have no Environmental Protection Agency (EPA) tolerance level, such as many fruits and vegetables.

Potato injury from glyphosate can occur from spray or particle drift, misapplication, or tank contamination. Particle drift often occurs when herbicides are applied under windy conditions (> 16 kph) and when environmental conditions favor volatilization and redisposition [21]. Many factors, including temperature inversion, affect particle drift, but the most important factor is the initial size of the droplet. Droplets smaller than 100 microns are considered highly driftable and can move over 61 m downwind in a 16-kph wind [22]. Felsot et al. [23] suggested that between 1% and 10% of the applied herbicide rate moved off-target during an application. However, Maybank et al. [24] reported that as much as 16% of the target dose drifted downwind from an unshielded sprayer. Most spray drift studies on crop injury in the United States have utilized sublethal doses in "controlled" experimental situations [23]. The objective of many simulated drift studies is to evaluate plant growth and development in response to low doses of a particular herbicide. Similarly, the intent of the rest of this chapter is to describe potato response to sublethal glyphosate doses.

3.1. Current-season symptomology

Glyphosate inhibits the synthesis of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme, which disrupts the shikimic acid pathway that produces aromatic amino acids such

as phenylalanine, tyrosine, and tryptophan used for protein synthesis and plant growth [25]. Visually, a sublethal glyphosate dose may cause chlorosis of the newest potato leaves as early as 7 days after treatment (DAT) depending on the glyphosate rate and potato growth stage [9]. They estimated that 30.5 g ae ha^{-1} glyphosate would be required to produce 5% visual injury. Glyphosate sprayed onto 10-cm plants caused new shoots to be produced from the tuber seed piece, which did not display injury symptoms. However, treated seed pieces were delayed in development compared to the nontreated. Plants at the hooking stage (BBCH-scale 40) appeared to be the most sensitive to glyphosate. They estimated the glyphosate dose resulting in 50% injury was approximately four and 53 times greater when potato plants were sprayed at the 10-cm height and bulking stage, respectively, compared to the hooking stage.

Hatterman-Valenti and Auwarter [26] reported chlorosis at the growing points, but indicated that this could be quite transient depending on glyphosate dose and environmental conditions. They also noted that little to no potato injury symptoms occurred when glyphosate was applied at the late bulking stage. Similarly, Crook and Hatterman-Valenti [27] reported no visible injury symptoms when glyphosate doses lesser than one-fourth of the lowest recommended single application rate of 846 g ha^{-1}were applied to "Red Norland" plants at the late bulking stage (BBCH-scale 47) but concluded that the lack of plant symptomology was because the determinant plants had flowered and stopped foliar growth.

3.2. Current-season tuber yield and quality

When glyphosate comes in contact with potatoes during the growing season, the herbicide enters the plant and is translocated to the growing points, both above and below ground. Daughter tubers form at the terminal end of stolons, which are lateral stems growing horizontally below ground from buds of the underground part of the stems. Morphologically, tubers are also modified stems that constitute the main storage organs of the potato plant [28]. Developing tubers act as a below ground growing point accumulating assimilates produced by the leaves and other exogenous compounds, including glyphosate, that is translocated by the plant. Tuber symptomology from glyphosate exposure may consist of skin cracking, tuber malformations, and tissue death, which may allow entry to secondary pathogens. Crook and Hatterman-Valenti [27] reported tuber cracking as early as 3 days after treating with glyphosate. Early tuber cracks and malformations from glyphosate-treated plants tend to increase as the tubers bulk or increase in size. This results in reduced marketability of tubers. Tuber cracking may be mistaken for growth cracks, but tuber cracks from glyphosate generally tend to cross the apical end of the tuber and often make an "X" configuration (Figure 1). Other tuber symptoms include elephant hide where small surface cracks make the tuber appear scaly. With red cultivars, the red skin color fades or has portions that appear as brown skin along with elephant hide. With the glyphosate dose at 215 g ha^{-1} and potatoes in the tuber initiation growth stage at application, some tubers continue to crack with little growth and look like large pieces of popcorn.

3.3. Simulated glyphosate drift trial

Worthington [29] may have been the first researcher to show that low doses of glyphosate would affect the growth and yield of potato mother plants. To better understand how potato

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Figure 1. Tubers from two plants where glyphosate drift was suspected. Tuber symptoms are typical from glyphosate exposure during plant hooking or tuber initiation growth stages.

growth stage could alter this affect, a two-year field study treated "Russet Burbank" plants at four growth stages—hooking (H)(BBCH-scale 40), tuber initiation (TI)(BBCH-scale 41), early bulking (EB)(BBCH-scale 43), and late bulking (LB)(BBCH-scale 47)—to evaluate glyphosate injury to the current-season crop. Plants within each of the growth stages received a sublethal dose of glyphosate, which corresponded to one-fourth, one-eighth, one-sixteenth, or one-thirty-second the lowest recommended single application rate for glyphosate at 846 g ha^{-1}. Plants at the H stage were an exception with only one sublethal dose (215 g ha^{-1}) applied at this stage. The last treatment of this 14-treatment trial, arranged as a randomized complete block with four replicates, was the nontreated check, which consisted of spraying the plants with a water plus ammonium sulfate solution. The middle two rows of each plot were harvested and graded in October. Data were subjected to analysis of variance (ANOVA) using PROC GLM (SAS version 9.2, SAS Institute Inc., Cary, NC) and, where appropriate, Fisher's protected least significant difference (LSD) tests, at a probability level of ≤ 0.05, were used for mean separation. Data could not be pooled across years for all yield and grade data and thus were analyzed separately.

3.3.1. Simulated glyphosate drift trial: Yield

The nontreated plots produced the greatest total yield in 2008, but this did not differ from the total yields when plants received sublethal glyphosate doses during LB or when plants received the lowest two glyphosate doses during TI or EB (Table 1). The greatest total yield in 2007 occurred when plants in the EB stage received the lowest glyphosate dose of 27 g ha^{-1}. However, similar total yields were also obtained with nontreated plants, plants that received the lowest two glyphosate doses during TI, plants that received glyphosate doses during EB, or when plants received the lowest three glyphosate doses during LB. Plants that received 215 g ha^{-1} glyphosate during H in 2007 had the lowest total yield, almost 5.5 times less than the nontreated and almost half the total yield, when compared to plants receiving the same glyphosate dose but at the TI stage. Similarly, Hutchinson et al. [30] reported that a sublethal glyphosate dose to potato at the H stage reduced yields more than 40% compared to the nontreated yields. However, in their study, they also subjected plants to sublethal glyphosate doses when the plants were only 10–15 cm tall and concluded that these plants would most likely recover even from relatively high glyphosate doses.

		Growth stage	Tuber yield			
		when	Marketable		Total	
	Rate	treated	2007	2008	2007	2008
	g ha^{-1}		MT ha^{-1}			
Glyphosate	215	H[a]	5.7 c[b]	15.7 c	9.9 d	29.3 bc
Glyphosate	215	TI	10.5 c	10.6 c	20.9 c	29.9 bc
Glyphosate	107	TI	26.6 b	13.7 c	41.8 ab	36.9 bc
Glyphosate	54	TI	39.2 ab	29.4 b	49.3 a	45.5 ab
Glyphosate	27	TI	47.3 a	41.5 ab	54.4 a	53.8 a
Glyphosate	215	EB	43.5 a	12.6 c	50.2 a	28.0 c
Glyphosate	107	EB	45.5 a	21.0 c	52.2 a	34.7 bc
Glyphosate	54	EB	49.0 a	39.1 ab	55.6 a	56.3 a
Glyphosate	27	EB	49.6 a	39.9 ab	55.8 a	55.8 a
Glyphosate	215	LB	28.2 b	40.3 ab	34.6 b	55.6 a
Glyphosate	107	LB	39.2 ab	40.7 ab	45.2 ab	57.2 a
Glyphosate	54	LB	44.6 a	39.8 ab	51.9 a	53.6 a
Glyphosate	27	LB	42.2 a	40.6 ab	48.4 a	53.4 a
Non-treated			50.5 a	43.4 a	55.1 a	60.2 a

[a] Abbreviations: H = hooking, TI = tuber initiation, EB = early bulking, LB = late bulking.
[b] Means within a column with a different letter are significantly different according to a Fisher's protected LSD test performed at the 0.05 level of probability.

Table 1. Effect of glyphosate dose on Russet Burbank tuber yield.

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Marketable yields mimicked total yields for most treatments (Table 1). Both years suggested that plants treated in the H and TI stages would have the greatest marketable yield reduction if plants would come in contact with sublethal doses of glyphosate. However, in 2007, marketable yield was not influenced if plants received ≤ 215 g ha^{-1} glyphosate during the EB stage, while in 2008, marketable yield was not influenced if plants received ≤ 215 g ha^{-1} glyphosate during the LB stage.

The response difference could be partially explained once the maximum and minimum air temperatures were plotted for the two growing seasons (Figure 2). In 2007, many of the days during the plant H and TI stages had maximum and minimum air temperatures greater than the 30-year average (normal). Thus, the lower set number and lower tuber number per plant in 2007 were understandable. In addition, 2007 air temperatures had been high just prior to the glyphosate dose at EB, but on the application day and the next 2 days, both maximum and minimum air temperatures were below normal. A similar event occurred during LB in 2008 along with a 6 °C drop in soil temperature (data not shown). It was concluded that these decreases in air temperature, especially at night, allowed a quicker recovery and the resumption of tuber bulking. Felix et al. [9] conducted a similar trial with "Ranger Russet" and concluded that injury was greater to potato plants receiving a glyphosate dose of 54 g ha^{-1} or more during the H or TI stages as compared to the other stages. Tuber yield at Paterson, WA, was reduced by 84% and 77% compared to the nontreated when plants received 107 g ha^{-1} glyphosate at H and TI, respectively, while at Ontario, OR, the yield reduction was only 54% and 52%. Thus, environmental conditions or other plant stresses may greatly influence plant recovery from sublethal glyphosate doses. To further confound matters, Hatterman-Valenti et al. [31] reported that russet cultivars respond differently to sublethal glyphosate doses with "Bannock" as the most sensitive cultivar of the four evaluated.

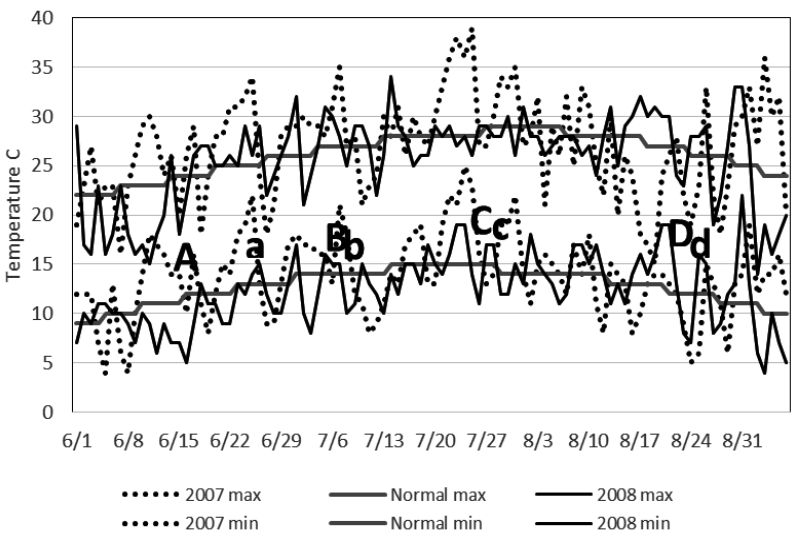

Figure 2. Minimum and maximum air temperatures along with normal minimum and normal maximum temperatures. Capital letters (A, B, C, D) represent when glyphosate doses were applied to potatoes during 2007. Small letters (a, b, c, d) represent when glyphosate doses were applied to potatoes during 2008.

3.3.2. Simulated glyphosate drift trial: Tuber grade

Tuber grade showed an increase in cull-sized tubers (< 113 g) compared to the nontreated when plants received ≥ 54 g ha^{-1} glyphosate at the TI stage but only in 2007 (Table 2). In 2008, the amount of cull-sized tubers were doubled and, often, tripled in comparison to 2007. However, tuber distribution as a percentage of the total yield showed that the nontreated had approximately 25% of the tubers at culls both years, suggesting that 2008 was a more productive year to grow "Russet Burbank" (Table 3). As grade size increased in 2007, plants that received the highest glyphosate dose at H, TI, and LB tended to produce few tubers in each grade level compared to the nontreated (Tables 2 and 3). As grade size increased in 2008, plants that received the two highest glyphosate doses at H, TI, and EB tended to produce few tubers in each grade level compared to the nontreated.

		Growth stage	Tuber yield									
		when	≤ 113 g		114–170 g		171–283 g		284–340 g		≥ 341 g	
	Rate	treated	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
	g ha^{-1}		MT ha^{-1}									
Glyphosate	215	H[a]	4.0 d[b]	13.1 bc	2.6 e	6.1 bc	2.4 c	5.3 c	0.4 c	2.0 c	0.2 d	2.9 a
Glyphosate	215	TI	9.8 b	16.6 bc	5.3 d	3.5 c	4.4 c	3.3 c	0.6 c	0.5 c	0.0 d	0.3 bc
Glyphosate	107	TI	14.4 a	23.1 a	10.2 ab	8.7 b	11.3 b	4.3 c	2.7 bc	0.4 c	1.6 cd	0.0 c
Glyphosate	54	TI	9.6 b	17.3 bc	10.6 a	13.6 a	17.5 ab	11.4 b	4.9 ab	1.9 bc	5.2 bc	2.1 ab
Glyphosate	27	TI	6.9 bcd	13.8 bc	8.7 bc	13.8 a	18.9 ab	18.2 a	7.5 a	4.1 a	11.5 ab	4.0 a
Glyphosate	215	EB	6.3 bcd	17.9 b	8.1 bc	6.5 bc	16.1 a	4.1 c	6.4 a	0.5 c	12.3 a	0.6 bc
Glyphosate	107	EB	6.6 bcd	15.4 bc	8.0 bc	13.2 a	17.2 a	7.8 bc	5.2 ab	0.9 c	14.8 a	0.4 bc
Glyphosate	54	EB	6.5 bcd	15.5 bc	8.7 bc	14.4 a	20.2 ab	17.5 ab	5.9 ab	3.0 ab	13.9 a	3.0 a
Glyphosate	27	EB	6.1 bcd	12.1 c	9.5 bc	12.8 a	19.6 ab	17.2 ab	6.7 a	4.1 a	13.5 a	5.0 a
Glyphosate	215	LB	6.3 bcd	15.6 bc	7.0 cd	14.8 a	12.5 ab	17.6 ab	2.6 bc	3.1 ab	5.1 bc	3.7 a
Glyphosate	107	LB	5.9 bcd	15.4 bc	8.8 bc	14.6 a	15.2 ab	17.6 ab	5.0 ab	3.8 a	9.7 ab	3.8 a
Glyphosate	54	LB	7.3 bc	13.8 bc	9.4 bc	15.1 a	16.7 ab	17.8 ab	6.1 ab	4.1 a	11.9 ab	3.1 a
Glyphosate	27	LB	6.1 bcd	13.4 bc	8.2 bc	14.5 a	16.4 ab	18.1 a	5.7 ab	3.1 ab	11.7 ab	4.4 a
Non-treated			4.6 cd	14.5 bc	7.4 bc	14.5 a	18.6 ab	18.9 a	8.6 a	3.5 ab	15.5 a	5.5 a

[a] Abbreviations: H = hooking, TI = tuber initiation, EB = early bulking, LB = late bulking.
[b] Means within a column with a different letter are significantly different according to a Fisher's protected LSD test performed at the 0.05 level of probability.

Table 2. Effect of glyphosate rate on Russet Burbank tuber grade.

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The effect of glyphosate on tuber growth is most evident when examining tuber distribution as a percentage of total yields (Table 3). In 2007, plants that received the highest glyphosate dose at H and TI had at least 70% of their tubers considered culls, while in 2008, plants that received the highest glyphosate dose at H and TI had 47%–64% of their tubers considered culls. Reductions in the 171–283-g grade are extremely important to processors of frozen French fries as this grade is ideal for long French fries. During both years, the nontreated plants had approximately 32% of their tubers in this category. However, plants in the H and TI stages that received sublethal glyphosate doses in 2007, with the exception of plants receiving 27 g ha^{-1}, had a lower percentage of tubers in the 171–283-g category than the nontreated. In 2008, plants in the H, TI, and EB stages that received sublethal glyphosate doses, with the exception of plants receiving 27 g ha^{-1} and plants in the EB stage receiving 54 g ha^{-1}, had a lower percentage of tubers in the 171–283-g category than the nontreated.

		Growth stage	Tuber yield									
		when	≤ 113 g		114–170 g		171–283 g		284–340 g		≥ 341 g	
	Rate	treated	2007	2008	2007	2008	2007	2008	2007	2008	2007	2008
	g ha^{-1}						%					
Glyphosate	215	H[a]	70 a[b]	47 abc	17 cd	18 b	10 cd	20 b	1 e	4 cd	1 f	9 a
Glyphosate	215	TI	76 a	64 a	14 d	20 b	8 d	11 c	1 e	1 e	0 f	1 cd
Glyphosate	107	TI	59 a	63 a	20 bc	24 ab	15 c	12 c	3 de	1 e	2 ef	0 d
Glyphosate	54	TI	41 b	37 cd	22 a	29 a	25 b	24 b	5 bcd	4 cd	4 de	4 bc
Glyphosate	27	TI	33 bc	25 e	21 ab	27 a	29 a	33 a	8 ab	7 ab	9 bc	7 ab
Glyphosate	215	EB	29 bc	60 ab	23 a	21 b	30 a	14 bc	7 bc	1 e	11 b	1 cd
Glyphosate	107	EB	31 bc	45 bc	20 bc	29 a	31 a	20 b	6 bcd	2 de	11 b	1 cd
Glyphosate	54	EB	33 bc	28 de	20 bc	28 a	28 a	32 a	6 bcd	6 ab	12 ab	6 ab
Glyphosate	27	EB	33 bc	23 e	21 ab	25 a	27 a	33 a	8 ab	8 a	10 b	10 a
Glyphosate	215	LB	41 b	28 de	22 a	27 a	26 ab	32 a	4 cde	5 bc	6 d	6 ab
Glyphosate	107	LB	32 bc	27 de	25 a	27 a	28 a	31 a	6 bcd	7 ab	9 bc	7 ab
Glyphosate	54	LB	34 bc	26 e	23 a	27 a	26 ab	33 a	7 bc	8 a	9 bc	6 ab
Glyphosate	27	LB	33 bc	25 e	22 a	27 a	28 a	34 a	7 bc	6 ab	10 b	8 a
Non-treated			24 c	25 e	20 bc	26 a	32 a	33 a	11 a	6 ab	14 a	9 a

[a] Abbreviations: H = hooking, TI = tuber initiation, EB = early bulking, LB = late bulking.
[b] Means within a column with a different letter are significantly different according to a Fisher's protected LSD test performed at the 0.05 level of probability.

Table 3. Effect of glyphosate rate on Russet Burbank tuber number as a percent of the total.

Tuber yield and grade data suggest that in addition to a general shift toward smaller-sized tubers, fewer tubers may have been produced, but this was only true in 2007 when plants in the H stage received 215 g ha^{-1} glyphosate (Table 4). However, the percentage of marketable tubers in 2007 was less than with the nontreated for all treatments, except when plants in the EB stage received 27 g ha^{-1} glyphosate. The average number of tubers per plant was higher in 2008, and all treatments were similar in tubers per plant. The percentage of marketable tubers in 2008 was less when plants in the TI and EB stages received ≥ 107 g ha^{-1} glyphosate and when plants in the H stage received 215 g ha^{-1} compared to the nontreated. These results suggest that glyphosate doses at 215 g ha^{-1} or higher may reduce tuber numbers per hill, but only if glyphosate enters plants during the critical tuber development stages of H or TI and when other stresses that occur do not maximize tuber set. On the other hand, marketable and total yield reductions (from reduced tuber size) may be expected if potato plants in the H or TI stages come in contact with ≥ 107 g ha^{-1} glyphosate or a lower dose if plant stress conditions simultaneously occur.

		Growth stage	Tuber yield			
		when	Marketable		Total	
	Rate	treated	2007	2008	2007	2008
	g ha^{-1}		%		no.	
Glyphosate	215	H[a]	29 e[b]	53 bc	2.9 d	10.2 b
Glyphosate	215	TI	23 e	34 d	6.6 c	11.9 ab
Glyphosate	107	TI	39 d	37 d	10.7 a	14.5 a
Glyphosate	54	TI	57 c	62 ab	9.3 ab	12.4 ab
Glyphosate	27	TI	66 b	74 a	8.4 bc	11.8 ab
Glyphosate	215	EB	66 b	39 d	7.7 bc	10.7 b
Glyphosate	107	EB	69 b	52 bc	7.9 bc	11.1 b
Glyphosate	54	EB	69 b	71 a	8.3 bc	12.5 ab
Glyphosate	27	EB	70 ab	76 a	8.3 bc	10.9 b
Glyphosate	215	LB	58 c	72 a	6.1 c	12.5 ab
Glyphosate	107	LB	67 b	72 a	7.1 bc	12.8 ab
Glyphosate	54	LB	65 b	74 a	8.2 bc	11.7 ab
Glyphosate	27	LB	67b	75 a	7.4 bc	11.8 ab
Non-treated			75 a	75 a	7.4 bc	12.4 ab

[a] Abbreviations: H = hooking, TI = tuber initiation, EB = early bulking, LB = late bulking.
[b] Means within a column with a different letter are significantly different according to a Fisher's protected LSD test performed at the 0.05 level of probability.

Table 4. Effect of glyphosate dose on Russet Burbank percent marketable and average number of tubers per plant.

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3.3.3. Simulated glyphosate drift trial: Conclusions

In summary, results from this study indicated that plants at the H and TI stages were most sensitive to glyphosate drift when evaluating current-season tuber yield and grade. Indirectly, this study also indicated that "Russet Burbank" potato yield and grade were not affected when treated with ≤ 215 g ha^{-1} glyphosate during EB or LB and environmental conditions conducive to quick recovery and/or no additional stresses were present. The tolerance of potato to sublethal glyphosate doses later in the growing season was reinforced by Haidar et al. [32] when they applied three sequential applications of 100 g ha^{-1} glyphosate following a rimsulfuron application to control *Orobanche ramosa* and found no negative effect on tuber yield or quality. However, application intervals were 15 days, and an increase of glyphosate rates to 200 g ha^{-1} produced nonmarketable tubers.

3.4. Glyphosate residues in seed production

Asexual seed production in potato is essential to preserve the genetic identity of potato cultivars. However, utilizing asexual production can cause problems with herbicide residues because, as the tubers are stored during a dormant period, the herbicide residues can also be stored. The seed potato crop is sensitive to many classes of herbicides, but the herbicide of most recent interest has been glyphosate. The reason for this is that millions of crop hectares globally now receive glyphosate herbicide treatments during the growing season because of the widespread acceptance of genetically modified crops in many countries. Glyphosate is a commonly used herbicide in agriculture because of low cost and effective control of grasses and broadleaf weeds.

3.4.1. How glyphosate injury occurs

Seed tubers can be exposed to glyphosate in a variety of ways. The primary way would be through phloem transport from the leaves and stems to the tuber during the previous growing season. However, some growers have reported that using contaminated water for in-furrow fungicide and insecticide treatments at planting caused effects similar to glyphosate-contaminated seed.

Exposure to glyphosate typically occurs through the leaves and stems. Glyphosate is translocated by the phloem to the metabolic sinks [33,34]. In potato, this would consist of the meristems, roots, and tubers, or the growing points above and below ground. New tubers developed by the plant, referred to as "daughter tubers," are a sink for assimilates produced by the leaves and for exogenous compounds (such as herbicides) that are translocated by the plant. The degradation of glyphosate within the plant can be slow as glyphosate is metabolized to aminomethylphosphonic acid [33]. This may be one of the reasons why glyphosate residues can be stored in tubers over time, until the next planting season.

3.4.2. Glyphosate uptake and translocation

Exposure to glyphosate can occur at any time after the shoots emerge from the soil. Hutchinson et al. [30] reported that visible injury tends to be greater when potatoes contact glyphosate

during vegetative growth, while visible injury from glyphosate is less apparent when tubers are bulking and are the primary sink. When the daughter tubers, or next field generation of tubers, were planted back the next season, tubers that received glyphosate during bulking had a reduced emergence rate, stem number, and marketable yield. Exposure to glyphosate during bulking may be unseen, but it can have dramatic negative effects on the next field generation of seed. Often the contact of glyphosate with potato in the bulking timing occurs at the same time that temperatures tend to be the highest. When temperature is high (24 °C day and 13 °C night), more glyphosate is absorbed and translocated compared to a low temperature (13 °C day and 4 °C night), causing greater injury [34] and potentially more glyphosate stored in tubers.

3.4.3. Testing for residual residues

Seed potato plants or mother plants can become exposed to glyphosate at any growth stage. Visual symptoms of glyphosate may be difficult to detect, especially if the rates are low or the exposure to glyphosate occurs during bulking. If glyphosate exposure is suspected, a laboratory test is the most accurate way to determine its presence. When taking a sample for residue analysis, both a leaf sample from the youngest leaves and a tuber sample should be taken because glyphosate will translocate to the growing points. Multiple plants should be sampled, and samples can be composited into a large sample to be tested. Timely sampling is important to detect glyphosate residues. In a study of young potato plants, glyphosate accumulation in meristematic tissue and roots was greatest between 4 and 8 days after exposure and was less thereafter [35]. Plants that have tubers, and are exposed to glyphosate, store the glyphosate in tubers. The effects of glyphosate will be seen when tubers are planted the next season.

3.4.4. Certification and winter testing

Symptoms of low glyphosate rates are difficult to observe visually and field inspections may not detect the symptoms. Potato seed certification typically makes multiple trips to inspect fields. Inspections are completed when the first signs of disease will be apparent. This timing is intended to check for virus, disease, cultivar purity, and other factors associated with seed quality. Typically, a final inspection is done prior to vine kill to determine if bacterial ring rot is present or any other diseases or infection sources that could compromise the quality of the seed. The postharvest, or winter test, is used for recertification. Inspections focus is primarily on virus, but other factors such as cultivar mixture, herbicide damage, vigor, and other diseases are noted. One of the challenges with glyphosate residues is that residues can inhibit sprouting of tubers. When tubers do not sprout in a winter test, inspectors should dig seed tubers and observe why they have not sprouted. Suspect tubers should be sent to a reputable laboratory to test for glyphosate residues.

3.5. Yield effects from glyphosate residue carryover in seed potato

Potato seed quality is always a concern when a potato field does not emerge properly. Glyphosate-contaminated seed produces a variety of obvious symptoms, but symptoms similar to glyphosate injury may be misdiagnosed as glyphosate. This is why laboratory testing

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for glyphosate is essential for proper diagnosis. The effects of glyphosate-contaminated seed that is planted can reduce yield and quality of potato. Additionally, the relationship between the seed producer and the buyer can be damaged when an herbicide contamination is found in a seed lot.

3.5.1. Germination of glyphosate-affected seed potatoes

A variety of problems can occur when tubers with glyphosate residues are planted. Hatterman-Valenti [36] reported only 18 g ha^{-1} glyphosate applied the previous growing season was needed to inhibit sprouts by 14% and 282 g ha^{-1} glyphosate inhibited sprouts by 95%. Glyphosate is so effective at inhibiting sprouts that it has been tested as a sprout suppressant to help tubers store longer in environments where refrigerated storage is not available [37]. If tubers are intended to be used for seed, sprout suppressants should never be used.

3.5.2. Symptomology

The first general observation of a potato field planted with glyphosate-contaminated seed is an erratic emergence pattern throughout the field. Being a seed-borne problem, a pattern in the field should not occur. Rather a scattering of plants with different rates of emergence should be observed (Figure 3). The erratic emergence pattern is found because tubers contain various amounts of glyphosate, and the higher the glyphosate level at the tuber eye, the slower the sprouts are to emerge from the soil. Plants with glyphosate residues will often express symptomology in new leaves through malformed leaves that appear to twist or bend in unnatural ways (Figure 4). Leaves have been noted to become chlorotic in some cases [38].

Figure 3. Erratic potato plant emergence pattern due to glyphosate-contaminated seed.

Figure 4. Plant symptoms from glyphosate-contaminated seed. Symptomology may be confused with those expected from drift of a plant growth regulator herbicide.

Figure 5. Multiple shoots emerging from a single eye of a glyphosate-contaminated tuber.

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When tubers are uncovered from the soil, more symptoms can be observed that can help identify the problem as glyphosate. In many cases, swelling of shoots or a "candelabra" of branching formation on the shoots may occur. Unique to glyphosate, multiple shoots coming from a single eye may be observed (Figure5). When glyphosate residue levels are at higher levels in tubers, shoots will not elongate, but a "cauliflower"-like formation of shoots will appear around the eye (Figure 6) [29,38]. If a field has these types of symptomology, carefully inspect the field because differences will be apparent by cultivar and the amount of glyphosate present in the tubers.

Figure 6. The "cauliflower"-like formation of shoots appearing around a single eye of a glyphosate-contaminated tuber with higher residue levels.

3.5.3. Residue testing

If glyphosate is suspected, the best way to confirm glyphosate residues in seed tubers is through laboratory testing. To test seed for glyphosate residues, use a credible laboratory that can detect glyphosate residues down to 0.01 parts per million (ppm). Select tubers for testing that have not emerged, because these tubers will likely have a higher concentration of

glyphosate, and this eliminates the potential of glyphosate drift contamination on newly emerged potato plants. Carefully remove the seed tubers from the soil and gently wash off all soil. Once tubers are clean, send the sample to the laboratory for analysis according to the laboratory directions. If results are positive, the presence of glyphosate is confirmed. The challenge from getting results from glyphosate residue testing is knowing the potential effects from the detected level of glyphosate. Robinson [38] reported that 0.015–0.036 ppm glyphosate was sufficient to reduce yield by 63% and tuber number by 38% compared to tubers with no glyphosate. However, this was a small study that compared only a few individual plants.

3.5.4. Yield loss

When herbicide injury occurs, a farmer expects to have some type of yield loss. With glyphosate-affected seed, a yield loss is often expected and, typically, a yield loss is found. The amount of reduction in yield will depend on the percentage of seed that contains glyphosate and the residue levels. But more importantly, the tuber size distribution is much wider than from a field that does not have glyphosate residues in the seed. Glyphosate-contaminated seed often produces plants that are slow to emerge compared to plants from uncontaminated seed. These normally emerging plants will outcompete the slower, or weaker, glyphosate-affected plants for sunlight and nutrients. This allows the stronger plant more resources, and as a result, tuber number and tuber size tend to be greater [38].

Research has demonstrated the amount of glyphosate, timing of glyphosate exposure to potato, and the environment has a direct effect on the seed and how it will perform when planted the next growing season. As expected, as glyphosate rates increase, so does reduction in emergence and yield. Rates studied have ranged from 8.5 to 423 kg ha^{-1} glyphosate [30, 36]. The cultivar "Ranger Russet" had the lowest total and marketable yield when glyphosate was applied at the bulking stage in comparison to earlier treatments [30]. Glyphosate applied to "Red LaSoda" and "Russet Burbank" during late bulking caused sprout inhibition with as little as 18 g ha^{-1} glyphosate, but 71 g ha^{-1} glyphosate was needed for a yield reduction [36]. Earlier exposure of the mother crop to glyphosate tended to have less effect on seed potato quality than later exposure [30].

4. Preventing potato injury from glyphosate

Some management strategies can prevent potato plants from being exposed to glyphosate. These include starting with clean seed that does not have abnormal malformation that could be from herbicide damage. If seed has possible herbicide injury symptoms, have it tested for glyphosate at a laboratory. Communicate with your neighbors who have fields near the potato field and let them know that potatoes are sensitive to glyphosate and special care should be taken to not drift on potato. If necessary, plant borders of a glyphosate-resistant crop around the potato field to help protect the field from unintended drift of herbicide. Have a dedicated sprayer for potatoes to eliminate any possible sprayer contamination problems. When applying pesticides, use proper storage of containers and spraying techniques. Farmers should

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be proactive to protect their potato crop. A small amount of herbicide can cause a great economical loss.

5. Conclusions

Glyphosate off-target movement onto potato may cause significant damage depending on the amount of glyphosate uptake into the plant, the cultivar and plant growth stage, the intended use of the daughter tubers, environmental conditions during and a few days after the off-target movement, and any other plant stress that would reduce plant recovery from a sublethal dose. Hutchinson et al. [39] reported that a 20% net return reduction in Idaho was equivalent to a loss of \$160 ha^{-1} based on total production costs at that time subtracted from gross returns as determined by an incentive-adjusted processing contract model. Therefore, the financial loss from glyphosate drift to a commercial potato field in the hooking or tuber initiation stage could be substantial. Plant injury symptomology may or may not be observed on the aboveground portion of the plant, and if symptoms are observed, tuber yield and grade reductions may or may not be affected. However, if glyphosate injury occurs during H or TI stages, visible tuber symptoms should occur.

By contrast, seed producers are at the greatest risk for serious financial loss and reputation consequences when glyphosate moves off-target and onto mother plants in the bulking stage with daughter tubers as the main sink for resources. Plant injury symptomology may be transient or not visible and daughter tubers often do not have visible injury symptoms. However, glyphosate residues are in these tubers and plant emergence may be delayed or prevented the following spring. Currently, certified seed producers hope affected seed will be identified during field plantings in winter nurseries. However, herbicides rarely drift onto more than a small portion of a field, so if tubers from affected areas were not selected for the winter nursery testing, the seed producer may be unaware of problems that may occur in the spring. Current research at North Dakota State University (NDSU) is directed toward identifying the threshold residue level in a seed piece that does not inhibit sprout growth along with a more economical yet reliable method to determine glyphosate residues in seed tubers. Seed producers are also being proactive by talking with neighbors and posting signs about the danger of glyphosate drift to potatoes grown for seed. Potatoes are not as sensitive as cereal crops to glyphosate, but the potential consequences from glyphosate off-target movement to potato are very significant.

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Chapter 3

Urban Impact on Selected Pre-Emergence Herbicides in Sediment cores

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Additional information is available at the end of the chapter

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Abstract

In order to determine if pre-emergence herbicide pollutant source, mixing with many others from residential and industrial activities, has an effect on pollutant degradation, three sediment cores were sampled in appropriate sites of the Garonne river near the city of Toulouse: two in rural surroundings, one upstream and one downstream from the urban area away from its influence, and one downtown close to Toulouse. Atrazine and DEA were analysed and, using DAR pesticide/metabolite ratio, an inferior metabolisation ratio was highlighted in the urban sediment compared to the rural ones, regardless of sedimentation rate, organic carbon content, topography or differences in the intensity of surrounding activities between rural cores.

Keywords: urban area, pesticides, reservoir, metabolites, DEA, atrazine

1. Introduction

Contamination of ecosystems by pesticides, especially aquatic ones, has become a major environmental concern for human health. Ecosystem contamination by agricultural practices and pest eradication procedures has been the subject of many studies, and more recently some have started to outline the impact of urban activities. Blanchoud et al. [1] estimated that 30% of surface water contamination by pesticide was due to applications, mainly of herbicides [2], in urban areas. Research concerning the Mid-Garonne area which was previously presented [2] in order to distinguish agricultural pollution by pesticide from urban one's was highlighted:

Toulouse city (1.100.000 inhabitants) is located in Mid-Garonne basin, surrounded by the third most intensive agricultural area of France, with no significant cities within an 80 km radius. Without topographical restraints, Toulouse city sprawls over the corn plain but the Garonne river flood plain, because of the flood risk, is essentially free of buildings – with the exception of Toulouse city centre.

Most of the herbicides used, excepted glyphosate, belong to s-triazine, substituted urea and anilide families (*inter alia*, for the USA [4] and for Europe [5]). One of their advantages is that some of them can be used for winter treatment in agricultural or urban procedures. However, some molecules such as atrazine have been forbidden since 2003 in France, or are under limitations as substituted ureas, i.e. linuron and isoproturon in the European Community (EC).

The river sediment is well known to be an integrative matrix for organic pollutants of aquatic environments [6,7]. Since there is little variation in the agricultural practice, an accumulation of pesticides is conceivable in river bed sediment caused by the direct agricultural and urban dissolved effluents as well as by the deposition of suspended particulate matter (SPM) contaminated by agricultural and urban use [8–10]. However, accumulation of these molecules is seldom studied in sediment and biota because no reliable and easily implemented method exists. Moreover, herbicide output from sewage treatment plants is estimated as at the same order as herbicide input [11]. Sewage treatment plants are mainly inefficient for retaining such molecules, with an intermediate hydrophobicity, i.e. log K_{ow} between 2 and 3.5, because of the use of standards appropriate to more hydrophobic molecules. Thus, pesticides will be insufficiently retained – like other pollutants with the same order of hydrophobicity, such as pharmaceuticals. Such antibiotic molecules could have an inhibiting effect on bacteria's activity [12], and monitoring such a flux could be a challenging target because of the molecules' number and their diversity and because of the fugacious but significant input events as consummation mode or storm water.

The aim of the present study is to determine the inhibiting conditions prevailing in urban sediment by following the pesticide biodegradation activity. Instead of monitoring chemical inputs for which the microbial inhibition is a multifactorial phenomenon crossing several contingencies [12,13], the authors focused on the pesticide/metabolite ratio or DAR ratio, considered as the result of such inhibitive conditions. In other fields, Adams & Thurman, [14] considered the DEA/atrazine ratio as the evidence of groundwater mobility. Based on Aelion & Mathur [15], crossing DAR with degradation activity, the authors will investigate the relationship between urban context and the organic chemical degradation.

Taking account of previous studies [3, 16–18], atrazine will be used because of its suitable extraction method for sediment [3], proven ubiquity in the Mid-Garonne River and the well-known relationship between atrazine and its metabolite desethylatrazine (DEA). The authors assume that deisopropylatrazine (DIA) is mainly due to low-O_2 conditions but, as previous studies indicated significant DEA concentrations in the sediment core, the DIA concentrations were not taken into account as uncertain. Moreover, atrazine is degraded in DEA under oxic conditions, corresponding to sediment surface layers. Due to their half-lives and K_{ow}, it is not consistent to consider deeper layers' degradation impacted by early diagenesis, bioturbation and sediment cementation [19,20].

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2. Material and methods

2.1. Sampling sites and period

Using sediment cores, the authors have sampled three sites – one corresponding to an urban area near the Toulouse sewage sludge output into the Garonne River, two corresponding to agricultural areas, respectively, upstream and downstream of Toulouse city, at a geodesic distance about 40 km (Figure 1).

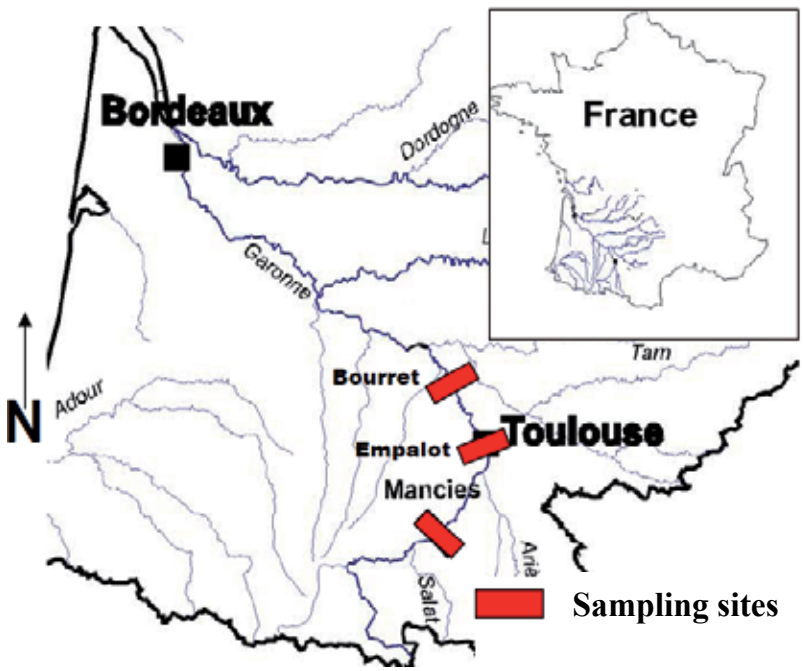

Figure 1. Map of sampling sites. Bourret and Mancies are 40 km geodesic distant from Toulouse.

Empalot weir is near Toulouse city downtown. This site was previously accurately investigated [21]. Suitable sedimentation characteristics and sedimentation rate were estimated about 0.41 cm/year by Devault et al. [18]. The first mention of Empalot weir dates from the Middle ages (1183 A.D.) because the weir was erected simultaneously with the Château Narbonnais's flour mill. The last 100-year flood (1900 A.D.) destroyed its deck which was rebuilt in 1922 A.D simultaneously with Ramier's hydropower station building.

Mancies dam, also named Carbonne dam, is a conventional hydropower one, which regulates the Garonne river's flow upstream from Toulouse city since 1965 A.D. This place was studied and described elsewhere [17]. Mancies dam is located at the boundary of the ancient regions of Volvestre and Comminges known for corn cultivation and a progressively predominant animal husbandry going towards the Pyrenees: upstream from Toulouse, no significant town lies on banks of the Garonne. At the coring place, sediment rate was estimated about 1.23 cm/

year [18]. Drinking water for the Toulouse urban area is drawn from the Garonne river close to the dam downstream.

Bourret oxbow is located about 80 km downstream from Toulouse city. Investigated by Steiger et al. [22] and Vervier et al. [23], in a conserved section of the Mid-Garonne, its sedimentation rate is especially low, about 1 mm/year, because of the topographic buffering of Garonne river flows. Indeed, Bourret oxbow is mentioned since 18th century. Riparian forest is 2-km-wide there at the thinnest place, but Bourret oxbow coring place is located at the meeting point between Garonne stream and the outlet of the 5-km-long oxbow lake, parallel to Garonne river but close to intensive agricultural Mid-Garonne activity – wheat and maize predominate. Nutriment and trace metal data elsewhere [24] highlight the limited urban impact on this site.

Sediment samples were collected during the winter low-water event corresponding to the beginning of herbicide application and to maximal sedimentation. The most marked low-water event of the winter was chosen. Thus, the sediment collected had never been above water level – consistent information mainly for Bourret station. Water temperature during winter was always about 5°C – a propitious temperature for pesticide degradation. The intensity of pesticide use in March corresponds to the annual average spreading level of pesticides.

2.2. Sample treatment

The cores were cut into sections: Section 1: 0 to 2 cm, Section 2: 2 to 6cm, Section 3: 6 to 14 cm, in order to monitor the 0–14 cm deep sediment surface layer where early diagenesis occurs [25]. Section 29 to 31 cm deep for all sites and Section 44 to 46 cm deep were also added except for the Empalot site.

For all layers, the different sediment fractions were separated according to the methods recommended by the Commission of Oslo and Paris (OSPAR). The components of the dried sediment were separated in an agate mortar and sifted with a 2 mm mesh (stainless steel sieves from Retsch GmbH and Co. KG (Haan, Germany)) to distinguish and eliminate coarse plant remains and gravel. In practice, coring was performed in sediments known to be free of large obstacles.

Total organic carbon content, due to organic matter, was estimated with a Leco CS 125 (St Joseph, Michigan). Conventional Kjeldhal method was used for organic nitrogen analysis on a Foss Analytical A/S's Tecator Kjelec-auto 1030 analyzer line (Hillerød, Denmark). Carbonate was estimated using Bernard's calcimeter.

2.3. Pesticide assay

2.3.1. Sample analysis

All solvents were of analytical grade for pesticide analysis ("Pestipur" by SDS, Peypin, France). Analytical grade anhydrous sodium sulphate was also from SDS. Pesticide standards (Mix 44) prepared by Dr. Ehrenstorfer GmbH (Augburg, Germany) were purchased from CIL, Sainte-Foy-la-Grande, France. Reference soil (Eurosoil7) was from Sigma-Aldrich (St Louis, Missouri,

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USA). Florisil cartridges used for purification were from Waters Corporation (Milford, Massachusetts, USA).

The protocol used is described in detail elsewhere [3]. Extraction was performed with a Dionex Accelerated Solvent Extractor (ASE 200) (Dionex, Salt Lake City, Utah, USA). Diatomaceous earth (Hydromatrix®) was from Varian, Palo Alto, California, USA. ASE extracts were analysed using HP5980 Series II gas chromatograph coupled to an MSD HP5971 mass detector (Agilent, Santa Clara, California, USA). Chromatographic conditions in the splitless mode (injection temperature: 280°C) were set up at an initial temperature of 45°C. The first step had a temperature increase rate of 35°C/min up to 180°C then the second step at 8°C/min up to 280°C and, finally, a 10 min plateau at 280°C. The detection conditions were temperature 300°C and EMV 2600V.

Limit of detection and limit of quantification are respectively 0.0001 and 0.001 μg/g.

3. Results and discussion

DEA pollution prevails in the three cores (Table 1) and especially in the fine fraction one (Figure 2): the metabolite is detectable and quantifiable for all samples in fine fraction and is only not detectable or quantifiable for the two Bourret's layers in coarse one. Inversely, atrazine is mainly undetectable (15 undetectable concentrations for 28 samples) – even if detection limits were similar for the two molecules.

		Fine fraction					Coarse fraction				
	Depth (cm)	Silts and clays (%)	TOC (%)	Renfield ratio	DEA (μg/g)	Atrazine (μg/g)	Sands and gravels (%)	TOC (%)	Renfield ratio	DEA (μg/g)	Atrazine (μg/g)
M1	0–2	91,2	2,7	8,20	0.07	0.26	1,2	6,57	15,63	T	0.02
M2	2–6	95,6	3,4	10,84	4.18	0.35	3,7	6,68	16,52	0.01	0.01
M3	6–14	94,5	4,3	11,98	0.43	0.81	7,2	8,35	18,12	0.01	0.01
M4	29–31	97,9	3,5	11,71	1.29	-	3,2	14	18,68	T	-
M5	44–46	98	2,60	10,95	0.02	0.01	6,6	3,97	15,07	0.01	T
E1	0–2	60,8	4,1	15,12	0.77	-	39	1,45	16,72	0.01	-
E2	2–6	73,2	2,8	10,20	5.44	0.31	27	1,41	14,11	0.05	0.13
E3	6–14	63,7	2,3	10,31	0.33	0.18	36	1,51	15,92	0.05	-
E4	29–31	52,8	1,8	9,76	-	0.20	47	1,25	13,83	0.02	0.02

		Fine fraction					Coarse fraction				
	Depth (cm)	Silts and clays (%)	TOC (%)	Renfield ratio	DEA (µg/g)	Atrazine (µg/g)	Sands and gravels (%)	TOC (%)	Renfield ratio	DEA (µg/g)	Atrazine (µg/g)
B1	0–2	94,1	2,2	8,91	22.37	-	5,9			T	-
B2	2–6	90,6	2,2	11,40	0.85	-	9,4	1,79	8,78	-	-
B3	6–14	82,1	0,66	12,83	0.38	0.07	18	1,71	4,78	0.02	-
B4	29–31	41,3	1,8	10,29	0.04	-	59	0,31	10,29	0.03	-
B5	44–46	29,3	1,6	12,35	0.08	-	71	0,33	12,35	0.11	-

Table 1. DEA and atrazine profiles (µg/g), granulometric data and total organic carbon content (%) and Renfield ratio (C/N, dimensionless) from Mancies (M), Empalot (E) and Bourret (B) sampling sites. Sedimentation rates are respectively estimated about 0.1, 0.41 and 1.23 cm/year.

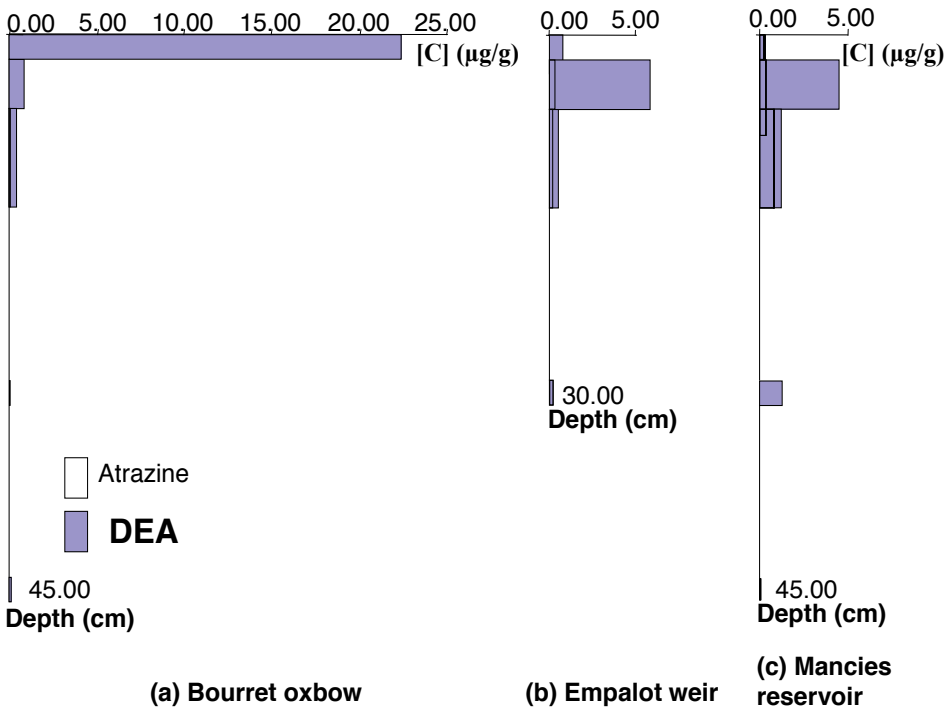

Figure 2. Representation of the contamination of the fine fraction in (a) Bourret oxbow, (b) Empalot weir and (c) Mancies reservoir. Sedimentation rates are respectively estimated about 0.1, 0.41 and 1.23 cm/year.

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Bourret oxbow surface layer is the most polluted layer (22.37 μg/g). Such concentration, even after considering all the core samples, argues that Bourret is the most polluted site (2.97 μg/g) even if atrazine concentration is only detectable or quantifiable for one sample, at 6–14 cm. Fine fraction surface layer, i.e. 0–2 cm deep, represents about 86.4% of total DEA stock from the core; 6–14 cm layer presents a significant contamination by atrazine – even it is the only one sample from Bourret were atrazine contamination is detected or quantified. At such depth, the authors hypothesize that bioturbation could be evoked: even if no evidence of benthic animal terrier was found, it could explain such an isolated result because the whole core allowed hypothesizing an oxyc atrazine degradation in the surface layer.

The core sampled in the Empalot urban weir presents an average concentration of about 1.75 μg/g; DEA contamination of the fine fraction is the least important compared to the other sites: non-DEA from fine fraction contamination represents 27.6% of total contamination of the whole core. Atrazine and its metabolites have sensibly the same proportion in the coarse fraction (0.41 μg/g for DEA, 0.36 μg/g for atrazine).

The contamination of the core sampled in the Mancies reservoir is globally limited to DEA pollution of fine fraction. Contamination by coarse fraction is minor compared to the DEA one (12.6%), with an intermediate score regarding the two others sites. Like the coarse fraction from the Empalot urban weir, atrazine and DEA presented similar average concentration (0.01 μg/g for each) but, by contrast to the Empalot site, concentrations observed are scarce and low, limiting the interpretation.

3.1. DEA/Atrazine Ratio (DAR)

DEA/atrazine molar concentration ratio could be used for groundwater pollution (DAR) [14,26–28] in order to determine point source from non-point source contamination and the timing and movement of atrazine in reservoirs and streams. For sediment, DAR was used for degradation investigation [15]. However, sediment variability depending on origin, transport, periodical or local pesticide uses (*inter alia*, [29–31]), needs to be pondered. Moreover, because of undetectable or unquantifiable concentrations, the choice of a substitutive value, in order to avoid division by zero, could be a critical parameter, which needs to be buffered. In the present case, detectable but unquantifiable concentrations were estimated at 0.005 μg/g, corresponding to the half of quantification limit, and undetectable concentrations were estimated about 0.001 μg/g.

In the present case, authors proposed to compare DAR performed for the fine fraction by DAR performed for coarse fraction.

Comparing average DAR for fine fraction (aDARff) to average DAR for coarse one (aDARcf) provides a notable similarity between rural sites, as opposed to strictly urban areas.

3.2. Statistic study

Non-parametric Spearman's rank correlation test has been performed, considering the variable natures. In order to have the same number of observations i.e. chemical concentration in

sediment, for each variable, i.e. cores or fractions, the deepest layer for the Bourret and Empalot sites were excluded.

The statistic study should first test the independence between DEA concentrations and atrazine ones and signals an independence for all the cores at $\alpha = 0.05$ (even $\alpha = 0.1$) for $n = 8$, incidentally justifying DAR use for sediment.

The second step seeks to determine if coarse fraction and fine fraction contaminations are dependent.

If the three site datasets confirm the dependence between coarse and fine fraction, both for atrazine and for DEA, the relationship between coarse and fine fraction characterises the Bourret and Mancies cores: $\alpha = 0.05$ for $n = 8$ each for their superior dependence; for Empalot core: $\alpha = 0.5$ (and not 0.05) for $n = 8$ lacks pertinence. Moreover, a striking similitude between Bourret and Mancies data appears: respective r_s are extremely close (0.53 each).

Such results are found when cores are compared: similarity between Mancies and Bourret is quite obvious ($\alpha = 0.005$ for $n = 16$) while similarity is seen between the Empalot core and the other ones but at a lesser scale, i.e. with $\alpha = 0.05$ (and not 0.005) for $n = 16$. r_s obtained when comparing Empalot to Bourret and then Empalot to Mancies were also close, highlighting the similitude between Mancies and Bourret cores.

3.3. Urban chemical mix impact on atrazine biodegradation

Considering statistical results, the authors propose to compare DAR obtained with the fine fraction to DAR obtained with the coarse one (1) since the fractions are not dependent, as the previous statistical analysis shows, (2) atrazine degradation is due to fine fraction which collects chemicals associated to organic matter, i.e. silts, clays and humic acids [32], and (3) because coarse fraction contamination is not due to contamination of lignin-rich allochthonous organic matter as the Renfield ratio shows (Table 1), and to use average concentration for DAR calculation, because of unquantifiable or undetectable samples. Authors followed equation (1):

$$\frac{\left\{[\mathrm{DEA}]/[\mathrm{Atrazine}]\right\}_{\mathrm{ff}}}{\left\{[\mathrm{DEA}]/[\mathrm{Atrazine}]\right\}_{\mathrm{cf}}} \tag{1}$$

where [DEA] and [atrazine] are the average concentrations observed in DEA and atrazine in the fine fraction (ff) is the numerator and coarse fraction is the denominator, for each core. For Bourret and Mancies, DAR ff/cf provides an obviously close result: 141 for each. Empalot's one is about 13 (Table 2).

Such a ratio could be justified by the lower degradation observed in the whole core, especially comparing Bourret and Mancies sedimentation rates (1 vs 12 mm/year), chemical vertical profiles and contamination levels (mainly in surface layer for the first and mainly in 6–14 cm for the second).

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	DARff	DARcf	DARff/DARcf
CM1	0,27	0,25	1,08
CM2	11,94	1,00	11,94
CM3	0,53	1,00	0,53
CM4	1290	5,00	258,00
CM5	2,00	2,00	1,00
Average Mancies	260,95	1,85	**141,05**
E1	770,00	10,00	77,00
E2	17,55	0,38	45,63
E3	1,83	50,00	0,04
E4	0,01	1,00	0,01
Average Empalot	197,35	15,35	**12,86**
B1	22370,00,	5,00	4474,00
B2	850,00	1,00	850,00
B3	5,43	20,00	0,27
B4	40,00	30,00	1,33
B5	80,00	110,00	0,73
Average Bourret	4669,00	33,2	**140,64**

Table 2. DAR depending granulometric fraction. DARff (dimensionless) correspond to fine fractions and DARcf for the coarse one.

Considering layer thickness and normalizing DEA and atrazine concentration like Koelmans et al. (1997) proposed (2), the result is still more obvious: Mancies and Bourret ratios stay close (respectively 117 and 145) compared to Empalot's one (about 4).

The main similarity between Bourret and Mancies is the surrounding rural landscape, limiting chemical pollution to that of agricultural activity: pesticides. It is obvious, moreover, that the pesticide level, considering atrazine, DEA or other pesticides [3, 16] is very similar to Empalot. Such difference, characterised by the lack of atrazine degradation, could be imputed to three sources:

- An input of contaminated fine fraction due to sewage sludge or sewer system. It is not a non-sense considering the works of Jiang et al. [33] and [34] inform us that pyrethroid adsorption on concrete is rapid, i.e. 85% and more of pyrethroids are adsorbed in the early days, and concrete undergoes structural deteriorations, providing possibly polluted concrete particles during rain wash-off events to surface water system. Consequently, pesticide transfer by runoff from concrete (between 30% and 60% in-lab transfer rate [35]) is mainly due to solid particles and not due to dissolved molecules whose size introduces them into fine fraction. It could justify why hydrophobic pesticides like atrazine and DEA

are more prone to wash-off than glyphosate from cement slab – but carbon organic content does not strengthen this hypothesis.

- A lack of degradation due to early diagenesis difference leading to the proposal of two hypothesis: (1) minor oxygenation in urban water compared to rural ones, but there is no evidence of it. Inversely, wastewater plant effluents are monitored and any significant clue support such hypothesis in dataset collected in situ or in sewage treatment actors. Moreover, the Empalot area was in detail studied elsewhere [21] during a period of less efficient effluent treatment compared to the authors' core sampling. Moreover, temperature of water was low, enhancing oxygen saturation content. (2) Difference in bioturbation activity – knowing that bioturbation, at global scale, prevails on abiotic early diagenesis [35]. There is no evidence of this, either in chemical profile or in the organic matter content: the lone example of possible bioturbation is in the core from Bourret.
- Metabolic brakes, leading to reduced atrazine biodegradation. Such phenomenon has never been previously demonstrated using pesticide/metabolite ratio even if [15] used DAR on polluted sediment and Harrison et al. [36] highlighted the site impact on microbial activity. In the present case, in similar conditions between urban and rural sites, the authors suggest that the lower metabolic yield highlighted by DARff/DARcf ratio could be due, at least partially, to the chemical mix typical of the urban area, especially because of antibiotic micro-pollution [37].

The present study should be completed in order to compare microbial activity in sediment influenced by urban mix of urban and agricultural activities, even if:

1. degradation gene estimation [38] is not necessarily an alternative factor able to supersede DAR – in this case, tfdA gene, coding for MCPA mineralization, was more pronounced in MCPA pollution even if mineralization rate, influenced by organic carbon content, finally favours agricultural soil as bioavailability favours urban ones.
2. Global degradation activity, monitored by labile organic matter, has been performed with sucrose, without providing significant differences. Such a molecule is acutely degradable, when atrazine could differ following enzymatic diversity and, likewise, early diagenesis conditions [39], which characterise microbial sediment environment [19], were not reproduced, nor urban water dissolved chemical mix. In the present case, sucrose metabolisation seems to be too rapid, for each sediment, for a valid interpretation – when atrazine degradation takes months in soil, and significantly longer in sediment [16].

4. Conclusion

Even if sedimentation rate, organic carbon content, topography or surrounding activities' intensity differ, inducing significant differences between cores from Bourret and Mancies, such samples from rural areas differentiate themselves clearly from the urban core from Empalot weir. This last one presents a less degraded level of atrazine in DEA, which could be attributed to chemical mix, including antibiotics. In this way, and while waiting for further data, the

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authors hypothesize that the vertical profile of DEA and atrazine is impacted by urban chemical mix from the Toulouse area, limiting atrazine metabolisation and leading to atrazine accumulation in a sediment core.

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Chapter 4

Triazine Herbicides in the Environment

Sarka Klementova and Lucie Keltnerova

Additional information is available at the end of the chapter

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Abstract

This chapter is a review of literature concerning the fate of chloro-s-triazine herbicides, particularly atrazine, in the environment. It addresses the distribution of such herbicides and their metabolites in the soil and in water bodies, including the conditions that affect their transport mechanisms. The biodegradation pathways regarding the microbial degradation are presented as well as modification mechanisms of the compounds in plants capable of tolerating their action. Studies on the influence of the compounds on animal and human physiological processes and health, that is distribution of atrazine in the animal organisms, effects on the regulatory platform in the liver, possible carcinogenesis and endocrine disruption risks are assessed. Toxicity tests used for evaluation of the toxicity of the compounds are critically reviewed. Possible methods for atrazine degradation, including advanced oxidation procedures (AOP techniques), are outlined.

Keywords: atrazine, s-triazine, fate in the environment, influence on animal and human physiological procesess, toxicity tests, methods of degradation

1. Introduction

Since the 1960s, the use of chemicals to control weed emergence and growth has steadily increased mainly in areas of land utilised for agriculture [1,2], but also along roads and railways [3] as well as in urban areas [4].

Many of the applied herbicides are compounds that are relatively water soluble and can thus be transported to bodies of surface water or leach into ground water resources [5].

Among herbicides, the chloro-s-triazine derivatives, and atrazine in particular, are the most heavily used worldwide, and are often therefore, detected in rivers, lakes and groundwater [6]. The main representants of the triazine herbicides are shown in Figure 1.

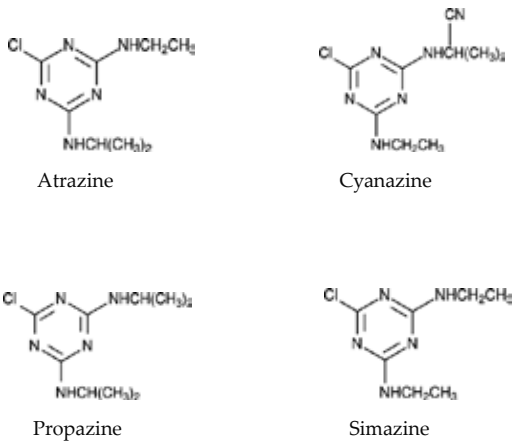

Figure 1. The main triazine herbicides.

Triazine herbicides belong to a category classified as persistent organic compounds [7] since they resist biological and chemical degradation.

Their persistence in natural bodies of water has led to a search of a way to degrade them into environmentally compatible compounds. The following techniques have been evaluated: photocatalytic decomposition on semiconductors [8-10], other advanced oxidation processes (AOPs) [10,11-13], homogeneous photocatalysis [14,15], photosensitised reactions [16,17] or photolysis by high energy UV radiation [11,16,18]. For major sources of pollution, that is wastewater from agricultural industries and pesticide manufacturers, in which the concentration of pesticides may reach levels as high as several hundreds of mg/dm^3, technologies for degradation that are low cost and relatively simple to implement have been proposed and developed [19]. Since degradation methods do not lead generally to full mineralisation of these herbicides, attention should also be paid to the toxicity tests of the resulting mixtures.

Herbicides, toxic by definition to some plant species, may display toxic effects to other species after short- or long-term exposure. The transformation products of herbicides represent an issue of emerging importance since in some cases they pose a greater threat to the environment than the parent molecules [20].

Low-level contamination of drinking water supplies is perceived as a serious potential health problem since many herbicides have been suspected of functioning as potential endocrine disruptors, that is substances interfering with the body´s endocrine system and resulting in adverse developmental, reproductive, neurological and immunological effects in both humans and wildlife [21,22]. Potential carcinogenic effects have been investigated in the laboratory as well as epidemiological studies [21-23].

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This chapter is a review of recent literature concerning the fate of atrazine in the environment, the influence of atrazine on animal and human physiological processes, toxicity tests for triazine herbicides and methods used for atrazine degradation.

2. The fate of atrazine in the environment

Atrazine, a white crystalline compound, is a selective herbicide. Its solubility in water is 33 mg/dm^3 at 22°C, pH=7 [24]. Atrazine is usually applied in a water spray at a concentration of 2.2 to 4.5 kg ha^{-1} [25] before the weed emerges.

Atrazine inhibits the photosynthesis of most plants. Its mode of action involves competitive inhibition for plastoquinone binding to the Q_B protein (32-kDa protein) in photosystem II, thus disabling the electron transport chain in the light-dependent reactions of photosynthesis [26,27].

Atrazine has been shown to have a high affinity for soil organic matter, its sorption correlating positively with organic carbon content [28]. The study also revealed that the sorption of atrazine on organic soil matter lowers its availibility to the biota.

Atrazine can be transported through the soil. Two distinct processes contribute to this transport: transport through the soil matrix,which is a slow process, and movement through large pores, which is much faster [29]. Correira et al. [30] evaluated the mobility of atrazine on Ultisol red clay soils, typical for humid moderate and tropic regions and concluded that the soils have a low sorption capacity for atrazine, which results in a high potential for leaching and runoff.

Many groups have studied the influence of organic matter in soil on atrazine sorption. Ben Hur et al. [31] examined the effects of dissolved organic matter (DOM) on atrazine sorption in soil. He reported that the higher the content of soluble organic matter the higher the atrazine affinity to the soil solid phase.

The role of humic substances in atrazine sorption on soils has been investigated in several studies. Several mechanisms of interactions of atrazine with humic organic matter have been hypothesised, such as proton transfer, electron transfer, hydrophobic interactions between humic material and atrazine, but no explicitely conclusive data have resulted mainly due to the large heterogeneity in humic materials [32,33].

In soils, atrazine degradation proceeds mainly via the microbial activity of soil microorganisms. The resulting hydroxylated and dealkylated intermediates can even be mineralised by some microorganisms to carbon dioxide. The sequence of such degradation as proposed by Sadowsky et al. [34] and de Souza et al. [35] is demonstrated in Figure 2.

Atrazine is tolerated by some plants. The detoxification of atrazine occurs through hydrolysis, non-enzymatic hydroxylation, enzyme-mediated N-dealkylation or conjugation with cystein or glutathion [36].

The ability of atrazine to undergo hydrolytic modification is connected with C_4 plants. C_4 plants are characterised by their initial incorporation of CO_2 into an anion of a 4-carbon organic acid; the anion is transported into specialised cells in which CO_2 is regenerated and enters the conventional C_3 pathway of the Calvin cycle. The ability of C_4 plants to perform hydrolytic modification has been shown to be closely correlated with their atrazine tolerance.

The results of Chang et al. [37] demonstrated that poplar (*Populus deltoides*) cuttings could absorb atrazine and metabolise it through hydroxylation and dealkylation into less toxic compounds.

In a growth chamber study, Lin et al. [38] examined the uptake and conversion of ^{14}C-atrazine by several grass species to examine efficacy of the so-called vegetative buffer strips. Multispecies vegetative buffer strips have been recommended as a potentially cost-effective conservation practice to reduce non-point source pollution of adjacent waterways. The study was conducted to compare atrazine degradation profiles in soil rhizospheres from different grasses and correlate the rates and degradation profiles with microbial activity.

The plants treated included seven grasses: orchardgrass (*Dactylis glomerata* L.), tall fescue (*Festuca arundinacea* Schreb), smooth bromegrass (*Bromus inermis* Leyss.), switchgrass (*Panicum virgatum)*, Illinois bundle flower (*Desmanthus illinoensis*), perennial ryegrass (*Lolium perenne* L.) and eastern gamagrass (*Tripsacum dactyloides*). All of the plant species significantly enhanced atrazine degradation, with eastern gamagrass showing the highest capability for promoting the biodegradation of atrazine in the rhizosphere (more than 90% of atrazine was degraded in the plant's rhizosphere compared with 24% in the control). N-dealkylation of atrazine was strongly correlated with increased enzymatic activity in β-glucosidase and dehydrogenase, which are microbial parameters used for the assessment of soil quality.

Results suggested that the efficacy of vegetative buffer strips in removing herbicides from surface runoff is related to the ability of plant species to promote rapid herbicide degradation. The authors also concluded that the microbial parameters widely used for the assessment of soil quality are promising tools for evaluating the overall degradation potential of various vegetative buffer designs for atrazine-soil remediation.

Atrazine uptake by green algae and diatoms was investigated by Tang et al. [39]. To inhibit the photosynthetic process of freshwater algae, atrazine must be absorbed intracellularly, the sorption is a prerequisite for its action at the chloroplast membrane.Tang et al. determined atrazine bioconcentration and uptake for eight freshwater green algae and diatoms. The results show that atrazine uptake was extremely rapid in all species examined, with nearly 90% of total uptake occurring within the first hour of exposure. Within each division, different species had different bioconcentration capacities, yet the accumulation of atrazine was consistently higher in green algae (5.43–12.73 ng/mg) compared to that in diatoms (0.33–1.69 ng/mg). Atrazine concentrations in the algal cells were much higher than in the medium, although the total amount of atrazine taken up by algae was small relative to the total atrazine in solution (1–3%). The ability of algal cells to accumulate atrazine was highly correlated with algal cell biovolume and surface area, and a strong relationship was observed between sensitivity to atrazine and bioconcentration, cell biovolume and surface area. In general, higher bioconcentration factors were associated with increased atrazine sensitivity.

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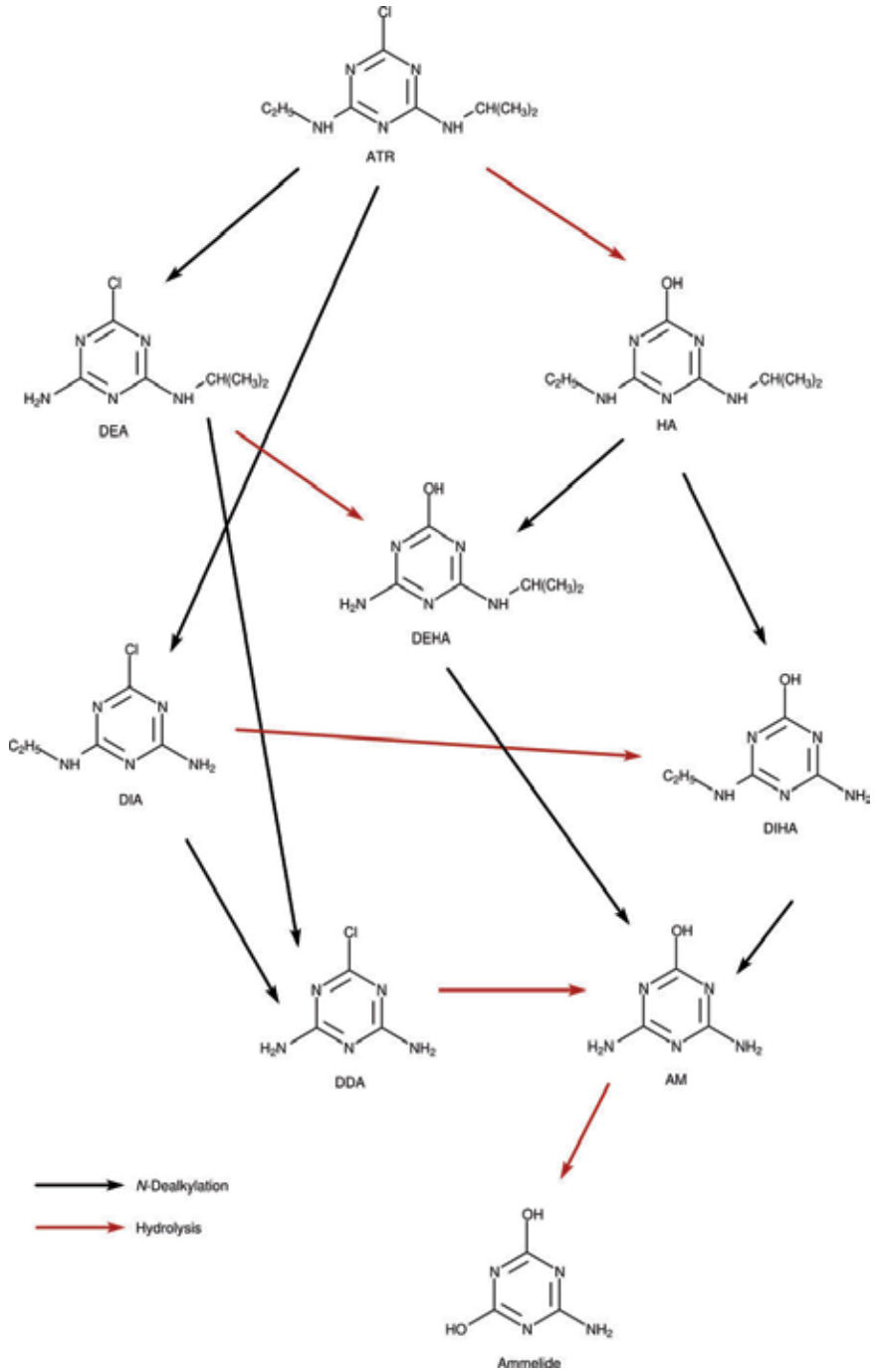

Figure 2. The major atrazine degradation pathways according to Sadowsky et al. [34] and de Souza et al. [35]. ATR – atrazine, DEA – desethyl atrazine, HA – hydroxyatrazine, DIA – desisopropyl atrazine, DIHA – desisopropyl hydroxyatrazine, DDA – didealkyl atrazine, AM – ammeline.

Triazine herbicides are considerably soluble in water and can therefore leach to ground waters or be washed to surface waters, which is why they are among the most often detected xenobiotics in aquatic ecosystems as shown by many studies such as those summarised by Scribner et al. [40].

The study conclusively shows that not only triazine herbicide themselves are found in significant amounts but also that their degradation products have been repeatedly detected; due to the persistence of the compounds, their concentrations in surface waters in agricultural areas remained elevated not only soon after application, but throughout the subsequent summer and into the autumn. Atrazine was shown to occur at the highest frequency (82.1%) and in the highest detectable concentration with a mean of 1.36 μg/L, whereas others were detected at significantly lower frequencies as well as concentrations, for example cyanazine was detected in only 47.2% of samples with a mean detectable concentration of 0.61 μg/L.

The study summarises results from monitoring both surface water bodies, such as reservoirs and streams, and ground water; the findings can be outlined as follows.

2.1. Reservoirs and streams

To ensure more accurate geographic and seasonal interpretation of the triazine and triazine metabolites sampling data, 147 stations in the Midwestern US were sampled in 1989, on 53 of them the sampling was conducted during the 1990s and 2002.

The data revealed that high concentrations of herbicides and their degradation products are mobilised with rainfall, then transported to streams with runoff. The majority of this transportation takes place during the first rainfall and runoff after the application of herbicides. Subsequent runoff tends to produce lesser peaks in concentration. Because of these flushes, the detections of herbicides in larger Midwestern streams tends to be seasonal with higher percentages of detections in spring and early summer and lower percentages in autumn and winter. Measurable amounts of atrazine occurred in 91% of the pre-application samples, providing an indication of its persistence in surface waters.

Three-dimensional images of the distribution of atrazine concentrations in Perry Lake during 1992 showed that recently applied atrazine is mixed with atrazine applied the previous year as water moved through the reservoir (Figure 3). Relations between atrazine and the degradation product, desethyl atrazine, indicated whether the atrazine in the reservoir had been flushed off the fields immediately prior to sampling or whether it remained present from the preceding year. Changes in atrazine concentrations in the reservoir resulted from several factors, including herbicide application, which fuelled and reset the system, and precipitation, which drove the system by flushing atrazine into the reservoir and determining the residence time of water in the reservoir. Concentrations varied between the main inflow and the public water supply intakes located at the opposite ends of the reservoir. The concentration range in the outflow varied much less than concentrations in the upstream parts of the reservoir because of mixing.

The frequency of occurrence, concentrations (both annual average and peak) and quantities of pesticides in streams or reservoir outflows is related to the magnitude of the herbicide used in

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the upstream drainage areas. In most cases, the annual loss of herbicides to streams represents about 5% of the amount applied. Pesticide losses are also affected by soil type, climate, land cover, other basin characteristics and management or application techniques.

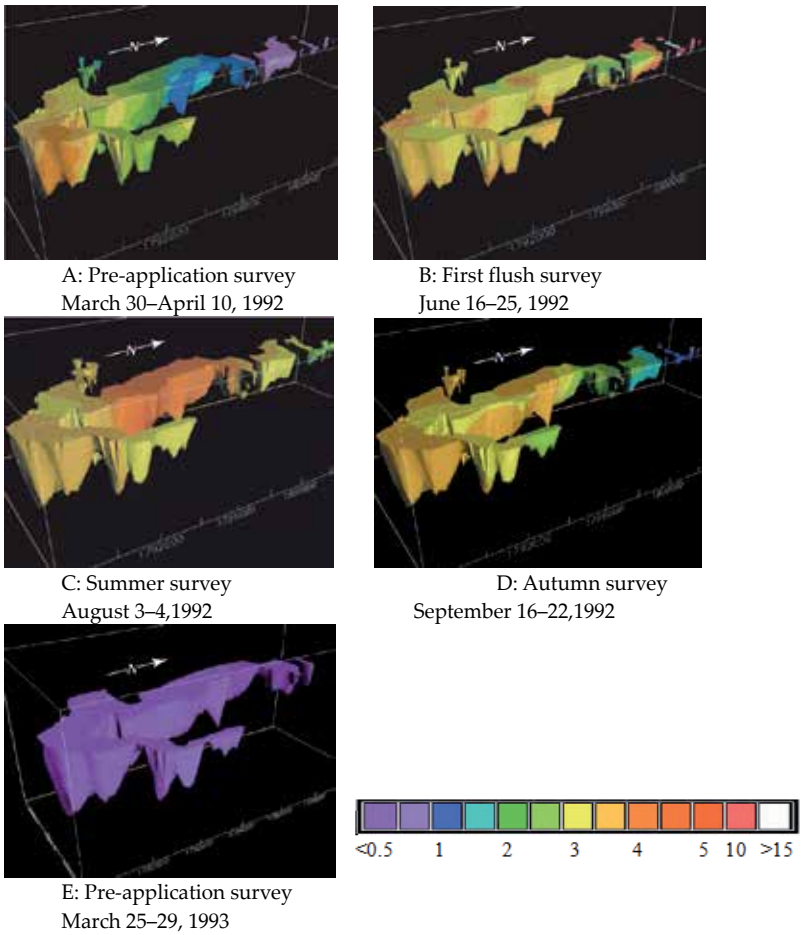

Figure 3. Three-dimensional computer images of atrazine concentrations in Perry Lake, northeastern Kansas as presented by Fallon et al. [41].

Triazine concentrations on the coloured scale are in μg/l.

2.2. Ground water

The study summarises an investigation of samples collected from 131 municipal wells in Iowa from 1995 to 1998. It compares the occurrence of herbicide degradation products with the occurrence of their parent compounds in ground water. An important finding of this study was the high frequency with which degradation products were detected in ground water. During 1995, more than one herbicide compound was detected in samples from 44

of the wells. Atrazine is the only herbicide for which the parent compound was found more often than any of its degradation products. This could be because of the greater environmental stability of atrazine compared to the other s-triazine parent compounds under investigation (simazine, propazine, cyanazine). Aquifer types presumed to have the most rapid recharge rates were those most likely to contain detectable concentrations of herbicides, indicating that groundwater age could be an important factor in explaining these variations in herbicide contamination.

As a continuation of the Iowa study, groundwater wells were sampled and analysed for 21 herbicides and 24 herbicide degradation products during 2001. The frequency of detection increased from 17%when only herbicide parent compounds were detected to 53% when both herbicide parent compounds and degradation products were found.

The groundwater samples collected during these studies consistently revealed that triazine herbicide degradation products were found more frequently than their parent herbicide; the frequency of herbicide detection was, in part, affected by a more sensitive analytical method. The study accentuates that groundwater age could be an important factor in explaining variations in herbicide contamination.

3. Influence of atrazine on animal and human physiological processes and health

The environmental pollution caused by pesticides has become a widespread problem. The high use of these chemicals has caused an increasing concern over their acute and chronic health effects. Persistent pollutants can contaminate organisms at all trophic levels, and may cause severe damage to the organisms through metabolites contained in contaminated bodies of water. The patterns of accumulation and effect of xenobiotics depend on the organism itself, on the properties of the compound, on the quantity of the compound present in the particular part of environment, and – last but not least – on the balance between bioassimilation and metabolic plus excretion rates [42].

Laboratory studies are usually based on tests of LD_{50} (lethal dose) values. An LD_{50} value represents the individual dose required to kill 50 % of a population of the animals tested (e.g. rats, mice, fish). LD_{50} values gives a measure of acute toxicity, thus determining the threshold concentration of the xenobiotic to classify acute hazards of the substance for the purposes of legislation.

To evaluate the impact of a pollutant on an organism the detection of biological effects is needed; such detection involves identifying morphological, molecular, biochemical, or physiological biomarkers [43].

Orally ingested atrazine is readily absorbed by the gastrointestinal tract. Experiments have shown that 20% of the dose administered orally to rats was excreted in feces within 72 h; the remaining 80% was detected in the bloodstream. After 72 h, 65% of the dose was eliminated in urine and the remaining 15% was detected in tissues such as the brain, liver,

kidneys, and lungs [44]. High concentrations of atrazine were found in the liver and kidneys of male mice [45]. This bioaccumulation is probably due to the ability of the substance to interact with the phospholipid components of biomembranes. The interaction prevents the excretion of atrazine until the substance undergoes a degradation leading to a higher solubility in aqueous solutions.

Since the liver is one of the target organs for atrazine, effect on the regulatory platform in the mechanism of liver homeostasis have been investigated. Kalmar and Greensmith [46] described the adaptation mechanism of the response to cellular stress, especially the oxidative stress, which is based on an activation of expression of heat shock proteins (HSPs); this mechanism helps a wide range of organisms from bacteria to mammals to survive environmental challenges and adapt to them. It may underlie the defence abilities of organisms to resist the small-molecule inducers of the heat shock response (HSR). This adaptation mechanism may be connected with so-called gap junctional intercellular communication and its role in the combat of liver-toxic compounds. As shown by Vinken et al. [47], gap junctions, that is aggregates of intercellular channels that permit the direct cell–cell tranfer of ions and small molecules, play a central role in the development of tissues as well as in the so-called bystander effect messaging cell death. They have also been shown to be the platform of communication between hepatocytes. The deletarious effects of toxic compound on the gap junctions are often accompanied by the triggering of oxidative stress in the tissue followed by the heat shock response.

A comprehensive survey of investigations on the health risks of triazine herbicides with respect to carcenogenesis is provided in a review by Jowa and Howd [21]. They collected the results of more than 200 studies on animals (rats, mice, dogs) as well as of human epidemiological studies on exposed populations, including occupational and residential risks. The data on occupational risk came from employees who underwent exposure during manufacturing; the residential risks were assessed from the data on the use of contaminted water and pesticide usage in corn crop production. The survey can be summarised as follows:

- The significant amount of research into the mechanism of mammary tumor formation in rats has investigated triazine effects on the estrus cycle. Both the *in vivo* and *in vitro* data suggest that atrazine and simazine disrupt ovarian cycling and that this mechanism can induce mammary tumors in the SD rat strain (SD rat = Sprague-Dowley rat, an outbred of alpino rat used extensively in medical research). This alteration of the estrous state through hormonal induction and estrogen mediated responses is associated with the incidence of mammary tumors in SD rats.

- Another suggested mechanism for chloro-s-triazine-mediated carcenogenicity is that it may be due to increased levels of estrogen and lower testosterone from the effect of chlorotriazine on aromatase activity. However, the induction of aromatase activity has not been established as a consistent effect. Moreover, long term reproductive studies have not provided particular evidence of feminisation of male rats (an expected concomitant of aromatase induction that would be demonstrated as developmental changes or mating success). The increase of pituitary tumors in rats is thought to be related to the mechanisme of estrogen mediated responses.

- Effects observed in experimental animal toxicity tests are assumed to be relevant to humans unless there is adequate evidence to the contrary. In the case of rat mammary tumors induced by atrazine and related chloro-s-triazine compounds, including metabolites, there is ample contrary evidence, since reproductive cycling in rodents is drastically different from that of humans. Differences between rodents and humans also exist in reproductive senescence. Thus, examination of the mechanistic basis for carcinogenicity of chloro-s-triazines has led to the deduction that the tumorigenic mechanism in rats is not relevant to humans.
- The epidemiologic studies have not indicated association of chloro-s-triazines with mammary carcinogenesis in humans. Evidence of assiociation with the occurence of other cancers is rather weak.
- There is little evidence that atrazine and its congeners are mutagenic. Putative human carcinogenic activity of triazines mediated through endocrine disruption has not been established by the observation of any direct binding to estrogen receptors or a competitive inhibition involving these receptors; there is the exception of some suggestive evidence that atrazine may interact with the GPR30 estrogen receptor (currently denoted as GPER) which is an integral membrane protein with a high affinity for estradiol though not for other endogeneous estrogens. There is no evidence that chloro-s-triazines behave like estrogens in their interaction with estrogen-sensitive tissues. Also, there was no increase in prolactin release with atrazine exposure.
- Hormonally active xenobiotics including atrazine have been identified as endocrine disrupting chemicals. These chemicals exert hormone-like activity in vertebrates and exposure to these compounds may induce both short- and long-term deleterious effects including functional alterations that contribute to decreased reproduction and fitness.There is clear evidence that the chloro-s-triazines have an endocrine influence, and that this influence is likely to be relevant to humans. Changes in circulating endocrine hormones have been observed regardless of rat strains in relation to all the triazines and it is a basis for assuming this to be a relevant endpoint for human risk assessment.

4. Toxicity tests for triazine herbicides

Water pollution consisting of toxic compounds represents a major cause of the failure of biological treatment plants, resulting in noncompliance with discharge permit limits. Experimental studies using nonspecific models at laboratory level are extensively used to predict the potential hazards of chemical and industrial waste regarding the environment, especially aquatic systems. These toxicity evaluation models have the advantage of generating quickly reproducible data at relatively low cost. The criteria of toxicity generally taken into consideration are death or changes in mobility, reproduction, growth, physiological functions, behaviour and genetic information.

Microbial tests have been widely used in toxicity screening procedures due to factors such as short exposure time, ease of handling and reproduciblity. In 1979, Bulich [48] proposed a

specific test for rapid assessment of the toxicity of aquatic samples using the light emitting bacterium *Vibrio fischeri* (former *Photobacterium phosphoreum*). This non-pathogenic marine bacteria emits light as part of cellular respiration, the light can be measured as luminescence. *Vibrio fischeri* have demonstrated high sensitivity across a wide variety of toxic substances. The organism´s response to toxicity is observed as a change in luminescence. In 1982, the system was developed commercially under the trade mark Microtox™ (Berckman Instruments Inc.) This test is now widely accepted as a standard bioassay [49] used also for chloro-s-triazine herbicide [1,50]. During the test, the 'effective concentration' (EC_x) is detected; the EC_x is an analogue to lethal dose LD_x, and represents the concentration at which the light emitted by the microorganisms is reduced by a specific percentage. Usually, EC_{10}, EC_{20} or EC_{50} is determined [1]. Kross et al. [1] determined the EC_{10}, EC_{20} and EC_{50} values for atrazine, desethyl atrazine and desisopropyl atrazine, the values are summarised in Table 1. The data show that the lowest concentrations necessary for a toxic effect are connected with atrazine, the concentration being almost an order of magnitude less than the toxic concentrations of the atrazine metabolites. Of the two atrazine metabolites, desisopropyl atrazine was found to be more toxic.

Nevertheless, as concluded by Lapertot et al. [51], Microtox© is basically inappropriate for surveying the toxicity of herbicides such as atrazine because *V. fischeri* is not a photosynthetic organism, and is not therefore by definition properly sensitive to herbicides, which are toxic in the short term, specifically to photosynthetic species.

	Compound		
	Atrazine	**Desethyl atrazine**	**Desisopropyl atrazine**
EC_{10}			
5 min	13.0 (5-20)	70.0 (37-100)	44.0 (22-75)
15 min	14.4 (0-32)	101.0 (75-125)	63.0 (43-92)
30 min	17.5 (0-37)	134 (120-160)	75.0 (56-102)
EC_{20}			
5 min	25.8 (15-35)	180.0 (130-220)	107.0 (74-155)
15 min	22.6 (7-37)	193.0 (160-220)	109.0 (88-155)
30 min	24.0 (5-45)	220.0 (200-250)	116.0 (96-150)
EC_{50}			
5 min	30	670	350
15 min	20	550	300
30 min	10	550	280

Table 1. Concentration of pesticides and metabolites inducing a toxic response in photoluminescent bacteria. Parentheses indicate 95% confidence intervals for concentrations. For EC_{50} data were extracted from a graphical representation. Data from Kross et al. [1].

For the aquatic environment, toxicity tests based on photosensitising aquatic organisms are considered more relevant. One of these organisms is a species of green alga, *Raphidocelis subcapitata*, a sensitive and environmentally relevant model. Klementová et al. [52] performed growth inhibition test in reaction mixtures of atrazine and atrazine degraded in photocatalytic reaction with UV light in the presence of immobilised TiO_2 as photocatalyst. All atrazine samples displayed dose–response relationships and the highest tested concentration (10 mg/l) of the initial atrazine concentraion treatments reached more than 80% inhibition (Figure 4). The study was focused on the comparison of the toxicity of the parent compound solution and the reaction mixtures after different irradiation times (photodegradation progress). The toxicity of samples to algae decreased exponentially with the time of irradiation in the presence of TiO_2 up to 3 h for both IC_{50} and IC_{20} values calculated for growth rate inhibiton and yield (Figure 5). After 3 h of irradiation the decrease in toxicity slowed down, this effect being more pronounced in IC_{20} values.There is a trend towards increasing variability between replicates in atrazine samples irradiated for longer time periods, which is particularly pronounced in samples irradiated for 3 and 5 h (less than 10% of the original amount of atrazine is present in the irradiated mixture), where the samples cause a moderate stimulation in lower concentration treatments (up to 550 μg/l of the initial atrazine concentration).

The results observed in the study for the non-irradiated atrazine samples are comparable with the results of Van der Heever et al. [53] who reported IC_{50} 385 μg/l, even though lower IC_{50} values for the same species were reported by Weiner et al. [54] and Pérez et al. [55] (48.77 μg/l and 196 μg/l, resp.)

The photochemical degradation on semiconducters such as TiO_2 has been established as an efficient tool for its removal from drinking and waste waters [8,9,18,56]. However, the toxicity of the mixtures of photodegradation products arising under distinct irradiation conditions is still subject to current research since there is evidence that degradation products of some compounds may elicit higher toxicity than the parent compound [20,57]. A decrease in the toxicity of atrazine after photocatalytic degradation has been described using Microtox bacterial bioluminiscence assay [18,58]. The data presented by Klementova et al. [52] show a similar trend with a significant decrease in toxicity to algae after 1 h (or longer) periods of irradiation when more than 65% of atrazine is degraded. The results indicate no formation of by-products with equal or higher toxicity to algae than the parent compound atrazine.

A study of a toxicity test was performed on another model relevant to the aquatic environment, RTgill cells [52]. In the study, cell viability was assessed using the combination of three fluorescent dyes that determine different cytotoxic mechanisms: Alamar Blue, which indicates the metabolic activity, carbon fluorescein diacetate acetoxymethyl ester, which is used for monitoring of cell membrane integrity, and neutral red, which indicates the energetic state of a cell and is used as an indirect criterion of the lysosomal membrane integrity. Experiments with fish RTgill cells showed no toxic effects for either atrazine or its reaction mixtures after photocatalytic degradation of atrazine in the presence of TiO_2.

The results of the study seem to correspond with the *in vivo* ecotoxicity data for atrazine reported in the US EPA Ecotox Database in which EC/LC_{50} of atrazine for rainbow trout in acute tests exceeded 10 mg/l [59].

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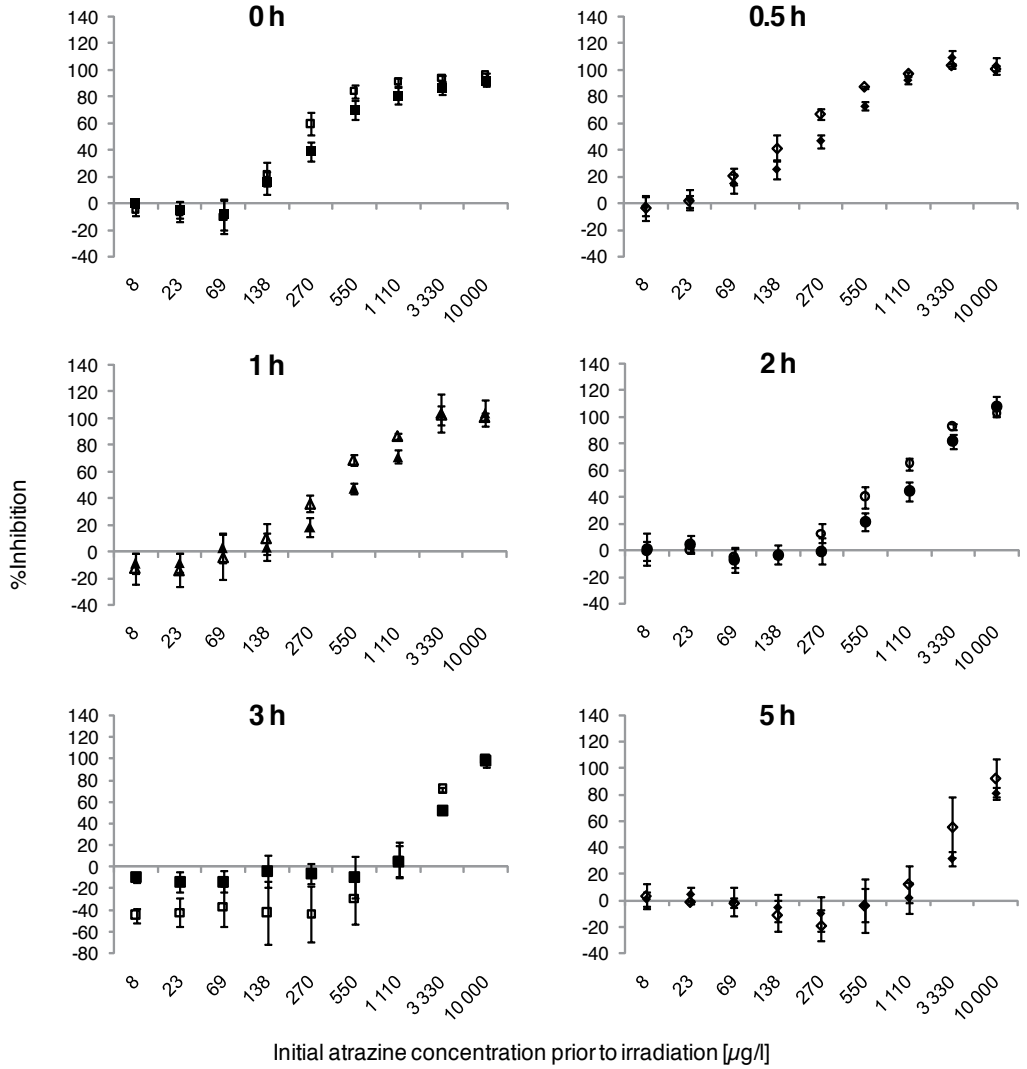

Figure 4. Inhibition of algal growth by atrazine samples with increasing duration of irradiation on TiO_2. The graphs show the initial atrazine concentrations [μg/l] in the non-irradiated sample (*0 h*), which is the theoretical maximum concentration of atrazine itself in the irradiated samples. Closed symbols show growth rate inhibition, open symbols yield inhibition and error bars standard deviation (*N*=5). Figure from Klementová et al. [52].

Wan et al. [60] reported acute LC_{50} values of atrazine 15 and 13 mg/l for 1- and 2–4-day exposures, respectively, for the same species. However, in the study of Waring and Moore [61] atrazine caused a significant reduction in gill Na+K+ATPase activity in Atlantic salmon (*Salmo salar*) smolts at environmentally relevant concentrations (2–10 μg/l). In a study by Prasad et al. [62], atrazine altered the hemocyanin metabolism, hydromineral balance, and gill function in crabs (*Oziotelphusa senex senex*). Exposure to 5 μg/l of atrazine led to osmotic disfunctions in mummichog fish larvae (*Fundulus heteroclitus*) and exposure to 40–80 μg/l resulted in behav-

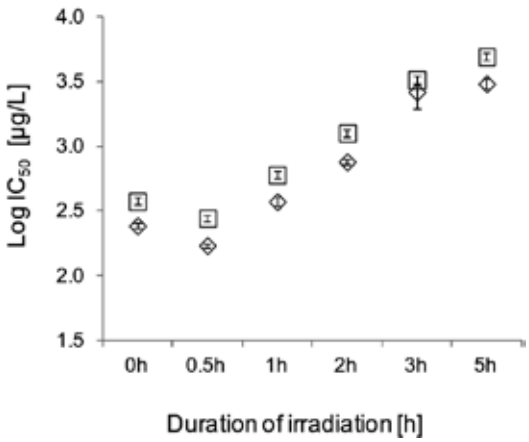

Figure 5. Decrease of atrazine toxicity to green alga *Raphidocelis subcapitata* with the duration of irradiation on TiO_2. The graph shows the logarithms of estimated inhibitory concentrations [µg/l] based on the nominal initial atrazine concentration in the non-irradiated sample (*0 h*), which is the theoretical maximum concentration of atrazine in the irradiated samples. Diamonds show growth rate inhibition and squares yield inhibition. Error bars denote standard deviation of the Hill's model fitting. Figure from Klementová et al. [52].

ioural and growth changes in red drum larvae *Sciaenops ocellatus*) [63]. Therefore, further experiments with fish embryos might provide additional insights into the toxic effects of atrazine and it photodegradation products.

5. Methods for atrazine degradation

The presence of chemically resistant and biorecalcitrant organic comtaminants in freshwater (surface water and ground water) attracts attention to developing technologies promoting easy and cost-effective degradation of these compounds. Among techniques for the treatment and purification of polluted waters, those based on advanced oxidation processes (AOP) are considered most promising. The AOP techniques are characterised by the ´*in situ*´ production of hydroxyl radicals (or other oxidative species. The production of hydroxyl radicals can be achieved in several ways, the most commonly used are Fenton process, photo-Fenton process, photodissociation of ozone or hydrogen peroxide and especially heterogeneous photocatalysis on semiconductors.

Also, some photosensitising reactions combining heterogeneous photocatalysis and photo-sensitisation have been used as AOPs.

Degradation can be achieved by UV photolytic processes that use low-wavelength high-energetic light.

5.1. Homogeneous photocatalysis, Fenton and photo-Fenton reaction

The Fenton reaction is the reaction of a mixture of ferrous ions and hydrogen peroxide providing hydroxyl radicals as shown in Eq. 1.

$$Fe^{2+} + H_2O_2 \rightarrow HO^{\bullet} + Fe^{3+} + OH^{-} \quad (1)$$

In this process, part of the radicals are consumed in the oxidation of ferrous ions, which results in the quick consumption of the added photocatalyst, ferrous ions.

In the photo-Fenton process, the ferrous ions are formed *in situ* photochemically, as shown in Eq. 2, the reaction of the ferrous ions with hydrogen peroxide proceeds as in the Fenton reaction (Eq. 1)

$$Fe^{3+} + H_2O \xrightarrow{h\nu} Fe^{2+} + HO^{\bullet} + H^{+} \quad (2)$$

The efficiency of Fenton type processes is influenced by several operating parameters such as the concentration of hydrogen peroxide, pH and the intensity of UV radiation. The main advantage of the photo-Fenton process is its ability to use sunlight for photochemical activation, thus avoiding the high costs of UV lamps. The disadvantages of the process are the low pH values required (to avoid iron precipitation) and the necessity of iron removal after the treatment. These processes have been used efficiently for different classes of pollutants including atrazine [64].

The photocatalytic action of metals such as ferric, copper and manganese ions on the degradation of triazine herbicides without the addition of hydrogen peroxide was also investigated [65]. The study revealed that cupric and manganese (II) ions exhibited only small activities, and only in high concentrations ($1*10^{-3}$ mol/l), whereas ferric ions positively affect the degradation at concentrations as low as $1.5*10^{-6}$ mol/l, and with increasing concentration of these ions the degradation rate significantly increased. The dependence of the rate constants of the degradation on the initial ferric ions concentration based on the data presented by Klementova [14] is given in Table 2. In all reaction mixtures of s-triazine studied, that is atrazine, propazine, simazine, significant photoreduction of ferric ions to ferrous ones was observed in spite of the saturation of the mixtures with the air; the steady state concentrations of ferrous ions were established in less than 10 min of irradiation and reached 22%, 70% and 85% of the initial concentration of added ferric ions for atrazine, propazine and simazine, respectively. This supports the conclusion that photoreduced metal ions act as a catalycally active form in the homogeneous photocatalytic degradation of triazines [14,65].

Initial concentration of added ferric ions (mol/l)	**Rate constant (min^{-1})**
$1.5*10^{-6}$	$3.0*10^{-4}$
$1.0*10^{-5}$	$9.0*10^{-4}$
$6.6*10^{-5}$	$1.7*10^{-3}$
$1.0*10^{-4}$	$4.8*10^{-3}$
$1.6*10^{-4}$	$1.3*10^{-2}$

Table 2. Dependence of the rate constant of atrazine in homogeneous photocatalytic degradation on the concentration of the ferric ions added.

5.2. O_3/UV, H_2O_2/UV techniques

Degradation techniques involving combination of H_2O_2/UV or O_3/UV often combined with photocatalysis in homogenenous as well as heterogeneous arrangement were applied to promote the degradation and mineralisation of many organic biorecalcitrant compounds including triazine herbicides [66-70].

5.3. Heterogeneous photocatalysis on semiconductors

Semiconductor photocatalysis uses solid catalytic systems while the substrate to be degraded is dissolved or dissipated in the solution (or gaseous phase) around the catalyst. Five distinct steps in the process of degradation of a reactant are, therefore, distinguished:

a. The transfer of liquid or gaseous phase reactant to the catalytic surface by the diffusion

b. The adsorption of the reactant on the catalyst surface

c. The reaction of the adsorbed molecules

d. The desorption of products

e. The removal of products from the interface region by diffusion

The photocatalytic reaction occurs in the stage in which the reactant is adsorbed by the catalyst surface; the activation of the reaction is triggered by incident photons.

Proper activation by irradiation depends on using an appropriate wavelength range corresponding to the band gap energy of the particular semiconductor (Figure 6).

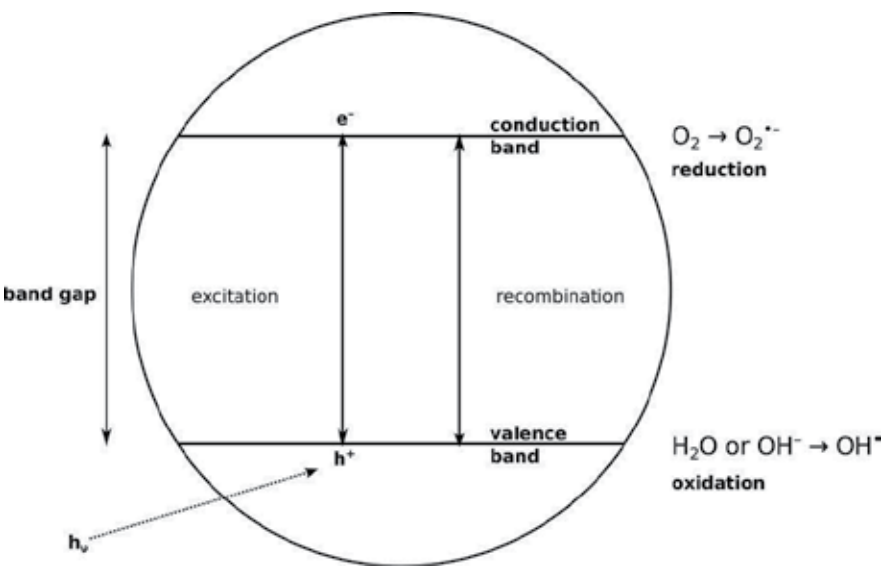

Figure 6. Scheme of oxidation species production in photocatalysis on semiconductors.

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The activation generates a pair of charge carriers, a hole and an electron; the charge carriers then react either with water molecules (), or with the dissolved oxygen (), in a series of reaction leading to production of hydroxyl radicals as shown in Eqs. 3–8.

$$h^+ + H_2O \rightarrow HO^\bullet + H^+ \tag{3}$$

$$h^+ + OH^- \rightarrow HO^\bullet \tag{4}$$

$$O_2 + e^- \rightarrow O_2^{\bullet -} \tag{5}$$

$$O_2^{\bullet -} + H^+ \rightarrow HO_2^\bullet \tag{6}$$

$$2HO_2^\bullet \rightarrow H_2O_2 + O_2 \tag{7}$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow HO^\bullet + O_2 + OH^- \tag{8}$$

Various metals oxides were used in semiconductor photocatlytic reactions. The most frequently used is TiO_2 [15,71, 72] but also other materials such as ZnO, CeO_2, WO_3, as well as semiconductor composites, doped or modified semiconductors [73-76].

The disadvantage of the photocalatysis on semiconductors lies in the fact, that though many catalysts were proposed, it is generally admitted that only TiO_2 gives reasonable results in pollutant degradation; unfortunately, photons required for overcoming the energy gap and thus initiating the photocatalytic process in TiO_2 must have a wavelength of less than 385 nm, which practically excludes the sun as a low-cost energy source since this radiation represents only about 5% of the sunlight ultraviolet and visible range reaching the earth's surface.

Doping a semiconductor with precious metals such as gold, platinum, silver or palladium may reduce the band gap but increases the cost of the catalyst.

Another option is a binding of sensitiser molecules on the catalyst. An example is a study of Granados-Oliveros [76] in which porphyrin derivatives with different metal centers were adsorbed on TiO_2 surface. Their delocalised macrocyclic structure, strong absorption in the visible region and excited state energy enabling electron transfer to the catalyst conduction band seem to make them very attractive for investigation though less likely candidates for large-scale usage.

Photocatalytic degradation of s-triazine herbicides on TiO_2 has been studied by many authors [15,52,71, 77-79]; atrazine was found to be degraded mainly to desethyl atrazine and desiso-

propyl atrazine; the hydroxy derivatives of these compounds as well as of the parent compound atrazine were present in the reaction mixtures. It means that photocatalytic degradation leads to the same compounds that have been distinguished as metabolites of atrazine biodegradation.

5.4. Photolysis

Photolysis means direct photochemical degradation with short-wavelength radiation which has enough energy to break bonds in a molecule.

The reaction includes only one reactant, the molecule that undergoes photolysis; therefore the reaction follows the first order kinetics.

To achieve a photolytic decomposition, highly energetic radiation is necessary, usually radiation of 254 nm is used.

The photolytic degradation of triazines has been studied by several authors [11,80,81]. The studies were focused on the kinetics of the degradation; analyses of products revealed that dechlorination is the main degradation pathway. Klementova and Piskova [80] reported on the basis of an analysis of dissolved organic carbon (DOC) that photolytic degradradation leads to the partial mineralisation of atrazine – about 20 % of DOC was mineralised in 90 min of irradiation at the intensity of $1.22*10^{-5}$ einstein/min.

6. Conclusions

Chloro-s-triazine herbicides, namely atrazine, have been shown to be distributed in the soil and water environment. Their biodegradation is slow and leads to dealkylated and hydroxylated derivatives, which – though seemingly have less deleterious effects on organisms – persist in the environment even longer than the parent compounds.

The harmful effects of triazine compounds on the health of humans appear to lie in their action as endocrine disruptors; evidence of possible carcenogenic or mutagenic effects on humans is rather weak but cannot been conclusively excluded.

AOP photochemical methods have proven to be a promising tool in disposing of these pollutant in contaminated bodies of water.

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Chapter 5

Biomonitoring the Environmental Quality by Bees

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Additional information is available at the end of the chapter

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Abstract

Modern farming techniques have increased the crop yield, but natural habitats of the pollinator were destroyed, affecting their populations compared to native vegetation. A simple, low-cost, and efficient way to determine the presence of insecticide residues from farming is the honeybee as a bioindicator. However in Brazil, there is another species of bee, the stingless bees. The insecticide toxicity analyses the beneficial insect species as pollinators which are performed to the *Apis mellifera*. Stingless bees are native to tropical and subtropical zones, and they are more sensitive to pesticides than honeybees. We present some results of contamination in these bees compared to Africanized honeybees, and pose an important question: Why does the pesticide industry not make assays with stingless bees too? When insecticides were in larger concentrations, bees did not feed. When the concentration of the insecticide was smaller, Africanized honeybees consumed the polluted honey, resulting in the death of some. Finally, we report several experiments concerning honeybees, and mainly stingless bees, and the effect of pesticides in them; results show stingless bees are more sensitive than honeybees. Our Bee Research Group studied this point, and we hope to contribute for understanding this relation between bee, pesticide, and environment.

Keywords: Pesticides, fipronil, thiamethoxam, bee, bioindicators

1. Introduction

Stingless bees are among the most common pollinators in tropical environments. Plant reproduction and pollinators are strongly linked, and the role of bees as pollinators becomes crucial in almost every terrestrial ecosystem. Improper use of pesticides has caused adverse effects on nontarget organisms, such as humans, domestic animals, wild organisms, pollina-

tors, and natural enemies. Insecticides, when used correctly, can control target organisms without compromising the populations of natural enemies and pollinators. Thus, biomarkers have been extensively used to reveal the exposure of organisms to various chemical compounds in the environment.

Decline of pollinator populations is mainly due to the non-sustainable use of chemicals for agricultural production. Excessive insecticide use on crops endangers the populations of pollinating bee species, including stingless bees.

Pollination is a fundamental factor of production in management of crops around the world, and the lack of pollinators for the plants may limit the quantity and quality of the fruit [1]. Therefore, it is essential for good agriculture development with global reach; it also prevents the environmental degradation at local level as well as global level to avoid declining of pollinators [2].

The total number of pollinators is estimated at 40,000 species, with 25,000 being bee species. About 73% of cultivated crops globally are pollinated by some type of bee [3]; 87 of 115 principal global crops benefit significantly from pollinators [4]. A decline of these species or inadequate pollination in some crops may decrease the production by 50%.

The decline of pollinator honeybee *Apis mellifera* worldwide is cause for concern, and several papers have evaluated the harmful effects of insecticides to this bee species. These studies are not frequent in the case of stingless bees because they do not occur in temperate zones [5, 6], although in Brazil we can find a great diversity of these stingless bees. Little research has been done to evaluate insecticide effects on Meliponini, Bombini, and Euglossini bees [7]. The pollinator activity is essential to preserve environment and high yield in agriculture [8].

Stingless bees are among the most common pollinators in tropical environments, and in some regions, these bees are dominant and visit several crops [9]. These insects are a diverse group, which include more than 400 species that present great variability in physiology, morphology, and size, from 0.2 mm in genus *Trigonisca* to more than 20 mm in some species of *Melipona* [10, 11]. The bees belonging to the genus *Tetragonisca* have featured ecological and economic importance. The meliponiculture is a way to preserve the fauna and flora, so the beekeepers can harvest honey, cerumen, and resins. This practice is widely spread, mainly, in the North and Northeast region of Brazil [12], but it increases yearly in other regions of the country.

Bees live in close contact with nature, harvesting pollen, nectar, water, and resins for their colony, so they require that all sources from these resources be pure and without contaminants [13]. The bees are susceptible to several insecticides commonly applied to protect crops, and these insects can be used to biomonitor the environmental quality to detect residues of some insecticides in plants, as well as to detect the toxicity level in bees [14]. Thus, the presence of bees and the quality of their products can be used in environmental biomonitoring, contributing to a better-quality environment for local human populations.

Studying pesticide effects in bees is fundamental; the farmer must learn to select and apply pesticides to control diseases and plagues without risks to the survival of beneficial insects [15]. The presence of pesticide residues is generally detected by physical, chemical, and biological

methods. Theoretically, any organism that is susceptible to an insecticide can be used in these assays, in any environmental sample, and thereby it is possible to make use of biomonitoring to detect some pollutants.

The evaluation and contribution of possible sublethal effects of pesticides in bees have been discussed by scientists and regulation committees [16]. Effects reported by these authors include alterations in learning behavior and the ability of orientation. The alteration in isozyme expression such as esterase that plays a role in metabolism of xenobiotic of bees is another way to use these insects in environmental monitoring.

Due to the importance of bees as pollinators, the usual interaction of beekeeping-agriculture, and the occurrence of areas with natural forests near agricultural areas, it is possible to study the sublethal effects of pesticides in bees. One way to do this is by electrophoretic analysis of esterase; verifying the presence or absence of an alteration in these isozymes can be useful in detecting the environmental contamination in regions in which the bees are visiting or by drifting during the pesticide application.

This review discusses environmental quality using bees to detect residues of pesticides and the alteration that can occur after contamination by sublethal doses of pesticides generally used in agriculture by developed methods with honeybee *Apis mellifera* and stingless bees.

2. Pesticides and biomonitoring

2.1. Pesticides

The first records that man used insecticides to reduce losses by insect attack to the crops are from 1000 B.C. and chemical control of plagues began in twentieth century with chemical 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT) [17]. Pesticides are classified according to target organisms; fungicides, herbicides, and insecticides are commonly used and are the principal focus of study [18]. The main chemical groups of insecticides of neurotoxic action, general name, and their action site are presented in Table 1.

Insecticides are classified according to their toxicity based on values of lethal dose LD_{50} for any of the routes of contamination (ingestion or contact) presenting the following toxicological categories: **I –** extremely toxic (LD_{50} < 50 mg/kg body weight), **II –** very toxic (LD_{50} 50–500 mg/kg body weight), **III –** moderately toxic (LD_{50} 500–5,000 mg/kg body weight), and **IV –** low toxicity (LD_{50} > 5,000 mg/kg body weight) [19].

Due to the feeding habits of bees, combined with the difficulty of evaluating the effect of chemicals on these insects in the embryonic and larval stages, many researchers seek to associate food with the supply of pesticides. In this sense, the experiment conducted directly provided *A. mellifera* colonies with sugar syrup contaminated with cypermethrin insecticide for 5 consecutive months [22]. During the 18 weeks of treatment, there was a mortality of bees in hives, but some also presented sublethal effects in laboratory tests on samples of bees, such as glycemia, changes in the ATPase enzyme activity, and other physiological disorders and behavioral changes.

Chemical group	General name	Action site
Carbamate	Propamocarb hydrochloride, iprovalicarb, oxamyl	Acetylcholinesterase inhibitors
Organophosphates	Acephate, cadusafos, chlorpyrifos, diazinon, dimethoate, disulfoton, ethione, ethoprophos, fenamiphos, fenitrothion	Acetylcholinesterase inhibitors
Pyrethroids	Acrinathrin, allethrin, alpha-cypermethrin, beta-cyfluthrin, beta-cypermethrin, bifenthrin, cyfluthrin, cypermethrin, d-allethrin, deltamethrin	Modulators of the sodium channel
Neonicotinoids	Acetamiprid, clothianidin, dinotefuram, imidacloprid, Thiacloprid, thiamethoxam	Agonists of nicotinic acetylcholine receptors

Source: Ref. [20].

The registration of all pesticides is performed after toxicity tests. Most of them possess recommendations on toxicity to *A. mellifera* bees. In some localities, there are laws prohibiting the use of pesticides on crops that are blooming (e.g., Ontario, Canada, where it was intended primarily to protect pollinating honeybees in orchards) [21].

Table 1. Main chemical groups of insecticides, general name, and action site

Testing the effect of endosulfan, deltamethrin, baytroid, and sevin, Abramson et al. [23] concluded that none of these products presented repellency effect when provided via food. Except for deltamethrin, the rest were highly toxic to the bees, with mortality 1 hour after feeding. Thompson [24] reported that deltamethrin did not cause mortality of bees but caused sublethal effects at low concentrations, such as hypothermia and loss of memory, making it impossible to return to the colony.

Knowledge about the mechanism of action of insecticides on pollinators is important particularly for bees to use them in a way that minimize lethal and sublethal effects on pollinators [25]. Besides the direct danger of pesticides applied to crops, there is the problem associated with pesticide residues in products of the colony. Thus, highly toxic residues can be found in the hives, jeopardizing the quality of bee products [21].

The food collection of these bees is essential for the maintenance of colony life; however, it exposes the workers to contamination in areas where pesticides have been applied. Therefore, bees require that all sources of their rewards are pure and free from contaminants, including pesticides [26].

The use of conventional pesticides to control pests results in reducing large-scale natural enemies, environmental pollution, resistance to chemical insecticides, as well as increased population of resistant pests [27]. Furthermore, they reduce the diversity of natural enemies of agricultural pests [27], as well as cause a decrease of beneficial insects.

Most synthetic insecticides are toxic to all animals, including man. Although many insecticides can be used safely, few are persistent in the environment and a small number of compounds have mutagenic action, carcinogenic and teratogenic effects in humans and domestic animals [28].

Organophosphate and carbamate pesticides have toxic effects resulting from accumulation of acetylcholine in the synaptic cleft, because the insecticide molecules bind irreversibly to the

catalytic site of acetylcholinesterase, leading to overstimulation of the acetylcholine receptors to produce neurotoxic symptoms [29]. In most bees, acetylcholinesterase is located in the head, especially in compound eyes and ocelli [30, 31]. The detection of anticholinesterase insecticides in bees is difficult to achieve due to their rapid hydrolysis in the body. Thus, one way to detect contamination with these insecticides can be accomplished by inhibition of acetylcholinesterase activity [29]. According to its toxic effect, the organophosphorus compounds can be subdivided into three groups: (a) those highly toxic to bees, such as methyl parathion, malathion, and azodrin, which should not be used in plantations with blossoms; (b) those highly toxic, but with little residual activity, such as mevinphos, that could be applied when bees were not collecting from flowers; and (c) those relatively nontoxic to bees, such as ethion and trichlorfon [32]. Integrated pest management programs determine which insecticides are compatible with a biological control agent and to identify the possible effect on them [32]. Biopesticide studies are frequent in an attempt to reduce the ecological impact that chemicals are causing on the environment [33].

The pesticide group known as bioinsecticides is formed by compounds from plant, animal, fungi, and bacteria, which have allelopathic action in various organisms. *Insect growth regulator insecticides* are biopesticides that have targeted specific characteristics or are stage specific, with a good safety margin for most of the nontarget biota, including invertebrates, fish, and birds, among others. They are relatively safe for humans and domestic animals. Insect growth regulator insecticides mimic juvenile hormone and/or ecdysone in cuticle formation process, and inhibit chitin synthesis in insects and action of the endocrine system [34]. These bioinsecticides are part of a generation of alternative compounds that have been used in agriculture, with a different mode of action of conventional insecticides, acting in specific systems of insects, characterizing them as selective products and low toxicity to mammals [35].

Neonicotinoid insecticides, developed in the 1990s, are registered for use in a wide variety of cultures and effective against insects such as beetles and other insects [36]. The current decline of pollinators has been attributed, at least in part, to the use of neonicotinoids on crops that offer attractive flowers for bees [37].

The class of neonicotinoids originated from the nicotine molecule, extracted from tobacco plants (*Nicotiana tabacum*). The first compound of this class being marketed, imidacloprid, was introduced in Europe and in Japan in 1990 by Bayer CropScience®, which, together with nitenpyram and acetamiprid, represents the cloronicotinil subclass, also known as first-generation neonicotinoids [38].

Commercially, products formulated based on thiamethoxam, the first insecticide of second-generation neonicotinoids, were provided by Syngenta® from 1998, being registered for use on cotton crops, coffee, citrus, soy, and rosettes, are indicated for the control of insect pests of occurrence in the shoot, as aphids, whiteflies, tripods, beetles, and some species of Lepidoptera [39]. Another interesting feature of the molecule is the versatility of use employed by foliar spraying, soil irrigation, and/or seed treatment [40, 41]. They are predominantly used in the application of the seeds in crops such as cotton, canola, and sunflower [42].

Thiamethoxam (3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1, 3,5-oxadiazinan-4-ylidene (nitro) amin) is a crystalline, odorless compound with 139.1°C melting point, low molecular weight (291.72 $g.mol^{-1}$), relatively high solubility in water (4.1 $g.L^{-1}$ at 25°C), and formula $C_8H_{10}ClN5O_3S$ [40]. The insecticide thiamethoxam is neurotoxic, damages the central nervous system, and inhibits the action of nicotinic acetylcholine receptors of insects by acting specifically on the α subunit of the receptor [43]. These receptors are found in many regions of the brain of bees, including areas involved in learning and memory processes [44]. Unlike acetylcholine, which is hydrolyzed by acetylcholinesterase, these compounds are not degraded immediately; therefore, nervous impulses are transmitted continuously and lead to hyperexcitation of the insect nervous system [45].

Maienfisch et al. [40] reported agonistic activity on nicotinic acetylcholine receptor mimics their docking sites, causing sublethal effects such as loss of memory and orientation [46]. Pettis et al. [47] added that these long-term effects may cause their death or make the colony more susceptible to disease.

In Brazil, a foliar neonicotinoid insecticide is permitted and suitable for various crops, including cotton, citrus, tomato, eucalyptus, apple, strawberry, and soybean [20]. However in 2012, after a series of protests in Europe in order to ban all forms of application of neonicotinoids such as imidacloprid, thiamethoxam, and clothianidin in crops that offer flowers for the bees, the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) declared temporary restriction of the use of the same insecticides and initiated a process of reassessment of these chemicals.

In 2013, the European Food Safety Authority (EFSA) published a document containing a compilation of studies that showed residual values of neonicotinoids found in materials stored in colonies of bees and pollen and nectar crops that offer attractive flowers to them. Inferences were made about the potential risk that the waste could pose to bees. Based on the results, it was concluded that many gaps on the risks that neonicotinoids pose to bees and other pollinators are yet to be understood. Thus, the final position of the European Union was to ban the use of neonicotinoid insecticides for 2 years (2013–2015), until more consistent results are presented [48].

Colony collapse disorder has been one of the biggest concerns about the decline of bee populations in the world [49], and the use of neonicotinoid insecticides has been one of the greatest attributes to this event [46]. The application of this new chemical class of insecticides on crops that offer attractive flowers to bees is growing worldwide [18], and the possibility of its neurotoxic [40] amounts to nontarget organisms, such as bees, justifies its intensification of studies that seek answers to these gaps in knowledge [36].

In recent years, several protests were held in Europe, in general, to ban the use of neonicotinoids on crops that offer flowers for the bees. In Brazil, these substances are sold in commercial formulations, and information use and toxicity can be accessed in the agriculture ministry [20].

2.2. Residues of insecticides

Detoxification is the cellular cleaning process that inactivates toxic compounds that pose risks to cellular health. Organophosphate pesticides, carbamates, and pyrethroids are detoxified by oxidases action with mixed functions, esterases and transferases [50].

The evaluation and contribution of possible sublethal effects of pesticides on bees have been discussed by scientists and regulatory authorities [16]. The effects considered by these authors include changes in learning behavior and orientation ability. Allied to the study of sublethal doses of pesticides, changes in isozyme expression as esterases that would be acting in xenobiotic metabolism of bees is a way of using these insects as bioindicators.

The carboxylesterases and cholinesterase are involved in insecticide resistance, as demonstrated by Whyard et al. [51] and Lee and Lees [52], who observed increased carboxyl esterase activity in *Culex tarsilis* and *Oryzaephilus surinamensis lines*, respectively, resistant to malathion. Among the cholinesterase involved in resistance mechanisms, there is acetylcholinesterase, which is specific to the central nervous system of insects. This esterase regulates acetylcholine levels in the synaptic regions between neurons.

Organophosphate and carbamate pesticides exert acute toxicity by inhibiting acetylcholinesterase (EC 3.1.1.7), a hydrolase serine found in the neuromuscular junction. Acetylcholinesterase is an important enzyme responsible for the rapid hydrolysis of acetylcholine in cholinergic synapses substrate, allowing precise control and modulation of nerve transmission. This leads, in susceptible species, to accumulation of the neurotransmitter acetylcholine and subsequent hyperpolarization of the postsynaptic membrane [45]. Carboxylesterases play a key role in the detoxification of certain hydrolytic organophosphate compounds and play an additional role as alternative binding sites of [53].

2.3. Bee susceptibility to insecticides

Toxicity studies commonly express means of results as LD_{50} and/or LC_{50} (lethal dose and concentration that kills 50% of a population, respectively). Brittain and Potts [54] reported that sublethal effects of pesticide contamination may be more harmful to the colonies in the long run than the lethal effects observed immediately after intoxication. Susceptibility to insecticides may be related to behavioral changes and memory loss that reduce the reproductive success of bees [55].

Bee response to pesticide contamination depends on a number of factors, such as body size, sociality, flight period, floral specialization, and nest behavior [54]. These authors also reported that there was an important correlation between the characteristics presented for each group of bee and environmental conditions provided. For instance, knowledge of the duration of the foraging activity of a bee and the particular plant flowering period will allow the presentation of evidence about the risks of exposure and, consequently, susceptibility for each bee species.

In small concentrations, cells damaged by insecticides can detoxify or be replaced by regenerating cells; however, with high concentrations of insecticides, affected cells are unable to efficiently detoxify and the regenerative cells are also impacted [56]. Another way to detect

bee sensitivity to residues of pesticides is through studies of the change in chromatin by analyzing the critical electrolyte concentration; this technique was developed by Vidal and Mello [57]. The analysis of critical electrolyte concentration checks whether change is occurring in gene expression after contamination with insecticide, leading to changes in the value of the critical electrolyte concentration which is due to inactivation or activation of genes after contamination.

Johnson et al. [58] treated bees with three pyrethroid insecticides (cyfluthrin, lambda-cyhalothrin, and tau-fluvalinate), in which one group was treated with an inhibitor of cytochrome P450 enzyme. These authors found that the toxicity of these insecticides was more significant with the pretreatment, concluding that this enzyme is important in bee tolerance to pyrethroid insecticides.

2.4. Toxicity of insecticides and biomonitoring with bees

Biomarkers have been used extensively to reveal the exposure of organisms to various chemicals in the environment. They are based on physiological, biochemical, anatomical, and behavioral parameters after exposure to pesticides [59].

In the existing literature, there are several studies using *A. mellifera* as a pollutant bioindicator insect in the environment. Toxicity data to verify the susceptibility of *A. mellifera* to 62 insecticides of six classes (carbamates, nicotinoids, organochlorines, organophosphates, pyrethroids, and miscellaneous) were performed [60]. Honeybees can be susceptible to individual insecticides, but they are not highly susceptible to insecticides overall or to specific classes of insecticides. Thereby, there is a great interest in using *A. mellifera* as a high-sensitivity bioindicator insect, because of their foraging activity and, consequently, their contact with pollutants present in the environment.

Hashimoto et al. [61] conducted various bioassays to detect changes in the activity on the esterase of Africanized *A. mellifera* after contamination by contact and by ingestion of the insecticide thiamethoxam neonicotinoid. In this study, it was found that five esterases (EST-1, EST-2, EST-3, EST-4, and EST-5) of these honeybees decreased in relative activity after infection with thiamethoxam, indicating that these isozymes exhibit a rapid response to poisoning by this neonicotinoid (Tables 2 and 3). Attencia et al. [62] evaluated the effects of parathion-methyl esterase in *A. mellifera* workers and found that, at a concentration of 0.01%, EST-1 activity was reduced by 75%, 14 and 21 days after the introduction of the insecticide. For esterases 3 and 4, there was 50% inhibition of its relative activity after 1-day release (Table 3). From these results, the authors suggested that inhibition of esterases 3 and 4 can be used to detect the presence of methyl-parathion residues in crops (Table 4).

Catae et al. [63] analyzed the effects of thiamethoxam in intestinal cells and Malpighian tubules in *A. mellifera* Africanized honeybees. Newly emerged workers were exposed up to 8 days with a diet containing sublethal doses of thiamethoxam LC_{50} equal to 1/10, i.e., 0.0428 ng a.i./L diet and found that the damage caused by thiamethoxam in the intestine was evident on bees exposed on the 1st day. Malpighian tubules presented abnormalities on the 8th day of exposure to the insecticide. Continuous exposure to sublethal doses of thiamethoxam can damage

organs that are used to metabolize the insecticide. *A. mellifera* Africanized honeybees newly emerged and exposed to a dose of 0.428 ng/day of thiamethoxam presented intoxication with sublethal dose of thiamethoxam, and Oliveira et al. [64] concluded that this can cause damage in the brains of bees.

	Ages/esterases																					
mg/mL	**Newly emerged**						**7 days**						**14 days**						**21 days**			
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
2.0	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
4.0	+	+	−	−	−	−	+	−	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−
4.1	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−
4.2	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−
4.4	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−

Modified from Ref. [61].

(+) = partial inhibition of esterase activity; (−) = absence of inhibition of esterase activity.

Table 2. Inhibition of relative activity of esterases detected in *A. mellifera* extracts of workers after contact with thiamethoxam

	Ages/esterases																					
mg/mL	**Newly emerged**						**7 days**						**14 days**						**21 days**			
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
4.0	+	+	−	−	−	−	+	+	−	+	+	−	+	−	−	+	+	−	+	+	−	−	−	−
2.0	+	−	−	+	+	−	+	+	−	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−
0.25	+	−	−	−	+	−	+	+	−	−	+	−	+	+	−	−	+	−	+	+	−	−	−	−
0.125	−	−	−	−	−	−	+	+	−	−	+	−	+	+	−	−	−	−	+	+	−	+	−	−
0.0625	−	−	−	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	+	+	−	+	−	−

Modified from [61].

(+) = partial inhibition of esterase activity; (−) = absence of inhibition of esterase activity.

Table 3. Inhibition of relative activity of esterases detected in extracts of *A. mellifera* workers after the application of thiamethoxam in the food

Studying *A. mellifera* honeybees, Yang et al. [65] noted that bees orally treated with imidacloprid insecticide were delayed in time to revisit a food source. This delay was dependent on the concentration of insecticide to which bees were exposed; at concentrations of 1,200 g/L many bees did not return to the food supply or to the colony. Rossi et al. [66] reported that low

doses of imidacloprid (0.809 ng/bee) caused cytotoxic effects on Malpighian tubules of Africanized *A. mellifera*.

		Esterase-1		Esterase-2	Esterase-3		Esterase-4	
Concentration	**Time (days)**	**Ac**	**But**	**But**	**Ac**	**But**	**Ac**	**But**
0.001%	1	*	*	*	*	*	*	*
	7	*	*	*	*	*	*	*
	14	*	*	*	*	*	*	*
	21	*	*	*	*	*	*	*
0.1%	1	*	*	*	--	*	*	-
	7	*	*	*	*	*	*	*
	14	---	*	+	*	+	*	+
	21	---	*	+	*	*	*	*
0.05%	1	---	-	-	---	-	---	-
	7	+	+	+	++	*	*	*
	14	*	*	*	*	*	-	*
	21	*	*	*	*	*	*	*
0.01%	1	*	*	*	*	*	*	*
	7	cd	cd	cd	cd	cd	cd	cd

Modified from Ref. [62].

+ =25% activity increase; ++ = 50% activity increase; - = 25% inhibition; -- = 50% inhibition; --- = 75% inhibition; * = no alteration; cd = colony death.

Table 4. Relative esterases activity in *A. mellifera* homogenates front to different concentrations of the methyl-parathion insecticide visualized with substrates 4-methylumbelliferyl acetate (Ac) and 4-methylumbelliferyl butyrate (But)

Kakamand et al. [56] indicated that deltamethrin, malathion, and thiamethoxam when orally administered to honeybees began to cause death of individuals after 4 hours, except for the deltamethrin 10-ppm dose, which began to kill honeybees 2 hours after the treatment. According to Kakamand et al. [56], the explanation of death after hours of poisoning is the difficulty of feeding and hydration of bees by paralysis of the digestive system or damage caused by insecticides to the intestine.

In a research conducted by Roat et al. [67], newly emerged bees and foragers from *A. mellifera* species were subjected to chronic treatment with fipronil insecticide at a dose of 0.01 ng (LD_{50}/100) for 3, 5, and 8 days, being subsequently subjected to cytochrome oxidase histochemical technique to assess the neural activity. Roat et al. [67] concluded that a basal marking in groups of newly emerged bees treated during 3 days similar to the control group, suggesting that neural activity in bees at this age is not altered by the presence of the insecticide, although symptoms of intoxication and changes in the survival of these individuals were noticed. A positive staining, most obvious, was observed only in the groups of forager bees treated during 3–5 days, causing changes in cell metabolism.

Arena and Sgolastra [68] compared the sensitivity of *A. mellifera* with 19 other species of bees by meta-analysis. Considering all species and pesticides evaluated, based on the LD_{50} and LC_{50}, Arena and Sgolastra [68] reported wide variation of the total radius of sensitivity measured between cases, and the statistics indicated *A. mellifera* is often more sensitive than other species. Nevertheless, considering the six chemical classes of insecticides used (carbamates, organochlorines, organophosphates, pyrethroid, neonicotinoids, and a mix of them), only for neonicotinoids all other species were more sensitive than *A. mellifera*. Hardstone and Scott [60] evaluated the susceptibility of *A. mellifera* and other insects, considering the same classes of insecticides, and pointed out that other species were more sensitive than *A. mellifera*.

Bees of genus *Tetragonisca* are rarely used in toxicity studies with insecticides. One of the studies of these bees was developed by Fermino et al. [69]. Species *Tetragonisca angustula* and *Tetragonisca fiebrigi* were used to evaluate the influence of nicosulfuron and paraquat herbicides on expression of isozyme esterase (EST – EC 3.1.1.1), malate dehydrogenase (MDH – EC 1.1.1.37), superoxide dismutase (SOD – EC 1.15. 1.1), soluble proteins, and brain cells by the critical electrolyte concentration technique (CEC).

Bioassays were performed by *in vitro* exposure to the herbicide in Petri dishes for 24 hours. Detected mortality was low being 2.75% for *T. angustula* and 5.8% for *T. fiebrigi* after contamination with nicosulfuron, and 2.5% for *T. angustula* and 1.25% for *T. fiebrigi* after contamination with paraquat (Table 5). However, changes in the expression of various isozymes and chromatin structure have been identified.

Authors verified that the herbicide nicosulfuron causes partial inhibition of esterases from *T. angustula* and *T. fiebrigi*. The herbicide paraquat promotes total inhibition of esterase relative activity in *T. fiebrigi* from the concentration of 1.5×10^{-5} g/mL, and in concentrations of 1.5×10^{-4} and 1.5×10^{-3} g/mL in *T. angustula*. Superoxide dismutase isozymes showed an increase in their relative activity after contamination with paraquat at 1.5×10^{-4} and 1.5×10^{-3} g/mL in both species. No changes were observed for MDH and soluble proteins. In the nerve cells, few changes were observed in gene expression after contact with the herbicides (Figures 1–4). According to the authors, Brazilian stingless bees *T. angustula* and *T. fiebrigi* have the potential to be used in biomonitoring for the presence of paraquat and nicosulfuron herbicides. *T. fiebrigi* has greater sensitivity to the herbicide paraquat than *T. angustula*.

Further studies are being developed by our research group with *T. angustula* and *T. fiebrigi* with insecticides fipronil, malathion, thiamethoxam, and growth regulators (neem and novaluron). Results obtained until now have shown that these stingless bees are susceptible to these insecticides and changes in the expression of esterases have been detected.

Bees *T. angustula* have esterases EST-3 and EST-4, whereas *T. fiebrigi* has EST-1, EST-2, and EST-4 [70]. Insecticide contamination promotes inhibition (decreased relative activity) of esterase EST-4 *Tetragonisca* bees, so this isozyme has the potential to become a bioindicator of environmental contamination with pesticides analyzed.

These studies have further shown that the *T. fiebrigi* bees are more susceptible to insecticides than *T. angustula* bees, so are more likely for population decline. A possible confirmation of this statement causes concern because *T. fiebrigi* bees have a more restricted distribution than

T. angustula bees. Camargo and Pedro [71] reported that the distribution of *T. fiebrigi* includes Argentina (Misiones, Tucumán); Bolivia (Santa Cruz); Brazil (Mato Grosso, Mato Grosso do Sul, Parana, Rio Grande do Sul, Brazil); Paraguay (Cordillera, Misiones). However, *T. angustula* is widely distributed in Americas: occurring from Mexico (Chiapas) to Brazil (in almost all regions of Brazil).

		T. angustula		*T. fiebrigi*	
Bioassay	**Concentration (g/mL)**	***N***	**Mortality (%)**	***N***	**Mortality (%)**
Control	----	56	0.0	62	0.0
Nicosulfuron	3.0×10^{-6}	60	0.0	63	0.0
	3.0×10^{-5}	57	6.5	79	1.25
	1.5×10^{-4}	60	0.0	73	8.75
	2.25×10^{-4}	46	8.0	55	9.83
	3.0×10^{-4}	60	0.0	54	10.0
Total		339	2.75	386	5.8
Control	----	60	0.0	40	0.0
Paraquat	1.5×10^{-6}	40	0.0	40	0.0
	1.5×10^{-5}	40	0.0	40	0.0
	1.5×10^{-4}	40	0.0	40	0.0
	1.5×10^{-3}	36	10.0	38	5.0
Total		216	2.5	198	1.25

Modified from Ref. [69].

N = number of bees analyzed.

Table 5. Mortality of *Tetragonisca angustula* and *Tetragonisca fiebrigi* in bioassays with the herbicides nicosulfuron (Sanson 40SC) and paraquat (Gramoxone 200) after 24-hour exposure

Other species of stingless bees have been evaluated for contamination with pesticides. Moraes et al. [5] evaluated the toxicity of some pesticides to *Scaptotrigona tubiba*. In the test of sprayed paper, delthamethrin, trichlorfon, and malathion showed an LC_{50} of 0.70, 0.26, and 0.015 ppm, respectively. *B. thuringiensis* had an LC_{50} higher than 336 ppm. For the topical application, delthamethrin, *B. thuringiensis*, and trichlorfon showed the respective LD_{50} of 0.73, 115.29, and 0.08 mg/bee. An LD_{50} higher than 0.04 mg/bee was inferred for malathion. All the insecticides were considered highly toxic by topical application route except *B. thuringiensis* that was relatively nontoxic.

Toxicity of pesticides by topical application in stingless bee species *Melipona beecheii, Trigona nigra,* and *Nannotrigona perilampoides* was performed by Valdovinos-Núñez et al. [72]. Results showed that for the three species, immature workers were more sensitive to pesticides than forager bees. These researchers also found that *M. beecheii* females were comparatively more resistant than males. However, queens were less resistant than the workers. Valdovinos-Núñez et al. [72] also assessed the toxicity of neonicotinoid insecticides for *N. perilampoides*. In this

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case, imidacloprid was more toxic than thiamethoxam and thiacloprid for stingless bee *N. perilampoides*.

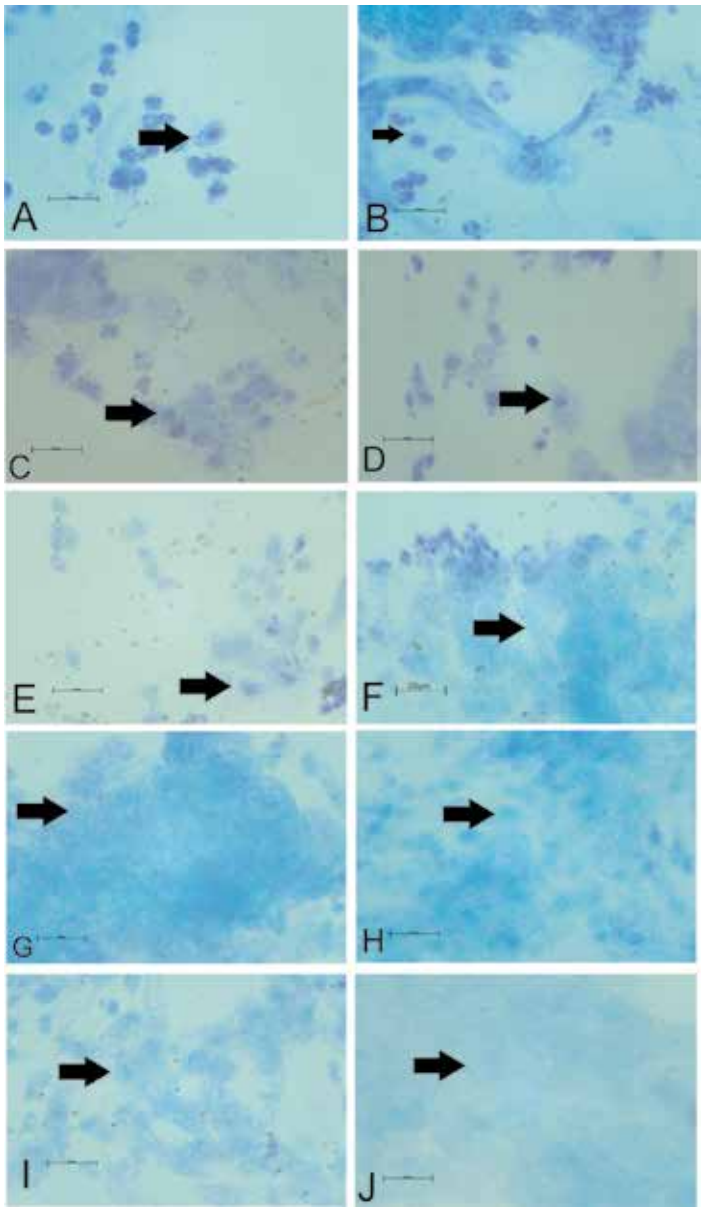

Figure 1. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca angustula* after treatment with water (A–C–E–G–I) (Control), and paraquat 1.5×10^{-3} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.12 mol/L $MgCl_2$; I and J: TB + 0.15 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20 μm. Courtesy Fábio Fermino.

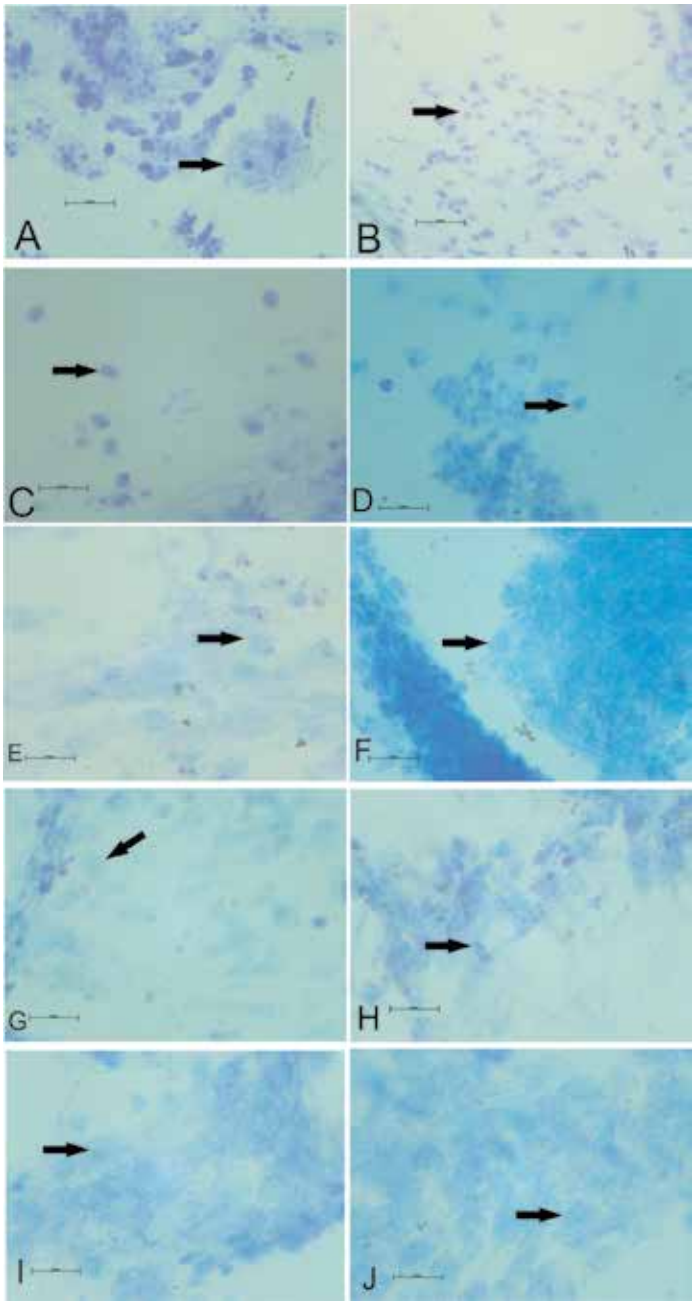

Figure 2. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca fiebrigi* after treatment with water (A–C–E–G–I) (Control), and paraquat 1.5×10^{-3} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.12 mol/L $MgCl_2$; I and J: TB + 0.15 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 50 μm. F = ommatidium presence in the lower left corner. Courtesy: Fábio Fermino.

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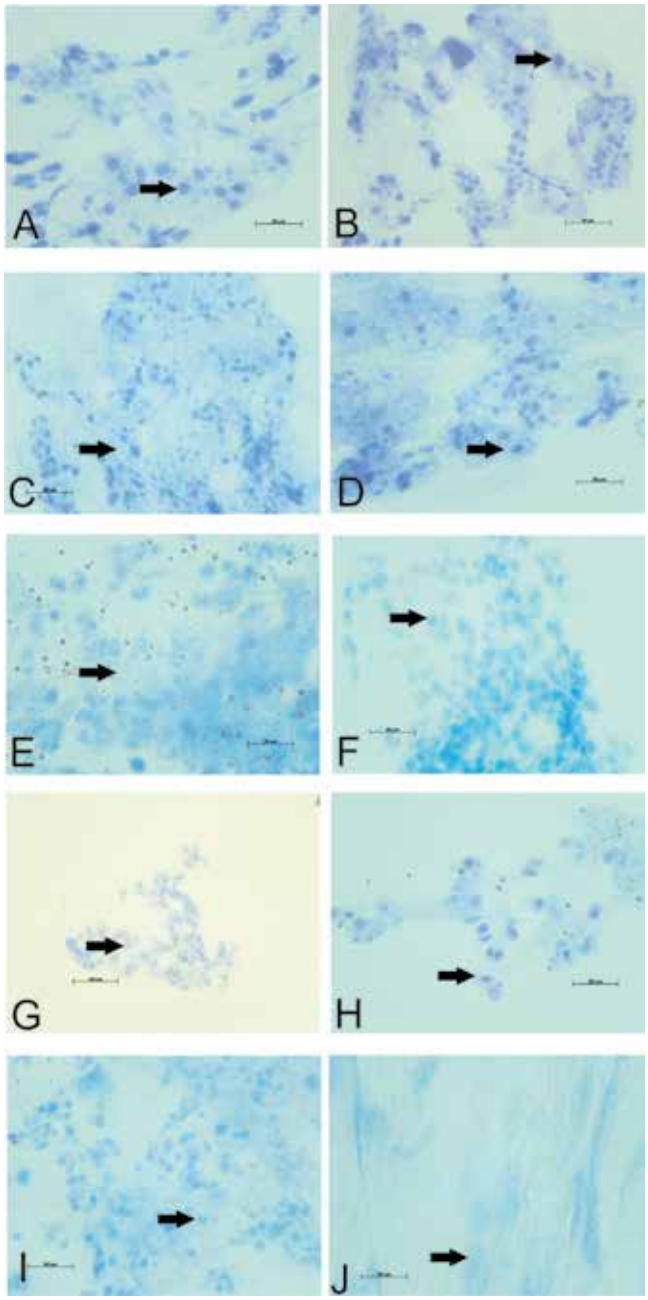

Figure 3. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca angustula* after treatment with water (A–C–E–G–I) (Control), and nicosulfuron 3×10^{-4} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.15 mol/L $MgCl_2$; I and J: TB + 0.20 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20μm. Courtesy: Fábio Fermino.

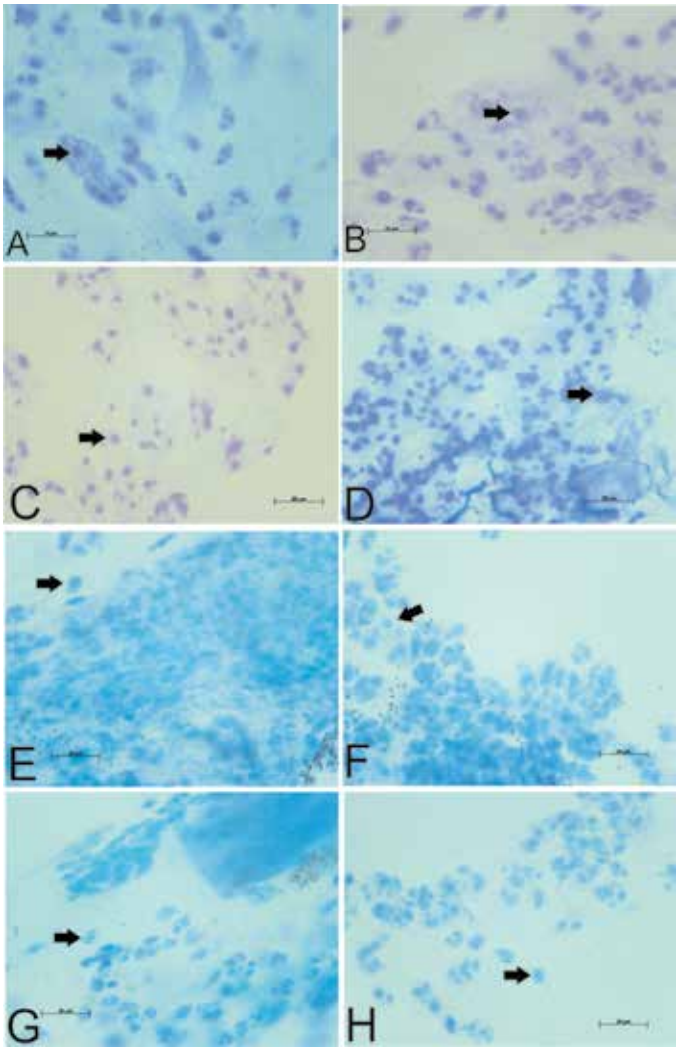

Figure 4. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca fiebrigi* after treatment with water (A–C–E–G–I) (Control), and nicosulfuron 3×10^{-4} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.12 mol/L $MgCl_2$; G and H: 0.20 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20 μm. Courtesy: Fábio Fermino.

Considering fipronil insecticide, Jacob et al. [74] estimated the dose and lethal concentration (LD_{50} and LC_{50}) for stingless bees *Scaptotrigona postica*. Results of LD50 and LC50 obtained after 24-hour exposure were 0.54 ng a.i./bee and 0.24 ng a.i./L diet, respectively. These values are considered highly toxic to stingless bees. Contamination of *S. postica* with a diet containing fipronil (0.1 μg/kg) and boric acid 0.75% wt/wt presented a reduction in the survival [73]. These compounds caused changes in Malpighian tubules, which had dilatation of microvilli, ribosome loss of the rough endoplasmic reticulum, and an increase of the electron dense matrix of the mitochondria.

Azadirachtin (triterpenoid), found in neem oil (*Azadiractha indica*), is considered an *Insect Growth Regulator Insecticide* and causes inhibition of development, changing the metamorphosis of insects. This compound can be used as a natural insecticide with specific effects on different stages of growth of insects [75].

Commercial neem oil was evaluated in coffee crop in Apucarana region northwest of Paraná, Brazil (23° 33′5″ South, 51° 27′41″ West) in 2009, to verify changes in the relative activity of esterases on stingless bee *T. angustula* [76]. A bioassay was carried out in the field using *T. angustula* hive placed at 100 m of coffee crop (*Coffea arabica* L.) when the flower buds were beginning to open (Figure 5 A and 5 B); a hive 100 m from the coffee crop was used as a control and did not have an application of neem oil (in the same geographical region). Neem oil was prepared according to the manufacturer's instructions (100 mL of commercial product in 10 mL of water) and applied to the coffee culture; only water was used in the control. Analyses of esterase isozymes were performed with adult workers *T. angustula* collected after 24, 48, and 72 hours after application of neem oil. The control sample bees were collected in the same period.

In this study, it was observed that exposure of bees *T. angustula* to neem insecticide on the field after 48 hours of contamination led to a decrease in activity of the esterases EST-3 and EST-4, compared with the control (Figure 6). After 72 hours, the relative esterase activity was similar to the control (Figure 6). Evaluation of soluble proteins using denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) presented an increase in intensity of bands between 25 and 120 kDa after 24 hours of contamination. This was probably due to an increase in the synthesis of proteins, which act in detoxification of these bees. However, this method allowed identifying the displayed peptides (Figure 7).

Behavioral observations of *T. angustula* visitors of pulverized coffee flowers with neem oil showed that bees were repelled by visited flowers [76]. Use of the insecticide, neem, promotes changes in the expression of esterase isozymes and soluble proteins of *T. angustula*, but does not cause mortality of these bees. Effects on *T. angustula* nests will probably be observed in the long term and may damage the meliponiculture as an alternative source of income for small farmers.

The first reports about the disappearance of bees occurred in 2006, which alerted the scientific and nonscientific community about these insects, which perform an essential role in pollination plants, and as a consequence other species. The importance of bees for the environment is unquestionable, yet the contradiction between increasing the amount of food for the human population using chemicals for pest control and reducing the use of these compounds has generated discussions and further studies.

In Europe and United States, honeybees are extremely important in the pollination of crops and the decline of these pollinators has promoted a series of actions including governmental actions. One such action would be a ban on spraying aircraft that can carry pesticide residues for drifting to areas of forests or places with beekeeping and/or meliponiculture.

Brazil still uses pesticides that have been banned in the European Union and the United States. Reports of colony collapse disorder (CCD) occur in several regions. In addition to the Afri-

canized honeybees that occur in Brazil, there are over 400 species of stingless bees of unquestionable importance for pollination of native and cultivated plants. Furthermore, Brazilian meliponiculture has increased and the handling of many species has occurred.

Studies presented in the text show that stingless bees are susceptible to pesticides and may be contributing to the decline of their populations; however, we do not have estimates. The effects of the use of these pesticides have been described at the level of mortality and changes at the molecular level; we still need to conduct population studies to detect whether the decline can be attributed to pesticide use.

Despite the need for employment of pesticides on crops, it is extremely important that humanity find a balanced way to produce food and maintain the health of nature.

Figure 5. A. View of coffee (*Coffea arabica*) crop flowering. B. View of *Tetragonisca angustula* nest entrance used in the bioassay in Apucarana, PR, Brazil. Courtesy: Mayra Cristina de Araujo.

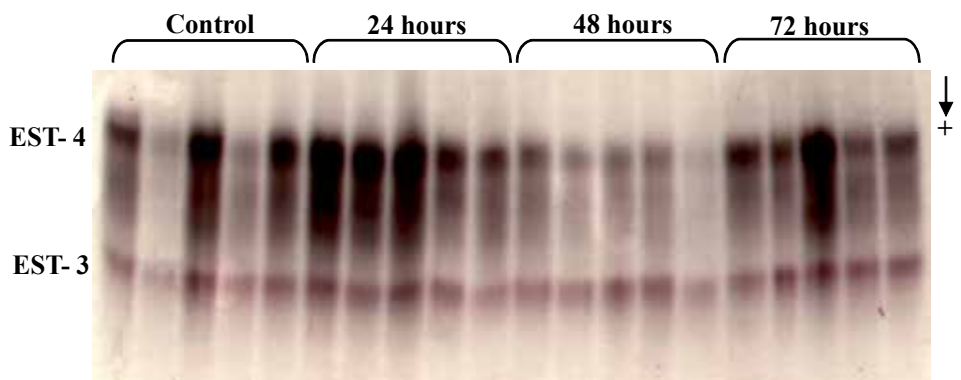

Figure 6. Electrophoresis profile of esterases in polyacrylamide gel electrophoresis (PAGE) of bee extracts *Tetragonisca angustula* after neem contamination. Courtesy: Mayra Cristina de Araujo.

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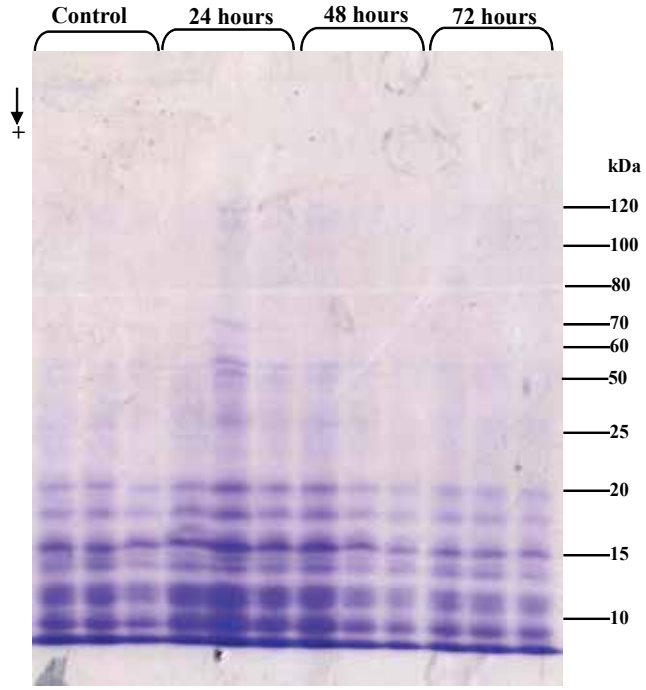

Figure 7. SDS-PAGE electrophoretic profile of *Tetragonisca angustula* extracts after neem contamination. Arrow indicates migration direction. KDa= molecular weight. Courtesy Mayra Cristina de Araujo

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Section 2

Herbicide Chemistry, Physiology of Action, and Safety

Chapter 6

Herbicides and Adjuvants

Zvonko Pacanoski

Additional information is available at the end of the chapter

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Abstract

Adjuvants are any substance either added in a herbicide formulation or added to the spray tank that modifies herbicidal activity or application characteristics, such as better mixing and handling, increasing droplet coverage, spray retention and droplet drying, increasing herbicide cuticle penetration and cellular accumulation reducing leaching of herbicide through the soil profile, etc. The interactions between herbicide adjuvants and herbicide activity, however, are not simple processes, and depend on factors that include crop/weed leaf surface, droplet characteristics, adjuvant type, chemical form of the herbicide, and environmental conditions. Understanding the complexity of these interactions is essential for optimum herbicide utilization, particularly in prolonging, enhancing and improving the efficacy; reduction of the critical rain-free period; minimizing herbicide leaching into groundwater; and decreasing harmful effects to non-target plants and animals.

Keywords: Herbicide adjuvants, history, classification, interaction

1. Introduction

Adjuvants (from Latin, *adiuvare:* to aid) are commonly used in agriculture to improve the performance of herbicides or other pesticides, including better mixing and handling, increased effectiveness and safety, better distribution, and drift reduction [1]. There are many definitions for adjuvants. According to the American Society for Testing Materials (ASTM) [2] "adjuvant is a material added to a tank mix to aid or modify the action of an agrichemical, or the physical characteristics of the mixture." Broadly defined, "an adjuvant is an ingredient that aids or modifies the action of the principal active ingredient" [3] or a "formulant designed to enhance

the activity or other properties of a pesticide mixture" [4]. Ferrell *et al*. [5] briefly describe adjuvants as "substances used with a pesticide to enhance its performance." Many other authors defined adjuvants, particularly herbicide adjuvants, in more detail. According to the Weed Science Society of America (WSSA) [6] "an herbicide adjuvant is any substance in a herbicide formulation or added to the spray tank to improve herbicidal activity or application characteristics" or "an adjuvant is any compound that can be added to a herbicide formulation to facilitate the mixing, application, or effectiveness of that herbicide" [7,8]. For Storrie *et al*. [9] adjuvants are "any substance either in an herbicide formulation or added to the spray tank, that modifies herbicidal activity or application characteristics." According to the last definition, adjuvants are already included in the formulations of some herbicides available for sale, or they may be purchased separately and added into a tank mix prior to use [10].

In order to be effective, herbicides must overcome a variety of barriers (morphological, biological, and environmental) to their entry into plants. For example, trichomes on the leaf surface can reduce herbicide efficacy by intercepting spray droplets before they contact the epidermal surface [11]. Environmental stress (e.g., hot, dry weather) may develop a thicker than normal wax layer, or increase other defensive structures such as reducing the plant's metabolic and transport processes that are required for adequate weed control.

Because of these reasons, adjuvants have been developed to assist herbicides, in that they:

- Allow better mixing and handling with herbicide active ingredient [12]
- Reduce or even eliminate spray application problems [13] (e.g., drift reduction) [14,15]
- Allow contact to the weed target, increase droplet coverage, spray retention, and droplet drying [16,17]
- Increase herbicide cuticle penetration and cellular accumulation [18,19]
- Significantly enhance and improve an herbicide's efficacy so that the concentration or total amount of herbicide required to achieve a given effect is reduced [20-26]
- Decrease the amount of herbicide applied and lower total costs for weed control [27,28]
- Enhance the formulation's ability to kill the targeted species without harming other plants [29]
- From an environmental aspect, can reduce leaching of herbicide through the soil profile [30,31]

However, it is important to note that in some circumstances, adding adjuvants will not significantly improve control [32]. Sometimes adjuvants can have negative effects, such as:

- Decreasing the activity of the herbicide (antagonistic effects) [33]
- Increasing the formulation's ability to spread or persist in the environment where it is not wanted [34,35], or otherwise
- Increasing harmful effects to non-target plants [36,37] and aquatic species [38,39]

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2. Historical background

The history of adjuvants in agriculture dates back to the 18th and 19th centuries when additives such as resins, tar, flour, molasses, and sugar were used with lime, sulfur, copper or arsenates to improve adherence and biological performance of active ingredients by modifying the physicochemical properties of the spray solution [40].

The first agricultural adjuvant was a soap solution (Gillette 1888, 1890) (cit. by Hazen, [41]). Soap solutions and kerosene were used in the United States to kill insect eggs or were added to arsenical solutions to increase toxicity to weeds [42]. Animal oil soaps were common adjuvants in use before 1900, as well (Gillette1889) (cit. by Hazen, [41]). They were derived from animals, fish, and whale oil and were used to enhance the pesticide performance. Sugars and glue were considered as stickers and many other materials followed as adjuvant research continued [43].

The modern era of synthetic organic pesticides began in the 1930s. The research behind medical (including antibiotics) and military uses funded research that led to the discovery of many pesticide families that are still in use today. An initial breakthrough in weed control occurred with the introduction of 2,4-D in the 1940s for broad-spectrum broadleaf weed control in corn and cereal crops [44]. Soaps and mineral oils were replaced by nonionic surfactants. Nitrogen fertilizers like ammonium sulfate (AMS) and urea ammonium nitrate (UAN) were also used to enhance the herbicidal activity while glycerin was introduced as humectant.

In the 1960s and 1970s, modern types of adjuvants such as crop oil concentrates (COC) were developed, which were used to reduce doses of atrazine and to lower spray volumes. Organosilicone-based adjuvants, nonionic surfactants (NIS), which have excellent wetting and spreading capability and enhance the penetration of post-emergent herbicides, were developed later [45].

3. Classification of adjuvants

There are over 3,000 adjuvants available for use. These can be grouped into three general types:

- Activators,
- Spray modifiers and
- Utility modifiers

4. Activators

Activators modify certain herbicide characteristics, including particle size and viscosity of the herbicide spray, evaporation rate, etc. Usually, they increase herbicide activity, herbicide

spread, absorption into plant tissue, and rainfastness, and decrease photodegradation of the herbicide.

There are three categories of activators: surfactants, wetting agents, and oils.

4.1. Surfactants (SURFace ACTive AgeNTS)

Surfactants (SURFace ACTive AgeNTS) are a type of activators designed to improve the dispersing/emulsifying, absorbing, spreading, wetting, sticking, and/or penetrating properties of the spray mixture [6]. Surfactants primarily influence the ability of herbicides to penetrate the leaf's waxy cuticle. Most herbicides are prepared in a solution of water. Water is a chemically polar material and thus can be repelled by the waxy surface of leaves. Water containing a surfactant reduces the surface tension of water on plants, spread in a wet thin layer over a waxy leaf surface, and allow the herbicide formulation to enter into the plant.

Surfactants can be classified in four groups on the basis of the ability to ionize the aqueous solution. Those groups are:

- Nonionic — are the most commonly used in agriculture and can be mixed readily with any herbicide. They produce little or no ionization in water (no electrical charge). Organosilicone and silicone surfactants are two types of nonionic surfactants.
- Cationic — are not often used with herbicides. They have a positive charge,
- Anionic — rarely used with herbicides, but mainly used in cosmetics, household cleaners, many domestic detergents, etc. They have a negative charge, and
- Ampholytic (amphoteric) — have a both positive and negative charge, that is, in aqueous solution are capable forming cations or anions.

4.2. Wetting agents

Wetting agents increase the ability of water to displace air or liquids from the leaf surface, allowing it to be wet by the herbicide. Wetting agents help spread the solution more evenly over the leaf.

4.3. Oils

Oils increase the retention time of a solution on leaves, allowing for an increase in herbicide uptake. Oils mostly contain emulsifiers to allow them to mix with water. Some claims regarding oils include reduced rainfast periods, more uniform droplet size (drift reduction), less spray evaporation, and better penetration of herbicide into waxy leaves.

All oils are basically mineral oils with different contents of surfactant in formulation (3%--20%). They can be classified as:

- Crop oils
- Dormant oils

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- Crop oil concentrates
- Vegetable oils
- Vegetable oil concentrate
- Modified vegetable oil, and
- Modified vegetable oil concentrate

4.3.1. Crop oils

Crop oils are emulsifiable petroleum oil-based products containing up to 5% w/w surfactant and the remainder of phytobland oil.

4.3.2. Dormant oils

Dormant oils are horticultural spray oils applied during the dormant phase of the targeted plant [2]. There are ''quick-break'' or dormant oils that use a very low amount (2%--5%) of emulsifier for dispersion into the spray tank [41].

4.3.3. Crop Oil Concentrates (COC)

COC are the most commonly used oils in agriculture. They were introduced to the market in the 1960s [45]. COC are emulsifiable petroleum oil-based products containing 5%--20% w/w surfactant and a minimum of 80% w/w phytobland oil [2]. COC enhanced activity of aryloxyphenoxy propionates, cyclohexadinones, triazines, phenoxy acid urea herbicides, imidazolinones, etc. [46,47,26].

4.3.4. Vegetable oils

Vegetable oils are also used as herbicide adjuvants. The base in formulation is oil from sunflower, soybean, oilseed rape, peanut, or corn, which is combined with surfactants in different content.

4.3.5. Vegetable oil concentrates

Vegetable oil concentrates are emulsifiable vegetable oil products containing 5%--20% w/w surfactant and a minimum of 80% w/w vegetable oil [2]. There are some vegetable oil concentrates used in the same manner as the crop oil concentrates, typically based upon canola or soybean oil, using 5%--10% emulsifier for dispersion [41].

4.3.6. Modified vegetable oil

Modified vegetable oil is oil extracted from seeds that have been chemically modified. Methylated seed oils (MSO) are vegetable oils mainly from oilseed rape or sunflower esterified with alcohol ethanol to get methyl esters.

4.3.7. Modified vegetable oil concentrate

Modified vegetable oil concentrate is an emulsifiable, chemically modified vegetable oil product containing 5%--20% w/w surfactant and remain chemically modified vegetable oil. Some of the best vegetable-based products are those modified (derivatized) to methyl and other lower alkyl esters such as methylated soybean oil, methyl sunflowerate, or ethyl canolate.

5. Spray modifiers

Spray modifiers affect the delivery and placement of the spray solution. They confine or alter the physicochemical characteristics of the spray solution [48], and make the herbicide spray easier to aim, reduce herbicide drift in the air, and cause the spray to more readily adhere to the plant. Spray modifiers include:

- Thickening agents (i.e., invert emulsions and polymers)
- Stickers
- Spreaders
- Spreader-stickers
- Foaming agents
- Humectants, and
- UV absorbents

5.1. Thickening agents

Thickening agents modify the viscosity (thickness) of spray mixtures. They control drift or slow evaporation after the spray has been deposited on the target area. Slowing evaporation is important when using systemic herbicides, because they can penetrate the plant cuticle only as long as they remain in solution. Invert emulsions, polymers, and *drift control agents* are three types of thickening agents commonly used in herbicide applications.

5.1.1. Invert emulsions

Invert emulsions are mixtures of inverting oil and water, having a mayonnaise-like appearance on the water surface and a snowflake-like appearance under the water surface. Depending on their solubility, herbicides dissolve in either the oil or water component. The oil in the case of *invert emulsions* reduces the evaporation, produces bigger particles, reduces drift problems and can be sprayed on wet foliage [49].

5.1.2. Polymers

Polymers are a very large, chain-like carbon molecules made up of monomers, up to 40,000 carbons in length, forming a thick mucus-like material which helps to break the surface tension of water and enhance sinking of herbicides [50,51].

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5.1.3. Drift control agents

Drift control agents modify spray characteristics to reduce spray drift, usually by minimizing small droplet formation. They are generally polyacrylamide or polyvinyl polymers [52].

5.2. Stickers

Stickers assists the spray deposit to adhere or stick to the the leaf surface and may be measured in terms of resistance to time, wind, water, mechanical action, or chemical action [2]. Stickers may be heavy petroleum fractions, water-soluble polymers, acrylic latex, epoxidized seed oils (similar to boiled linseed oil, which dries on exposure to air), or alkylphenol condensates called resins. Stickers are commonly used in field crops (like corn and soybeans) where residue on leaves is not a problem. They are usually used for application of fungicides and insecticides rather than herbicides.

5.3. Spreaders

Spreaders are compounds that cause the surface tension of the herbicide to be reduced in such a way that it easily spreads into a very thin film over a leaf surface. Spreaders increase the efficiency of the herbicide dramatically. Typically, the alcohol ethoxylates [53] such as tridecanol ethylene oxide allow a spread diameter increase of two to three times. They may contain fatty acids, latex, aliphatic alcohols, crop oils such as cottonseed, or inorganic oils.

5.4. Spreader-stickers

Spreader-stickers are essentially combinations of stickers and spreaders. They provide additional retention of herbicide in wet conditions. They are usually used with contact insecticides and fungicides for which complete coverage is critical.

5.5. Foaming Agents

Foaming Agents are compounds that facilitate formation of foam for reducing drift and evaporation. These agents are used infrequently for drift control of herbicide applications.

5.6. Humectants

Humectants, like stickers, increase the amount of time that the herbicide is on the leaf, in a form available for uptake [41]. When water evaporates from the spray droplet and the herbicide becomes a crystalline residue, it is no longer available for uptake into the leaf. Humectants keep the spray deposit moist and in true solution, and therefore extend the time that it is available for absorption [54].

5.7. UV absorbents

UV absorbents protect herbicides from the deleterious effect(s) of sunlight. They may do this by either physical or chemical processes, such as by increasing the rate of herbicide uptake into the cuticle, or by absorbing the UV-light themselves.

6. Utility modifiers

Utility modifiers help minimize handling and application problems. They do not directly improve efficacy, but widen the conditions when an herbicide can be used or maintain the integrity of the spray solution. For example, utility modifiers reduce foaming, increase solubility, modify pH, or reduce spray drift.

Types of modifiers include emulsifiers, dispersants, stabilizing agents, coupling agents, co-solvents, compatibility agents, buffering agents, antifoam agents, and ammonium fertilizers.

6.1. Emulsifiers

Emulsifiers are molecules with one hydrophilic and one hydrophobic end. They make it possible for water and oil to become finely dispersed in each other, creating a stable, homogeneous, smooth emulsion. Most crop oils contain emulsifiers to allow them to mix with water and some contain various levels of surfactants.

6.2. Dispersants

Dispersants are chemicals that are sprayed on a surface oil slick to break down the oil into smaller droplets that more readily mix with the water. These water soluble dispersants have been found to be unique and highly effective dispersants for water insoluble agricultural suspension concentrate formulations.

6.3. Stabilizing agents

Stabilizing agents act as thickening or gelling agents that increase the viscosity of the final product. These agents stabilize emulsions, either by adsorbing to the outer surface of oil droplets. Stabilization can be achieved in agricultural suspension and emulsion through the use of fine-particle-size solids and fine liquid droplets in the disperse phase along with appropriate dispersants and wetting agents.

6.4. Coupling agents

Coupling agents are compounds which provide a chemical bond between two dissimilar materials, usually an inorganic and an organic. Organosilanes are well-suited in this application because of the ability to incorporate an organic-compatible functionality and an inorganic-compatible functionality within the same molecule.

6.5. Cosolvents

Cosolvents are defined as water-miscible organic solvents that are used in liquid herbicide formulations to increase the solubility of poorly water-soluble substances or to enhance the chemical stability of an herbicide.

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6.6. Compatibility agents

Compatibility agents allow simultaneous application of two or more ingredients. They are most often used when herbicides are applied in liquid fertilizer solutions.

6.7. Buffering agents

Buffering agents are used to change the pH and hardness of the water and to increase the dispersion or solubility of herbicides in alkaline or acid waters used in making up an herbicide solution. Ammonium sulfate (AMS) is sometimes added to reduce hard water problems.

6.8. Antifoam agents

Antifoam agents reduce foaming in spray mixtures that require vigorous agitation. They are particularly useful in soft water. Antifoam agents are usually siliconebased and used at 0.1% or less of the total spray volume [55].

6.9. Ammonium fertilizers

Ammonium fertilizers are often added to spray solutions with foliar applied herbicides. The two most common ammonium fertilizers used are ammonium sulfate (AMS) and urea ammonium nitrate (UAN) solution (28-0-0). The exact mechanism of action for ammonium fertilizers is not known although increased herbicide uptake into plant has been reported [26].

7. Positive interaction between herbicide efficacy and adjuvants

Surfactants are the most widely used and probably the most important of all adjuvants [56]. They can be especially effective in improving the biological activity of many herbicides [57-59]. Nonionic surfactants (NIS) improved the effect of nicosulfuron [58] and enhanced glyphosate absorption, which was 20 times greater and the spread of spray drop was 200 greater than with no adjuvants added [60].

Several researchers have observed that adjuvant efficacy is dependent on the herbicide being applied and the characteristics of the target weed species [61-63]. For example, MSO increase foliar absorption and efficacy of many herbicides, including primisulfuron, rimsulfuron, imazethapyr, quinclorac, and several graminicides for grass weed control [21,64-66]. MSO was the only adjuvant used with foramsulfuron that provided acceptable giant foxtail control (*Setaria faberi*) [32]. Stagnari *et al*. [67] found strong influence of mineral and vegetable oil on clodinafop-propargyl and diclofop-methyl + fenoxaprop-p-ethyl on *Lolium multiflorum*, *Avena ludoviciana*, and *Phalaris minor*.

NIS have been effective in improving the activity of several sulfonylurea herbicides, including primisulfuron, rimsulfuron, and thifensulfuron, as well [57,58,68]. MSO and COC have been shown to further enhance the effectiveness of several herbicides on certain weed species, including nicosulfuron [23]. These adjuvants enhanced the effectiveness of chlorimuron and

imazethapyr on purple nutsedge (*Cyperus rotundus* L.). Chlorimuron controlled *Cyperus rotundus* more effectively with COC than with a NIS or organosilicone surfactant (OSS), but imazethapyr was more effective with OSS or COC than NIS [24]. Seed-oil-based crop oils and organosilicone adjuvants combined with halosulfuron provided 100% control of *Cyperus rotundus* L. 8 weeks after treatment (WAT) compared with <90% control when halosulfuron was combined with the nonionic or paraffin-based crop oil adjuvants [69]. Similar results were found in studies of McDaniel *et al*. [70] who reported that >90% control of yellow nutsedge (*Cyperus esculentus* L.) in container landscape plants was achieved with late-spring applications of halosulfuron at 18 g/ha combined with 0.5% (v/v) rate of either the soybean crop oil Scoil®, or the sunflower *(Helianthus annuus* L.) crop oil Sun-It II. Increased control with nicosulfuron on yellow foxtail [*Setaria glauca* (L.) Beauv.] and large crabgrass [*Digitaria sanguinalis* (L.) Scop.] with MSO compared with other oils and surfactant adjuvants has been reported by Nalewaja *et al*. [23]. Young and Hart [71] reported that isoxaflutole applied with MSO provided greater giant foxtail (*Setaria faberi* Herrm.) control compared with isoxaflutole applied with NIS or COC. The addition of an organosilicone (OSL) adjuvant to primisulfuron spray solution increased foliar herbicide absorption, spray retention, and control of *Setaria faberi* Herrm. compared with adding a NIS to the spray solution [21].

The addition of AMS or UAN to the spray solution can enhance herbicide effectiveness by further increasing herbicide absorption [26,72] which gives better result up to 12%--13.5% than use of herbicide alone [73]. For instance, thifensulfuron absorption into velvetleaf (*Abutilon theophrasti* Medicus) was increased from 4% to 45% when 28% UAN was added to the spray solution [61,74,75]. Addition of AMS and potassium phosphate to the spray solutions of MSMA and dalapon enhanced control of *Sorghum halepense* (L.) Pers. and *Cyperus rotundus* L. [76,77]. Wills [78] reported that AMS and potassium phosphate each increased the phytotoxicity of glyphosate. Wills and McWhorter [79] further reported that the monovalent cations NH_4^+ and K^+ in combination with anions including NO_3^-, Cl^-, and CO_3^{2-} increased the phytotoxicity of glyphosate. Glyphosate isopropylamine, bentazon sodium, 2,4-D dimethylamine, and dicamba sodium were all equally effective when AMS was added to the spray tank before or after the herbicide. The benefit of AMS in enhancing herbicide efficacy was greatest when used with spray water high in cations [80]. Also, several studies have indicated that AMS may be used to overcome an antagonism between two herbicides [81,82]. The antagonism between bentazon and sethoxydim was overcome with the addition of AMS and by changing the adjuvant from a COC to a highly concentrated oil-based adjuvant [83,81]. Applying UAN (0.4 or 0.8 g/ha) and organosilicone-based nonionic surfactant (OSL/NIS) or methylated seed oil/organosilicone (MSO/OSL) adjuvant with bispyribac enhanced efficacy and reduced the time period required to affect bispyribac efficacy on barnyardgrass [*Echinochloa crus-galli* (L.) Beauv] [84]. Bunting *et al*. [32] reported 90% or greater giant foxtail (*Setaria faberi* Herrm.) control with the addition of MSO or MSO plus 28% UAN. Twenty percent control of giant foxtail was obtained when a COC or a NIS was added to foramsulfuron, whereas control increased to 90% and 85%, when 28% UAN was added to COC or NIS, respectively. Density, fresh and dry weight of *Trianthema portulacastrum, Cyperus rotundus* and *Coronopus didymus* 20 and 40 days after sowing and at harvest of maize decreased significantly when foramsulfuron + isoxadifen-ethyl was applied at 1125 g/ha a.i. plus 3% UAN solution as adjuvant as compared to herbicide

alone. Finally, UAN used as an adjuvant reduced the herbicide dose up to 10% without compromising maize yield loss due to weeds [85]. Considering the wide-spread use of tribenuron-methyl, the identification of the most appropriate adjuvant for tribenuron-methyl against different weed species was found to be necessary [86]. The activity of tribenuron-methyl was significantly enhanced by NIS (20% isodecyl alcohol ethoxylate plus 0.7% silicone surfactants), an anionic surfactant (25.5% alkylethersulfate sodium salt), and a vegetable oil (95% natural rapeseed oil with 5% compound emulsifiers) on *Sinapis arvensis, Tripleurospermum inodorum, Papaver rhoeas*, and *Chenopodium album,* and only minor differences were observed among the tested adjuvants [87]. Besides sulfonylureas, an addition of adjuvants greatly improved the efficacy of saflufenacil, a new PPO-inhibited herbicide. For example, ED_{90} values for field bindweed (*Convolvulus arvensis* L.) control at 28 days after treatment (DAT) were 71, 20, 11, and 7 g/ha for saflufenacil applied alone, or with NIS, COC, or MSO, respectively. MSO was the adjuvant that provided the greatest enhancement of saflufenacil across all broadleaf weed species tested — *Taraxacum officinale, Convolvulus arvensis, Thlaspi arvense, Lamium amplexicaule, Lactuca serriola*, and *Capsella bursa-pastoris* [88]. Murphy *et al*. [89] investigated the influence of different adjuvants on flamprop-M-isopropyl efficacy in controlling of *Avena* spp. The mean results from six trials (five wheat, one barley) showed that the addition of adjuvants, "Swirl" and "Dobanol 25–7" was beneficial, increasing wild oat floret control from a mean value of 80% to 92% at current recommended rates (flamprop-M-isopropyl, 600 g/ha; "Swirl," 2.5 L/ha). However, combinations of flamprop-M-isopropyl and "Dobanol 25-7" gave superior levels of control even at lower a.i. application rates. For example, a mean level of 96% control of *Avena* spp. was obtained at 300 g/ha a.i. with 1200 g/ha "Dobanol 25-7"; with even better control at higher rates of application of both components.

8. Interaction between herbicide absorption/translocation and adjuvants

Considering environmental factors, rain shortly after an herbicide application is one of the most detrimental issues for herbicide performance. Adjuvants have been shown to improve the rainfastness of herbicides and the effect on rainfastness should be considered when selecting an adjuvant [90,91]. A number of studies have been published that outline the beneficial effects of OSL adjuvants in reducing the critical rain-free period after the foliar herbicidal application. Field and Bishop [92], Reddy and Singh [93], and Roggenbuck *et al*. [63] reported that the addition of an OSL adjuvant to glyphosate reduced its critical rain-free period. The reduction of the critical rain-free period was attributed to decreased liquid surface tension of glyphosate caused by the OSL adjuvant and subsequent promotion of stomatal infiltration of glyphosate into the plant.

Studies with ^{14}C-labeled glyphosate have demonstrated that plants absorb as little as 22% of the amount applied; however, the addition of surfactant improved absorption up to 35% [94]. For instance, the OSL adjuvants produced rapid absorption of the ^{14}C-glyphosate into the redroot pigweed (*Amaranthus retroflexus* L.) leaves, reaching maximum absorption within 0.5–1.0 h after application (HAT). The conventional adjuvants produced slower absorption of the ^{14}C-glyphosate, as the maximum absorption was not achieved until at least 24 HAT in redroot

pigweed, remaining similar until 72 h [60]. Non-silicone surfactant (NSS) "Browndown" increased the speed and quantity of glyphosate uptake, with no adverse effects on herbicide translocation. At the recommended rate (0.25% v/v), this surfactant reduced spray retention compared to OSS, "Pulse" (0.1% v/v), but provided faster brown-out of foliage and equivalent herbicide efficacy on glyphosate-tolerant ryegrass (*Lolium perenne* L.) in spring [95]. Surfactants of higher ethylene oxide (EO) content provided greater uptake enhancement in wheat, broad bean, and common lambsquarters for glyphosate, whereas those of lower EO content were more beneficial for 2,4-D uptake [96]. Addition of the water conditioning agent Quest (0.25% v/v) to glyphosate spray mixtures diminished the influence of simulated rain events following glyphosate application [97]. OSS increased rainfastness of primisulfuron on velvetleaf (*Abutilon theophrasti* Medicus) more than other adjuvants, although no differences in velvetleaf control occurred under rain-free conditions [98]. NIS (20% isodecyl alcohol ethoxylate plus 0.7% silicone surfactants), an anionic surfactant (25.5% alkylethersulfate sodium salt), and a vegetable oil (95% natural rapeseed oil with 5% compound emulsifiers) significantly improved the rainfastness of tribenuron-methyl on *Tripleurospermum inodorum*, with differences among the adjuvants being more pronounced when rain occurred shortly after herbicide application. The effect of the vegetable oil on tribenuron-methyl's rainfastness was significantly lower than that of the surfactants with rain at 1 HAT, while no significant differences among the three adjuvants were observed when rain occurred at 2 and 4 HAT [87]. The addition of UAN decreased the rainfast period from 8 h (registered rainfast period) to 1 or 4 h (99 to 100% control) when either the between bispyribac application and wash-off during a rainfall event [84].

In contrast to surfactants, water repellent adjuvants increase surface tension, thus inhibiting wetting of the leaf surface. The water repellent DC 1-6184 may have some utility for reducing corn injury when isoxaflutole is applied to corn foliage at early growth stages [99]. These results are consistent with the observation of Nelson and Penner [100] that DC 1-6184 applied in combination with herbicide safener R-29148 and isoxaflutole reduced injury to spike-stage corn (28%) as compared with isoxaflutole applied alone (53%) or isoxaflutole applied with only R-29148 (37%). Penner and Fausey [101] found that DC 1-6184 consistently reduced retention of flumioxazin spray on plant foliage by increasing the number of droplets that bounced off the foliage. Flumioxazin spray had the greatest retention of all herbicide treatments on tomato when DC 1-6184 was included. Also, the same water repellent, DC 1-6184, reduced isoxaflutole retention on tomato, wheat, and cabbage [100].

9. Interaction between herbicides, environment, and adjuvants

From an environmental aspect, adjuvants can weakly bind herbicides and release them slowly in order to prolong the efficacy of herbicides and to minimize their leaching into groundwater. Enersol 12% adjuvant resulted in a 13%–18% reduction in leaching of dicamba and bromacil in five pore volumes of leachate. The leaching of simazine was significantly decreased when charcoal, three humic substances (Enersol SP 85%, Enersol 12%, and Agroliz), and a synthetic polymer (Hydrosorb) were used. However, the decrease in leaching was significantly greater when using Enersol SP 85% or Enersol 12% (24%–28%) than when using the other adjuvants

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(12%–16%) [30]. In a study by Locke *et al*. [102] nonionic, cationic, and anionic adjuvants generally increased the water solubility of cyanazine, atrazine, and norflurazon (10%--91%). Cyanazine and atrazine sorption (K_d) was reduced in most soils with nonionic adjuvant (ranged 1.18--4.50 and 1.59--4.28, respectively) compared with water alone (1.36--5.59 and 1.75--4.59, respectively), whereas norflurazon sorption was increased with nonionic adjuvant (ranged 3.88--8.76 in water; 4.66--9.82 in adjuvant). Similarly, more cyanazine and atrazine were desorbed by solutions containing adjuvant than in water, indicating that adjuvants may be useful in remediating some soils contaminated with certain herbicides.

10. No or negative interaction between herbicides and adjuvants

In many situations, as mentioned earlier, adjuvants can significantly enhance an herbicide's effect [25]. However, it is important to note that in some circumstances, adding adjuvants will not significantly improve control. For example, several studies have shown that the addition of AMS to herbicides increases the control of *Abutilon theophrasti*; however, control of other species, such as *Chenopodium album* is not always improved [74, 75,103]. Leafy spurge (*Euphorbia esula* L.) control with annual picloram or picloram plus 2,4-D treatments was similar whether applied alone or with a variety of adjuvants in the field [104]. Addition of laffmul DA and ethoxylated castor oil (EO 40) – both nonionic crop oil concentrates surfactants reduced the efficacy of glufosinate ammonium and 2,4-D Na salts in controlling of *Cyperus rotundus* and *Oxalis latifolia* [105]. Addition of sulfuric acid and/or of AMS to spray solution does not increase herbicide activity of glyphosate [106]. This claim is corroborated by results of Breeden *et al*. [107] who reported that AMS additions to glyphosate, while not decreasing effectiveness, did not improve efficacy over glyphosate applied alone to sicklepod [*Senna obtusifolia* (L.) Irwin and Barneby]. The addition of two polysaccharide adjuvants decreased the percentage of the spray volume in small diameter spray droplets (<141 mm) and either had no effect or increased glyphosate efficacy [108]. One disadvantage to the use of surfactants with glyphosate is the postapplication effect. Surfactants tend to reduce the translocation efficiency of glyphosate within the plant [94]. Studies with water-stressed plants have shown that surfactants do enhance absorption, even under stress, but they decrease movement of the herbicide once it is inside the plant tissue [94]. Glyphosate application rate was more important than adjuvant addition or sprayer type, with the higher rates of application providing greater control [109].

Sometimes adjuvants can decrease the killing power of the herbicide (antagonistic effects). The efficacy of sethoxydim or clethodim on large crabgrass [*Digitaria sanguinalis* (L.) Scop.] was antagonized by the addition of halosulfuron with NIS or COC. Simalarly, combinations of sethoxydim and halosulfuron with COC or MSO were antagonistic on smooth crabgrass (*Digitaria ischaemum* Schreb. ex Muhl.) [33].

Some adjuvants can increase harmful effects to non-target plants. Imazamox applied at 108 g/ha plus 1% (v/v) MSO applied in the fall consistently injured all wheat cultivars more than the same rate with NIS at 0.25% and 54 g/ha imazamox regardless of adjuvant and timing [37]. Injury caused by these treatments ranged from 23%to 70% for all cultivars. Adjuvant affected

cotton injury from CGA 362622. NIS resulted in increased cotton injury at 29%, whereas COC increased cotton injury to 37%. [110]. Crooks *et al*. [111] reported similar injury from CGA 362622 with either NIS or COC. Flumioxazin did not injure wheat or cabbage except when the silicone adjuvant was added, which increased retention of the spray solution [112].

Sometimes adjuvants can have negative effects, such as increasing the formulation's ability to spread or persist in the environment where it is not wanted. According to Kucharski and Sadowski [113], the addition of adjuvants caused an increase of the residues of active ingredients in the soil and roots of sugar beet compared to plots with a reduced dose of herbicide without adjuvants. Swarcewicz [114] and Swarcewicz *et al.* [34] described experiments in which influence of adjuvants on trifluralin degradation were tested. 50 DAT residues of trifluralin amounted to 38% of initial dose and in treatments with adjuvants residues ranged from 42% to 49% of initial dose. In a similar experiment Kucharski [35] also proved that the addition of adjuvants slowed down the degradation and increased the level of phenmedipham residue in the soil. Some adjuvants can have adverse effects on aquatic species, and certain types can be extremely toxic to fish and shellfish [39]. Parr [38] reports that some adjuvants caused noticeable alterations in fish gill tissue, and that the toxicity of these adjuvants increased as exposure time increased.

11. Conclusion

The agricultural adjuvants market, in terms of value, is projected to reach $3,183.04 million by 2019, at a CAGR of around 5.6% from 2014 [115]. Numerous factors such as, easy application, modern production practices, new product offerings, increased availability, increasing infestation of pests and diseases, and government regulations to protect the environment from hazardous chemical usage are the major drivers of the agricultural adjuvants market. Adjuvants are quietly helping to revolutionize the agrochemical business as they are the best tools for farmers to improve application, facilitate the right dosage, and achieve more cost-effective, better targeted, and environmentally acceptable pest control. Agricultural adjuvants play an essential role in the performance of most herbicides, fungicides, and insecticides, and function by transforming the dosage from preventative, high-dose applications to low dosages, specifically targeted for curative applications.

From all previous research mentioned, it can be concluded that the herbicide-adjuvant--plant-environment interaction is a complex system. Understanding the different roles of adjuvants in the behavior of herbicides is essential for their optimum utilization. Adjuvants can improve the biological activity of the herbicide active ingredient, the performance of the spray application, and the economics of herbicide applications, but in some circumstances adjuvants can manifest negative effects. Therefore, there is no universal adjuvant that can improve the performance for all herbicides, against all weeds, or under all environmental conditions. The herbicide and adjuvant selected and the relative amounts used must be tailored to the specific conditions of each application.

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Chapter 7

Binding Mode Identification for 7-keto-8-Aminopelargonic Acid Synthase (AtKAPAS) Inhibitors

Nam Sook Kang, Jung-Sup Choi and In-Taek Hwang

Additional information is available at the end of the chapter

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Abstract

In this study, we determined the 3D structure of Arabidopsis thaliana KAPAS by homology modeling. We then investigated the binding mode of compounds obtained from the in-house library using computational docking methods. From the flexible docking study, we achieved high dock scores for the active compounds denoted in this study as compound 3 and compound 4. Thus, we highlight the flexibility of specific residues, Lys 312 and Phe 172, when used in active sites.

Keywords: KAPAS, Herbicides, Homology modeling, Protein docking

1. Introduction

Agricultural research efforts for discovery of herbicides acting on new target sites are increasing due to demand from farmers and multinational companies in many countries. However, new modes of action have not been turned to commercial success for the past 10 years. We have recently reported 7-keto-8-aminopelargonic acid synthase (KAPAS, also known as 8-amino-7-oxononanoate synthase, ANONS) and have suggested the potential KAPAS inhibitor triphenyltin. Research on herbicides has advanced during the past 50 years to the point that herbicides can now protect crops and improve the quality and quantity of agricultural products. However, the successful development of herbicides has decreased recently owing to new environmental regulations and lack of discovery. To overcome this problem, there is an urgent need for new herbicidal targets and new techniques [1].

While the traditional approach to discover lead compounds heavily depends on serendipity given the poor understanding of biological modes of actions, the structure-based approach utilizes the structure of appropriate target proteins, which have well-known binding sites for possible rational designs. The introduction of new herbicides with either a new mode of action or of a novel chemical class has lingered. However, discovering a chemical structure that could enter the pest, be transported within it, inhibit a key target, get away from detoxification, and also be modified to allow it to fulfill increasing regulatory criteria with respect to environmental compatibility has been required. The structure-based approach uses only appropriate target proteins instead of the entire plant for *in vivo* testing [2]. In order to perform a structure-based assay, it is necessary to determine a potent target and to have a thorough understanding of the mechanism of action of the target.

Several enzymes in plants are known to be essential enzymes, meaning that they are crucial for the plant's survival. Disrupting a single essential enzyme leads to severe disorder of metabolic processes in the plant, ultimately causing a lethal outcome. The enzyme 7-keto-8-aminopelargonic acid synthase from *Arabidopsis thaliana* plants (*At*KAPAS), introduced in this research, and is a new potent herbicide target, which is involved in the early steps of the creation of the biotin biosynthesis pathway. *At*KAPAS as a pyridoxal 5′-phosphate-dependent enzyme catalyzes the decarboxylative condensation of L-alanine with pimeloyl-CoA in a stereospecific manner to form KAPA, coenzyme A, and carbon dioxide in the first committed step of biotin biosynthesis. Inhibiting *At*KAPAS leads to significant changes in the phenotype, such as growth inhibition, severe growth retardation, and the creation of lethal phenotypes [3].

Although the physiological systems of humans and plants are different in various ways, the misuse of agricultural chemicals can be extremely harmful to humans. Therefore, use of herbicides must follow strict toxicity regulations that are in place to prevent harm to humans and other life. As mentioned above, the novel herbicidal target 7-keto-8-aminopelargonic acid synthase functions in the initial steps of the biosynthetic pathways of biotin (vitamin H) in plants and microorganisms. Because biosynthetic steps of biotin exist only in plants, we expect that the inhibition of the potent target *At*KAPAS will not affect the human metabolic system [1, 3]. A few publications have also reported beneficial effects of *At*KAPAS as a potential herbicidal target. Hwang et al. described the possibility of *At*KAPAS as a potential herbicide target enzyme and chemical validation of triphenyltin acetate as a lead compound for the *At*KAPAS inhibition in vitro and in vivo [1]. They also suggested *At*KAPAS can be a useful target for the rational design of inhibitors in the hope of developing new herbicides.

In this chapter, we aim to obtain potential *At*KAPAS inhibitors using the knowledge-based computational informatics method in this research. We described the 3D-structure of *At*KAPAS via theoretical method and the binding mode for *At*KAPAS and its inhibitors obtained from *in vitro* assay for in-house compounds.

2. Building a homology model of AtKAPAS

To apply the structure-based drug design (SBDD) method using current knowledge of protein and drug interactions, a three-dimensional protein structure is necessary [4]. Because the

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known protein crystal structural information of 7-keto-8-aminopelargonic acid synthase from an experiment was absent, a homology model of *At*KAPAS was constructed from its amino acid sequence (Table 1).

10	20	30	40	50	60
madhswdktv	eeavnvlesr	qilrslrpic	Msrqneeeiv	ksranggdgy	evfdglcqwd
70	80	90	100	110	120
rtsvevsvsi	ptfqkwlhde	psngeeifsg	dalaecrkgr	fkkllfsgn	dylglsshpt
130	140	150	160	170	180
isnaaanavk	eygmgpkgsa	licgyttyhr	Llesslaqlk	kkedclvcpt	gfaanmaamv
190	200	210	220	230	240
aigsvaslla	asgkplknek	vaifsdalnh	Asiidgvrla	erqgnvevfv	yrhcdmyhln
250	260	270	280	290	300
sllsnckmkr	kvvvtdslfs	mdgdfapmee	lsqlrkkygf	llviddahgt	fvcgengggv
310	320	330	340	350	360
aeefnceadv	dlcvgtlska	agchggfiac	skkwkqliqs	rgrsfifsta	ipvpmaaaay
370	380	390	400	410	420
aavvvarkei	wrrkaiwerv	kefkelsgvd	Isspiislvv	gnqekalkas	ryllksgfhv
430	440	450	460	470	480
mairpptvpp	nscrlrvtls	aahttedvkk	Litalsscld	fdntathips	flfpkl

Table 1. Single letter amino acid sequence for *At*KAPAS.

To obtain the 3D-structure of *At*KAPAS, a hierarchical protein structure modeling approach was adopted based on secondary-structure enhanced profile-profile threading alignment (PPA) and iterative implementation by the threading assembly refinement (TASSER) program [5]. We obtained five candidate models for the three-dimensional structure of *At*KAPAS and then performed molecular dynamics simulations with the use of the CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field (version 27.0) [6] with default parameters interfaced with Accelrys Discovery Studio 3.1. To identify binding sites, we collected the crystal structures of homologous proteins with *At*KAPAS as templates from the RCSB Protein Data Bank (PDB) based on three different categories: structural similarity, binding site similarity and functional similarity. Ten different protein crystal structures originating from different species but structurally similar to *At*KAPAS were selected. The binding site sequence and conformation of the target protein are particularly important here compared to other sites. Therefore, we found 10 different PDB hits which are similar in terms of their binding site to *At*KAPAS. A comparison between each PDB hit and the *At*KAPAS biding site sequence was done. The results are denoted using root mean square deviation (RMSD) values ranging from 1.83 to 3.51, as shown in Table 2.

PDB code	Sequence	RMSD
1FC4	36.3	1.83
1DJE	39.5	1.66
2BWO	39.6	1.59
2WKA	32.2	2.42
3KKI	30.3	2.64
3DXV	16.6	3.26
3DXW	16.8	3.25
2OAT	15.7	3.38
1GBN	15.6	3.43
1MLY	15.5	3.51

Table 2. Sequence similarity and RMSD between 10 reference PDB proteins.

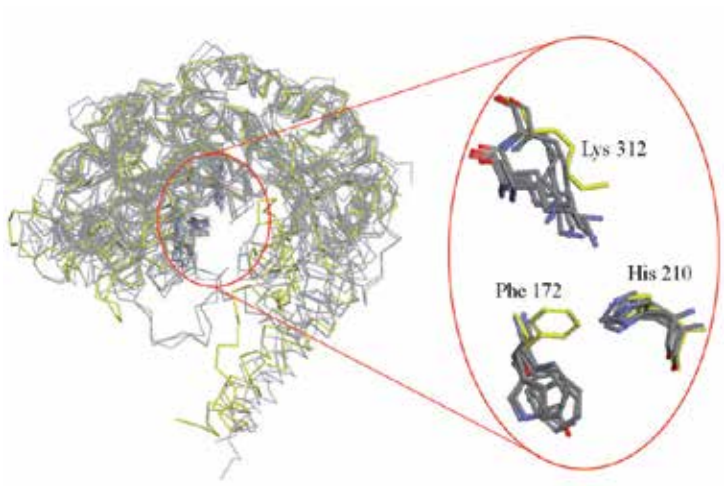

Figure 1. The superimposed 10 known enzymes (PDB code: 1FC4, 1DJE, 2WKA, 2BWO, 3KKI, 3DXV, 3DXW, 2OAT, 1GBN and 1MLY). The yellow-colored structure represents our homology model, and the overlapping predominantly important residues in active site are elaborated on the right side of the entire protein alignment.

To identify the binding mode of *At*KAPAS, 10 different enzymes (PDB code; 1FC4 [7], 1DJE [8], 2WKA [9], 2BWO [10], 3KKI [11], 3DXV [12], 3DXW [12], 2OAT [13], 1GBN [14] and 1MLY [15]) with different functions originating from different species were superimposed (Fig. 1). Consequently, we found that several residues were crucial for protein–ligand interactions. His 210 mainly forms a π–π interaction or a π–cation interaction with the ligands, and all of the reference proteins contain histidine residue at a position homologous to the *At*KAPAS. Adjacent to His 210, Phe 172 forms a π–π interaction with compounds that have an aromatic ring moiety as well. 50% of proteins have phenyl residue at a similar position; however, its

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conformation was considerably different. As shown in Fig. 1, conformation of Phe 172 residue of our homology model, shown in yellow, is uniquely folded toward the active site cavity as opposed to the phenyl residues of the reference PDB. To confirm the effect of Phe 172, we undertook a flexible docking evaluation. Lys 312 also plays an important role as a hydrogen bond donor in the active site. All reference enzymes showed the lysine residue at an analogous location which led to π–cation interaction and hydrogen bonding interaction with its ligand. In addition, due to its comparatively free long aliphatic chain, lysine residue was very flexible. Lysine residues superimposed onto the analogous site verified its flexibility. By superimposing the binding sites of other similar proteins, we reached the conclusion that the residues His 210, Phe 172 and Lys 312 play major roles at the binding site. This research will therefore highlight the ligand binding site residues and its flexibility to search for potent hits.

3. Rigid docking

As a structure-based drug design, we used an automated docking method [16]. To dock the compounds as shown in Table 3 into the protein active site, we used the rigid docking method implemented in Discovery Studio 3.1 (Accelrys, Inc.), which adopts a Monte-Carlo algorithm to generate ligand conformations and docks the generated ligands into the active site using a shape-based filtering method. The rigid docking process consists of two main steps: defining a binding cavity and docking ligands onto the defined cavity [17]. The *At*KAPAS protein as obtained from the homology model needs to be prepared before the docking process is initiated. To prepare the protein, the CHARMM force field was assigned and the docking cavity was defined using the advanced define and edit binding site tool module. Ligands also should be prepared with low-energy conformations so as to be docked onto a 'clean' protein. The protocol 'Generate Conformations' in Discovery Studio 3.1 was used to obtain three-dimensional conformations of each ligand. In-house 17 ligands were generated by the protocol 'Generate Conformation' with the conformation method 'BEST', and the CHARMM force field was applied. For an interaction filter, Lys 312 was selected.

4. Flexible docking

Various docking methods are utilized by researchers. Each approach was developed by focusing on different aspects of docking. One of the factors determining the accuracy of docking is protein flexibility. Much emphasis has been placed on the conformational changes of protein binding sites, where different ligands form interactions. The Flexible Docking protocol of Discovery Studio 3.1 allows receptor flexibility during the docking of ligands [18-20]. To confirm the flexibility of the selected residues in the *At*KAPAS active site, three different sets of residues were defined as follows: flexible docking 1 (Lys 312), flexible docking 2 (Phe 172), and flexible docking 3 (Lys 312 and Phe 172). For the flexible docking 1 set, only the residue Lys 312 was assigned to move when flexible docking was underway. 17 pre-processed ligands were docked into the prepared *At*KAPAS homology model. The 'BEST'

conformation method was selected to generate three-dimensional ligands with stable energy levels, and other parameters were left with the default values. The residue Phe 172 was selected for the second flexible docking trial with the same parameters used with the flexible docking 1 set. To validate the effect of both residues, Lys 312 and Phe 172 were set as the flexible docking 3 group, and these residues were moved while flexible docking was underway.

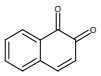
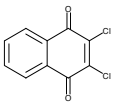
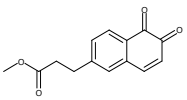
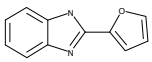
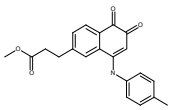
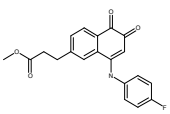
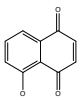
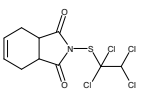
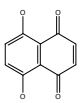
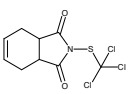
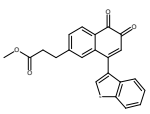
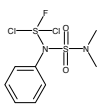
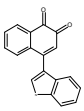
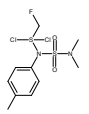
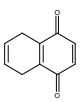
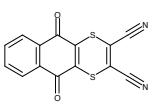
Compounds	Structure	pIC_{50}	Compounds	Structure	pIC_{50}
1		5.48	10		4.95
2		5.36	11		4.38
3		6.23	12		5.68
4		6.20	13		5.02
5		4.25	14		5.36
6		4.27	15		5.97
7		4.39	16		5.90
8		4.33	17		5.36
9		4.95			

Table 3. The structures of the 17 compounds used and their biological activities.

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5. *In Vitro* assay

Pimeloyl CoA was synthesized according to a previously described method [21]. *At*KAPAS activity was determined according to the method described previously [22] using a linked assay by monitoring the increase in absorption of NADH at 340 nm using a microplate spectrophotometer (Benchmark Plus, Bio-rad, USA), thermostatically controlled at 30°C. A typical assay contained 20 mM potassium phosphate (pH 7.5), 1mM α-ketoglutarate, 0.25 mM thiamine pyrophosphate, 1 mM NAD^+, 3 mM $MgCl_2$, 0.1 unit of α-ketoglutarate dehydrogenase, and 2 to 10 μg of *At*KAPAS in a total volume of 200μL. L-Alanine and pimeloyl-Co A were added to give the desirable final concentrations. Prior to analysis, enzyme samples were dialyzed for 2 hours at 4°C against 20 mM potassium phosphate (pH 7.5) containing 100 μM PLP. The KAPAS concentration in all analyses was 10 μM in 20 mM potassium phosphate (pH 7.5) and the concentrations of each compound were 0.1 to 250 μM. Reference cuvettes contained all other compounds except inhibitor.

6. Results and discussion

Interesting results were obtained from the docking processes undertaken in this study. The rigid docking output scores of the in-house compounds are shown in Table 4. The most active compounds, in this case compound 3 and compound 4, obtained high dock scores of 104.21 and 105.47, respectively. Moreover, active ligands which have IC_{50} (μM) values 1.07 and 1.26 (compound 15 and compound 16, respectively) formed a stable docking pose (Fig. 2) with high dock scores 106.03 and 72.5, respectively. However, other active compounds, specifically compound 1, compound 9, and compound 10, showed rather low dock scores, as shown in Table 3.

Compound	Dock score	pIC_{50} [a]	Compound	Dock score	pIC_{50} [a]
1	40.40	5.48	10	45.79	4.95
2	67.64	5.36	11	49.91	4.38
3	104.21	6.23	12	63.13	5.68
4	105.47	6.20	13	52.84	5.02
5	55.73	4.25	14	52.99	5.36
6	42.30	4.27	15	106.03	5.97
7	50.67	4.39	16	72.51	5.90
8	51.67	4.33	17	52.31	5.36
9	58.72	4.95			

[a]: $pIC50 = -\log IC_{50}$

Table 4. The result of rigid docking.

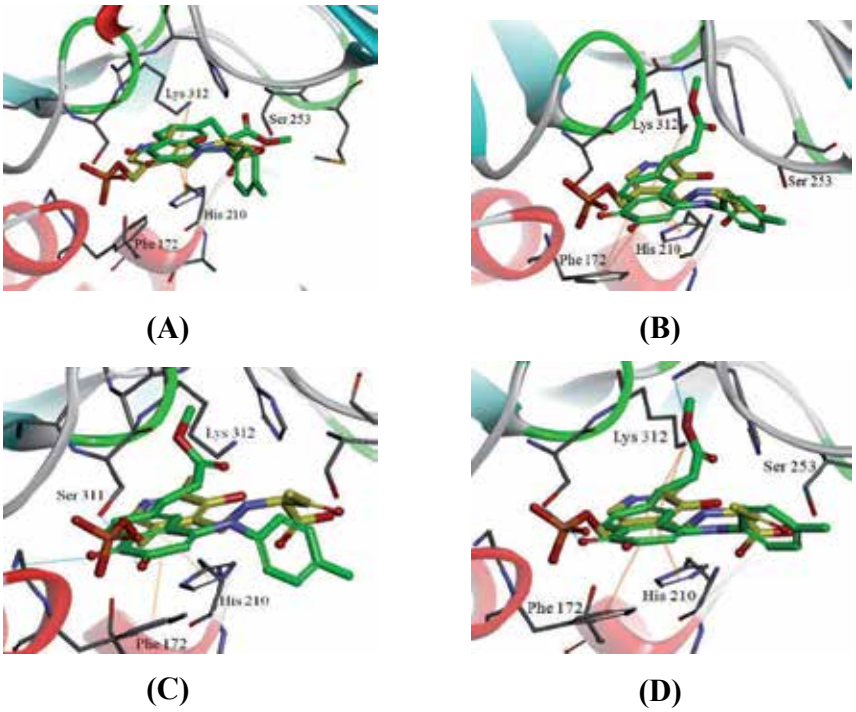

Figure 2. The docking pose from the process of rigid docking and flexible docking (A~D). The blue dashed line represents hydrogen bonding interaction and the orange dashed line represents the π–cation and π–π interaction. The docked ligand, compound 3, is colored with green and the crystal ligand of 1FC4 colored with yellow is overlapped as a reference compound. The docking pose from the process of rigid docking is shown in (A). From the process of flexible dock set 1, the docking pose of compound 3 was obtained as shown in (B). The docking pose shown in (C) achieved from the process of flexible docking set 2. In addition, the docking pose of compound 3 shown in (D) obtained from the process of flexible docking set 3.

To obtain a better docking result, we used a flexible docking strategy, as mentioned in the experimental section. In the result for flexible docking 1, which stipulated that the Lys 312 residue of the *At*KAPAS model is set to move, most of the compounds formed a stable conformation and formed interactions with the *At*KAPAS homology model (Table 5).

According to the docking result, the diverse conformation of Lys 312 directly affects the pose of the ligands and the related activity. The Lys 312 residue forms a hydrogen bond or undergoes the π-cation interaction mostly with the oxygen moiety of the ligand by flexibly moving through the protein cavity (Fig. 2). Interestingly, we obtained relatively high dock scores for compound 1, compound 9, and compound 10. This was unobtainable with the rigid docking process. The correlation coefficient between pIC50 and the dock score for the flexible docking 1 set was 0.72, as shown in Fig. 3.

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Compound	Dock score	pIC_{50}
1	74.00	5.48
2	83.14	5.36
3	134.03	6.23
4	125.27	6.20
5	53.40	4.25
6	50.83	4.27
7	59.46	4.39
8	54.40	4.33
9	105.54	4.95
10	76.74	4.95
11	74.36	4.38
12	83.56	5.68
13	76.92	5.02
14	80.04	5.36
15	131.67	5.97
16	96.88	5.90
17	75.41	5.36

Table 5. The result of flexible docking set 1.

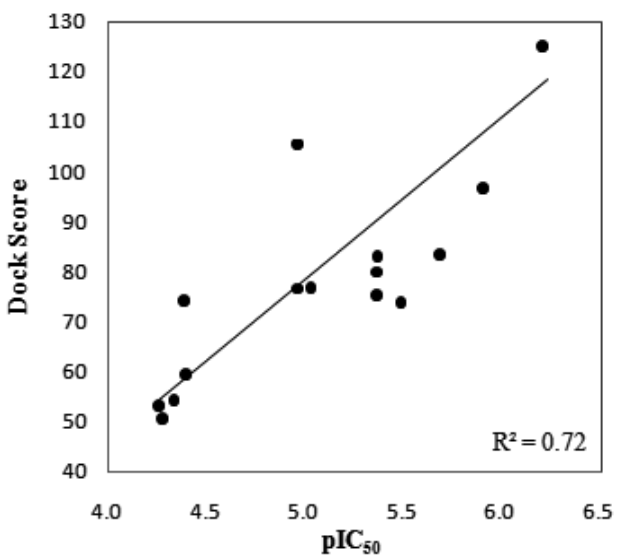

Figure 3. Correlation of IC_{50} and dock score for the flexible docking set 1.

By flexibly moving the residue Phe 172 while the flexible docking 2 process was underway, a better result than the flexible docking 1 process was obtained (Table 6). The flexibility of the Phe 172 residue has a significant effect on the ligand binding at the *At*KAPAS active site. The phenyl ring is particularly important to form the π–π interaction with compounds. According to the docking result, high dock scores of 119.91 and 113.06 were the result with the most active compounds: compound 3 and compound 4. In addition, active compounds with IC_{50} values lower than 1.3 μM received dock scores higher than 90 with a stable conformation. Conversely, the inactive compounds of compound 5 through compound 8 as well as compound 11 obtained relatively low dock scores (Table 6).

Compound	Dock score	pIC_{50}
1	75.60	5.48
2	85.84	5.36
3	119.05	6.23
4	113.06	6.20
5	48.11	4.25
6	44.80	4.27
7	53.49	4.39
8	49.60	4.33
9	72.97	4.95
10	74.43	4.95
11	57.66	4.38
12	85.24	5.68
13	79.99	5.02
14	67.73	5.36
15	113.29	5.97
16	90.84	5.90
17	65.49	5.36

Table 6. The result of flexible docking set 2.

The correlation coefficient between pIC50 and the dock score for the flexible docking 2 set was 0.85, as shown in Fig. 4.

The two specific *At*KAPAS residues, Lys 132 and Phe 172, can be moved mutually for the flexible docking 3 process. The result shows the clear discrepancy between an active compound and an inactive compound (Table 7). The flexible docking 3 results showed a higher correlation between pIC50 and the dock score compared to flexible docking 2, whereas it had a slightly lower R^2 value than the flexible docking 2 result (Fig. 5). Because the two residues were set to move at the same time, more diverse results could be obtained. As a result of several flexible docking processes, this study emphasizes the flexibility of several residues. Noticeably higher

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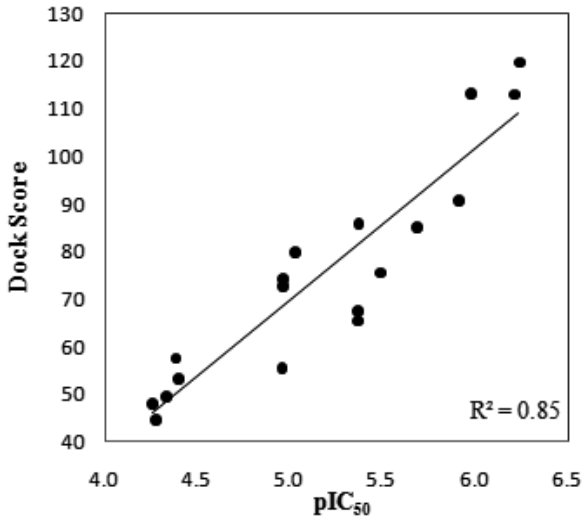

Figure 4. Correlation of IC_{50} and dock score for the flexible docking set 2.

dock scores were obtained from flexible docking as compared to rigid docking. The rigid Lys 312 residue mostly tends to form π–cation interaction with the aromatic moiety of compounds, allowing a certain amount of space for compounds during the rigid docking process. However, the flexible Lys 312 forms either the π–cation interaction or the hydrogen bonding interaction with hydrogen bond acceptors. The flexibility of the residue, including Lys 312 and Phe 172, allows more space for compounds, thus offering a better docking pose and dock scores.

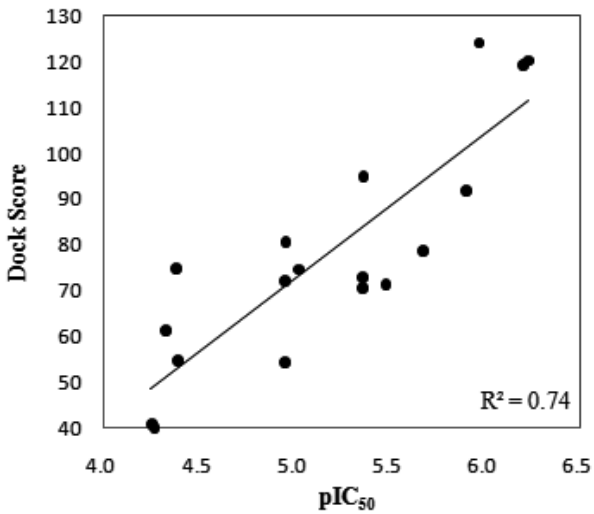

Figure 5. Correlation of IC_{50} and dock Score for the flexible docking set 3.

Compound	Dock score	pIC_{50}
1	71.47	5.48
2	95.12	5.36
3	120.48	6.23
4	119.46	6.20
5	40.92	4.25
6	40.03	4.27
7	54.90	4.39
8	61.54	4.33
9	80.87	4.95
10	72.13	4.95
11	75.00	4.38
12	78.83	5.68
13	74.82	5.02
14	73.04	5.36
15	124.28	5.97
16	91.92	5.90
17	70.70	5.36

Table 7. The result of flexible docking set 3.

7. Summary

In this study, we determined the 3D-structure of *At*KAPAS by homology modeling. We then investigated the binding mode of our in-house library using computational docking methods. In the rigid docking of the in-house compounds as shown in Table 3, the most active compounds, in this case compound 3 and compound 4, obtained high dock scores of 104.21 and 105.47, respectively. However, some active compounds showed rather low dock scores. To obtain a better docking result, we used a flexible docking strategy. From the flexible docking study, we achieved high dock scores and stable binding conformations for the active compounds denoted in this study. Thus, we highlight the flexibility of specific residues, Lys 312 and Phe 172, when used in active sites. Furthermore, we are going to optimize compound 3 and compound 4 using this homology model for *At*KAPAS. Following the initial identification of a lead chemical, intensive research and testing needs to be followed to optimize its structure to understand its action and provide data on its environmental compatibility.

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Chapter 8

Modes of Action of Different Classes of Herbicides

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Additional information is available at the end of the chapter

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Abstract

The mode of action of herbicides is important for understanding the management, classification, organization, and hierarchy of the herbicides. It also provides an insight into herbicide resistance, which continues to be a problem in sustainable agricultural management. The overuse of herbicides, just like other pesticides such as insecticides, has led to increased development of resistance among weeds, causing injury and destruction of useful plants in agriculture, land management, and other related industries. This chapter focuses on the main theme while providing in-depth analysis of the different modes of action of various classes of herbicides. The modes of action of herbicides are as variable as their chemical compositions as they focus on controlling susceptible plants through various biochemical means. Depending upon the specific mode of action at work, it may involve a plant enzyme or a biological system that the herbicide may interrupt, thus injuring or disrupting the regular plant growth and development and causing eventual plant death. Having an in-depth knowledge of the mode of action of herbicides is important in choosing a specific herbicide for a specific crop, understanding the injury symptoms, and devising an appropriate crop-management strategy.

Keywords: Herbicides, mode of action, resistance, translocation, regulator

1. Introduction

Herbicides or weedkillers belong to a class of pesticides that are used in the management of undesired plants in the areas of agriculture, landscaping, forestry, gardening, and industry [1, 2]. Weeds cost billions of dollars' worth of damage each year to crops, particularly corn and soybean in the United States and Canada, to which the maximum quantity of herbicides are applied [3-5]. Similar economic and environmental losses have been associated with nonindigenous plant species in Southeast Asia [6,7]. The control of weeds and other unwanted plants in a cost-effective manner is very important to agriculture and other related industries. Herbicide use, though essential for limiting and eliminating the weed populations, poses its

own set of problems and risks; its use must be minimized to account for the desired economic and environmental effects [8-10]. The problem associated with weed control is amplified due to herbicide resistance that some of the weed species have developed over the course of time due to the overuse of herbicides or their evolution (process of natural selection) toward favorable conditions [11]. Weeds may be resistant to specific herbicides (selective) or may be resistant to a broad spectrum of herbicides (nonselective) [11-13]. These inherent features have evolved based upon the various mechanisms such as absorption, metabolism, translocation, detoxification, and site of action, which confer resistance to the weeds [14-16].

In order for a successful weed elimination and control strategy to be effective, all of the above conditions must be met. Most of the herbicides, as described in this chapter, interact and interfere with the metabolic machinery and other biochemical pathways of weeds and cause irreversible damage, tissue injury, leading to the eventual death and elimination of the weeds [17]. Herbicides may vary based upon their complex chemical structures, characteristics, and properties with other members of their family, and are grouped according to their mode of action and target specificity [18-20]. The mode of action of herbicides includes inhibition, interruption, disruption, or mitigation of the regular plant growth [21-23].

Herbicides are classified based upon different aspects, such as mode of action, site of action, chemical families, time of application, selectivity, translocation, etc. [24-26]. It is important to note here that even a particular herbicide-resistant weed could be susceptible to a specific herbicide provided the amount and the rate of application are appropriate. On the other hand, excessive use of herbicides could damage the crop and also impart resistance to the same weeds which were intended for control or elimination. Therefore, it is important to strike a balance between these strategies and find the optimum medium for the best and maximum effect. Based upon the time of application, herbicides are classified as preemergence or postemergence [27] as shown in Figure 1. When applied preemergent, they may be effective against grassy weeds or broad-leaf weeds [28,29]. On the other hand, when applied postemergent, they may be selective (specific target) or nonselective (broad target) [28]. At the preemergent stage, the herbicide may be applied to the soil or even the seeds may be treated with them. With postemergent applications, the seedlings are sprayed with specific herbicides so as to eliminate weeds. Selectivity is defined as the capacity of a herbicide to kill a target plant without harming or killing the nontarget plants [30]. Selective herbicides are highly specific and are best suited for the control of a specific weed associated with a specific crop; most of the herbicides used in agriculture and related industries are highly selective. Upon contact, they act by getting absorbed and translocated into the xylem or the phloem of the weeds, by inhibiting or disrupting the metabolic machinery or other biosynthetic pathways, and by injuring or killing the weeds [31]. Nonselective herbicides, on the other hand, have a limited use in agriculture and other related industries, but they are effective in land-reclamation projects where the land needs to be cleared of all vegetation or where the weeds may be localized, away from the plants of interest. Glyphosate, however, has been used worldwide as a nonselective herbicide, but it acts more selectively when used in association with genetically engineered crops, which have been developed for resistance against glyphosate [21,32,33]. The selectivity or nonselectivity of herbicides depends upon various factors, such as plant physi-

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ology, soil topography, environment, timing of application, rate of application, and application technique [26]. The classification of herbicides is equally important for managing and understanding herbicide resistance, which continues to be a problem in sustainable agricultural management [24,25]. The overuse of herbicides, just like other pesticides such as insecticides, may lead to increased development of resistance among plants, causing injury and destruction of useful plants in both agriculture and land management [6]. Understanding the reasons for classifying the herbicides based upon their modes of action, instead of the chemical family or the site of action, will help to understand the reasons behind the development of resistance due to their overuse.

Plants interact differently with different herbicides based upon their absorption, translocation, metabolism, and physiological response. The mode of action may be prevalent at the tissue or cellular levels and the tissue-injury symptoms are similar for a specific group. Herbicides are also selective in their mode of action, crop/weed favorability, and soil topology. Herbicides may either be applied directly to the foliage or be added to the soil during plowing/tilling [34]. Herbicides may have a vertical or horizontal translocation movement of the chemicals representing different groups [35-37]. There are other herbicides that kill upon contact on the foliage and are potent enough such that they do not require translocation either way. Plants are intact systems that consist of organs, tissues, cells, and molecules, which are reservoirs of organized biochemical processes that take place uninterrupted. Herbicides may be absorbed by the plants via the roots (soil-based herbicides) or the shoots (spray-based herbicides) [38]. The metabolic activity requires the movement of sap through the xylem (translocation of water and nutrients) and phloem (translocation of sugars) [39,40]. When the herbicides penetrate the cell walls of the weeds, they cause tissue injury and permeate the sap, in the process, interrupting various biochemical pathways. Upon interaction with the herbicides, weeds are killed by the dysfunction of their biochemical processes.

Traditionally, herbicides were mixed with the soils, but at present, the trend is leaning toward spraying herbicides and also developing herbicide-resistant crop varieties [6,41]. Given the environmental concerns associated with aerosols, soil pollution, and water-system contamination, developing environment-friendly herbicides has become a priority [42,43]. The United States Environmental Protection Agency (US EPA) has many regulations and guidelines in place for the proper manufacturing, sale, and use of herbicides (www.epa.com). The rate of application of herbicides and their strategic placement are of prime importance and depend upon the kind of weeds that need to be killed. Herbicides with higher rates of absorption and retention (during spraying) require a less volume and a less potency as compared to their counterparts. The weather/temperature conditions (mild, temperate, or tropical) determine the effectiveness of herbicides on a specific crop. Along with the temperature, the humidity and the plant vigor also play important roles in designing the herbicide application strategy [44,45]. An understanding of the leaf-surface coverage area, leaf-surface properties, and the chemical properties of the herbicide is essential for maximum success [46-48].

Herbicides are grouped based upon their chemical structures, which consist of a base-specific molecule surrounded by a side chain or a group(s) [49]. A modification of a functional group leads to a modification in the activity, selectivity, and persistence of herbicide and also

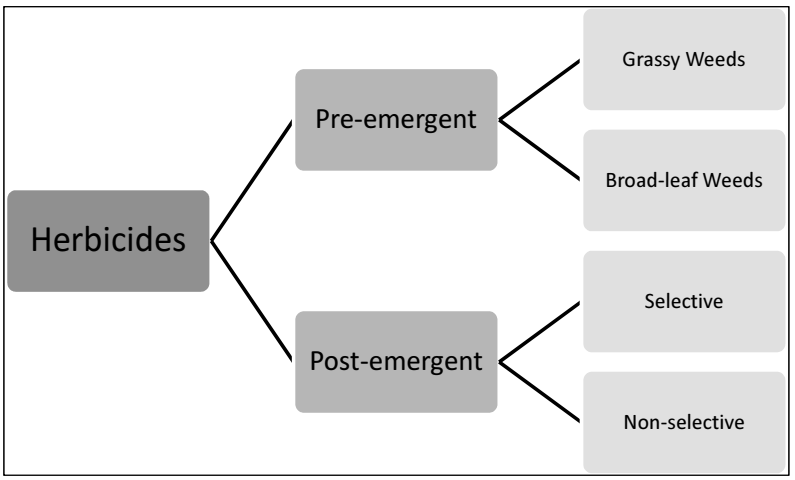

Figure 1. Herbicide classification and application

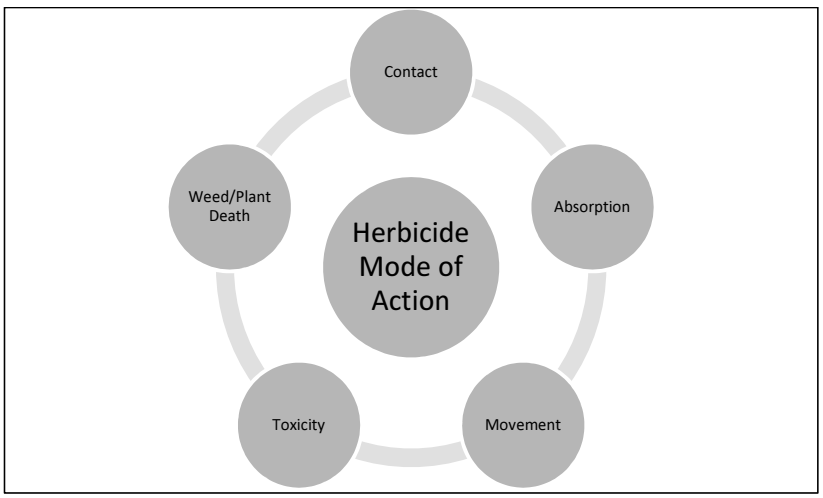

Figure 2. Herbicide mode of action – stages

determines its mode of action. The overuse of herbicides has increased the chances of damage and injury to the non-target weeds/plants while also causing groundwater contamination [6,10,50). This chapter describes the different modes of action of herbicides based upon whether they are inhibitors, regulators, or disruptors of various biosynthetic pathways and hence classified accordingly. Slight variations have been observed in the group-classification system in scientific publications but those are of minor concern. The mechanism of the action of herbicides for killing a weed is well understood and requires that, in order for it to be effective, the herbicide must undergo the following processes, in the sequential order, listed below [51] and also as illustrated in Figure 2:

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1. Contact – Must come in contact with the weed
2. Absorption – Must be absorbed by the weed
3. Movement – Must move to the site of action in the weed
4. Toxicity – Must possess or acquire sufficient level of toxicity or potency to kill the weed
5. Death – Must cause injury to the weed leading to eventual death

2. Herbicide resistance

Herbicide resistance is a widespread problem in agriculture and related industries [11,21]. The resulting loss of crops can be significant for farmers and other growers. Instead of using a passive approach to herbicide-resistance management, the use of proactive strategies based upon the latest advancements and knowledge will deliver the best results [52-54]. The results of a successful herbicide-resistance management strategy can be quantified in terms of both the cost-effectiveness and the total output of the harvest [55]. The maximum loss occurs during the initial stage of the crop's planting, particularly when the soil and/or the seeds are not treated with herbicides [56,57]. It is at this stage that the seeds are the most vulnerable and the weeds are the most robust and must be treated accordingly [34,58]. If left untreated or not appropriately treated, the weeds can get out of control, take over the crop/plants, and also become resistant leading to long-term and devastating economic consequences for farmers and growers [59,60]. Therefore, it is important to select a weed-resistant variety of crop instead of attempting to deal with the populations of weeds, which may be resistant to such herbicides, thus increasing the overall cost of cultivation [61,62].

Weeds have been shown to have acquired unprecedented resistance to several herbicides due to their indiscriminate use over the years [21,63]. This may be due to the result of mutation or substitution of a single base, thus changing the reading frame of the amino acid and the amino acid itself [64-68]. This modification, usually in the quinone-binding region of the peptide, decreases the binding capacity of the herbicide and renders it to be vulnerable and less effective [49,69,70]. If the mutation is at the gene level, it may be a single-gene mutation or a multigene mutation and would impart the weed a higher or lower level of resistance, respectively [71-74]. Recent advances have shown that slight alterations in the binding affinity of the herbicides to the crops, via different pathways, particularly the photosynthetic pathway, have led to the development of various effective herbicide strategies [69,75,76]. Despite the concerns of genetically modified (GM) crops, the recombinant DNA technology is one such tool that allows for the development of crop-safe and effective herbicides, which kill only the weeds when applied to the entire crop [77,78]. However, the processes of natural selection and gene transfer may allow the weeds to acquire herbicide resistance quickly [21,79]. On the other hand, identifying resistance among weeds is an extremely challenging task, which requires different tools at one's disposal [12]. Some of the observational tools, necessary for controlling the weed and other undesirable plants during cultivation, include:

1. Loss of consistent control of the weed population

2. Effect of environment/weather conditions on the weed population
3. Rate of application of herbicides on the weed population
4. Soil topography (effect of overuse or crop rotation on the weed population)
5. Use of herbicides with a similar mode of action
6. Use of herbicides with related chemical families

Herbicide resistance, like antibiotic resistance, is a common occurrence among weeds and is a result of overuse of a specific herbicide and soil conditions [21,80]. In order to avoid and overcome the development of herbicide resistance, different herbicides must be used at different points in time on the same crop [81]. Crop rotation is also recommended to maintain soil integrity and soil vigor [29]. The genotype of different plants lends them to interact differently due to conformational variations and compatibility with various chemical groups [82]. For example, Kochia and pigweed are resistant to triazine and ALS-inhibiting herbicides [83]. Certain genetically modified (GM) crop plants, which may inadvertently come in the path of the herbicides (carry-over) may able to overcome the harmful effects of these herbicides [11,84,85]. Cross-resistance is another concerning issue in agriculture and related industries as some weeds and pests have acquired resistance to several herbicides, which are related by their mode of action [86-88]. For example, herbicides belonging to separate chemical families but within the same mode of action [e.g., acetolactate synthase (ALS) inhibitors] may acquire herbicide resistance against both the chemical families. Crop rotation on the same piece of land is highly influential and beneficial in minimizing the development of herbicide resistance as the soil comes in contact with various microorganisms and interacts differently with different chemicals in the surrounding micro-ecosystem [21,29]. The crop rotation allows for different herbicides to be used, which reduces the selection for resistance. The weed-control effect is the maximum at the emergence stage and the minimum at the maturity stage, as the plant goes through the stages of emergence, seedling, vegetative, flowering, and maturity, respectively [89].

3. Mode of action

The modes of action of herbicides are as variable as their chemical compositions and focus on controlling susceptible plants through various biochemical means. The weed-control effect is the maximum at the emergence stage and the minimum at the maturity stage, as the plant goes through the stages of emergence, seedling, vegetative, flowering, and maturity, respectively [89]. Depending upon the specific mode of action at work, it may involve a plant enzyme or a biological system that the herbicide may interrupt, thus injuring or disrupting the regular plant growth and development and causing eventual plant death. Extensive research in the area of herbicides has led to their classification based upon their modes of action into various groups, which are discussed in detail in this chapter. It is important to note that the modes of action discussed in this chapter, though comprehensive, are not exhaustive by any measure. Newly discovered groups and unexplained (unknown) groups continue to be added to the list, reflecting slight variations of classification, as new research knowledge continues to emerge. Also, some scientists may have a slightly different method of classification as well as different

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groups, but those are only slight variations. What is important, however, is that having an in-depth knowledge of the mode of action of herbicides is necessary in choosing a specific herbicide for a specific crop, understanding the injury symptoms, and devising an appropriate crop-management strategy and using an appropriate herbicide. Even though this chapter focuses on the herbicide's mode of action, it also introduces the reader to a vast array of topics such as herbicides, herbicide resistance, group-numbering system for designating various herbicides based upon their mode of action, etc. Herbicides belonging to a specific group have the same mode of action even though they may belong to a different chemical family. Finally, the environmental fate of herbicides (persistence, degradation, mobility) with specific reference to the groundwater, water sterilization, soil contamination, and environmental and public health concerns will enlighten the reader on the importance of using the herbicides diligently and according to the regulations, guidelines, and labeling. The modes of action, as discussed in this chapter, are listed below:

1. Lipid biosynthesis inhibitors
2. Amino acid biosynthesis inhibitors
3. Plant growth regulators
4. Photosynthesis inhibitors
5. Nitrogen-metabolism inhibitors
6. Pigment inhibitors
7. Cell-membrane disruptors
8. Seedling-growth inhibitors

3.1. Group 1: Acetyl Coenzyme A Carboxylase (ACCase) inhibitors

Also known as lipid biosynthesis inhibitors, these herbicides inhibit the ACCase enzyme activity and are used typically for controlling grass during the cultivation of broadleaf crop varieties or crop rotation. The ACCase enzyme catalyzes the primary step in the fatty-acid synthesis, thus blocking the production of phospholipids necessary for synthesizing the lipid bilayer, which is indispensable for cell structure and function [90-92]. The chemical family of aryloxyphenoxypropionate, cyclohexanedione, and phenylpyrazolin operates by inhibiting the ACCase enzyme. These herbicides are also known by their chemical family nicknames – FOPs, DIMs, and DENs [93]. Many broadleaf crop varieties, including grasses, have a natural resistance to these herbicides due to a strong and less-sensitive ACCase system [94].

3.2. Group 2: Acetolactate Synthase (ALS) inhibitors

Also known as amino acid synthesis inhibitors, these herbicides inhibit the action of the acetolactate synthase (ALS) enzyme. Also known as acetohydroxy acid synthase (AHAS), ALS catalyzes the first step in the synthesis of the branched-chain amino acids, such as leucine, isoleucine, and valine [95]. These are also referred to as the AHAS inhibitors or branched-chain amino acid inhibitors. Comprising the imidazolinones, pyrimidinylthiobenzoates, sulfonyla-

minocarbonyltriazolinones, sulfonylureas, and triazolopyrimidines chemical family, the ALS inhibitors are a part of the largest group of herbicides, which function with the amino acid synthesis inhibitor mode of action [96]. They may show cross-resistance to other herbicides and act by reducing the production of the branched amino acids in the presence of the ALS enzyme, causing plant wilting and, ultimately, plant death.

3.3. Group 3: Root growth inhibitors

Also known as the seedling root growth inhibitors, these herbicides inhibit cell division as part of their mode of action, which, ultimately, blocks root extension and growth. They are applied preemergent or preplant in vegetables and ornamentals. Their site of action is the microtubule and is marked by the assembly of the herbicide–tubulin complex inside the microtubules. This complex inhibits the polymerization of microtubules during assembly but remains unaffected during depolymerization [97]. A loss in the structure and function of the microtubule causes cell death by compromising the cell-wall formation due to the nonalignment of the spindle fibers and nonseparation of the chromosomes during mitotic cell division. This group is represented by the benzamide, benzoic acid [dimethyl-2,3,5,6-tetrachloroterephthalate (DCPA), dinitroaniline, phosphoramidate, and pyridine chemical family, which act by causing the loss of microtubule formation leading to the obstruction of cell division and elongation as evidenced by the swelling of the root tips.

3.4. Group 4: Plant growth regulators

Also known as synthetic auxins, this group includes hormone-based herbicides and is used to keep broadleaf weeds out during the cultivation of corn, wheat, and sorghum. The mode of action of the endogenous indole acetic acid (IAA) is mimicked by the herbicides belonging to the chemical family represented by benzoic acid, phenoxycarboxylic acid, pyridine carboxylic acid, and quinoline carboxylic acid [98]. The specific molecular binding site responsible for the IAA activation is yet to be established and remains unknown. All of these chemicals disrupt the nucleic acid metabolism and the cell-wall integrity by activating the adenosine triphosphate (ATP)ase proton pump, which increases the enzyme activity in the cell wall [99]. These regulators mimic the IAA activity, thus increasing the transcription, translation, and the protein biosynthesis activities within the cell leading to uninhibited vascular growth, causing cell bursts and ultimate cell and plant death.

3.5. Groups 5, 6, and 7: Photosynthesis inhibitors – Photosystem II (PSII) inhibitors

The mode of action of these herbicides is the inhibition of the photosynthetic pathway, specifically the Photosystem II (PSII). Due to their excessive use, some weeds have become resistant to these herbicides developed on this metabolic principle. Group 5 is represented by the chemical family of triazine, triazinone, phenylcarbamates, pyridazinones, and uracils. Group 6 is exemplified by nitriles, benzothiadiazinones, and phenylpyridazines. Group 7 comprises phenyl urea and amides. All of these groups represent different binding schemes as compared to each other with several similarities. All of these PSII herbicides inhibit the photosynthetic pathway by binding the Q_B-binding site of the D1 protein complex present in

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the chloroplast thylakoid membrane. The binding disrupts the electron transport system (ETS) from Q_A to Q_B and also blocks the CO_2 fixation, ATP generation, and nicotinamide adenine dinucleotide hydrogen phosphate ($NADPH_2$) production required for various biochemical pathways as part of the plant growth and development [70,100]. As a result of the blockage at the level of the electron transport chain (ETC), the plant is unable to reoxidize Q_A, which generates the triplet-chlorophyll (^3Chl), which forms singlet-oxygen ($^1O_2$) upon reacting with molecular oxygen (O_2). The unsaturated fatty acids and lipids release hydrogen in the presence of triplet-chlorophyll (^3Chl) and singlet-oxygen ($^1O_2$) while forming a lipid radical, thus causing lipid peroxidation. Lipid peroxidation causes the lipids in the bilayer and other proteins to be oxidized, producing reactive oxygen species (ROS) [70,100]. Some of these herbicides also cause the disruption of the carotenoid, anthocyanin, and protein biosynthetic pathways and affect the transcription machinery as well. The oxidation, ultimately, causes the loss of chlorophyll and other pigments like the carotenoids from the cell membranes exposing the cells and cell organelles to harsh conditions leading to their collapse, disintegration, and eventual plant death [101,102]. Due to their overuse, some weeds have acquired resistance to these PSII inhibitor herbicides, such as atrazine and metribuzin [103].

3.6. Groups 8 and 15: Shoot-growth inhibitors

Also known as seedling shoot growth inhibitors, the herbicides designed with this mode of action are applied as part of the soil preparation and act effectively before the grass and broadleaf weeds emerge. The site of action of the Group 8 herbicides is at the location of the lipid synthesis machinery in the cell membrane. These Group 8 herbicides are represented by the chemical family of phosphorodithioates and thiocarbamates and inhibit the biosynthesis of lipids, fatty acids, proteins, isoprenoids, flavonoids, and gibberellins [104]. The site of action of the Group 15 is at the very-long-chain fatty acid (VLCFA) location in the cell membrane [105,106]. Group 15 herbicides are represented by the chemical family of chloroacetamide, acetamide, oxyacetamide, and tetrazolinone. These herbicides conjugate with acetyl COA and certain sulfhydryl-containing molecules via thiocarbamate sulfoxides, which inhibit the long-chain fatty acids during the seedling shoot growth stage of the plant and affect the weeds' preemergence.

3.7. Group 9: Aromatic amino acid inhibitors

The mode of action of these herbicides is as an amino acid synthesis inhibitor. This mode of action is specific to glyphosate (glycines), which are nonspecific herbicides that act by inhibiting the amino acid synthesis. These herbicides kill or cause injury to any plant that they come in contact with and hence are approved only for use in glyphosate-resistant crops, such as corn, cotton, canola, and soybean. Glyphosates inhibit the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme necessary in the generation of the EPSP from shikimate-3-phosphate and phosphoenolpyruvate as part of the shikimic acid pathway [107]. As a result, the necessary levels of the aromatic amino acids (tryptophan, tyrosine, and phenylalanine) are depleted, thus compromising the biosynthetic metabolic pathways leading to eventual plant death [108]. As a herbicide, glyphosate use is the most prevalent in the world due to its broad-spectrum

properties and for being environmentally- and user-friendly. Glyphosates are available as ammonium salts, diammonium salts, dimethylammonium salts, isopropylamine salts, as well as potassium salts. The ease of access to the target (broad-spectrum), and the ease of translocation (via the xylem and the phloem), and the failure of the weeds to overcome their effect make the Group 9 herbicides as ideal candidates for killing weeds associated with the glyphosate-resistant crops.

3.8. Group 10: Glutamine-synthesis inhibitors

The mode of action is nitrogen metabolism-based and is specific to glufosinate, which is nonspecific in nature. These herbicides can be used in glufosinate-resistant crop cultivation during postemergence of the resistant seedlings. Glufosinate and bialophos (phosphinic acids) inhibit the activity of the enzyme, glutamine synthetase (GS), which converts glutamate and ammonia to glutamine [109,110]. GS plays a key role in nitrogen metabolism (nitrogen fixation and nitrate/ammonia nutrition) by re-assimilating the ammonia generated during respiration. These herbicides disintegrate the proteins by disrupting the optimum activity of GS leading to an accumulation of ammonia, which lowers the pH gradient on either sides of the cell membrane. This causes the disruption of various cell functions, particularly shutting down the PSI and PSII systems leading to the uncoupling of the photophosphorylation [100,111]. Since GS is located in important organelles like the chloroplast and cytoplasm, these herbicides are highly effective in controlling weeds and other undesired plants.

3.9. Groups 12, 13, and 27: Pigment synthesis inhibitors

Also known as carotenoid biosynthesis inhibitors, these herbicides destroy the green pigment, chlorophyll, which is necessary for photosynthesis in the plants. These herbicides are also known as bleachers as they impart a white color to the plant tissues after coming in contact with the plant foliage leading to cell and tissue injury and ultimately killing the weeds. As part of their mode of action, these herbicides inhibit the pigment synthesis, specifically the catalysis of the 4-hydroxyphenyl pyruvate dioxygenase (HPPD) enzyme and are also referred to as the HPPD-inhibitors. Group 12 is represented by the chemical family of amides, anilidex, furanones, phenoxybutan-amides, pyridiazinones, and pyridines. These herbicides containing these chemicals disrupt the carotenoid biosynthetic pathway by inhibiting the function of the phytoene desaturase enzyme [112]. Group 13 is represented by the chemical family, Isoxazolidinone, and the site of action is at the location of the diterpene synthesis. Group 27 is represented by the chemical family, Isoxazole, and they are also HPPD inhibitors. Carotenoids play a key role in quenching the oxidative control of singlet O_2 ($^1O_2$) among healthy plants. Upon being treated with these herbicides containing the pigment synthesis inhibitors (Groups 12, 13, and 27), the level of carotenoids is highly reduced leading to the presence of unbound lipid radicals. These lipid radicals compromise the uptake of the membrane lipids and fatty acids, causing lipid peroxidation, which renders the chlorophyll, other cell membrane lipids, and some proteins dysfunctional. As a result of membrane leakage, the cell contents are exposed and destroyed rather rapidly causing wilting and eventual plant death.

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3.10. Group 14: PPO inhibitors

The PPO inhibitors act by disrupting the cell membranes and hence their mode of action is categorized as cell membrane disrupters and their site of action is the cell membrane. Most of these herbicides are applied at the postemergence stage while some are used at the preemergence stage of the seedling. The mode of action of these herbicides depends upon the inhibition of protoporphyrinogen oxidase (PPO) enzyme (also known as PPG oxidase or Protox inhibitors), which catalyzes the chlorophyll and heme biosynthesis [protoporphyrinogen IX (PPGIX) to protoporphyrin IX (PPIX) oxidation catalysis]. These herbicides are represented by the chemical family, diphenylether, aryl triazolinone, *N*-phenylphthalimides, oxadiazoles, oxazolidinediones, phenylpyrazoles, pyrimidindiones, and thiadiazoles. As the PPO is inhibited, it causes the over-accumulation of the PPIX, which interacts with the light in the chlorophyll and produces the triplet-PPIX. Upon reacting with O_2, triplet-PPIX forms $^1O_2$ causing the hydrogen-bond disruption in the unsaturated fatty acids and lipids in the membrane, which causes lipid peroxidation [113]. As a result of the cell membrane disruption, the lipids and proteins become oxidized causing the chlorophyll and other pigments to leak and causing cell disintegration, wilting, and eventual plant death.

3.11. Group 22: Photosynthesis inhibitors – Photosystem I (PSI) inhibitors

Upon contact with the plant foliage, these herbicides act by penetrating and destroying the cell lipid bilayer leading to the breakdown of the cell membranes and hence their mode of action is categorized as cell membrane disrupters. These herbicides are nonselective in nature and are applied usually prior to harvesting the crop. These herbicides are represented by the bipyridilium chemical family and are also known as PSI electron diverters as they accept electrons from PSI and, in the process, generate herbicide radicals. Upon interacting with O_2, the herbicide radicals form superoxide radicals, which, in the presence of the superoxide dismutase enzyme, form hydrogen peroxide (H_2O_2) and hydroxyl radicals [114]. These radicals disrupt the unsaturated fatty acids, chlorophyll, lipids, and proteins in the cell membrane. As a result, the cell membrane is disrupted beyond repair causing leakage of the cell cytoplasm, which leads to wilting and eventual plant death.

4. Conclusion

Due to the overuse of herbicides, agricultural weeds and other undesirable plants develop resistance, which must be contained or eliminated in order for the maximum output of the harvest. Herbicide resistance occurs due to the overuse of herbicides over the years. It is important to follow the lead in developing a proactive and robust herbicide resistance management strategy for minimizing the agricultural loss. Similar techniques can be extrapolated to the land management, ornamental, and other related industries to minimize the evolution of the herbicide-resistant varieties of weeds and other pests. Based upon the available body of knowledge, recent advances in agricultural research, and latest techniques, it is advisable to use different herbicides (different modes of action) at different times of the year

and with different crops so that the weeds do not develop resistance to the herbicides as quickly as that have been reported. Deciding which herbicide is the most effective and the most environmental friendly option for a specific crop can be a daunting task particularly with so many products competing for attention in the multibillion dollar herbicides market. Most of these herbicides are developed based upon similar weed control and pest management strategies and are designed based upon their mode of action. Rearranging various chemical groups within the chemical family, which most of the companies tend to do while developing their key products, may not be the best strategy as the weeds tend to develop a quick resistance to such chemicals or groups of chemicals. Therefore, it is imperative that the herbicides be designed to obtain the maximum effect with regard to their mode of action so as to control or eliminate weeds and destroy their capacity to acquire the herbicide resistance.

Group	Mode of Action	Site of Action	Chemical Family
1	Lipid-Synthesis Inhibitors	ACCase Inhibitor	Aryloxyphenoxypropionate (FOPs), Cyclohexanedione (DIMs), Phenylpyrazolin (DENs)
2	Amino-Acid Synthesis Inhibitors	ALS Inhibitors	Imidazolinones, pyrimidinylthiobenzoates, sulfonylaminocarbonyltriazolinones, sulfonylureas, triazolopyrimidines
3	Root-Growth Inhibitors	Microtubule Inhibitors	Benzamide, benzoic acid (DCPA), dinitroaniline, phosphoramidate, pyridine
4	Plant-Growth Inhibitors	Site of Action Unknown	Benzoic acid, phenoxycarboxylic acid, pyridine carboxylic acid, and quinoline carboxylic acid
5	Photosynthesis Inhibitors	Photosystem II Inhibitors	Triazine, triazinone, phenylcarbamates, pyridazinones, and uracils.
6	Photosynthesis Inhibitors	Photosystem II Inhibitors	Nitriles, benzothiadiazinones, and phenylpyridazines
7	Photosynthesis Inhibitors	Photosystem II Inhibitors	Phenyl, urea, and amides
8	Shoot-Growth Inhibitors	Lipid-Synthesis Inhibitors	Phosphorodithioates and thiocarbamates
9	Amino-Acid Synthesis Inhibitors	EPSP Synthase Inhibitors	Not designated by any specific chemical family
10	Nitrogen-Metabolism Inhibitors	Glutamine-Synthesis Inhibitors	Not designated by any specific chemical family
12	Pigment-Synthesis Inhibitors	HPPD Inhibitors	Amides, anilidex, furanones, phenoxybutan-amides, pyridiazinones, and pyridines
13	Pigment-Synthesis Inhibitors	Diterpene-Synthesis Inhibitors	Isoxazolidinone
14	Cell-Membrane Disrupters	PPO Inhibitors	Diphenylether, aryl triazolinone, *N*-phenylphthalimides, oxadiazoles,

Group	Mode of Action	Site of Action	Chemical Family
			oxazolidinediones, phenylpyrazoles, pyrimidindiones, and thiadiazoles.
15	Shoot-Growth Inhibitors	Very-Long-Chain Fatty Acid (VLCFA) Inhibitors	Chloroacetamide, acetamide, oxyacetamide, and tetrazolinone.
22	Cell-Membrane Disrupters	PSI Inhibitor	Bipyridilium
27	Pigment-Synthesis Inhibitors	HPPD Inhibitors	Isoxazole

Table 1. Modes of action of different classes of herbicides

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Chapter 9

The Role of White-rot Fungi in Herbicide Transformation

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Additional information is available at the end of the chapter

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Abstract

Understanding herbicide transformation is necessary for pesticide development for their safe and efficient use, as well as for developing pesticide bioremediation strategies for contaminated soil and water. Recent studies persuasively demonstrated the key role of soil white-rot fungi in biotransformation of various anthropogenic environmental contaminants. However, often this common knowledge is not associated with specific metabolic processes of fungi and therefore cannot be transformed into specific recommendations for agricultural practice. The given review offers a systematic collection and analysis of the current knowledge about herbicide transformation by white-rot fungi at the cellular and molecular levels. Special attention is given to the role of oxidative enzymes such as laccases, lignin peroxidases, and manganese peroxidases in the biotransformation processes.

Keywords: White-rot fungi, biotransformation, herbicides, oxidases, metabolic fate

1. Introduction

Fungi are unique organisms that colonize all areas of the environment – air, water, and soil. This group lists more than 1.5 million species and is remarkably flexible, occupying all biocenoses from the arctic tundra to the deserts. Biodiversity and specific genetic and molecular organization of fungi provided background for their key role in nature, i.e., maintaining of ecosystems' equilibrium. One of the most important groups playing a key role in the carbon cycle in nature is Wood Degrading Fungi, due to its ability to degrade or even mineralize lignin – widely present and one of the most stable biopolymers. They belong to Basidiomycota and Ascomycota and possess the unique ability to degrade components of xylem cell walls (cellulose, hemicellulose, lignin, and compounds forming these biopolymers). According to Anastasi et al. [1], this group is divided into white-rot fungi (WRF) or white rotters, brown-rot fungi, and soft-rot fungi because of the appearance of rotten wood.

The ability to degrade lignin and its aromatic compounds is mostly attributed to white-rot fungi [2]. The white-rot decay of wood is performed by the combined action of oxidoreductive metalloenzymes, heme peroxidases, and laccases, encoded by multigene families as well as organic acids, secondary metabolites, and surfactants secreted by WRF [3]. This assembly is considered the Lignin Modifying System (LMS) [4]. It should be mentioned that extracellular ligninolytic enzymes (laccases, lignin peroxidases, manganese peroxidases, and versatile peroxidases) are nonspecific and can act both alone as well as using a redox mediator that enhances the range of potential substrates and provides the possibility of effective oxidation of xenobiotics. The WRF also produces reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [5]. WRF, their LMS and ROS are involved in the degradation of lignin and carbohydrate components of wood commonly accomplished by production of carbon dioxide and water. Both WRF and LMS are capable of in vitro oxidizing and degrading a broad range of xenobiotics: polycyclic aromatic hydrocarbons (anthracene, benz[a]pyrene, naphthalene, and phenanthrene); polychlorinated phenols (2,4-di-, 2,4,5-, and 2,4,6-tri-, and pentachlorophenols), chlorinated guaiacol and benzoate derivatives, 2,4,6-trichlorophenoxyacetate, and chlorinated biphenyls; stable polymers (polyacrylate, polyacrylamide, polycaprolactam, and polyethylene), 2,4-dichloroaniline, dioxins, explosives nitrates, dyes. The degradation of xenobiotics by WRF as well as enzymatic aspects of these processes is well documented and summarized in several recent reviews [1,6-10]. However, there are contradictory data reported on the role of ligninolytic enzymes in pesticide degradation. There was no relationship between WRF degradation of the dye Poly R-478, a presumptive test for ligninolytic potential, and degradation of the highly available pesticides, diuron, metalaxyl, atrazine or terbuthylazine in liquid culture [11]. Moreover, it was also shown that no degradation of the herbicide picloram by *Ganoderma lucidun* and *Trametes sp.* occurred under liquid stationary conditions in spite of the fact that both extra- and intracellular laccases were produced and, in the case of *Trametes sp.*, the enzyme production level improved especially for secreted laccase [12].

Recent findings have highlighted the molecular aspects of ligninolytic enzymes' functioning [13-15]. Genes-encoding ligninolytic enzymes of the white-rot fungi have been found to undergo differential regulation in response to different environmental signals and stimuli such as carbon and nitrogen concentration in cultural media, presence of xenobiotics and heavy metals, temperature regime, and various lengths of daylight. The analysis of MnP, LiP, and laccase gene promoter regions revealed the presence of xenobiotic response mechanism (XRE – xenobiotic responsive element), suggesting that these enzyme expressions can be similar in the presence of xenobiotics [15]. It was shown that compounds such as paracetic acid, ethanol, sodium arsenite, 2,4-dichlorophenol, and N,N-dimethylformamide enhanced the MnP production [16]. Moreover, a list of available aromatic compounds including xenobiotics (1-hydroxybenzotriazole, 2,5-xylidine, o-toluidine, 3,5-dihydroxytoluene, dimethylphenol, caffeic acid, caffein, guaiacol, hydroquinone, etc.) that demonstrated the similar effect on laccase production was generated by Piscitelli et al. [17]. The data available confirmed that regulation of the expression of genes-encoded ligninolytic enzymes is a highly complex process. However, the constant progress in molecular and genomic techniques gave new insights on the role of regulating elements in the differential expression of ligninolytic enzymes

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in WRF. Further studies will elucidate the mechanisms of ligninolytic enzyme transcriptional regulation and provide deeper understanding of this complicated process.

Thus, the potential of ligninolytic enzymes in degradation of herbicides has not been well characterized yet, especially at the molecular level. Most of the data available correspond to the studies of different herbicide degradation by WRF, their individual ligninolytic enzymes and oxidative enzymes – redox mediator systems that are successful or less successful [6,9,10,18-21]. Few attempts have been made to propose the mechanisms of pesticide degradation (based on pentachlorophenol degradation pathways and ligninolytic enzymes action). The aim of this review is to summarize the data about herbicide degradation by WRF and their ligninolytic enzymes.

2. Modern herbicides and common regularities of their transformation

Approximately 2 million tons of pesticides are used worldwide each year [22] and play a significant role in modern agricultural practices. Approximately half of this volume is herbicides that are routinely applied to crops at rates varying from g to kg ha^{-1}. In 2010, about 907 million kg of active ingredients of herbicides was applied in the world (FAO data), and this figure continues to grow. 2019 estimates demonstrate that the herbicides market will experience both the highest growth rate as well as the highest volume traded in the next years as compared with other pesticides. The expected annual growth rate of herbicides for the given period is computed to be 6.1% [23].

Although attempts to reduce pesticide use through organic agricultural practices and the use of other technologies continue, direct and indirect exposure to pesticides is still an important health risk factor. About one-third of the agricultural products are produced by using pesticides [24]. Without pesticide application the loss of fruits, vegetables, and cereals from pest injury would reach 78%, 54%, and 32%, respectively [25].

The emergence of herbicide-resistant (HR) genetically engineered crops in 1996 made it possible for farmers to use a broad-spectrum herbicide, glyphosate, in ways that were previously impossible. From 1996 through 2011, 0.55 billion hectares of HR corn, soybeans, and cotton were grown in the USA, and in 2011, an estimated 94% of the soybean area planted, 72% of corn, and 96% of cotton were planted to HR varieties, respectively, which led to a 239 million kg increase in herbicide use [26].

Priority pesticides vary significantly for different regions and crops, and have evolved with time. The era of organic synthetic pesticides started approximately 70 years ago from DDT, 2,4-D, and such compounds as HCH, dieldrin were added to the most actively used compounds at the second wave. The assortment of modern pesticides is highly variable in trademarks and based on relatively wide (but much shorter) row of successfully commercialized active ingredients. However, the bulk of the world market is formed by a very small number of compounds, even taking into consideration their variability for different regions (really – for main crops of these regions). Atrazine, glyphosate, acetochlor, metolachlor,

tefluthrin, cyfluthrin, and, maybe, mesotrione should be considered as priority pesticides for environmental/health risks due to their wide application as protection tools in cereal agriculture. It should be noted, however, that the integral impact of two parameters, i.e., manufacturing volume and toxicity of active compounds, determines danger of different agrochemicals and necessity in their efficient decontamination.

Only a lesser part of applied pesticides reaches the target organism, with the remainder being deposited on the soil and nontarget organisms, as well as moving into the environment [27]. The metabolic fate of pesticides is dependent on their physico-chemical characteristics, field abiotic conditions, and plant and microbial communities. Transformation of pesticides includes abiotic processes (such as photolysis, hydrolysis, oxidation, and rearrangements) and chemical/biological reactions. The variety of biotransformation processes for herbicides should be considered in connection with specific features of the microenvironments in and near target organisms involved into metabolic pathways. So the key stage for determining further biotransformation of herbicides is their adsorption (and adsorption of their intermediate metabolites) to soil and soil colloids. These processes are highly important to regulate the dynamics of action for modern herbicide preparations. The ratio between free and adsorbed forms of herbicides determines the rate of their abiotic transformation. Nevertheless, enzymatic transformation (i.e., biotransformation) is the major driver of detoxification.

The classic concepts of pesticide metabolism [28,29] divide their transformation into three phases. In the first phase, the parent compounds are transformed through oxidation, reduction, or hydrolysis to more water-soluble and usually less toxic products. As a whole, oxidation (hydroxylation, dealkylation, and deamination), hydrolysis (esters, amides, and nitriles) and reduction reactions are considered as main factors for this phase. The main process of the second phase is the conjugation of the obtained derivatives to a sugar (typically glucose), glutathione, or amino acid with further increased water solubility, reduced toxicity, and support of internal transport of the metabolite for final transformation. The third phase provides further conjugation and results in nontoxic final products of metabolic pathways of the pesticides.

An important factor in the transformation of pesticides in soil is a complementary action of plants and microorganisms on them. The roles of plants may be simply characterized as reduction of toxicity, whereas microorganims are responsible for deep destruction and mineralization. The line of enzymes and catalyzed reactions for microorganisms is much wider as compared with plants. Several processes, such as dehalogenation or C-P bond cleavage, are associated mainly with microbial metabolism of pesticides. (On the other hand, however, glutathione conjugation is a typical tool for plant transformation of pesticides.) The common opinion in modern remediation biotechnology is that the tasks of detoxification cannot be solved at the plant level alone and should be based on the detailed analysis of the most efficient microbial participants of this process. A significant additional factor of interest concerning microbial detoxification is the lower cost of such technologies as compared with the alternative ones [30].

Pathways of pesticide destruction have been described in many works, both at the levels of the species responsible and the enzymes involved. Currently, the existing information is

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systematized in several sources, and Biocatalysis/Biodegradation Database of the University of Minnesota (EAWAG-BBD; http://eawag-bbd.ethz.ch) seems the most informative available tool. This database contains information on microbial biocatalytic reactions and biodegradation pathways for primarily xenobiotics. This permanently maintained and updated system collects data about hundreds of pathways, enzymes, and microorganisms, thousands of reactions and compounds of environmental interests. In addition, this database contains two supporting tools. The Pathway Prediction System predicts microbial catabolic reactions using substructure searching, a rule-base, and atom-to-atom mapping. The biotransformation rules are based on reactions found in the EAWAG-BBD or in the scientific literature. The Biochemical Periodic Table provides an overview of microbial interactions with different chemical elements. Individual element pages contain a summary of published data about microbial interactions with the selected element.

It should be noted that efficient recommendations for microbial remediation require integral knowledge about potential of individual enzymatic reactions and specific features of their interactions for different microbial species. Current information about genetic regulation of coupled reactions may significantly improve bioremediation technologies as well as contribute to empiric data about multistep detoxification with the use of different microorganisms. That is why the further consideration of fungal destruction of herbicides will provide data on integrated potential of multienzyme systems from different detoxificators rather than data about elementary catalytic reactions, but first of all about.

3. Effects of herbicides on soil fungi

There are two main teqniques of herbicide application in the field. The first one is foiliar spray, and the second is soil application. In case with soil application, the herbicide is introduced directly into the soil and so can affect soil microorganisms. However, even in case with foliar application, significant amounts of these chemicals reach the soil. Therefore, although herbicides are very useful in farming, under certain circumstances they may turn into pollutants, affecting soil microflora and deteriorating the quality of soil if there are sensitive organisms and/or if the degradation products are toxic. Among various indicators used in monitoring soil biological activity, microbial community structure seems to be the most preferred due to its sensitivity to the environmental changes. To address these concerns, the impacts of herbicides on soil microbial communities are widely studied and discussed.

In general, the recommended field rate of herbicide had no major effects on soil microorganisms, but excessive doses retard the reproduction rate of some groups of microflora and may reduce enzyme activity and populations of various microorganisms in soil, including fungi (Table 1) [31-35]. No significant changes in soil microflora were detected using phospholipid fatty acid (PLFA) profiles' analysis after atrazine, bentazon, or glyphosate application by Banks and coauthors [35]. Crouzet and coauthors [33] tested the herbicide mesotrione in chernozem soil at the rates from 0.45 to 45 mg/kg and recorded only small genetic structural shifts in the bacterial and fungal communities. Maximum dissimilarity of the bacterial and fungal genetic

structures between control and herbicide-treated soil did not exceed 12% and 28%, respectively. Martinez et al. [36] did not demonstrate any significant changes in the multiplication of bacteria and fungi following an application of sulfentrazone. Allievi and Gigliotti demonstrated no significant differences in number of aerobic bacteria in soil attributable to cinosulfuron treatment at the field rate 0.42 μg/kg after 1 and 4 weeks of incubation under laboratory conditions [31]. Possible effects of the herbicide on the specific group of microorganisms of the microbial community resulting in eventual counterbalance by the development of another group were further tested. To execute this, the individual microbial strains were isolated and their sensitivity in relation to cinosulfuron was tested. Among eighteen studied strains of aerobic bacteria from uncultivated soil, a fourth of the tested strains underwent some growth inhibition in the presence of the herbicide, and for one strain total and permanent inhibition was observed. In the case of fungi, however, only two of seventeen fungi strains underwent temporary growth inhibition. In the case of isolates from agricultural soil, neither bacterial nor fungal isolates were sensitive to the studied herbicide. The herbicide cinosulfuron was concluded to negatively affect only a few aspects of the microbial community in soil ecosystems, even at concentrations higher than those currently in use. Baćmaga and coauthors [37] also reported on the absence of adverse effects of the herbicide metazachlor at the recommended dose (0.3 mg/kg) on soil microorganisms including oligotrophic bacteria, *Azotobacter* spp. bacteria, organotrophic bacteria, actinobacteria, and fungi. When applied at excessive doses, metazachlor inhibited significantly the reproduction of all analyzed microorganisms, including fungi.

Herbicide	Effect at field rate	Effect at excessive rates	Ref.
Auxin growth regulators			
2,4-D	No effect	Stimulation	[38]
Amino acid biosynthesis inhibitors			
EPSP synthase inhibitors			
Glyphosate	No effect	nd	[35]
	Stimulation	nd	[39]
	No effect	No effect	[40]
ALS inhibitors			
Cinosulfuron	No effect	Temporary growth inhibition or no effect	[31]
Imazethapyr	No effect	Inhibition	[38]
Metsulfuron-methyl	Stimulation	nd	[41]
Nicosulfuron	Inhibition	Inhibition at intermediate doses, no effect at high doses	[42]
Sulfosulfuron	Stimulation	Inhibition	[43]
	No effect	nd	[44]
Glutamine synthetase inhibitors			
Glufosinate	Inhibition	Inhibition	[45]
Photosynthesis inhibitors			

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Herbicide	Effect at field rate	Effect at excessive rates	Ref.
Systemic herbicides inhibiting PSII			
Atrazine	No effect	nd	[35]
Isoproturon	Inhibition	nd	[46]
	Inhibition	nd	[44]
Linuron	No effect	No effect	[47]
Metribuzin	No effect	nd	[44]
Contact herbicides inhibiting PSII			
Bentazon	No effect	nd	[35]
Contact herbicides inhibiting PPO			
Brominal	Inhibition	Inhibition	[32]
	Temporary inhibition	Temporary inhibition	[48]
Sulfentrazone	No effect	nd	[36]
Lipid biosynthesis disrupters (ACC inhibitors)			
Clodinafop	No effect	nd	[44]
Pigments biosynthesis inhibitors (HPPD inhibitors)			
Mesotrione	No effect	Slightly modified the fungal genetic structures	[33]
Seedling growth inhibitors			
Alachlor	No effect	Inhibition	[49]
Butachlor	Stimulation	Inhibition	[50]
Metazachlor	No effect	Inhibition	[37]
Napropamide	Inhibition followed by stimulation	nd	[51]

ACC – acetyl-CoA carboxylase; ALS – acetolactate synthase; EPSP – 5-enolpyruvylshikimate-3-phosphate; HPPD – p-hydroxyphenylpyruvate dioxygenase; PPO – protoporphyrinogen oxidase; PSII – photosystem II
nd – no data

Table 1. Influence of different herbicides on soil fungi

The negative influence of the herbicides on fungi was also reported by Kucharski and Wyszkowska [43], who tested herbicide Apyros 75 WG (a.i. sulfosulfuron), and by Zhang and coauthors [38], who studied the effect of imazethapyr in two agricultural soils. The ratio of fungi/bacteria in the imazethapyr-treated soil tended to decrease in the initial 15 d incubation period when compared to the control, and then recovered after 30 d of incubation. Stimulation of bacterial and suppression of fungal population due to isoproturon application was reported by Nowak et al. [46]. Omar and Abdel-Sater studied the effect of soil treatment with brominal on population counts of bacteria, actinobacteria, and cellulolytic fungi in soil and found out that the herbicide significantly decreased the total number of cellulolytic fungi and most fungal species while bacterial populations in soil treated with the herbicide was promoted at field application rates and inhibited only at higher levels [32]. Pampulha et al. demonstrated a significant decrease of soil bacteria, fungi, and actinobacteria populations 40 days after glufosinate application [45].

The evidences of no effect or positive effect of the herbicides on fungi growth were also numerously demonstrated. Araújo et al. [39] proved that soil pollution with glyphosate increased populations of fungi and actinobacteria while depressing counts of the other bacteria. Kucharski and Wyszkowska [43] demonstrated a stimulating effect of sulfosulfuron on fungi in the objects treated with the recommended dose of the herbicide 8.9 μg/kg. Treatment of soil with 2,4-D butyl ester at the extremely high dose of 1000 mg/g caused a decline in culturable microbial counts, with the exception of fungal numbers, which increased over the incubation time [34]. At that, when herbicide concentration increased, the Gram-negative/Gram-positive bacteria ratio decreased dramatically in the studied soils. Soil treatment with linuron at the dosages of 4–400 mg/kg did not change the fungal numbers significantly in two agricultural soils as compared to the corresponding controls [47]. Sørensen et al. explained the observed phenomenon by the presence of linuron-degrading fungi, including different species of *Cunninghamella*, *Mortierella*, *Talaromyces*, *Rhizopus*, *Rhizoctonia*, and *Aspergillus* [52]. Along with linuron-degrading fungi, there are some soil bacteria which are able to use herbicide as a source of C and N, resulting in a significant increase in bacterial counts [53]. The latter is confirmed by increased bacterial numbers in soils treated with the high dosage of linuron [47].

He et al. studied the effects of metsulfuron-methyl on soil microorganisms by the method of microbial inoculation culture and found an inhibiting effect of the herbicide on the aerobic heterotrophic bacteria, whereas the number of tolerant fungi increased greatly in the rhizosphere after the application of metsulfuron-methyl [41]. Impact of another sulfonylurea herbicide, nicosulfuron, on the structure, abundance, and function of the soil microbial community using standardized methodologies (PLFAs, taxa-specific qPCR, and enzyme activities) was investigated by [42]. Soil concentrations of nicosulfuron exceeding 1 μg/g resulted in significant reduction of the total PLFAs, although significant reductions of the bacterial PLFAs were observed only at nicosulfuron concentration levels above 10 μg/g. A different picture was evident for fungal PLFAs with significant reductions observed only at intermediate herbicide concentration levels (1–10 μg/g) compared to the control. Besides, qPCR analysis demonstrated that fungi showed the highest sensitivity to nicosulfuron and their abundance was reduced even at the lowest concentration levels of the herbicide (0.25–1 μg/g). Finally, field experiments showed that nicosulfuron applied to the field at dose rates ×1, ×2, and ×5 of the recommended did not significantly affect either the soil microbial biomass or the abundance of fungi and bacteria or enzymatic activity. No significant changes in fungal numbers due to clodinafop introduction into the soil were observed by [43]. Wardle and Parkinson [40] reported that bacterial propagules were temporarily enhanced while actinobacteria and fungal propagule numbers were unaffected by glyphosate. Min et al. [50] reported the influences of the herbicide butachlor on microbial populations, respiration, nitrogen fixation, and nitrification and on the activities of dehydrogenase and hydrogen peroxidase in paddy soil. The results showed that the number of actinobacteria declined significantly after the application of butachlor at different concentrations ranging from 5.5 to 22 mg/kg, while that of the other bacteria and fungi increased. However, at higher butachlor concentrations the growth of fungi was retarded, and the growth of anaerobic hydrolytic fermentative bacteria, sulfate-reducing bacteria, and denitrifying bacteria was stimulated. Treatment of soil with another acetanilide herbicide, napropamide, resulted in decrease of populations of

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bacteria, while the populations of fungi displayed the decreasing, recovering, and increasing trend [51].

Detailed examination of the observed effects of the herbicides on soil fungi associated with the mode of action of herbicides (Table 1) does not reveal any interrelationships between herbicide identity and their toxicity to fungi. Though herbicides inhibiting amino acid synthesis (ALS and glutamine synthetase inhibitors), contact herbicides inhibiting PPO, and seedling growth inhibitors are seemingly the most toxic, a detailed systematic study needs to be conducted to prove or disprove this observation. Moreover, currently, no general pattern of soil microbiota responses has been inferred regarding herbicide doses applied, exposure time, soil type, or other environmental factors [40,54]. The latter results very likely from the fact that up to now most studies dealing with pesticide soil microbial toxicity were performed using methods that were not well standardized, which did not allow their comparative meta-analysis, and focused on the independent assessment of effects on population, diversity, or functional endpoints, which did not provide a comprehensive view of the toxicity of the pesticide [42]. Standardization of the advanced methodologies available in soil microbial ecology is a necessary step toward harmonization of datasets and is a prerequisite for their integration in the regulatory framework of pesticide soil microbial toxicity assessment [55]. Standards for a number of methods have been already developed and others are under development at the International Standard Organisation (ISO) by TC190/SC4/WG4 and can be found elsewhere [42]. These include:

- Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates (ISO/TS 22939)
- Determination of soil microbial diversity. Part 1: method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis (ISO/TS 22843 part 1)
- Determination of soil microbial diversity. Part 2: method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method (ISO/TS 22843 part 2)
- Method to directly extract DNA from soil samples (ISO11063)
- Estimation of abundance of selected microbial gene sequences by quantitative real-time PCR from DNA directly extracted from soil (ISO/DIS 17601)

Therefore, there is a global need for more complex investigations of the functional diversity responses and degrading activity of soil microbial communities in order to provide deeper insight for herbicide risk assessment. The combined utilization of the above standardized molecular and biochemical methods that provide data of different resolution levels guarantee an accurate estimation of pesticide-driven effects on soil microbes [42].

4. Transformation of the herbicides by white-rot-fungi

It is well documented that a wide range of pollutants including pesticides are transformed and degraded by WRF: pentachlorophenols, isoproturon, derivative of isoxaflutole, atrazine,

simazine, propazine, lindane, atrazine, diuron, terbuthylazine, metalaxyl, DDT, dieldrin, aldrin, heptachlor, chlordane, etc. [11,56-65]. This list may be expanded given the strong evidence for WRF degradation potential toward different classes of pollutants. The data on herbicide degradation by WRF are summarized partly in Table 2. It should be mentioned that a large number of works were performed using stationary conditions on liquid media and solid system fermentation conditions. However, there are contradictory data about level of herbicide degradation, role of ligninolytic enzymes in this procedure, and mechanism of degradation as well.

Fungus	Herbicide	Cultivation		Disappearance, %	References
		Type	Days		
Agrocybe semiorbicularis	Atrazine	Stat	42	40	[11]
	Diuron		42	70	
	Terbuthylazine		42	60	
Auricularia auricola	Atrazine	Stat	42	16	[11]
	Diuron		42	10	
	Terbuthylazine		42	37	
Cerrena maxima	Atrazine	Sub	40	83	[66]
Cerrena maxima& Coriolus hirsutus	Atrazine	Sub	40	78	[66]
Coriolopsis fulvocinerea	Atrazine	Sub	40	88	[66]
Coriolus hirsutus	Atrazine	Sub	40	91	[66]
Coriolus versicolor	Atrazine	Stat	42	86	[11]
	Chloronitrofen		12	30	[67]
	Diuron		42	99	[11]
	Nitrofen		12	80	[67]
	Terbuthylazine		42	63	[11]
Dichotomitus squalens	Atrazine	Stat	42	25	[11]
	Diuron		42	21	
	Terbuthylazine		42	52	
Flammulina velupites	Diuron	Stat	42	6	[11]
	Terbuthylazine		42	30	
Ganoderma lucidum	Bentazon (5 mM)	Stat	10	88	[68]
	Bentazon (20 mM)		10	55	[69]
	Bentazon (50 mM)	Sol	10	90	[68]
	Diuron (30 μM)	Stat	10	55	[69]
	Picloram		10	0	[12]
Hypholoma fasciculare	Atrazine	Stat	42	57	[11]
	Diuron		42	71	
	Terbuthylazine		42	97	
Phanerochaete chrysosporium	Atrazine	Stat	14	0	[57]
		Stat	10	60	[70]

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Fungus	Herbicide	Cultivation		Disappearance, %	References
		Type	Days		
			42	20	[11]
	Bentazon	Sol	33	55	[71]
			20	65	[72]
	Diketonitrile (derivative of isoxaflutole)	Stat	15	42	[74]
	Diuron	Stat	10	94	[75]
			42	3	[11]
	Isoproturon	Bio-beds	28	78	[76]
			100	>99	[76]
	MCPA	Sol	20	75	[73]
	Propazine		8	45	[70]
	Simazine		8	5	[70]
	Terbuthylazine		42	53	[11]
			8	95	[70]
Pleurotus ostreatus	Atrazine	Stat	42	15	[11]
	Diuron		42	12	
	Terbuthylazine		42	30	
Stereum hirsutum	Atrazine	Stat	42	57	[11]
	Diuron		42	80	
	Terbuthylazine		42	88	
Trametes sp.	Picloram	Stat	10	0	[12]
Trametes versicolor	Diketonitrile (derivative of isoxaflutole)	Stat	15	34	[74]

Stat – Stationary conditions on liquid media
Sub – Submerged cultivation on liquid media
Sol – Solid-state cultivation

Table 2. Degradation of herbicides by white-rot fungi

Several fungi, such as *Agrocybe semiorbicularis, Auricularia auricula, Coriolus versicolor, Dichomitus squalens, Flammulina velupites, Hypholoma fasciculare, Pleurotus ostreatus, Phanerochaete velutina*, and *Stereum hirsutum* have shown the ability to degrade various herbicides like atrazine, diuron, and terbuthylazine with different efficiencies [72]. *Coriolus versicolor, Hypholoma fasciculare*, and *Stereum hirsutum* degraded more than 86% of diuron, atrazine, and terbuthylazine in 6 weeks. They were also the most active in ligninolytic enzymes' production. However, the ability of WRF to degrade aromatic herbicides, diuron, atrazine, and terbuthylazine, did not correlate with their ligninolytic activity determined in the Poly R-478 decoloration test (which is used as an indicator of ligninolytic activity). The possible explanation of these results was the difference in LME patterns produced by fungi in liquid cultures. Interesting that under field trials the most effective strain *S. hirsutum* was inactive in herbicide

degradation and the other strains *C. versicolor* and *H. fasciculare* demonstrated 30% of chloropyriphos degradation in 6 weeks [72].

White-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* converted up to 35–40% of diketonitrile (a soil transformation product of the herbicide isoxaflutol) to inactive benzoic acid analogue after 15 days under stationary conditions on liquid media [74]. The level of ligninolytic enzymes, such as laccases, produced during fermentation seemed to be correlated with herbicide degradation, confirming the role of these enzymes in degradation processes. However, the authors underlined that induction of laccase production sixfold via addition of 2,5-xylidine did not lead to any significant diketonitrile cleavage increase.

It was shown that *Coriolus hirsutus*, *Cerrena maxima*, *Coriolopsis fulvocinerea*, and co-cultured *Coriolus hirsutus*/*Cerrena maxima* [66] can degrade atrazine under submerged cultivation; the herbicide removal was 77–91% after 40 days' cultivation. It is interesting to mention that negligible amounts of atrazine were found to be absorbed on mycelium. The activity of laccase was rather high, allowing the proposal of laccase participation in atrazine degradation by these fungi. This hypothesis was supported by the study of atrazine degradation in the presence of laccase inducers (guayacol and syringaldezine) under submerged cultivation [77]. The efficiency of herbicide degradation was higher in induced cultures by 78–98% and the highest level of atrazine removal was achieved for *Coriolopsis fulvocinerea* using guaiacol as an inducer.

Hiratsuka et al. [67] reported that *Coriolus versicolor* IFO 30340 degraded 30% of chloronitrofen (CNP) and 80% of nitrofen (NIP) after 12 days' cultivation under stationary conditions on liquid media. The herbicide degradation rate depended on the nitrogen concentration in the media and was higher under low nitrogen conditions, suggesting that the lignin degradative system was responsible for the herbicide degradation. However, LiP, MnP, and laccase as well as culture filtrate did not oxidize herbicides. Neither chloronitrofen nor nitrofen were oxidized by the laccase – redox-mediator system using HBT, which is a well-known laccase redox-mediator. These results draw the conclusion that extracellular ligninolytic enzymes were not involved in the initial step of CNP or NIP degradation by *Coriolus versicolor* IFO 30340. The sequential identification of products formed during the metabolism of CNP and its intermediates by *C. versicolor* enabled the authors to propose four different pathways for the degradation of CNP: aromatic hydroxylation, oxidative dechlorination, reductive dechlorination, and the reduction of the nitro group to amine. The aromatic hydroxylation to form 2,4,6-trichloro-3-hydroxy-4′-nitrodiphenyl ether and the oxidative dechlorination to form 2,4-dichloro-6-hydroxy-4′-nitrodiphenyl ether were assumed to catalyze by cytochrome P450-type enzyme(s) because these paths were efficiently shut off by the exogenous addition of piperonyl butoxide, a P450 inhibitor. The conversion of CNP to NIP by *Coriolus versicolor* IFO 30340 should be reductive dechlorination. Reductive dechlorination reactions were involved in the degradation of pentachlorophenol by *P. chrysosporium* [60]. CNP was also converted to 2,4,6-trichloro-4′-aminodiphenyl ether by *C. versicolor*. The reductive dechlorination and nitro-reduction reactions were also found as initial reactions in CNP degradation, which were enhanced upon the addition of the cytochrome P450 inhibitor. Aromatic hydroxylation and oxidative dechlorination were also observed during the fungal conversion of NIP; however, the products formed were not identified – they were assumed to be either 2, 4-dichloro-3-

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hydroxy-4′-nitrodiphenyl ether or 2, 4-dichloro-6-hydroxy-4′-nitrodiphenyl ether and 2-chloro-4-hydroxy-4′-nitrodiphenyl ether or 2-hydroxy-4-chloro-4′-nitrodiphenyl ether, respectively. The fungal conversion of NIP was also effectively inhibited by piperonyl butoxide.

Based on the result obtained, the authors assumed that cytochrome P450 played an important role in lowering the ionization potential of environmentally persistent aromatics and in providing suitable substrates for ligninolytic one-electron oxidizing enzymes for effective degradation. When diphenyl ether, 4-chlorodiphenyl ether, and 4-nitrodiphenyl ether were added to the fungal culture, 4-hydroxydiphenyl ether, 4-chloro-4′-hydroxydiphenyl ether, and 4-nitro-4′-hydroxydiphenyl ether were identified as the major products, respectively. 4-chlorophenol and 4-nitrophenol were detected in trace amounts from 4-chlorodiphenyl ether and 4-nitrodiphenyl ether, respectively, but the counterpart hydroquinone was not observed. These data suggest that the formation of phenolic products from either the A or B ring of CNP might be derived via a different pathway, and that the direct ether cleavage might not have occurred. These findings gave evidence that fungi degraded herbicides via different pathways using their multiple metabolic systems.

Ganoderma lucidum was shown to be resistant to the herbicides diuron and bentazon [69]: the upper limits were 80 μM and 20 mM, respectively. This finding can be explained by higher toxicity of the metabolites formed during diuron transformation. It was reported previously that some of the metabolites resulting from fungal transformation of diuron may be even more toxic than the parent compound [78]. *G. lucidum* was able to efficiently remove 55% of diuron and 88% of bentazon after 10 days' cultivation in liquid cultures. Both bentazon and diuron strongly improved the production of laccase by the fungus inducing one of the two laccase isoforms. Native PAGE analysis of the extracellular enzymes revealed that the improvement in the laccase activity in response to the herbicides was not due to the expression of a new laccase, but that it was due to the overproduction of an already existing isoform in the noninduced cultures. Similar results were obtained with *Trametes versicolor* and *Abortiporus biennis* [79], where their constitutive laccases were overproduced in the presence of paraquat, a quaternary nitrogen herbicide. The electrophoretic analysis of extracellular enzymes from *G. lucidum* showed that laccase1 was the dominant enzyme under noninduced conditions. Interestingly, the herbicides induced only the laccase2 isoform while the laccase1 was suppressed in these cultures. Such results suggest that laccase2 is, probably, the isoform more intensely involved in the defense system of the fungus, considering that both herbicides strongly inhibited the fungus growth. These observations show that these types of enzymes have, at least in part, an important role in the degradation of pollutants under in vivo conditions.

The comparative study of herbicide bentazon degradation by *Ganoderma lucidum* in liquid and solid-state cultures using corn cob as substrate has been performed [68]. The fungus was more resistant to herbicide and more efficient in its degradation in solid-state cultures in comparison with liquid cultures: 50 mM against 20 mM and 90% against 55%, respectively. The authors proposed two, not mutually exclusive, possible explanations: a lower availability of herbicide due to its adsorption to the insoluble substrate corn cob for this observation and the higher

activities of both laccase and Mn peroxidase in solid-state cultures compared to the liquid cultures, where the high laccase activity was detected. However, no metabolite products were found in the combined aqueous and methanolic extracts. The *G. lucidum* crude filtrates containing laccase and Mn peroxidase were shown to degrade bentazon in vitro. The experiments with addition of Mn^{2+}, ABTS, Tween 80, and H_2O_2 to crude filtrates demonstrated synergisms in bentazon degradation, suggesting that both laccase and Mn peroxidase were involved in its degradation. It is well known that ABTS mediates the oxidation of non-phenolic compounds of lignin [80] and the presence of unsaturated fatty acids (Tween 80) improves the oxidation process catalyzed by Mn peroxidases and laccases due to the production of lipid peroxyl or alkoxyl radicals [81]. The hypothetical mechanism of bentazon degradation may be the following Mn peroxidase and laccase generated lipid peroxyl or alkoxyl radicals; in the presence of these radicals Mn peroxidase oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes bentazon, whereas laccase uses ABTS as redox-mediator for bentazon oxidation. However, no degradation of picloram *G. lucidum* and *Trametes sp.* were observed in liquid cultures, maybe due to its high substitution of the aromatic ring [12]. This herbicide enhanced the production of laccase by *Trametes sp.*, whereas the enzyme production by *G. lucidum* was suppressed. The authors assumed that enzyme production inhibition could be occurring at the mRNA level after picloram has entered the cell or by enzyme modification before or after secretion [12]. The exposition of *G. lucidum* and *Trametes sp.* to picloram revealed a peculiar mechanism of transitory bioaccumulation of herbicide by both fungi.

The most studied WRF is *P. chrysosporium*, which was shown to degrade a wide range of herbicides under different conditions. MCPA and bentazon were degraded by *P. chrysosporium* at 65% and 75%, respectively, in 20 days [73]. *P. chysosporium* degraded isoproturon belonging to phenylurea groups [73,76], atrazine [70], and also diuron [82]. However, according to [57], no atrazine degradation was observed by this fungus in liquid cultures. The degradation efficiency of *P. chrysosporium* was higher in solid-state cultures in comparison with liquid ones [71,73]. Two mechanisms of herbicides degradation were proposed: the action of ligninolytic enzymes and the action of intracellular enzymes in particular cytochrome P450. In [75], the degradation of diuron by *P. chrysosporium* was studied including the identification of products formed and the evaluation of cytochrome P450's role. Two findings were of great importance: the considerable amounts of diuron, DCPMU [1-(3,4-dichlorophenyl)-3-methylurea], and DCPU [1-(3,4-dichlorophenyl)urea] found in fresh mycelia and the inhibition of diuron degradation by ABT (1-aminobenzotriazole), a cytochrome P450 inhibitor. These results confirmed the intracellular mechanism of this herbicide degradation resulting in N-demethylation. However, after 5 days concentrations of DCPMU and DCPU were higher in cultural filtrates than in mycelia extracts suggesting possible involvement of lignolytic enzymes in degradation of these metabolites. According to da Silva Coelho-Moreira et al. [75], enzymatic crude extracts supplied with combinations of veratryl alcohol H_2O_2 and Mn^{2+} did not degrade the herbicide, it is possible that DCPMU and DCPU can be further transformed by MnP.

P. chrysosporium is also able to transform atrazine, its transformation product and other s-triazine herbicides [70]. The first and main step in the chlorinated-s-triazine degradation

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pathway by the fungus was mono-N-dealkylation. Hydroxyatrazine was the main degradation product found in soils treated with atrazine and in liquid cultures. *P. chrysosporium* actively transformed hydroxyatrazine to an unknown compound that accumulated in the culture medium. It was established that the presence of both alkyl groups and chlorine at the 2-position are necessary for the mono N-dealkylation of atrazine by *P. chrysosporium*. Consequently, formation of desethylhydroxyatrazine in liquid cultures should result from hydrolysis of deethylatrazine. Experiments with terbuthylazine, atrazine, and simazine also show that the removal of the ethyl side chain is the preferential reaction, and might depend on the mass of the second alkyl group. In other words, compounds with a high-mass group linked to one amino substituent are expected to undergo a higher N-dealkylation affecting the other chain. The symmetric compounds propazine and simazine were also degraded at a slower rate than atrazine. Neither LiPs nor MnPs transformed atrazine and its N-dealkylated metabolites. It was shown that atrazine N-dealkilation decreased in the presence of cytochrome P450 inhibitor. Moreover, herbicide degradation was supported by mycelium. Therefore, the cytochrome P450 involvement in atrazine degradation was assumed. These data are in line with previously published study of atrazine degradation by *Pleurotus pulmonarius*, which involved such enzymes as lipoxygenase, peroxidase, and cytochrome P-450 [83]. Mn^{2+}, which activates these enzymes, stimulated atrazine transformation to N-dealkylated and propylhydroxylated metabolites whereas antioxidants and inhibitors of lipoxygenase and peroxidase (nordihydroguaiaretic acid) as well as cytochrome P-450 (piperonyl butoxide) suppressed its degradation.

To analyze data presented in Table 2, rate of herbicide disappearance was calculated as the ratio of disappearance (%) to the duration of degradation (days), followed by an average value calculation for every herbicide (Fig. 1). Taking into consideration the effect of cultivation conditions on herbicide degradation by fungi, only data on stationary conditions on liquid media were treated this way.

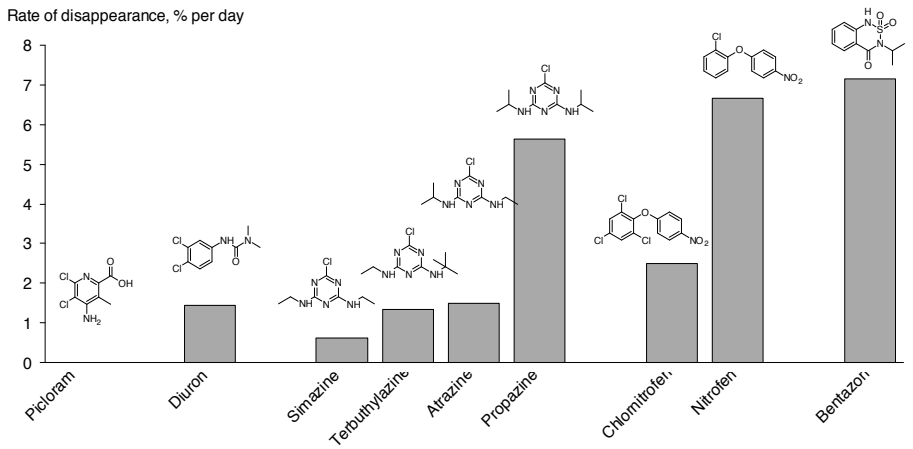

Figure 1. Relationship between rate of disappearance of herbicides and their structure.

Obtained results correspond well to the study [70], where it was established that the presence of alkyl groups is necessary for the degradation of s-triazine herbicides by *P. chrysosporium via* mono N-dealkylation. Moreover, ability of WRF fungi to degrade s-triazines seems to enhance along with increase in the amount of exactly branched alkyl groups. However, detailed quantitative structure–degradation activity studies should be conducted to prove or disprove this preliminary observation. Another important conclusion is a marked negative influence of chlorine in the herbicide molecule on the degradation rate, which can be seen from the comparison of degradation rate of nitrofen (one atom of chlorine) and clornitrofen (three atoms of chlorine) and the highest degradation rate of bentazon, which is the only chlorine-less herbicide in the presented range (Fig. 1). Therefore, data presented in Fig. 1 demonstrate clearly the barest necessity of further QSAR studies. Together with knowledge on main enzymatic pathways of herbicide degradation, the latter will improve significantly the preliminary assessment of degradation ability of WRF in relation to the herbicide of known structure.

The contradictory data about participation of ligninolytic enzymes in the herbicide degradation and transformation did not allow establishing their precise role in these processes [18,75, 81,84,85,86]. We summarized the data about efficiency of individual ligninolytic enzymes, their mixtures, and enzymes – redox-mediator systems in herbicide degradation in Table 3. As can be seen, no degradation of diketonitrile, diuron, atrazine, chloronitrofen, nitrofen, glyphosate was observed for MnP and LiP crude extracts and purified enzymes from *P. chrysosporium*, *Trametes versicolor*, and *Coriolus versicolor* even in the presence of redox-mediators [67,69,70,74,75,81]. However, MnP from *P. chrysosporium* degraded Irgarol 1081 up to 37% after 24 h [87] and LiP from *P. chrysosporium* degraded bentazon up to 100% after 4 h [71]. Moreover, bentazon was effectively transformed by laccase with catechol, laccase, and MnP crude extracts with redox-mediator ABTS, recombinant MnP [69,71,88]. Analysis of the data summarized in Table 3 draw to the conclusion that MnP, laccase, and laccase – redox-mediator systems are the most efficient tools for degradation of a wide range of herbicides – diketonitrile, glyphosate, Pesticide Mix 34, chloroxuron, atrazine, and dymron [74,81,86,89], however, with few exceptions, namely, choronitrofen and nitrofen [67]. It should be underlined that efficiency of laccase – redox-mediator systems toward different herbicides strongly depends on the redox-mediator used, which in turn depends on mechanisms of the mediators' oxidation by enzyme and the reactivity of the mediators' intermediates.

Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.
Laccase	Atrazine	No	25°C, pH 4.5	240	0	*Coriolopsis fulvocinerea*	Koroleva & Gorbatova (unpublished data)
		$[Ru(bpy)_2Cl_2]$			0		
		$[Ru(phpy)(phen)_2]PF_6$			0		
		HBT			70		
		Syringaldezine			0		
	Bentazon	Catechol	25°C, pH 4.0	0.5	100	*Polyporus pinsitus*	[88]
	Chloronitrofen	No			0	*Coriolus versicolor*	[67]
		HBT			0		

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Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.
	Diketonitrile (derivative of isoxaflutole)	ABTS	pH 3.0		0.3–0.4 nmol /(h unit)	*Trametes versicolor*	[74]
	Dymron	No	37°C	24	0	*Trametes versicolor*	[89]
		ABTS	60°C	24	>90		
		HBA			90		
		MeHBA			90		
		NNDS			>90		
	Glyphosate	No	pH 6.0, Mn^{2+} + H_2O_2 + Tween 80	24	90	*Trametes versicolor*	[81]
		No	pH 6.0, Mn^{2+} + Tween 80		90		
	Nitrofen	No			0	*Coriolus versicolor*	[67]
		HBT			0		
Laccase, immobilized	Chloroxuron	No	30°C, pH 4.5	0.5	80	*Trametes versicolor*	[86]
		3-HAA		0.5	80		
		HBT		0.3	100		
		Syrinaldehyde		0.5	80		
LiP	Atrazine	No	30°C, pH 5, veratryl alcohol + Mn^{2+} + H_2O_2	1	0	*Phanerochaete chrysosporium*	[70]
	Bentazon	No	pH 3.5, veratryl alcohol + H_2O_2	4	~100	*Phanerochaete chrysosporium*	[71]
	Chloronitrofen	No			0	*Coriolus versicolor*	[67]
		No			0	*Phanerochaete chrysosporium*	
	Glyphosate	No	pH 3.0, veratryl alcohol + Mn^{2+} + H_2O_2 + Tween 80	24	0	*Trametes versicolor*	[81]
	Nitrofen	No			0	*Coriolus versicolor*	[67]
		No			0	*Phanerochaete chrysosporium*	
MnP	Atrazine	No	30°C, pH 5, veratryl alcohol + Mn^{2+} + H_2O_2	1	0	*Phanerochaete chrysosporium*	[70]
	Bentazon	No	pH 4.5, Mn^{2+} + Tween 80	168	~700	*Aspergillus oryzae*	[71]
	Chloronitrofen	No			0	*Coriolus versicolor*	[67]
	Glyphosate	No	pH 4.5, Mn^{2+} + H_2O_2 + Tween 80	24	100	*Nematoloma frowardii*	[81]

Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.
		No	pH 4.5, Mn^{2+} + Tween 80		100		
	Irgarol 1051	No	30°C, Mn^{2+} + glucose + glucose oxidase	24	37	*Phanerochaete chrysosporium*	[87]
	Nitrofen	No			0	*Coriolus versicolor*	[67]
	Pesticide Mix 34	No	35°C, pH 4.5, Mn^{2+} + H_2O_2 + Tween 80	144	20-100	*Nematoloma frowardii*	[81]
Lac+MnP	Bentazon	ABTS	Mn^{2+} + H_2O_2 + Tween 80	24	98	*Ganoderma lucidum*	[69]
LiP+MnP	Atrazine	No	39°C, veratryl alcohol + Mn^{2+} + H_2O_2	24	0	*Phanerochaete chrysosporium*	[57]
		No	30°C, pH 5, veratryl alcohol + Mn^{2+} + H_2O_2	1	0		[70]
	Diketonitrile (derivative of isoxaflutole)	No	30°C, pH 3 or 5, H_2O_2	12	0	*Phanerochaete chrysosporium*	[74]
		1-HBT			0		
		3-HAA			0		
		ABTS			0		
	Diuron	No	pH 3.0, veratryl alcohol + Mn^{2+} + H_2O_2	24	0	*Phanerochaete chrysosporium*	[75]

Irgarol 1051 – derivate of s-triazine herbicide
3-HAA – 3-hydroxy-antranilic acid
1-HBT – 3-hydroxybenzotriazole
HBA – 4-hydroxybenzoic acid
MeHBA – methyl-4-hydroxybenzoic acid
NNDS – 1-nitroso-2naphtol-3,6-disulfonic acid
Laccase iimmobilized – Laccase iimmobilized on an electrospun zein polyurethane nanofiber via cross-linking with glutaraldehyde

Table 3. Degradation of herbicides by ligninolytic enzymes produced by white-rot fungi

In the study of atrazine degradation with purified laccase from *Coriolopsis fulvocinerea*, no herbicide degradation was observed (Koroleva & Gorbatova, unpublished data). The screening of redox mediators (syringaldezine, $[Ru(phpy)(phen)_2]PF_6$, $[Ru(bpy)_2Cl_2]$, HBT) revealed that only HBT caused the decrease in atrazine concentration in the system laccase–atrazine–redox-mediator. A more detailed study of components of the model system "atrazine/laccase/HBT" showed that HBT itself reacted with atrazine and other chlorine-containing atrazine derivatives directly, without laccase involvement, and did not interact with the atrazine

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hydroxy derivatives. It is known that HBT in aqueous solution can pass into ionic form. Therefore, it has been suggested that two products can form, both consisting of HBT and atrazine, with the formation of (-N-O-C-) bonds in position (2) of atrazine. Addition of laccase to a solution of HBT/Atr resulted in the formation of several products, one of them having a retention time matching that of HBT-Atr compound. In enzymatic reactions, two other products formed with retention times of 15.3 min and 19.4 min, which were identified as deethylatrazine (DEA) and the compound formed by the interaction of DEA and HBT. Thus, the addition of enzyme resulted in the formation of new products different from that formed in the reaction of HBT with atrazine. The model system "atrazine/laccase/HBT" was studied at different molar ratios of atrazine/mediator (9:1 to 1:9) and at two different concentrations of enzyme (0.02 μm and 1.0 μm). The deepest atrazine conversion – up to 70% in 10 days – was observed at HBT/Atr ratio of 9/1 and enzyme concentration of 0.02 μm. Proton nuclear magnetic resonance (^{1}H-NMR) and HPLC-MS/MS allowed confirming the product identification in the model systems "Atr/HBT" and "Atr/HBT/laccase": the formation of Atr-HBT in the "Atr/HBT" system, and DEA and DEA-HBT in the "Atr/HBT/laccase" system. Atr-HBT existed in two forms: protonated (M.W. 315 g/mol) and diprotonated (M.W. 316 g/mol). In the reaction "Atr/HBT/laccase" DEA is formed, as well as protonated (M.W. 287 g/mol) and diprotonated (M.W. 288 g/mol) forms of the product DEA-HBT. Based on the data obtained for the five established structures of the products, we have proposed the atrazine oxidation scheme by the "laccase/HBT" system (Fig. 2), which includes nonenzymatic and enzymatic stages (Fig. 3).

During the nonenzymatic stage, a product consisting of atrazine and HBT is formed. As the substrates and the products in the "Atr/HBT" system are in equilibrium, the addition of laccase to the reaction causes the oxidation of HBT and the formation of HBT radical. The HBT radical reacts with the Atr-HBT compound and triggers the dissociation of the (-NH-CH-) bonds, resulting in the formation of DEA-HBT and ethyl alcohol. In turn, DEA-HBT decomposes to form two products: DEA and HBT. The ability of HBT to form tautomeric forms and to directly react with atrazine suggested that HBT would degrade in the reaction mixture. However, under the proposed scheme, during the hydrolysis of DEA-HBT, DEA and HBT formed. This may be one of the reasons for the effectiveness of HBT as a redox-mediator in laccase – redox-mediator system.

The high potential of WRF as well as their ligninolytic enzymes in herbicide transformation is well documented. Nevertheless, the mechanisms of degradation and degradation pathways for many herbicides are still not explored. Further studies are needed to elucidate the mechanism of herbicide degradation by WRF and ligninolytic enzymes and identify the metabolites formed.

5. Bioremediation technologies based on application of white-rot-fungi or their extracellular enzymes

The increasing use of agricultural chemicals including herbicides results in the accumulation of these compounds and their derivatives in soil and water. Many herbicides have medium-

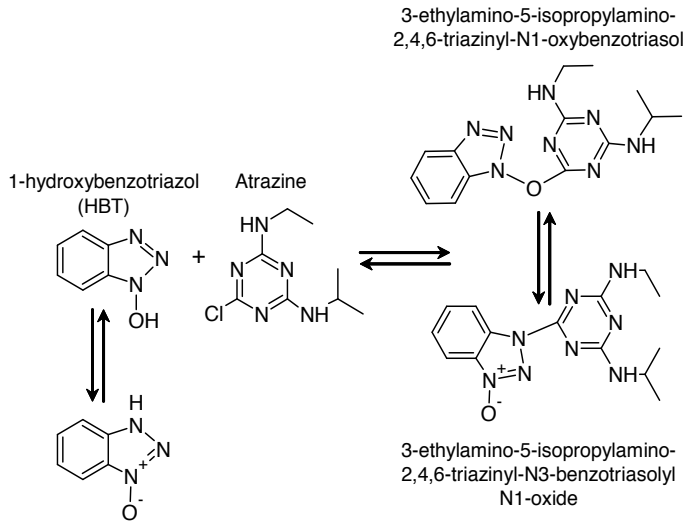

Figure 2. Scheme of atrazine oxidation in an "Atr/HBT" system.

to long-term stability in soil and so their persistence has a significant impact on the functioning of soil ecosystems. Biological decomposition of herbicides is the most important and effective way to remove these compounds. Therefore, bioremediation is now regarded as a promising strategy for the rehabilitation of polluted environments because of its cost efficiency and environmental friendliness. A detailed examination of the advantages and the disadvantages of bioremediation as well as comparison of bacteria and white-rot fungi in terms of their usage for bioremediation can be found in [9,90]. Filamentous fungi in general and white-rot fungi in particular are generally more tolerant to high concentrations of organic and inorganic toxicants as compared to bacteria [8,91]. On the other hand, white-rot fungi possess great powers of endurance under environmental stresses [91-93]. Finally, white-rot fungi are unique among eukaryotic or prokaryotic microorganisms, because they possess a very powerful extracellular oxidative lignin-modifying enzyme system, which has broad substrate specificity and is able to oxidize a fair amount of organic pollutants [91]. So, white-rot fungi are likely to be powerful prospective agents in soil bioremediation technologies [90,91]. Table 2 gives some examples of white-rot fungi that have been demonstrated to be able to degrade herbicides effectively.

Currently, more than ten species of white-rot fungi can be considered as the effective degraders of different herbicides (Table 2). Among them, *Ph. chrysosporium* and *T. versicolor* have become the most commonly used indicators in herbicide biodegradation studies due to their good degrading capacity, fast growth, and easy handling in culture [19]. Achieved efficiency of the herbicide degradation by white-rot fungi is usually very high; *Ph. chrysosporium* has been shown to decrease 2 μM atrazine in growth medium by 48% within the first 4 days of incubation [58]. Koroleva and coauthors reported that *Cer. maxima*, *Coriolopsis fulvocenerea*, and *C. hirsutus* consumed up to 50% atrazine in 5-day cultivation in the presence of the xenobiotic and at least 80–92% in 40 days [66]. According to the data presented by Bending and coauthors, maximum degradation of herbicides by *T. versicolor*, *H. fasciculare*, and *S. hirsutum* after 42 days

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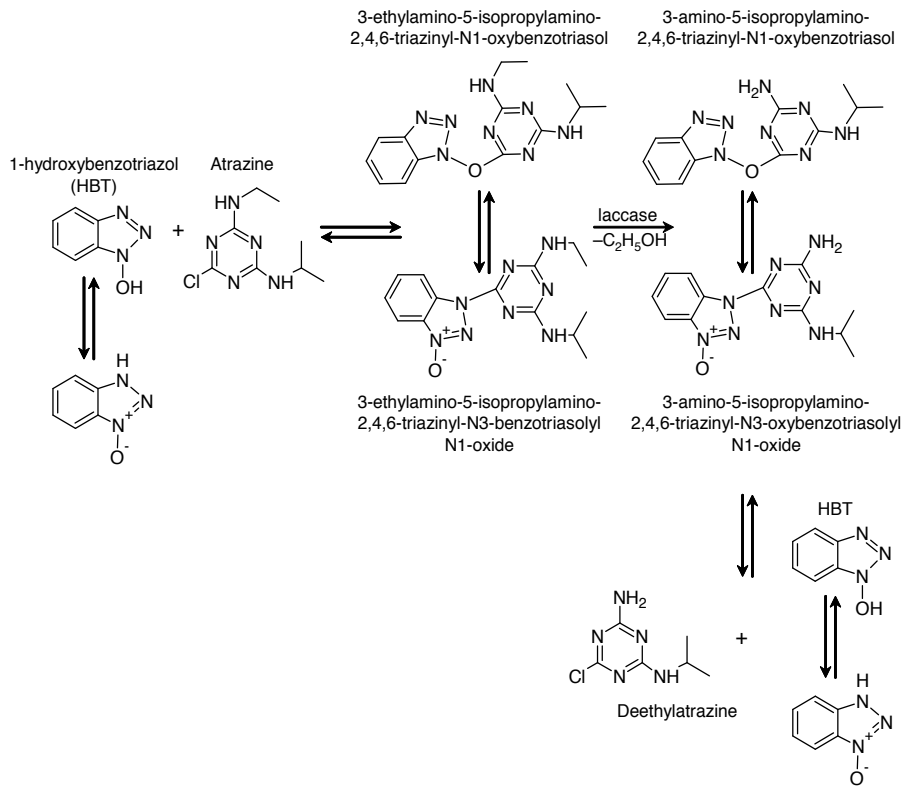

Figure 3. General scheme of atrazine oxidation in an "Atr/HBT/laccase" system.

of cultivation was above 86% for diuron, atrazine, and terbuthylazine and about 44% for metalaxyl [72]. However, the degradation efficiency depends greatly on the initial concentration of the herbicides. After 10-day cultivation of *G. Lucidum*, residual concentration of diuron was 48% when initial concentration of the herbicide was 30 μM and increased to 81% when initial concentration of the herbicide was 80 μM. Corresponding values for bentazon initial concentrations 5 and 20 μM were 61% and 85%, respectively [68]. The observed phenomenon most likely results from fungi inhibition at the excessive rates of herbicide application.

In spite of high degradation potential of white-rot fungi demonstrated in lab settings, fungi are rarely agents of choice for environmental biotechnology. The most important problem is that many research studies examine only destruction of single xenobiotic, whereas in reality mixtures of xenobiotics differing in their structure and mode are subjects for detoxification in the environment [90]. The latter can be toxic for the fungi, resulting in significant inhibition of their growth and, in turn, in the target herbicide degradation. For example, Maceil and coauthors studied effects caused by picloram on the white-rot fungi *G. lucidum* and *Trametes* sp. They found oxidative stress in the fungi induced by the herbicide and inability of the studied fungal strain to degrade picloram effectively [12]. Taking into consideration real contamination of soil with mixtures of xenobiotic compounds, studies of such multitarget

degradation have to be specially addressed [94,95]. Additionally, low bioavailability of xenobiotic and preferential use of carbon compounds other than the contaminant of interest is often among potential reasons for the general lack of success of bioremediation strategies [94]. Thus, researches are needed to develop and engineer bioremediation technologies that are appropriate for sites with complex contaminants [90].

Bending et al. [11] studied degradation of the herbicides diuron, atrazine, and terbuthylazine in the so-called biobeds inoculated with white-rot fungi. Biobeds are on-farm pesticide bioremediation constructions developed in Sweden to retain pesticide spills occurring during filling the spraying equipment and facilitate natural attenuation and are currently being evaluated in a number of other European countries [96]. Biobed matrix was prepared by mixing together barley straw, topsoil, and compost [97]. When *Cor. versicolor*, *H. fasciculare*, and *S. hirsutum* were grown in biobed matrix, they were all able to degrade the herbicides, although there were differences in the relative degrading capacities of the fungi in liquid and biobed media. Wirén- Lehr et al. studied the degradation of isoproturon in biobeds with and without inoculation with *Ph. chrysosporium*. They determined that after 28 days in biobeds inoculated with the fungus, total extractable isoproturon decreased by 78%, and after 100 days by >99%, i.e., the herbicide had disappeared in the biobeds, while in noninoculated biobeds that value after 100 days was 76% [76].

To confine white-rot fungi within the toxic environment, a new methodology, which uses growing on potato dextrose agar only or dextrose agar enriched with adsorbent materials, was explored for the removal of xenobiotics from wastewaters [98-100]. This methodology, assuming combined adsorption of organic toxicants followed by their removal, debarred mycelium entrance in the contaminated medium, and excreted fungal enzymes could degrade only the contaminants that entered the medium. The advantage of this methodology is that it avoids additional contamination of the environment with fungal hyphae and exudates, scarce aeration for fungal activity, the continuous contaminant supplying for fungal activity, and the fungus can be easily removed with the agar medium. The developed methodology was successfully employed for simultaneous removal of five coexisting xenobiotics including herbicide linuron from wastewaters, using isolates of *T. versicolor* and *S. hirsutum* as biodegradation agents [101]. Treatments with *T. versicolor* removed linuron from wastewater completely or almost completely, with removal percentages varying from 95% to 100%, depending on adsorbent material used. *S. hirsutum* did not show a great potential to degrade linuron, although after 20 days, the amount of compound removed by this fungus was statistically greater than the control in some cases. Of special importance was that the wastewater used in the study was a real leachate collected from a municipal landfill. Loffredo et al. [100] demonstrated also degradation of linuron from a similar municipal landfill leachate by the described approach, using the fungus *P. ostreatus*.

An approach assuming an introduction preliminary inoculated matrix rather than fungal inoculum itself seems to be very promising with respect to the contaminated soil as well. Recently, some companies have included the use of ligninolytic fungi for soil remediation into their programs, for example, "EarthFax Development Corp." in the USA and "Gebruder Huber Bodenrecycling" in Germany [90]. EarthFax Engineering, Inc. and its affiliate EarthFax

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Development Corp. have demonstrated the degradation of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs) in soil under pilot-scale conditions through the use of sawdust thoroughly colonized with the white-rot fungus *P. ostreatus*. After 282 days of the experiment, the degradation values of the dioxins varied from 61% to 80%, depending on the PCDD structure [http://www.earthfax.com]. The obtained results indicate the clear necessity of further examination of inoculated-matrix-based approach to develop technologies of remediation of the herbicide polluted environments. Overall, future research should be geared toward narrowing the gaps between fungal-based bioremediation in laboratory and environmental applications [20,102].

Although the mechanisms involved in herbicide degradation by white-rot fungi are not clearly understood, most scientists emphasize the role of the extracellular enzymes of LMS in the degradation of the herbicides by WRF [8,9,103,104]. An alternate pathway of detoxification is the use of a cytochrome P450 monooxygenase system, independent of the production of ligninolytic peroxidase enzymes [105]. To date, the latter was clearly proved only for the fungus *Ph. chrysosporium* and so, the capacity of extracellular lignin-modifying enzymes to degrade herbicides has been mainly investigated [18].

The three principal classes of these enzymes, namely lignin peroxidases, manganese peroxidases, and laccase, are likely able to degrade not only phenols, chlorophenols, and aromatic amines but also non-phenolic compounds such as phenylureas, phenylamides, and s-triazines [100], and the presence of redox active mediators can enlarge the range of compounds that could be oxidized by these enzymes [106]. Table 3 gives some examples of herbicide degradation by the above enzymes. Laccase from *Ph. chrysosporium* converts the diketonitrile isoxaflutole to the acid in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) acting as a redox mediator at pH 3 [74]. LiP and MnP produced by *Ph. chrysosporium* degraded isoproturon in both in vivo and in vitro experiments [73]. MnP from *Ph. chrysosporium* oxidized bentazon in the presence of Mn(II) and Tween 80 [71]. The herbicide glyphosate was degraded by laccase of *N. frowardii* MnP and *T. versicolor* in the presence of ABTS as a mediator [81]. These reports clearly show the potential application of extracellular enzymes of white-rot fungi in the treatment of soil and wastewater contaminated with herbicides [18].

Although application of LiP, MnP, and laccase for degradation many organic pollutants including aromatic compounds, pentachlorophenol, dyes, chlorophenol, urea derivatives, etc., is well known [21,107], only a few papers concerning herbicide degradation specifically are available. Bollag suggested that it is possible to enhance the natural process of xenobiotic binding and incorporation into the humic substances by adding laccase to the soil [108]. Chlorinated phenols and anilines were transformed in soil by oxidative coupling reactions mediated by laccase or peroxidase [109]. The herbicide bentazon was incubated with laccase or peroxidase in the presence of guaiacol, which was used as a model humic monomer. Although bentazon did not react significantly with guaiacol in the presence of the enzymes solely, the reaction of the herbicide with guaiacol was almost complete in 30 min in the presence guaiacol and ferulic acid, which are the electron donor co-substrates in most of the oxidative coupling reactions [88]. Laccase from *C. unicolor* displayed a lower efficiency in oxidizing the herbicides 2,4-D and simazine, but the enzyme oxidized efficiently 2,4-DCP, a derivative of 2,4-D [110]. González Matute and coauthors demonstrated the ability of extracellular enzymes

of *A. blazei* to degrade the herbicide metsulfuron-methyl [111]. Crude enzyme preparation was obtained from spent compost, which was the residual compost waste generated by the fungi cultivation industry. The degradation of the herbicide was confirmed using bioassay experiments with oil rape (*Brassica napus* L.) as the plant indicator. The detoxifying capacity of the preparations containing lignolytic enzymes and products of coal solubilization by *T. hirsuta* and *T. maxima* in respect to the herbicide atrazine was demonstrated by Klein et al. [112].

Pizzul et al. conducted degradation tests using purified MnP from *N. frowardii* and LiP and laccase from *T. versicolor* in combination with different mediators in order to estimate transformation of glyphosate and Pesticide Mix 34. The latter included the herbicides atrazine, chlorotoluron, chloroxuron, diuron, fenuron, isoproturon, linuron, metamitron, metazachlor, metobromuron, metolachlor, metoxuron, metribuzin, monolinuron, prometryn, simazine, terbuthylazine, and terbutryn. Authors demonstrated that MnP and laccase were very efficient in the transformation of glyphosate and led to (aminomethyl) hosphonic acid formation (metabolite of glyphosate) and accumulation. In addition, simultaneous degradation of 22 pesticides in a mixture was obtained by the action of MnP in the presence of Tween 80 and $MnSO_4$, with degradation values varying from 20% to 100% [81].

However, real contaminated environments contain usually a wide number of different chemical species, some of which can inhibit fungal growth and/or reduce enzymatic activity [106]. To preserve the enzyme's activity and stability over time, immobilization of the enzyme can be used. Immobilized enzymes have usually a long-term and operational stability, being very stable toward physical, chemical, and biological denaturing agents. Furthermore, they may be reused and recovered at the end of the process [85,91]. Immobilization of laccase from *T. versicolor* onto a hydrophilic PVDF microfiltration membrane allowed obtaining the membrane grafted with 220U enzyme activity used in a filtration module to transform a phenylurea herbicide derivative 2-HF (*N'*,*N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea) from waste water. No 2-HF was found in permeate 5 min after the beginning of the experiment [85]. Laccase from *T. versicolor* immobilized on an zein polyurethane nanofiber *via* cross-linking with glutaraldehyde completely degraded the phenylurea herbicide chloroxuron within 30 min in the presence of 1 mM 1-hydroxybenzotriazole [86].

Both LiP, MnP and laccase may behave as powerful catalysts in the biodegradation of herbicides. However, their full-scale application for remediation of polluted environments is still limited. The latter may derive from several drawbacks and disadvantages of the enzymes application such as enzyme instability in the environment and loss of their activity. Immobilization of the enzymes is likely to be a promising way to develop a successful approach for the remediation of the herbicide polluted sites.

6. Conclusion

The high potential of WRF as well as their ligninolytic enzymes in herbicide transformation is analyzed in the present review. Analysis of literature data on degradation rate of herbicides by WRF demonstrated enhancing WRF degradation capacity along with increase content of branched alkyl groups in the herbicide molecule. However, detailed quantitative structure–

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degradation activity studies should be conducted to prove or disprove this preliminary observation. Therefore, the mechanisms of herbicides degradation by WRF for many herbicides are still not explored and degradation pathways are not established, including the identification of the metabolites formed.

The ligninolytic enzymes MnP and laccase were shown to behave as powerful catalysts in the biodegradation of herbicides. However, their full-scale application for remediation of polluted environments is still limited. The latter may derive from several drawbacks and disadvantages of the enzymes application such as enzyme instability in the environment and loss of their activity. Immobilization of the enzymes is likely to be a promising way to develop a successful approach for the remediation of the herbicide polluted sites.

The potential of ligninolytic enzymes in the degradation of herbicides is beginning to be characterized at the molecular level. The constant progress in molecular and genomic techniques has provided new insights on the role of regulating elements in the differential expression of ligninolytic enzymes in WRF. Further studies will elucidate the mechanisms of ligninolytic enzymes' transcriptional regulation and provide deeper understanding of this complicated process.

It should be noted that efficient recommendations for microbial remediation need integral knowledge about potential of individual enzymatic reactions and specific features of their interactions for different microbial species. Current information about genetic regulation of coupled reactions may improve significantly bioremediation technologies, as well as empiric data regarding multistep detoxification with the use of different microorganisms.

Analysis presented in this review confirms the important role of white-rot fungi as participants in herbicide decontamination in the environment and the prospects of the development of new biotechnological preparations on the basis of fungal enzymes. The most important tasks in the development of bioremediation technologies and recent results of key stakeholders in this field are discussed.

7. Abbreviations

ABTS – 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)

ACC – acetyl-CoA carboxylase

ALS – acetolactate synthase

DEA – deethylatrazine

DCPMU – 1-(3,4-dichlorophenyl)-3-methylurea

DCPU – 1-(3,4-dichlorophenyl)urea

^{1}H-NMR – proton nuclear magnetic resonance

HPPD – p-hydroxyphenylpyruvate dioxygenase

HR – herbicide-resistant crop

EPSP – 5-enolpyruvylshikimate-3-phosphate

LiP – lignin peroxidase

LMS – lignin modifying system

MnP – Mn peroxidase

PCDD – polychlorinated dibenzo-p-dioxin

PCDF – polychlorinated dibenzo furan

PCR – polymerase chain reaction

PLEL – phospholipid ether lipids

PLFA – phospholipid fatty acid

PPO – protoporphyrinogen oxidase

PSII – photosystem II

ROS – reactive oxygen species

WRF – white-rot fungi

XRE – xenobiotic responsive element

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Chapter 10

Herbicide Metabolism in Weeds — Selectivity and Herbicide Resistance

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Additional information is available at the end of the chapter

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Abstract

The metabolic detoxication/bioactivation pathways, the levels and activity of enzymes, and endogenous cofactors mediating these reactions in crops have been well documented; however, much less evidence has been accumulated in weed species. The herbicide metabolism as a selectivity factor is summarized with special attention to acetyl-CoA carboxylases (ACCase)-inhibiting aryloxyphenoxypropionate, protoporphyrinogen IX oxidase (PPO) inhibitor, carotenoid biosynthesis inhibitor clomazone, and acetolactate synthase (ALS) inhibitor imidazolinone and sulfonylurea herbicides in various weed species. The metabolism-based herbicide resistance related to these herbicide classes is also discussed along with the role and level of metabolizing enzymes and cofactors in weed species.

Keywords: Herbicide, metabolism, selectivity, resistance, weed species

1. Introduction

Metabolism, or biotransformation of herbicides, resulting in detoxication or bioactivation of the parent molecules, is a major factor in herbicide resistance and selectivity in plants. Selectivity, the principal basis of herbicide usage, is influenced by many factors such as application methods, differential absorption, translocation, sequestration in plants, and, at subcellular levels, differences in active site sensitivity, as well as rate of metabolism. Among the factors affecting the active internal concentration of the herbicide, the rate of metabolism seems to be of major importance in the selective action, which, in turn, depends on the activity of detoxifying enzymes and concentration of endogenous substrates. Plants metabolize herbicides through various intermediates, mostly to more polar products and insoluble bound residues. The metabolism of herbicides may occur as a three-phase process in plants. Phase I is primary metabolism to convert biologically active molecules into less active compounds

(detoxication) but occasionally into more phytotoxic metabolites (bioactivation). Phase I reactions include oxidation, reduction, and hydrolysis and yield phenolic, *N*-demethylated, carboxylic acid compounds. In phase II reactions, phase I products are converted to even less toxic water-soluble conjugates by glycosyl, glutathione, or amino acid conjugation. In phase III metabolism, conjugates from phase II are transformed to practically nontoxic secondary conjugates or insoluble bound residues.

Much is known about herbicide metabolism in crop plants and the effect of biotransformation products on the biological activity. Metabolism that confers herbicide tolerance in crops also occurs in weeds. Metabolic pathways and rates in both crops and weeds must often be considered together to understand the metabolic basis for crop selectivity. The metabolic detoxication/bioactivation pathways, the levels and activity of enzymes, and the endogenous cofactors mediating these reactions in crops have been well documented [1–3]; however, much less evidence has been accumulated in weed species. On the other hand, repeated use of herbicides with similar chemistry may lead to the selection of herbicide-resistant biotypes with an enhanced capacity to degrade herbicides. Weed species that have evolved resistance to herbicides due to enhanced metabolic capacity have been a major issue [4]. Target-site resistance develops by mutation within a gene coding for an herbicide target-site enzyme or by overproduction of the target enzyme. Non-target-site resistance involves mechanisms that minimize the amount of herbicidally active molecule reaching the target site by reduced uptake and translocation, increased sequestration, and enhanced metabolism.

This chapter provides an overview of herbicide metabolism in weeds as a selectivity factor with special attention to ACCase-inhibiting aryloxyphenoxypropionate, protoporphyrinogen IX oxidase (PPO or protox) inhibitor, carotenoid biosynthesis inhibitory clomazone, and acetolactate synthase (ALS) inhibitor imidazolinone and sulfonylurea herbicides. Moreover, the metabolism-based herbicide resistance is also examined with the abovementioned chemistry of herbicides. Finally, the role and level of metabolizing enzymes and cofactors in weed species are discussed.

2. Metabolism and selectivity of ACCase inhibitor aryloxyphenoxypropionate herbicides in weed species

Aryloxyphenoxypropionates such as diclofop-methyl, fenoxaprop-ethyl, fluazifop-butyl, haloxyfop-methyl, and quizalofop-ethyl are highly selective postemergence herbicides for the control of graminaceous weeds. These herbicides are known inhibitors of the acetyl-CoA carboxylases (ACCase) which are crucial for the biosynthesis of fatty acids catalyzing the production of malonyl-CoA from acetyl-CoA and CO_2 [5].

Diclofop-methyl selectivity between tolerant wheat and susceptible wild oat (*Avena fatua*) may be a function of its placement on the plant and the rate of herbicide metabolism, but the ability of wheat to detoxify the herbicide by aryl hydroxylation was found as the primary selective factor [6]. Diclofop-methyl was hydrolyzed rapidly to diclofop acid in both species (Table 1). Neither compound accumulated in the tissues of either species. The major reactions in wheat are the aromatic ring hydroxylation of the 2,4-dichloro-phenyl moiety of the herbicide,

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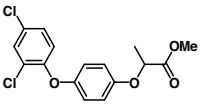
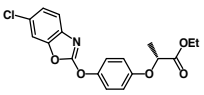
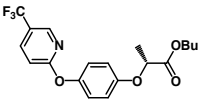
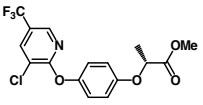
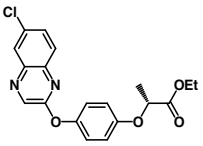
followed by conjugation to form an acidic aryl glycoside. Metabolism of diclofop acid in wheat is catalyzed by cytochrome P450-dependent hydroxylase [7]. In wild oat, a carbohydrate conjugation of the carboxylic group in the diclofop produced a neutral glycosyl ester. The sugar moiety was not characterized positively in either conjugate. The fate of foliarly applied diclofop-methyl was determined in intact plants of barnyardgrass (*Echinochloa crus-galli*), a susceptible grass, proso millet (*Panicum miliaceum*), a moderately susceptible grass, and longspine sandbur (*Cenchrus longispinus*), a tolerant grass [8]. Plant extracts contained diclofop-methyl, diclofop, and unknown water-soluble conjugates. The amounts of water-soluble metabolites were consistent with the tolerance of these species to the herbicide. The ratio of unhydrolyzed diclofop-methyl to diclofop was higher in sensitive species except for tolerant soybeans, indicating that soybeans may possess an additional mechanism or mechanisms for detoxication. The results support the hypothesis that differential metabolism provides a basis for selectivity to foliarly applied diclofop-methyl.

Following widespread applications of ACCase-inhibiting herbicides, resistance to these graminicides developed [4]. In cereal and leguminous grain crop areas of South Australia, a large number of populations of annual ryegrass (*Lolium rigidum*) have developed resistance to diclofop-methyl following selection with this herbicide. However, several diclofop-resistant biotypes also exhibit resistance to various herbicide chemistries such as the aryloxyphenoxy-propionate and cyclohexanedione graminicides, the sulfonylurea herbicides, the dinitroaniline type trifluralin, and, to a lesser extent, the triazinone metribuzin. Mechanism of resistance to the postemergent graminicide diclofop-methyl was corroborated using resistant (SLR 31) and susceptible (SLR2) biotypes of rigid ryegrass (*Lolium rigidum*) [9]. In both biotypes, diclofop-methyl was rapidly demethylated to the herbicidally active metabolite diclofop acid, which, in turn, was metabolized to ester and aryl-*O*-sugar conjugates. Resistant plants had a slightly greater capacity to form inactive sugar conjugates. Despite these differences, resistant plants retained 20% of the herbicidally active diclofop acid 8 days after treatment (DAT) (Table 1), whereas susceptible plants, which were almost lethally injured, retained 10% more diclofop acid. The small differences in the amount of the active and inactive metabolites are unlikely to account for a 30-fold difference in sensitivity to the herbicide at the whole-plant level. The diclofop-related physiological and biochemical differences between susceptible ryegrass biotype SLR 2 and resistant ryegrass biotype SLR 31 seem to be their differential abilities to recover membrane polarization, the higher portion of diclofop acid in shoots of susceptible plants, and a higher capacity of shoots of resistant plants to form conjugated metabolites. Target site-based resistance is the most commonly reported mechanism of resistance to the ACCase-inhibiting herbicides [10]. A resistant VLR 69 biotype of *Lolium rigidum*, resistant to members of at least nine herbicide classes with five mode of action, and a susceptible biotype, VLR 1 with no history of herbicide application, were used to clarify mechanisms responsible for diclofop-methyl resistance. It was found that in addition to enhanced metabolism of PS II-, ALS-, and ACCase-inhibiting herbicides, VLR 69 contains an ACCase that is insensitive to the aryloxyphenoxypropanoate herbicides. This enzyme is between 4- and 29-fold resistant to the aryloxyphenoxypropanoate herbicides, but showed no resistance to sethoxydim and tralkoxydim of ACCase inhibitory cyclohexanedione herbicides. In addition to possessing a resistant ACCase, the resistant biotype shows enhanced metabolism of diclofop-methyl. The susceptible biotype had metabolized 51% of the herbicide to products other than diclofop acid 48 hours

after treatment (HAT), whereas the resistant biotype had metabolized 62% of the herbicide (Table 1).

Herbicide	Structure	% of metabolized herbicide (% of acid form)	Weed species	Ref.
Diclofop-methyl		97 (20) 1 DAT	*Avena fatua*	[6]
		53 (28) 1 DAT	*Ecbinocbloa crus-galli*	[8]
		52 (20) 1 DAT	*Panicum miliaceum*	
		74 (13) 1 DAT	*Cencbrus longispinus*	
		97 (30) 8 DAT	*Lolium rigidum*	[9]
		95 (20) 8 DAT	biotype SLR 2a	
			biotype SLR 31b	
		62 (49)[c] 2 DAT	*Lolium rigidum*	[10]
		51 (38)[c] 2 DAT	biotype VLR 69d	
			biotype VLR 1e	
Fenoxaprop-ethyl		28 (n/a) 3 DAT	*Digitaria ischaenum*	[11]
		92 (85) 1 DAT	*Digitaria ischaenum*	[12]
		96 (35) 3 DAT	*Echinochloa phyllopogon*	[14, 15]
		96 (10) 3 DAT	susceptible biotype	
			resistant biotype	
Fluazifop-butyl		98 (73) 24 DAT	*Agropyron repens*	[16]
		90 (76) 10 HAT	*Digitaria sanguinalis*	[18]
		92 (63) 10 HAT	susceptible biotype	
			resistant biotype	
Haloxyfop-methyl		74 (25) 2 DAT	*Sorghum bicolor*	[19]
		80 (6) 2 DAT	*Setaria glauca*	
Quizalofop-ethyl		92 (38) 5 DAT	*Elytrigia repens*	[20]
		87 (42) 5 DAT	Biotype 2f	
			Biotype 10g	
		62 (29) 4 DAT	*Setaria glauca*	[21]

[a]Susceptible biotype.

[b]resistant to herbicides including diclofop-methyl, haloxyfop-methyl, haloxyfop-ethoxyethyl, fluazifop-butyl, quizalafop-ethyl, fenoxaprop-ethyl, trifluralin, alloxydim, sethoxydim, chlorsulfuron, metsulfuron methyl, and triasulfuron.

[c]% of diclofop-methyl plus diclofop acid in parenthesis.

[d]resistant to members of at least nine herbicide classes with five mode of action.

[e]susceptible biotype with no history of herbicide application.

[f]less sensitive on the basis of leaf chlorosis.

[g]sensitive on the basis of leaf chlorosis.

Table 1. Metabolism of aryloxyphenoxypropionate herbicides in weed species

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Basis for sensitivity differences among small crabgrass (*Digitaria ischaemum*), oat, and wheat to fenoxaprop-ethyl may be due to differences in the metabolism of fenoxaprop-ethyl among these species [11]. Differential response of crabgrass and oat may be partially explained by differences in absorption and translocation. Metabolism studies indicated that roughly two-thirds of recovered radioactivity in wheat was bound to insoluble constituents or was converted to polar metabolites (Table 1). The amount of these fractions was found only 28% and 40% in crabgrass and oat, respectively. Both unextractable and polar metabolites represent detoxified forms of the parent herbicide. The metabolites proposed were the *O*-glucoside of hydroxy-6-chloro-2,3-dihydro-benzoxazol-2-one, hydroxy-6-chloro,2,3-dihydro-benzoxazol-2-one, and hydroxy-6-chloro-2,3-dihydro-benzoxazol-2-one. The results indicated that the primary selective action of fenoxaprop-ethyl may be due to differences in the metabolism of fenoxaprop-ethyl among crabgrass, oat, and wheat. In another attempt to reveal detoxification pathways of the fenoxaprop-ethyl in wheat, barley, oat, and crabgrass, *in vitro* metabolism studies with excised shoots were carried out [12]. The fenoxaprop-ethyl was rapidly hydrolyzed to fenoxaprop in all four species. However, in oat and crabgrass, the radioactivity remained mainly in the form of fenoxaprop, while in wheat and barley, fenoxaprop underwent rapid displacement of the phenyl group by glutathione (GSH) and/or cysteine resulting in production of *S*-(6-chlorobenzoxazole-2-yl)-glutathione (GSH conjugate), *S*-(6-chlorobenzoxazole-2-yl)-cysteine (cysteine conjugate), and 4-hydroxyphenoxy-propanoic acid. The GSH conjugate also may be catabolized to form *S*-(6-chlorobenzoxazole-2-yl)-cysteine (cysteine conjugate) which was subsequently metabolized to an unidentified metabolite which was speculated to be the *N*-glucoside of the cysteine conjugate (*S*-(6-chlorobenzoxazole-2-yl)-cysteine *N*-glucoside). 4-Hydroxyphenoxy-propionic acid was further metabolized to yield a glucoside conjugate, which upon hydrolysis with acid or β-glucosidase yielded 4-hydroxyphenoxy-propionic acid. The results indicate that GSH, cysteine, and glucose conjugation have a major role in the metabolic detoxification and selectivity mechanism of fenoxaprop-ethyl in grass species. Later, the same authors reported that the amounts of glutathione (GSH) and cysteine are higher in grass species that are moderately tolerant, such as wheat, and moderately susceptible, such as barley and triticale, to fenoxaprop-ethyl than in species that are very susceptible to the herbicide, such as oat, wild oat (*Avena fatua*), yellow foxtail (*Setaria glauca*), large crabgrass (*Digitaria sanguinalis*), and barnyardgrass (*Echinochloa crus-galli*) [13]. *In vitro* experiments at physiological pH demonstrated that fenoxaprop-ethyl may conjugate with GSH nonenzymatically. Therefore, the nonenzymatic conjugation of fenoxaprop-ethyl with glutathione may be an important mechanism for tolerance of some grasses to this herbicide.

The possible mechanism(s) of resistance to fenoxaprop-P-ethyl in late watergrass (*Echinochloa phyllopogon*), a noxious weed of rice, was examined by comparing the absorption, translocation, metabolism of fenoxaprop-ethyl and ACCase susceptibility to fenoxaprop acid (FA) in resistant (R) and susceptible (S) biotypes [14, 15]. Studies of the *in vitro* inhibition of ACCase minimized any differential active site sensitivity as the basis of resistance to fenoxaprop-ethyl. R biotype absorbed more herbicide differences between 3 and 48 h after application than S biotype, but no differences were found in the translocated amounts. Fenoxaprop-ethyl was rapidly de-esterified by hydrolysis in plants to yield FA, which is the active form of the parent compound, and is followed by the formation of water-soluble metabolites. The R biotype

produced 5-fold less FA and approximately 2-fold more nontoxic (polar) metabolites 48 hours after treatment than the S biotype (Table 1). The enhanced GSH and cysteine conjugation was considered as the major mechanism of resistance of the R biotype against fenoxaprop toxicity.

Only few details are available on metabolic fate of fluazifop-butyl in weed species. Fluazifop acid was the major metabolite in quackgrass *(Agropyron repens* = *Elymus repens*) plants treated with fluazifop-butyl, comprising 73% of the extractable radioactivity 24 days after treatment (DAT) [16]. Similarly, in *E. repens*, less than 25% of extractable radioactivity contained metabolites other than fluazifop and fluazifop acid 2 days after treatment [17].

A population of crabgrass (*Digitaria sanguinalis*) has evolved resistance to the herbicide fluazifop-P-butyl following treatment six times of this herbicide. The resistant biotype was found resistant to other aryloxyphenoxypropanoate herbicides such as haloxyfop-methyl and quizalofop-P-ethyl, and to a lesser extent to the cyclohexanedione herbicide sethoxydim, but was not resistant to clethodim [18]. ACCase enzymes extracted from plants of both resistant and susceptible biotypes were equally sensitive to both chemistries of herbicides. Absorption of fluazifop-butyl and translocation of the radiolabel from ^{14}C fluazifop-butyl were similar in plants of both biotypes. Leaves of both resistant and susceptible species rapidly hydrolyzed fluazifop-butyl to fluazifop acid. However, a more rapid rate of metabolism of fluazifop acid was shown in the resistant plants to unknown metabolites. Enhanced metabolism of fluazifop acid which is more phytotoxic than its ester analog was postulated as a mechanism of resistance.

Metabolism of haloxyfop-methyl in intact plants of shattercane (*Sorghum bicolor*) and yellow foxtail (*Setaria glauca*) resulted in a rapid hydrolysis of the parent herbicide to haloxyfop acid in all three species [19]. Haloxyfop-methyl levels 48 and 96 h after application were below 25%, and significant quantities of polar products were detected. Nevertheless, in shattercane, which is more susceptible than yellow foxtail, a higher level (30% 2 DAT) of haloxifop acid was observed than in the yellow foxtail (~10%) at the same time (Table 1). This may explain the observed selectivity between the tolerant and the susceptible weed species.

Selective action of quizalofop-ethyl in a sensitive (Biotype 10) and a less sensitive (Biotype 2) quackgrass (*Elytrigia repens*) biotypes was partially attributed to differential rates of metabolism [20]. Conversion of the relatively inactive applied form (quizalofop-ethyl) to the biologically active form (quizalofop) occurred rapidly and further conversion of quizalofop to polar metabolites occurred more slowly. However, in both biotypes, the level of quizalofop was similar regardless of time, indicating that differential sensitivity of acetyl-CoA carboxylase, which is the molecular target of quizalofop, could be involved in determining the overall response of the biotypes to quizalofop. De-esterification of quizalofop-P-ethyl into quizalofop-P acid was rapid in the treated leaf of yellow foxtail (*Setaria glauca*) [21]. Further metabolism resulted in a glucose conjugate and a phenolic metabolite. Four days after treatment, the parent herbicide accounted for 38% of all metabolites, while the acid, the glucose conjugate, and the phenolic derivative accounted for 29%, 28%, and 4% of all metabolites, respectively (Table 1).

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3. Metabolism and selectivity of protoporphyrinogen IX oxidase inhibitory herbicides in weed species

Protoporphyrinogen oxidase (PPO), the last common enzyme in heme and chlorophyll biosynthesis, is the target of several classes of herbicides acting as inhibitors in both plants and mammals [22]. PPO inhibitor herbicides inhibit the enzyme, protoporphyrinogen oxidase (called also as protox), which is essential for the synthesis of chlorophyll. Susceptible plants accumulate toxic levels of protoporphyrinogen IX (proto IX) which reacts with oxygen and light to form singlet oxygen. Singlet oxygen causes rapid lipid peroxidation, membrane destruction, desiccation, and death. PPO inhibitors belonging to different chemical families have been developed as wide-spectrum agricultural herbicides. PPO inhibitory diphenyl ethers, *N*-phenylphthalimides, oxadiazoles, oxazolidinediones, phenylpyrazoles, pyrimidindiones, thiadiazoles, and phenyltriazolinones are all herbicidally active molecules. These compounds have been shown as environmentally safe molecules exhibiting low mammalian toxicities, low application rates with broad herbicidal activity controlling both monocot and dicot weeds, rapid onset of action, and long-lasting effect [23].

Diphenyl ether herbicide types, acifluorfen and lactofen, are used postemergence in soybean for selective control of annual morning glory and other broadleaf weed species. The rate of metabolism of acifluorfen was inversely related to susceptibility of plants such as common ragweed (*Ambrosia artemisiifolia*) > common cocklebur (*Xanthium pensylvanicum*) > soybean to the herbicide [24]. The more rapid penetration and translocation, coupled with slower metabolism of acifluorfen by the weed species in comparison to soybean, may account for the difference in susceptibility of the weeds and soybean to acifluorfen. However, metabolites were not identified in this study. Another research with [chlorophenyl-^{14}C]- and [nitrophenyl-^{14}C]acifluorfen showed that the diphenyl ether bond was rapidly cleaved by the attack of homoglutathione (hGSH) in soybean leaves and resulted in two major types of metabolites [25]. Catabolism of the initially formed hGSH conjugate (*S*-(3-carboxynitrophenyl)-γ-glutamyl-cysteinyl-β-alanine) of carboxynitrophenyl moiety of the herbicide yielded a cysteine conjugate (*S*-(3-carboxy-4-nitrophenyl)cysteine), while the 2-chloro-4-trifluoromethylphenol formed after diphenyl ether cleavage by hGSH conjugated to D-glucose which was further metabolized by malonyl conjugation, resulting in the malonyl-glucose metabolite. Experiments to clarify selectivity of acifluorfen and lactofen in pitted morning glory (*Ipomoea lacunosa*) and ivyleaf morning glory (*Ipomoea hederacea*) showed that translocation and metabolism of acifluorfen were minimal in both morning glory species [26]. However, decreased absorption of acifluorfen may account for greater tolerance of ivyleaf morning glory to acifluorfen. The translocation and metabolism of lactofen were also minimal in both morning glory species. Apparently, pitted morning glory and ivyleaf morning glory convert lactofen to acifluorfen inside treated leaves. The degree of this conversion was similar for both species. Quantities of other lactofen metabolites were less than 3% in both morning glory species. The results indicated that pitted morning glory would be more effectively controlled by acifluorfen than lactofen on the basis of differential penetration of these two herbicides into treated leaves.

The phenyl-triazolinone, carfentrazone-ethyl, is a selective postemergence herbicide against troublesome weeds such as morning glories (*Ipomoea spp*) and velvetleaf (*Abutilon theophrasti*) in soybean fields. The herbicide was more rapidly metabolized in the crop than in the weed species, with 27%, 54%, and 60% of the parent compound remaining in soybean, ivyleaf morning glory (*Ipomoea hederacea*), and velvetleaf, the most sensitive species, respectively (Table 2) [27]. The free acid metabolite, carfentrazone, was produced by all species and accounted for 21–27% of the absorbed herbicide. At least two unidentified metabolites were observed in each species. Unknown metabolites were four to five times more abundant in soybean than in the weed species. Both carfentrazone-ethyl and carfentrazone were potent inhibitors of PPO with high binding constants (258 nM and 285 nM, respectively). Based on metabolism studies, the selectivity of carfentrazone ethyl was partially attributed to the lower accumulation of proto IX in soybean than in the weeds which was associated with the enhanced ability of soybean to metabolize more carfentrazone than the weeds.

Sulfentrazone, also a phenyl-triazolinone herbicide, exhibits activity toward weeds commonly associated with soybeans. Consistent with field observation, sicklepod (*Senna obtusifolia*) exhibited considerable tolerance to sulfentrazone, and coffee senna (*Cassia occidental*) showed relatively high sensitivity to this herbicide in greenhouse tests [28]. There is little sulfentrazone metabolized in the roots of sicklepod and coffee senna, indicating that most of the radioactivity reaching the foliage is the parent molecule. There were differences in the ability of sicklepod and coffee senna to metabolize sulfentrazone in the foliage. After 9 h, only 8% of the parent compound remained in sicklepod, whereas coffee senna contained almost 83% (Table 2). The tolerance of sicklepod to sulfentrazone is primarily due to a relatively high rate of metabolism of the herbicide compared to coffee senna. The primary detoxification reaction appeared to be oxidation of the methyl group on the triazolinone ring, resulting in the formation of the more polar hydroxymethyl derivative. Although the biological activity of the hydroxymethyl and carboxylic acid derivatives of sulfentrazone is not known, it has been reported that the methyl group on position 3 of the triazolinone ring is necessary for maximum biological activity and that its replacement by other substituents resulted in 3- to 6-fold decrease in biological activity. Further studies on the metabolism of root-absorbed sulfentrazone in peanut, prickly sida (*Sida spinosa*), and pitted morning glory (*Ipomoea lacunosa*) indicated that all plant species are able to metabolize sulfentrazone [29]. One day after treatment, 5%, 27%, and 32% of the radiolabeled sulfentrazone in the shoots of peanut, prickly sida, and pitted morning glory, respectively, was present as unmetabolized sulfentrazone. The initial concentrations of sulfentrazone in the shoots at 3 and 6 HAT correspond to reported tolerance levels; peanut was the most tolerant among these species, whereas prickly sida and pitted morning glory were moderately tolerant and susceptible to the herbicide, respectively. On this basis, tolerance in peanut is largely due to its ability to rapidly metabolize sulfentrazone.

The *N*-phenylphthalimide, flumioxazin, a preemergence herbicide, is used for weed control in peanut. Flumioxazin also acts by inhibiting protoporphyrinogen oxidase. Experiments to investigate the basis of differential tolerance of peanut, ivyleaf morning glory (*Ipomoea hederacea*), and sicklepod (*Senna obtusifolia*) to root-absorbed flumioxazin were conducted [30]. No translocation of absorbed radiolabel from the herbicide was observed at all harvest times.

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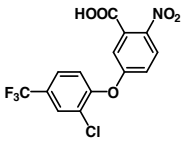
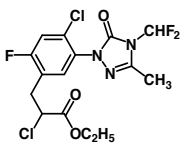
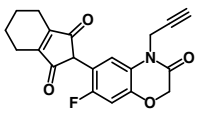
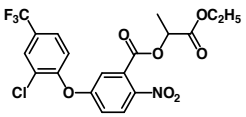
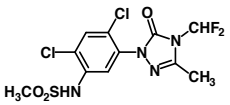
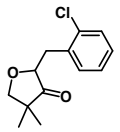
Herbicide	Structure	Metabolized herbicide, % (time after treatment)	Weed species	Ref.
Acifluorfen		44 (7 DAT)	*Ambrosia artemisiifolia*	[24]
		55 (7 DAT)	*Xantbium pensylvanicum*	
		4 (4 DAT)	*Ipomoea lacunose*	[26]
		11 (4 DAT)	*Ipomoea hederacea*	
Carfentrazone-ethyl		46 (1 DAT)	*Ipomoea hederacea*	[27]
		39 (1 DAT)	*Abutilon theophrasti*	
Flumioxazin		59 (3 DAT)	*Ipomoea hederacea*	[30]
		76 (3 DAT)	*Senna obtusifolia*	
Lactofen		19 (4 DAT)	*Ipomoea lacunose*	[26]
		19 (4 DAT)	*Ipomoea hederacea*	
Sulfentrazone		73 (1 DAT)	*Sida spinosa*	[29]
		68 (1 DAT)	*Ipomoea lacunosa*	
		92 (9 HAT)	*Senna obtusifolia*	[28]
		17 (9 HAT)	*Cassia occidentalis*	
Clomazone		50 (4 DAT)	*Amaranthus hybridus*	[35]
		60 (4 DAT)	*Amaranthus retroflexus*	
		55 (4 DAT)	*Amaranthus lividus*	
		35 (3 DAT)	*Amaranthus retroflexus*	[36]
		54 (3 DAT)	*Abutilon theophrasti*	
		84 (7 DAT)	*Echinocloa oryzoides*	[37]
		N/Aa	*Echinocloa phyllopogon*	[38]
		N/Ab	resistant biotype	
			sensitive biotype	

[a]Resistant plants accumulated 6- to 12-fold more of the monohydroxylated metabolite than susceptible plants.

[b]susceptible plants accumulated 2.5-fold more of the phytotoxic metabolite 5-ketoclomazone.

Table 2. Metabolism of protoporphyrinogen oxidase (PPO) inhibitor herbicides and metabolism of the carotenoid biosynthesis inhibitor clomazone in weed species

Ivyleaf morning glory contained the highest portion (41%) of unmetabolized herbicide 3 DAT, whereas sicklepod and peanut contained only 24% and 11% parent herbicide, respectively (Table 2). Results indicated a slower metabolism of flumioxazin by susceptible ivyleaf morning glory as compared with tolerant sicklepod and peanut. Nevertheless, no metabolites were reported in this study. Under aerobic conditions in an aquatic environment, hydrolytic cleavage of flumioxazin and separation of the phthalimido and benzoxazin moieties at the amine bridge are the major degradation reactions [31]. Metabolism studies in rats revealed that cytochrome P450-related monooxygenases are involved in the metabolism, yielding 3-OH- and 4-OH-flumioxazin derivatives [32, 33].

4. Metabolism of carotenoid biosynthesis inhibitor clomazone in weeds

Clomazone belongs to the group of isoxazolidinones, and acts by inhibiting the biosynthesis of photosynthetic pigments of both chlorophyll and carotenoids. Clomazone is not a protox inhibitor herbicide. Clomazone inhibits the 1-deoxy-D-xylulose 5-phosphate (DXP) synthase, the first enzyme of the non-mevalonate isoprenoid pathway in plastids which generates isopentenyl pyrophosphate for the biosynthesis of terpenes and terpenoids [34]. As a consequence of clomazone action impaired chloroplast development and pigment loss occur in susceptible plants. At higher light intensities, reactive singlet oxygen initiates membrane lipid peroxidation in the absence of carotenoids or at extremely reduced carotenoid levels.

Differential metabolism or differential rate of metabolism of clomazone did not appear to explain the tolerance of soybean (48% clomazone metabolized in 4 days) and smooth pigweed (*Amaranthus hybridus*, 50%) or the susceptibility of redroot pigweed (*Amaranthus retroflexus,* 60%) and livid pigweed (*Amaranthus lividus,* 55%) to this herbicide (Table 2) [35]. Tolerant soybeans and *Amaranthus hybridus* absorbed less root-applied clomazone than sensitive species after 4 days. It was postulated that clomazone may be a proherbicide that is metabolized to an active form by both tolerant and susceptible plants and differences at the site of action (carotenoid biosynthesis) may account for the selectivity. Differences in clomazone uptake, distribution, and metabolism among corn, soybean, smooth pigweed (*Amaranthus hybridus*), and velvetleaf (*Abutilon theophrasti*) were either insignificant or poorly correlated to selectivity and, therefore, cannot account for the tremendous differences in clomazone sensitivity among these species [36]. These observations indicate, indirectly, that differences at the site of action may account for selectivity.

Rice, a relatively tolerant species, and early watergrass (*Echinochloa oryzoides*), a relatively susceptible species, were exposed to clomazone to determine biotransformation of the herbicide [37]. More metabolized residue was measured in watergrass compared to rice (84% vs. 68%). Metabolism yielded hydroxylated derivatives, β-D-glucoside conjugates, and several other unidentified polar metabolites in both plants, but higher metabolite concentrations were detected in watergrass. The level of 5-ketoclomazone, the active metabolite of the parent compound, was significantly higher in early watergrass than in rice (21 and 5.7 pmol/g, respectively). Selectivity of clomazone between rice and the weed species is likely due to

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differential metabolism, when the susceptible watergrass metabolized higher amount of clomazone than the tolerant rice. This result suggested that metabolism activated clomazone to herbicidally more active derivative. *Echinochloa phyllopogon* (late watergrass) is a major weed of California rice that has evolved cytochrome P450-mediated metabolic resistance to different herbicides with multiple modes of action. Evaluation of the differential clomazone metabolism with resistant and susceptible biotypes of late watergrass was carried out to explore whether enhanced oxidative metabolism also confers clomazone resistance in *E. phyllopogon* [38]. Late watergrass plants hydroxylated mostly the isoxazolidinone ring of clomazone, and clomazone hydroxylation activity of resistant biotypes was higher than that of susceptible plants. The major metabolites were the mono- and dihydroxylated derivatives of the isoxazolidinone ring. In resistant plants, 6- to 12-fold more monohydroxy metabolite was detected than in susceptible plants. On the other hand, susceptible plants accumulated 2.5-fold more of the herbicidally active 5-ketoclomazone (Table 2). Oxidative metabolism appears to confer multiple herbicide resistance to *E. phyllopogon* with cross-resistance to clomazone by enhanced herbicide metabolism and less concentration of the phytotoxic active metabolite in resistant plants.

5. Metabolism and selectivity of acetolactate synthase (ALS) inhibitor herbicides in weed species

The endogenous ALS (also known as acetohydroxy acid synthase, AHAS) gene is involved in the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine), catalyzing the formation of 2-acetolactate or 2-aceto-2-hydroxybutyrate [39]. ALS is the site of action of several structurally diverse classes of herbicides such as sulfonylureas, imidazolinones, and triazolopyrimidine sulfonamides [40]. ALS inhibitors are quite unique inhibitors since they do not show structural similarity to the natural substrates, such as pyruvate and α-ketobutyrate, cofactors, such as thiamine diphosphate and flavin adenine dinucleotide, and allosteric effectors, such as valine, leucine, and isoleucine, of the enzyme. Inhibition of ALS results in deficiency of the amino acid pool and triggers a decrease in protein biosynthesis, which eventually leads to reduced rate of cell division. This process eventually kills the plants after showing symptoms in meristematic tissues where biosynthesis of amino acids primarily takes place [41].

5.1. Metabolism of imidazolinone herbicides in weeds

The imidazolinones are important ALS-inhibiting herbicides. The most significant members of this chemistry are imazamethabenz-methyl, imazaquin, imazethapyr, imazapyr, and imazamox. The basic structural requirements for this class include an aromatic/pyridine ring with 5′-carboxylic acid or carboxylic ester function as well as an adjacent *ortho*-imidazolinone ring. The crop selectivity of imidazolinones is primarily related to the differential metabolism of the herbicide between the crops and targeted weeds [40]. The wide range of selectivity of this herbicide series is probably a function of the balance of oxidative and hydrolytic metabolism at substituents other than the imidazolinone ring. Imidazolidinone-resistant crops

contain a selective mutation in the ALS gene which encodes an ALS enzyme that no longer binds these herbicides, although metabolism may play a role in determining the level of tolerance of the resistant crop.

Imazamethabenz-methyl, actually a racemic mixture of *meta* and *para* 2-imidazolinone toluates in the ratio of 3:2, is an imidazolinone-type herbicide controlling wild oat (*Avena fatua*) in maize and wheat. The unesterified carboxylic acids are also herbicidally active, exhibiting no selectivity. The initial metabolism by hydrolytic activation of the esters of the separate isomers resulted in hydrolysis of ester only in wild oat among these species 2 weeks after foliar treatment. The hydrolysis of herbicidally more active *meta*-isomer produced a 2- to 3-fold greater concentration of the free acid than the *para*-isomer [42]. In maize and wheat, detoxication takes place *via* rapid oxidation of the aryl methyl group to the corresponding alcohol followed by glucose conjugation. Apparently, the primary mechanism of resistance to imazamethabenz-methyl in wild oat is due to reduced metabolism of imazamethabenz-methyl to the biologically active imazamethabenz. *Meta*-isomer was metabolized to the acid form to a greater extent in the susceptible biotype than in the resistant biotype [43].

Imazaquin is a broad-spectrum herbicide developed for the use in soybean. Imazaquin can be used both preemergence and postemergence on both broadleaf and grass weeds. In order to understand the selectivity between crop plant and weed absorption, translocation and metabolism of imazaquin in soybean (*Glycine max*), common cocklebur (*Xanthium strumarium*), and velvetleaf (*Abutilon theophrasti*) were investigated [44]. Imazaquin was metabolized rapidly by soybean and velvetleaf but appeared to be metabolized slowly by cocklebur. The order of tolerance of these three species to imazaquin was soybeans > velvetleaf > cocklebur. This order of tolerance was directly correlated in young plants with the half-life of imazaquin within the tissue. Soybean metabolized imazaquin more rapidly (half-life 3 days) than velvetleaf (half-life 12 days), which was more rapid than cocklebur (half-life 30 days) (Table 3). Velvetleaf exhibited increased tolerance to imazaquin with age, which was attributed partially to greatly reduced absorption of the herbicide by older leaves and more rapid metabolism of the herbicide. The rate of metabolism was greatest in older plants, the half-life of imazaquin decreasing from 12 days at the cotyledonary stage to 4.5 days at the four-leaf stage. However, no identified metabolite was reported. Basis for greater imazaquin tolerance of entireleaf morning glory (*Ipomoea hederacea*) than pitted morning glory (*Ipomoea lanucosa*) to postemergence applications of imazaquin initiated uptake, translocation, and metabolism studies [45]. Entireleaf morning glory metabolized slightly more imazaquin than pitted morning glory in treated leaves. The difference of tolerance of these species is attributed to reduced absorption and translocation and increased metabolism of the herbicide in the entireleaf morning glory (Table 3). Metabolism data indicate that imazaquin susceptibility of pitted and entireleaf morning glory represent a moderate susceptibility similar to velvetleaf. Results indicated that imazaquin was translocated by both xylem and phloem in pitted and entireleaf morning glory. However, less absorption and translocation of imazaquin and/or its metabolites in entireleaf morning glory than in pitted morning glory probably contribute to its greater tolerance to foliar applications.

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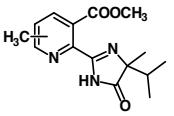
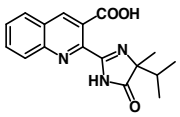
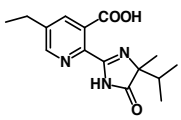
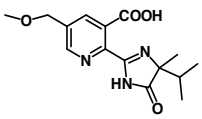
Herbicide	Structure	Half-life	Metabolized herbicide (%)	Weed species	Ref.
Imazamethabenz-methyl			72%[a] 14 DAT	*Avena fatua*	[42]
			49%[b] 7 DAT 54%[c] 7 DAT	*Avena fatua* susceptible biotype resistant biotype	[43]
Imazaquin		30 days 12 days		*Xanthium strumarium* *Abutilon theophrasti*	[44]
			30% 8 DAT 42% 8 DAT	*Ipomoea lacunose* *Ipomoea hederacea*	[45]
Imazethapyr		14 h 24 h 32 h		*Desmodium tortuosum* *Cassia obtusifolia* *Amaranthus retroflexus*	[46]
			14%[d] 8 DAT 47%[e] 8 DAT	*Euphorbia esula*	[47]
			81%[f] 14 DAT 68%[f] 14 DAT	*Ambrosia artemisiifolia* *Ambrosia trifida*	[48]
Imazamox		42 h 84 h		*Secale cereal* *Aegilops cylindrica*	[49]
		7.7 h		*Myriophyllum spicatum*	[50]

[a]28% Parent herbicide and 8% acid.

[b]51% parent herbicide and 13% acid.

[c]28% parent herbicide and 8% acid.

[d]% of metabolized herbicide in the leaves.

[e]% of metabolized herbicide in the roots.

[f]% of metabolized herbicide in the treated leaf.

Table 3. Metabolism of imidazolinone herbicides in weed species

Sicklepod (*Cassia obtusifolia*) and Florida beggarweed (*Desmodium tortuosum*) are troublesome weeds in peanut and soybean production. Evaluation of the differential response of these species as well as redroot pigweed (*Amaranthus retroflexus*) following foliar and/or root applications of imazethapyr showed that redroot pigweed was the most sensitive, with sicklepod and Florida beggarweed being intermediate [46]. The half-life of foliar-applied imazethapyr was 6.6 days in soybean, 6.5 days in peanut, 14.4 days in Florida beggarweed, 24.0 days in sicklepod, and 32.1 days in redroot pigweed (Table 3). The tolerance of these species to foliar-applied imazethapyr was related to the half-life of foliar-applied imazethapyr within the plants. Nevertheless, no identified metabolites were reported. Leafy spurge (*Euphorbia esula*) is an introduced, herbaceous, perennial weed that infests large areas of

rangeland in Canada. Metabolism studies revealed that greater than 90% of the imazethapyr was unmetabolized 2 DAT in leafy spurge [47]. Crown, roots, and adventitious shoot buds had metabolized an average of 61, 36, and 47% of the imazethapyr, respectively, while only 14% was metabolized in the treated leaf 8 DAT. The primary metabolite was postulated as 5-hydroxyethyl-imazethapyr. Two metabolites of imazethapyr were observed in both common (*Ambrosia artemisiifolia*) and giant ragweed (*Ambrosia trifida*) [48]. These metabolites were identified as the α-hydroxyethyl analog of imazethapyr and its glucose conjugate. Common ragweed showed a consistently higher rate of imazethapyr metabolism to the glucose conjugate than giant ragweed.

Jointed goatgrass (*Aegilops cylindrica*) and feral rye (*Secale cereal*) respond differently to imazamox. Jointed goatgrass appeared to be susceptible, and feral rye was tolerant to foliar application [49]. Biological half-lives for imazamox in jointed goatgrass and feral rye were determined on a whole-plant basis. The half-life of imazamox was 42 h in feral rye and 84 h in jointed goatgrass (Table 3). Differential response can be attributed to differences in both translocation and metabolism. Feral rye translocated more imazamox to root tissue and exuded a large proportion of radiolabel into the sand media. Eurasian water milfoil (*Myriophyllum spicatum)* is a submersed invasive species. Approximately 70% of the absorbed imazamox was found in the bound fraction 24 HAT, while 10% detected as soluble metabolites [50]. Only 19% remained as intact imazamox. The metabolism study indicated 69% of absorbed ^{14}C-imazamox was found in the bound fraction 144 HAT, while 12% appeared as soluble metabolites, and only 21% as intact imazamox. Based on predicted values, the half-life of imazamox in Eurasian water milfoil was short (7.65 h) (Table 3).

5.2. Metabolism of sulfonylurea herbicides in weeds

Sulfonylureas represent a great advance in crop protection and have revolutionized herbicide research and weed control in the 1980s by introducing an unprecedented mode of herbicide action. The high potency of sulfonylureas decreased the previously applied high herbicide rates from kg/ha to as low as 1 g/ha. These molecules possess remarkably low mammalian toxicity, and advantageous environmental properties. The target site for sulfonylurea herbicides is also the ALS, the first common enzyme responsible for the biosynthesis of the branched-chain amino acids. Sulfonylureas are generally extremely potent inhibitors of ALS, regardless of plant species, and differential sensitivities at the target site play little, if any, role in their selective action [2]. Rather, differential metabolism has been implicated in their crop selectivity.

A major factor responsible for the selectivity of chlorsulfuron as a postemergence herbicide for small grains is the ability of the monocot plants to metabolize the herbicide [51]. Tolerant monocotyledonous plants such as wheat, oats, barley, wild oats (*Avena fatua*), annual bluegrass (*Poa annua*), johnsongrass (*Sorghum halapense*), and giant foxtail (*Setaria faberii*) rapidly metabolize chlorsulfuron to a polar, inactive product. This metabolite was characterized as the O-glycoside of chlorsulfuron in which the phenyl ring underwent hydroxylation followed by sugar conjugation. Sensitive broadleaf cleavers (*Galium aparine*) showed no metabolism of chlorsulfuron. Metabolism of chlorsulfuron took place in both roots and shoots in the root-

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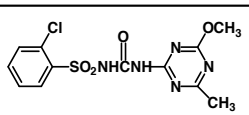
treated moderately susceptible chamomile (*Matricaria chamomilla*) and in susceptible Johnny–jump-up (*Viola tricolor*) at different rates [52]. The more sensitive Johnny–jump-up metabolized less herbicide to a polar water-soluble sugar conjugate in shoots than chamomile. The basis for differences in response of eastern black nightshade (*Solanum ptycanthum*), a tolerant species, and velvetleaf (*Abutilon theophrasti*), a susceptible species, to foliar-applied chlorsulfuron is also the differential rate of degradation [53]. Little metabolism of chlorsulfuron occurred in the sensitive, broadleaf species of velvetleaf. The rapid metabolism of chlorsulfuron in eastern black nightshade would appear to be responsible for the tolerance. The ability of flax (*Linum usitatissimum*) and black nightshade (*Solanum nigrum*) to metabolize chlorsulfuron was studied to determine if metabolism contributes to their tolerance to chlorsulfuron [54]. Shoot-treated plants metabolized more than 90% of parent herbicides. The major metabolite contained hydroxylated 4-methyl group of the triazine ring of chlorsulfuron. A second major metabolite was determined to be a carbohydrate conjugate of the hydroxymethyl derivative. Plants were more tolerant to 4-hydroxymethyl chlorsulfuron applications than to chlorsulfuron. These results suggest that metabolism may be the basis of selectivity to chlorsulfuron for tolerant broadleaf plants as well as for grasses. In the case of grasses, metabolism occurs on the phenyl ring [51], whereas in broadleaves, it occurs on the heterocyclic ring.

Resistance to ALS inhibitor herbicides in weeds was first discovered in 1987 [55, 56]. Since then numerous weed species have become resistant to sulfonylureas and imidazolidines. Several mutations in the ALS gene are capable of conferring resistance to ALS inhibitor herbicides [57]. Metabolism studies with cross-resistant (SLR31) and two susceptible (VLR1 and VLR6) biotypes of rigid ryegrass (*Lolium rigidum*) proved that there was no difference in the metabolite profiles from susceptible and resistant ryegrass [58]. Metabolism rate of chlorsulfuron in cross-resistant ryegrass SLR31 was approximately double than in the susceptible biotype (VLR1). Half-life of chlorsulfuron was 6 h in the cross-resistant biotype as compared to 12 h in the susceptible species (Table 4). The increased rate of detoxification of chlorsulfuron in the cross-resistant SLR31 biotype was found to be solely related to chlorsulfuron resistance since the diclofop-methyl-resistant biotype VLR6, which in turn, is susceptible to chlorsulfuron, metabolized chlorsulfuron at the same rate as the chlorsulfuron and diclofop-methyl-susceptible biotype VLR1. Metabolism of chlorsulfuron in wheat took place at a higher rate than any ryegrass biotype having half-life of 2 h. Major metabolite of chlorsulfuron metabolism was identical in wheat and ryegrass biotypes, the glycosylated derivative of chlorsulfuron hydroxylated in the phenyl ring [51]. The metabolic detoxication of chlorsulfuron was faster in both roots and shoots of the resistant rigid rygrass biotype SR4/84 than in susceptible biotype SRS2, and the chlorsulfuron sensitivity between these biotypes could be explained by differential rate of metabolism [59]. Despite the correlation between enhanced metabolism and reduced plant sensitivity, no quantitative agreement can be described. The 4-fold increase in rate of metabolism (R/S, 4) resulted in 23-fold increase of chlorsulfuron tolerance of the resistant biotype (R/S, 23). Environmental factors can also affect plant injuries and metabolism rates caused by ALS-inhibiting herbicides. The major pathway of the chlorsulfuron metabolism proposed was a phenyl ring hydroxylation followed by glycosylation, while the sulfonylurea bridge cleavage results in 2-chloro-benzenesulfonamide and 2-amino-4-methyl-6-methoxy-triazine metabolite as minor products.

A biotype VLR69 of rigid ryegrass (*Lolium rigidum*) resistant to some ALS exhibited greater capacity to detoxify chlorsulfuron than the susceptible VLRl population [60]. The half-life of chlorsulfuron in the resistant culms was 3 versus 6 h in the susceptible culms. The difference in rate of metabolism between the two biotypes was similar to that observed between the resistant biotype SLR31 and susceptible biotype VLR1 [58]. Uptake of chlorsulfuron into the cut shoots was similar for both biotypes. The metabolites observed in VLR69 have the same high-performance liquid chromatography (HPLC) elution profile as those of SLR 1 and VLRI, indicating that the enhanced detoxification was not due to the production of novel metabolites. A biotype of *Lolium rigidum* Gaudin (VLR69) showed multiple resistances to at least nine dissimilar herbicide chemistries. ALS, the target site for chlorsulfuron, was found sensitive to chlorsulfuron in VLR69 biotype, and only about 5% of the population contained a chlorsulfuron-resistant ALS. Studies supported the enhanced chlorsulfuron metabolism in the resistant biotype. While 22% of the herbicide was metabolized 6 HAT by the resistant biotype, only 8% was metabolized by the susceptible biotype VLR1 (Table 4) [61].

Chlorimuron-ethyl is a highly active sulfonylurea herbicide for preemergence and postemergence use in soybeans. Studies on soybean selectivity to chlorimuron-ethyl showed that the selectivity was not based on differential active site sensitivity [62]. ALS from tolerant soybeans is just as sensitive to chlorimuron-ethyl as ALS preparations from diverse sensitive weeds. While the metabolic half-life of chlorimuron ethyl in soybean was 1–3 h following foliar application, in redroot pigweed (*Amaranthus retroflexus*), common cocklebur (*Xanthium pensylvancium*), and common morning glory (*Ipomoea purpurea*), half-life values ranged between 24 and >48 h. It is interesting to note that the methyl ester analog of chlorimuron-ethyl is metabolized much more slowly by soybeans (half-life > 12 h) (Table 4). The metabolism of chlorimuron-ethyl in soybean seedlings yielded homoglutathione conjugate as the primary metabolite by displacement of the pyrimidinyl chlorine with the cysteine sulfhydryl group of homoglutathione [63]. Minor metabolite of chlorimuron-ethyl was its de-esterified free acid. These metabolites were found inactive against plant ALS, and the tolerance of soybean to chlorimuron-ethyl was demonstrated as a result of rapid metabolism to herbicidally inactive products.

Herbicide	Structure	Half-life, h or metabolized herbicide, %	Weed species	Ref.
Chlorsulfuron		92% 1 DAT	*Poa annua*	[51]
		92% 1 DAT	*Avena fatua*	
		>80% 1 DATa	*Sorghum halapense*	
		>80% 1 DATa	*Setaria faberii*	
		0%	*Galium aparine*	
		69% 6 DAT	*Viola tricolor*	[52]
		18% 6 DAT	*Matricaria chamomilla*	
		81% 3 DAT	*Solanum ptycanthum*	[53]

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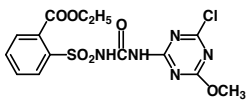
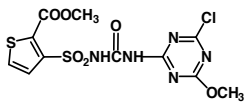
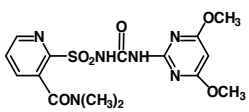
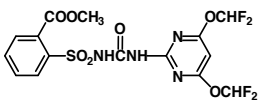
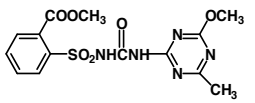
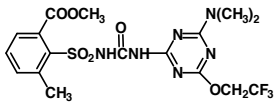
Herbicide	Structure	Half-life, h or metabolized herbicide, %	Weed species	Ref.
		7% 3 DAT	*Abutilon tbeophrasti*	
		92% 1 DAT	*Linum usitatissimum*	[54]
		92% 1 DAT	*Solanum nigrum*	
		31% 8 HAT	*Lolium rigidum*	[58]
		54% 8 HAT	biotype VLR1[b]	
		33% 8 HAT	biotye SLR31[c]	
			biotype VLR6[d]	
		1[f] and 3[g]	*Lolium rigidum*	[59]
		4[f] and 13[g]	biotype SR4/84	
			biotype SRS2	
		3	*Lolium rigidum*	[60]
		6	biotype VLR69[b]	
			biotype VLR1[e]	
		22% 6 HAT	*Lolium rigidum*	[61]
		8% 6 HAT	biotype VLR69[b]	
			biotype VLR1[e]	
Chlorimuron-ethyl		>30	*Xanthium pensylvanicum*	[62]
		>30	*Amaranthus retroflexus*	
		>48	*Xanthium pensylvanicum*	[63]
		24–48	*Amaranthus retroflexus*	
		20–24	*Ipamoea purpurea*	
Thifensulfuron-methyl		50% 3 DAT	*Abutilon theophrasti*	[65]
		50% 3 DAT	*Anoda cristata*	
		>48	*Amaranthus retroflexus*	[63, 64]
		>48	*Chenopodium album*	
		>48	*Abutilon theophrasti*	
		30	*Ipomoea purpurea*	
Nicosulfuron		31% 3 DAT	*Sorghum halapense*	[66]
		>72	*Sorghum bicolor*	[67]
		36	*Eriochloa villosa*	
		39% 3 DAT	*Sorghum halapense*	[70]
		60% 3 DAT	*Echinochloa crus-galli*	
		59% 3 DAT	*Setaria faberii*	
		70% 3 DAT	*Solanum ptychanthum*	
		7% 3 DAT	*Brachiaria platyphylla*	[68]
		47% 3 DAT	*Cynodon dactylon*	[69]

Herbicide	Structure	Half-life, h or metabolized herbicide, %	Weed species	Ref.
		58% 3 DAT	*Zoysia japonica*	
		51% 3 DAT	*Lolium arundinaceum*	
		37% 3 DAT	*Agrostis stolonifera*	
		36% 3 DAT	*Poa annua*	
Primisulfuron		1.5	*Echinochloa crus-galli*	[71]
		12	*Cynodon dactylon*	
		3.5	*Digitaria sanguinalis*	
		>24	*Pueraria lobata*	
		6.0	*Panicum texanum*	
		>72	*Sorghum bicolor*	[67]
		<4	*Eriochloa villosa*	
		48% 3 DAT	*Sorghum halapense*	[70]
		24% 3 DAT	*Echinochloa crus-galli*	
		29% 3 DAT	*Setaria faberii*	
		54% 3 DAT	*Solanum ptychanthum*	
		90% 3 DAT	*Brachiaria platyphylla*	[68]
Metsulfuron-methyl		0% 4 DAT	*Oxytropis sericea*	[74]
		0% 4 DAT	*Astragalus mollissimus*	
Triflusulfuron		36	*Brassica napus*	[75]
		80	*Matricaria inodora*	
		61	*Veronica persica*	
		7	*Chenopodium album*	

[a]Estimated value.

[b]susceptible to diclofop-methyl and chlorsulfuron at normal field rates.

[c]resistant to both diclofop-methyl and chlorsulfuron.

[d]resistant to diclofop-methyl but susceptible to chlorsulfuron.

[e]this biotype is resistant to herbicides in 10 chemical classes, including the sulfonylureas and imidazolinones.

[f]half-life in shoots; [g]half-life in roots.

Table 4. Metabolism of sulfonylurea herbicides in weed species

Thifensulfuron-methyl differs from most other sulfonylurea herbicides in several respects. It is a short-residual herbicide, by virtue of its high susceptibility to microbial degradation in the soil. Soybeans metabolize thifensulfuron-methyl relatively rapidly, with half-life of 4–6 h (Table 4) [64]. The very sensitive species, including velvetleaf (*Abutilon theophrasti*), pigweed (*Amaranthus retroflexus*), and lambsquarters (*Chenopodium album*), metabolize the herbicide

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much more slowly, with half-lives greater than 48 h. Morning glory (*Ipomoea purpurea*), which is moderately sensitive, metabolizes thifensulfuron-methyl somewhat more rapidly but still more slowly (half-life of 30 h) than soybeans. The primary metabolite of thifensulfuron-methyl in soybean seedlings is its de-esterified free acid (thifensulfuron acid), which is herbicidally inactive and inactive against ALS. Soybean tolerance to thifensulfuron-methyl results from rapid de-esterification to inactive free acid. Further metabolism studies with tolerant soybean, moderately tolerant soybean, and spurred anoda (*Anoda cristata*) as well as susceptible velvetleaf (*Abutilon theophrasti*) showed that differential rate of metabolism seems to be a contributing factor in the selectivity of thifensulfuron-methyl between the two soybean cultivars and velvetleaf [65]. The metabolic basis for the moderate tolerance of spurred anoda to thifensulfuron-methyl was not clear. The two soybean cultivars metabolized 62–70% of absorbed thifensulfuron methyl at 3 days after treatment, while velvetleaf and spurred anoda metabolized about 50% of the absorbed herbicide (Table 4). The major metabolite formed in all species appeared to be de-esterified thifensulfuron acid.

Johnsongrass (*Sorghum halapense*) is a major perennial weed infesting many crop production areas. Nicosulfuron can be used to selectively control both seedling and rhizome johnsongrass in corn. Selectivity studies showed that 40% of nicosulfuron applied to the leaf surface of corn was absorbed into the leaf and the herbicide was rapidly metabolized with a half-life of 2 h (Table 4) [66]. Within 20 h, the parent herbicide was almost completely metabolized. Johnsongrass absorbed similar amount as corn; however, there was no perceptible metabolism of nicosulfuron in the treated leaves up to 24 h and 3 DAT; only 31% was metabolized. Research carried out to elucidate mechanism(s) of nicosulfuron and primisulfuron selectivity in corn, woolly cupgrass (*Eriochloa villosa*), and shattercane (*Sorghum bicolor*) revealed that corn absorbed less than one half the nicosulfuron and primisulfuron that woolly cupgrass and shattercane absorbed [67]. Corn rapidly metabolized nicosulfuron and primisulfuron, with a half-life of less than 4 h (Table 4). Shattercane metabolized the herbicides more slowly, with a half-life greater than 72 h for nicosulfuron and 36 h for primisulfuron. Nicosulfuron and primisulfuron half-lives were greater than 72 h and less than 4 h, respectively, in woolly cupgrass. Selectivity with nicosulfuron and primisulfuron is likely based on metabolism to non-phytotoxic compounds. Corn tolerance to nicosulfuron and primisulfuron was also attributed to reduced herbicide penetration and translocation below the treated leaf.

Broadleaf signalgrass (*Brachiaria platyphylla*) is sensitive to nicosulfuron and resistant to primisulfuron, but corn is resistant to both. By 72 HAT, broadleaf signalgrass under conditions of high light and temperature had metabolized nearly 90% of the primisulfuron absorbed, but less than 7% of the nicosulfuron absorbed was metabolized during the same time (Table 4) [68]. Corn rapidly metabolized both herbicides. These results suggest that differential activity of nicosulfuron and primisulfuron on broadleaf signalgrass may be based on differential rates of metabolism to non-phytotoxic compounds; uptake and translocation differences agree with the differential broadleaf signalgrass activity. In addition, environment has the potential to affect rates of sulfonylurea absorption, translocation, and metabolism. In nicosulfuron selectivity studies, relative tolerance of grasses from high to low was Bermuda grass (*Cynodon dactylon*) = zoysiagrass (*Zoysia japonica*)> tall fescue (*Lolium arundinaceum*) > creeping bentgrass

(*Lolium arundinaceum*) > annual bluegrass (*Poa annua*) [69]. At 72 HAT, annual bluegrass metabolized 36% of absorbed nicosulfuron, which was less than Bermuda grass, tall fescue, and zoysiagrass that metabolized 47–58% (Table 4). Creeping bentgrass metabolism of nicosulfuron was similar to annual bluegrass. Tall fescue had similar levels of metabolism to Bermuda grass and zoysiagrass, averaging 67%, at 168 HAT but produced fewer metabolites. Turfgrass tolerance to nicosulfuron is associated with relative herbicide concentrations in shoots and differential species metabolism.

The physiological basis for nicosulfuron and primisulfuron selectivity in corn, johnsongrass (*Sorghum halapense*), barnyardgrass (*Echinochloa crus-galli*), giant foxtail (*Setaria faberii*), and eastern black nightshade (*Solanum ptychanthum*) was established [70]. The levels of sensitivity were as follows, corn tolerant to both herbicides, seedling johnsongrass sensitive to both herbicides; barnyardgrass sensitive to nicosulfuron and tolerant to primisulfuron, giant foxtail sensitive to nicosulfuron and tolerant to primisulfuron, and eastern black nightshade tolerant to nicosulfuron and sensitive to primisulfuron. Selectivity of nicosulfuron and primisulfuron in corn, johnsongrass, barnyardgrass, and giant foxtail can primarily be attributed to differential rate of herbicide metabolism. Both herbicides were more rapidly metabolized by tolerant species. However, selectivity of these herbicides in eastern black nightshade could not be explained by differential herbicide absorption, translocation, or metabolism. The tolerance of eastern black nightshade to nicosulfuron and its sensitivity to primisulfuron were directly related to the sensitivity of ALS toward these molecules. ALS from eastern black nightshade was more sensitive to primisulfuron. Studies on the tolerance to primisulfuron of weeds such as Bermuda grass (*Cynodon dactylon*), crabgrass (*Digitaria sanguinalis*), kudzu (*Pueraria lobata*), Texas panicum (*Panicum texanum*), and particularly the tolerant barnyardgrass (*Echinochloa crus-galli*) showed that barnyardgrass was the fastest at metabolizing primisulfuron with an *in vivo* half-life about 1.5 h followed by crabgrass (3.5 h), Texas panicum (6 h), Bermuda grass (12 h), and kudzu (>24 h) (Table 4) [71]. The mechanism of tolerance of barnyardgrass was determined to be the metabolism and not an insensitive target enzyme, ALS. Two major classes of metabolites were produced and were found to be non-inhibitory to ALS. The metabolism proceeds through ring hydroxylation of the pyrimidine moiety followed by glycosylation.

Metsulfuron-methyl is an effective herbicide for use against broadleaf weeds and some grasses but is safe for use on wheat. Metabolism of metsulfuron-methyl in wheat and barley yielded a phenolic derivative formed after phenyl ring hydroxylation, a glucosyl conjugate of the phenolic metabolite as well as a hydroxymethyl derivative from hydroxylation of the methyl substituent of the triazine ring [72]. Hydrolysis of the sulfonylurea bridge resulted in several other unconjugated metabolites. Nevertheless, no de-esterified metabolites were detected. On the other hand, soybean seedlings do not metabolize metsulfuron-methyl and are correspondingly quite intolerant of this herbicide (GR50 < 0.5 g/ha). Soybeans exhibit an interesting specificity for de-esterification. The ethyl ester of chlorimuronethyl is de-esterified, while the methyl ester of metsulfuron-methyl is not, even though both compounds possess a phenyl ring. It was speculated that soybeans are incompetent to de-esterify ortho phenyl methyl ester sulfonylureas but are capable of this reaction with certain higher phenyl esters and even the methyl ester of thiophene sulfonylureas [73]. The locoweeds, woolly loco (*Astragalus mollissi-*

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mus) and silky crazyweed (*Oxytropis sericea*), contribute to livestock poisoning in the western United States. Silky crazyweed compared to woolly loco was more than 10 times as sensitive to increasing rates of herbicide. Nevertheless, no metabolism of metsulfuron was observed in these broadleaf weed species [74]. Selectivity differences between these locoweed genera to metsulfuron most likely are due to sensitivity differences at sites of action.

Triflusulfuron-methyl is a postemergence sulfonylurea herbicide for the control of annual and perennial broadleaf weeds and grasses in sugar beets. The mechanism of selectivity was studied by comparing the response of sugar beets with that of sensitive weeds such as rapeseed (*Brassica napus*), scentless false mayweed (*Matricaria inodora*), and common field-speedwell (*Veronica persica*) and a moderately tolerant lambsquarters (*Chenopodium album*) [75]. A good correlation between metabolism and plant tolerance does exist. Sugar beets metabolize triflusulfuron methyl very rapidly (half-life of < 1 h), while lamb's-quarters have an intermediate rate (half-life of 7 h) and sensitive weeds have slow rates of metabolism (half-lives of > 35 h) (Table 4). The initial metabolism of triflusulfuron methyl in sugar beets involves nucleophilic attack by glutathione at the urea carbonyl group, producing the *S*-(*N*-triazolylcarbamoyl)glutathione conjugate plus 7-methyl saccharin and its free acid which are all herbicidally inactive. Although the data strongly suggest enzymatic involvement, attempts to determine if glutathione-*S*-transferase was involved failed.

6. Metabolizing enzymes in weed species

Metabolism of herbicides in weed species generally produces identical metabolites that were formed in crop plants. Metabolizing enzymes such as glutathione-*S*-transferases (GSTs), cytochrome P450 oxygenases (P450s), glycosyl, and malonyl transferases more or less are well characterized in crop plants [76]. However, only few details have been published on these metabolizing enzymes and cofactors in weeds. The only exception is the glutathione-*S*-transferase superfamily which is widely studied in weeds. GSTs are a ubiquitous group of enzymes catalyzing the conjugation of electrophilic substrates with the tripeptide glutathione (GSH). GSH conjugation has been established as a major detoxication reaction in the metabolism of several classes of herbicides. An atrazine-resistant biotype of *Abutilon theophrasti* was reported to have 4-fold higher GST activities toward the herbicide than those found in the susceptible biotype [77]. The role of GSTs in the selectivity of chloroacetanilide herbicides has been well described in numerous monocot and dicot weed species [78-83].

Both monocot and dicot weeds contain the level of non-protein thiols (mostly GSH) comparable to that of maize (Table 5) [80]. Since the activity of GST enzymes toward herbicidal substrates in weed species is inferior to that of maize, we can conclude that the contribution of nonenzymatic GSH conjugation can be substantial in the metabolism of herbicide in weed species.

The involvement of cytochrome P450 monooxygenases in herbicide detoxication and selectivity has been well demonstrated in plants [84]. However, only few cytochrome P450-mediated herbicide metabolisms were carried out with microsomes from weed species.

Weed species[a]	GSH nmol g^{-1} fresh wt	GST(CDNB) nmol mg^{-1} prot h^{-1}	GST(acetochlor) pmol mg^{-1} prot h^{-1}	Cyt P450 pmol mg^{-1} prot
Avena fatua	267 ± 17	2869 ± 160	922 ± 85	41 ± 11
Bromus secalinus	492 ± 28	676 ± 43	684 ± 79	N/A
Bromus inermis	N/A	N/A	N/A	ND[b]
Echinocloa crus-galli	391 ± 24	170 ± 23	517 ± 49	17 ± 8
Amaranthus retroflexus	140 ± 11	56 ± 8	356 ± 39	10 ± 4
Abutilon theophrasti	309 ± 27	59 ± 7	ND	51 ± 24
Xanthium strumarium	273 ± 12	70 ± 9	874 ± 76	ND
Maize[c]	488 ± 16	986 ± 92	4567 ± 347	67 ± 14

[a]7-day-old etiolated weed seedlings.

[b]ND, not detectable.

[c]4-day-old etiolated maize seedlings.

Table 5. Glutathione contents, GST activities, and cytochrome P450 levels of monocot and dicot weeds and maize

Microsomes from naphthalic anhydride-treated and untreated shattercane and johnsongrass catalyzed the hydroxylation of bentazon [85]. The results indicated that bentazon hydroxylation in shattercane and johnsongrass is mediated by a constitutive and an inducible cytochrome P450 monooxygenase enzyme. Primisulfuron, but not nicosulfuron, was hydroxylated in woolly cupgrass (*Eriochloa villosa*) microsomes [86]. Neither nicosulfuron nor primisulfuron was hydroxylated in shattercane (*Sorghum bicolor*) microsomes. Bentazon and primisulfuron inhibited nicosulfuron hydroxylation in corn microsomes. Bentazon, but not nicosulfuron, also inhibited primisulfuron hydroxylation in the corn microsomes. This indicates that the three herbicides can interact at the same cytochrome P450(s) in corn. Primisulfuron hydroxylation was not inhibited by either bentazon or nicosulfuron in woolly cupgrass microsomes. This suggests that the cytochrome P450(s) for primisulfuron hydroxylation are different between corn and woolly cupgrass.

The role of cytochrome P450 monooxygenases in enhanced metabolism of resistant weed species has also been documented [87, 88]. Cytochrome P450 levels in *Avena fatua* and *Abutilon theophrasti* were found comparable to P450 content of maize (Table 5) [89]. Cytochrome P450 content in the microsomal membrane fraction of *Avena fatua* was 2.4-fold greater than in *Echinochloa crus-galli*. Among dicotyledonous plants, *Abutilon theophrasti* contained 5.1-fold higher level of P450 as compared to that of *Amaranthus retroflexus*.

Since the primisulfuron metabolism in barnyardgrass proceeds through hydroxylation of the pyrimidine moiety followed by formation of glycosyl conjugate we can assume that glycosyl transferases are also present in weed species [71].

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Chapter 11

Bioherbicides

Zvonko Pacanoski

Additional information is available at the end of the chapter

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Abstract

Bioherbicides are biologically based control agents useful for biological weed control. Hence, bioherbicides have been identified as a significant biological control strategy. Bioherbicides have many advantages such as clearly defined for target weeds, no side effect on beneficial plants or human health, a lack of pesticide residue build-up in the environment, and effectiveness for control of some herbicide-resistant weed biotypes. More importantly, it has been demonstrated that mixtures of some bioherbicides and synthetic herbicides can be more effective. Apart from many bioherbicide benefits, some factors have been noted to restrict the development of bioherbicides into profitable products. They involved environmental, biological and technical–commercial restrictions.

Keywords: Bioherbicide (inundative) approach, advantages, restrictions

1. Introduction

Development of alternative weed control methods is needed to help decrease reliance on herbicide use. Biological weed control is an alternative option for weed problems, particularly in agriculture and forestry. It is based on the use of natural enemies, particularly insects and pathogens to control weeds, as a sustainable, low cost and more environmentally acceptable method of weed control. One of the approaches to biological weed control using pathogens, mainly fungi, is inundative, bioherbicide approach.

Bioherbicides are phytopathogenic microorganisms or microbial phytotoxins useful for biological weed control applied in similar ways to conventional herbicides [1–3]. The active ingredient in a bioherbicide is, however, a living microorganism. Most commonly the organism is a fungus; hence the term mycoherbicide is often used in these cases [4]. Although the use of fungi and bacteria as inundative biological control agents (bioherbicides) has been

recognized as a significant technological weed control alternative [5–9], it can be argued that it serves a more important role as a complementary component in successful integrated management strategies [10], and not as a replacement for chemical herbicides and other weed management tactics [11]. Actually, in many situations, bioherbicides can be used as the sole option for the management of one or two target weeds, i.e. as a minor supplement to conventional chemical herbicides [12].

However, according to many authors, bioherbicides offer many advantages in comparison with synthetic herbicides. They include:

- a high degree of specificity of target weed;
- no effect on non-target and beneficial plants or man;
- absence of residue build-up in the environment; and
- effectiveness for managing herbicide-resistant (HR) weed populations [7,9,12–16].

Except numerous advantages of bioherbicides, some circumstances have been noted to restrain the progress of bioherbicides into profitable outputs. These include:

- biological restrictions (host changeability, host scope resistance mechanisms and interaction with other microorganisms that affect efficacy) [17];
- environment restrictions (epidemiology of bioherbicides reliant on optimal environmental conditions) [18–20];
- technical restrictions (wholesale production and formulation development of reliable and effective bioherbicide) [17,21]; and
- commercial restrictions (market capacity, patent protection, confidence and adjustment) [21–23].

2. Biological weed control

Biological control is the deliberate use of natural enemies to reduce the population of a target weed to below a desired threshold [24,25] and can be divided into two main approaches:

1. classical approach, in which a natural enemy is exported from its native range to an introduced (weedy, invasive) range of a plant, [24,26,27], and
2. bioherbicide approach, in which a natural enemy is used within its native range to control a native or naturalized weed [28–30].

2.1. Classical (inoculative) approach

The biocontrol approach using an imported pathogen to control a native or naturalized weed with minimal manipulations has been termed the inoculative or classical biocontrol method [31]. The classical approach is directed mainly towards the control of exotic weeds, which have

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spread in the introduced area in the absence of natural enemies. Control is achieved by the importation and release of highly host-specific pathogens virulent to the target weed in its native region [32]. These agents feed on the weed, reproduce and gradually suppress the weed as their population grows.

A highly successful biological control programme was implemented in Hawaii in the 1970s when a white smut fungus (*Entyloma ageratinae* sp. nov.) was introduced from Jamaica to control the exotic weed mistflower (*Ageratina riparia* (Regel) K. & R.), which was invading Hawaiian indigenous forest. The effect was rapid, with 95 per cent control after 3–4 years [33]. Western Australian golden wattle (*Acacia saligna* (Labill.) H.L. Wendl.) is regarded as the most important invasive weed that threatens the Cape Fynbos Floristic Region of South Africa, a unique ecosystem. In about 8 years, the introduction of the rust disease (*Uromycladium tepperianum* Sacc.) from Australia had become widespread in the province and tree density was decreased by 90–95% [34].

Another widely acclaimed example of biological control success is the use of a rust fungus (*Puccinia chondrillina* Bubak & Syd.) to control rush skeleton (*Chondrilla juncea* L.) in Australia. The rust fungus was introduced from the Mediterranean. *Puccinia chondrillina* was also introduced into the western United States to control a *Chondrilla juncea* L. biotype. However, unlike in Australia, the rust was only partially successful [35]. An example of successful classical biological control is that of rust fungus (*Puccinia carduorum* Jacky), imported from Turkey and released into the northeastern United States (Virginia and Maryland) in 1987 to control musk thistle (*Carduus thoermeri* Weinm.). The rust fungus has spread widely from its original introduction to the western states of Wyoming and California [36–38]. Baudoin *et al.* [36] found that *Puccinia carduorum* reduces musk thistle density by rushing agedness of rust-infected musk thistle and diminishes seed production by 20– 57%. Rust fungus (*Puccinia jaceae* var. *solstitialis*), imported from Bulgaria and Turkey, was released in California, United States, in 2003 for biological control of yellow starthistle (*Centaurea solstitialis* L.). The host range tests on this pathogen were extensive [39,40].

Trujillo *et al*. [41] have introduced septoria leaf spot (*Septoria passiflorae* Syd.) for the biological control of the exotic weed banana poka (*Passiflora tripartita* (Juss.) Poir var. *tripartita* Holm-Nie. Jörg. & LAW), at different forest sites in Hawaii, which resulted in over 50 per cent biomass reduction of the weed 3 years after inoculation.

Klamathweed beetle (*Chrysolina quadrigemina* Suffrian), introduced from Australia, proved especially effective for common St. Johnswort (*Hypericum perforatum* L.) weed control on California rangeland. Populations of the beetles quickly grew and spread. After 5 years, millions were collected from original release sites for redistribution throughout the Pacific Northwest. Ten years after the first release, H. perforatum populations in California were reduced to less than 1% of their original size [25]. Another example of successful classical biological control is the introduction of the black dot spurge beetle (*Aphthona nigriscutis* Foudras) from Hungary as a biological control agent for leafy spurge (*Euphorbia esula* L.). Release of these insects has resulted in a 99 per cent reduction in spurge stand density in one

area and a corresponding 30-fold increase in grass biomass in pasture and rangeland after 4 years [42,43].

2.2. Bioherbicide (inundative) approach

Opposite to classical (inoculative) approach, the bioherbicide (inundative) approach uses indigenous plant pathogens that are isolated from weeds and are cultured to produce large quantity of infective material [28]. These are utilized at amounts that will provoke tremendous levels of infection, leading to elimination of the target weed before economic damages happen [29]. A development of this strategy involves application of weed pathogens in a manner similar to herbicide applications. Bioherbicide inoculum is susceptible to unfavourable environmental conditions after spraying, and viability needs to be maintained for as long as is necessary to achieve infection following application [30]. Once in the field, the inundative application of inoculum is timed to coincide with the most favourable environmental conditions and susceptible growth stage of the weed, so that a disease epidemic occurs and the weed population is suppressed [44,45]. Once the weed problem has been removed, natural constraints ensure that the pathogen population returns to a low level once again.

The bioherbicide (inundative) approach has been successfully implemented for a number of important agricultural, invasive and exotic weeds. Many examples dedicated to positive bioherbicide implementation are elaborated in the Section "Bioherbicide case studies".

3. History of bioherbicides

Utilization of plant pathogens for weed control was first reported in the early 1900s, but the concept of using bioherbicides to control weeds attracted wide interest among weed scientists and plant pathologists after the Second World War. The earliest experiments simply involved fungus *Fusarium oxysporum* Schlecht. against prickly pear cactus (*Opuntia ficus-indica* (L.) Mill.) in Hawaii. In the 1950s, the Russians mass-produced the spores of *Alternaria cuscutacidae* Rudakov and applied them to the parasitic weed dodder (*Cuscata* spp.). In 1963, the Chinese mass-produced a different fungus (*Colletotrichum gloeosporioides* f. sp. *cuscutae*) for the same weed (*Cuscata* spp.). They called their mycoherbicide "LuBao" and an improved formulation is still in use today. Official date of bioherbicide control of weeds commenced in the late 1960s with an ambitious programme to find out a pathogen or pathogens for sorrels or docks (*Rumex* spp.) in the United States [46] and blackberries (*Rubus* spp.) in Chile [47]. From the 1970s there has been a considerable number of prosperous bioherbicide projects [8,24,48]. The number of scientific articles on bioherbicide research has enlarged excessively since the early 1980s. The number of weeds aimed for control as well as the number of potential pathogen candidates studied has increased. Registered and unregistered uses of bioherbicides have also increased considerably. In addition, the numbers of US patents published for the bioherbicidal technology and bioherbicide handling have increased, perhaps anticipating an increased dependence on bioherbicides in the future [49].

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4. Bioherbicide case studies

Considering the research effort expended in this area, some bioherbicides are commercialized (Devine®, Collego®, BioMal®, Camperico®, Myco–TechTM®, Woad Warrior®, Smolder®, Dr. bioSedge®, Biochon®, StampOut® [13,22,28,50–55] and many are underway to develop and register. Plant pathologists and weed scientists have identified approximately 200 plant pathogens that are candidates for development as commercial bioherbicides [48,56]. Some examples are presented below.

Culture filtrates of *Plectosporium tabacinum* (van Beyma) M. E. Palm, W. Gams et Nirenberg, isolated from naturally infected cleavers plants, provided 80–90% control of *Galium* spp. under field conditions. [57]. *Fusarium oxysporum* (PSM 197), a potential mycoherbicide for controlling *Striga* spp. in West Africa, showed significant reductions in the total number of emerged plants of *S. asiatica* (91.3%), *S. gesneroides* (81.8%) and *S. hermonthica* (94.3%) [58]. Hemp sesbania (*Sesbania exaltata* [Raf.] Rydb. ex A. W. Hill), one of the 10 most troublesome weeds in soybean in Arkansas, Louisiana and Mississippi [59] was 90% controlled with the isolates of the fungus *Colletotrichum truncatum* [14–16]. The level of control was similar to those achieved with the synthetic herbicide acifluorfen in the same crop [15]. A *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar:Fr. (MV) bioherbicidal isolate IMI 361690 provided >85% control of *Chenopodium amaranticolor* Coste & Reynier, *Senna obtusifulia* L., *Sesbania exaltata* (Raf.) Cory and *Datura stramonium* L. [60]. Other MV isolates have bioherbicidal activity for the control of *Carduus acanthoides* L. and *Euphorbia esula* L. [61,62]. Trichothecenes produced by an MV isolate from Italy could inhibit seed germination of the parasitic plant *Orobanche ramosa* [63]. Recently, MV was shown to be highly virulent against *Portulaca oleracea, Portulaca portulacastrum, Euphorbia maculata* and *Euphorbia prostrata* in commercial tomato (*Lycopersicon esculentum* L.) fields in the southeastern United States [64]. *Phomopsis amaranthicola*, an indigenous plant pathogen, provided up to 100% control of several *Amaranthus* species [65–67]. Host range testing of this organism has not shown infection of soybean, corn, sorghum or wheat. Mintz *et al*. [68] evaluated another fungal pathogen, *Aposphaeria amaranthi* Ell. & Barth. (later renamed as *Microsphaeropsis amaranthi* (Ell. & Barth.) [69], as a potential bioherbicide for several *Amaranthus* species (*A. retroflexus, A. spinosus*, *A. hybridus* and *A. albus*). In this context, in field experiments, eight *Amaranthus* species treated with *Microsphaeropsis amaranthi* and a mixture of *Microsphaeropsis amaranthi* and *Phomopsis amaranthicola* had severe disease ratings of 15 days after treatment (DAT), and mortality ranged from 74% to 100% [70]. *Drechslera avenacea* is a potential bioherbicide for *Avena fatua* control in dryland wheat crops in southern Australia. Maximum disease severity (DS) (1.1 lesions per mm^2 of leaf tissue) was recorded following the application of 1×10^5 spores per mL and exposure of weeds to a 12- to 16-h dew period at 20–25°C [71]. The fungus *Pyricularia setariae* applied at the concentration of 10^5 spores mL^{-1} reduced fresh weight of *Setaria viridis* (L.) Beauv. by 34% 7 DAT when compared with controls, whereas a concentration of 10^7 spores mL^{-1} reduced fresh weight by 87%. More importantly, *Pyricularia setariae* caused 80% fresh weight reduction of *Setaria viridis* (L.) Beauv. biotype resistant to sethoxydim, compared with 17% achieved with sethoxydim [72]. *Sesbania exaltata* [Raf.] Rydb. ex A.W. Hill was effectively controlled by 85, 90 and 93% of *Colletotrichum truncatum* (Schwein.) Andrus & Moore at inoculum concentrations of 2.5, 5.0 and 10.0×10^6 spores mL^{-1}, respectively

[73]. *Taraxacum officinale* was controlled by 70–80% and 90% by biocontrol strains of *Phoma macrostoma* applied as granular fungal inoculums to soil at the rate of 63g/m^2 and 125g/m^2, respectively [74]. The fungus *Phoma macrostoma* exhibits control of broadleaved weeds *Taraxacum officinale* and *Cirsium arvense* while showing no effect on grasses or cereal crops and is now being developed as a biological herbicide for weeds in turfgrass (lawns, golf courses, public grounds), agriculture (cereal crops) and agro-forestry (reforestation nurseries) [75]. Kadir *et al*. [76] have demonstrated the efficacy of *Dactylaria higginsii* as a bioherbicide agent for *Cyperus rotundus* L. in field trials. They have also reported that *Dactylaria higginsii* disease could help reduce interference from *Cyperus rotundus* L. and improve yield in greenhouse-grown tomato [77]. Morales-Payan *et al*. [78] estimated the bioherbicidal efficacy of *Dactylaria higginsii* in several field trials in Florida and Puerto Rico. According to their results, application of *Dactylaria higginsii* at 8 and 18 days after emergence (DAE) or 8, 18 and 25 DAE reduced the yield of pepper to 24 and 31%, respectively, compared to weed-free control plots. Similarly, Semidey *et al*. [79] have reported that onion yield was higher in plots sprayed three times with *Dactylaria higginsii* as compared to the yield from one or two applications. The potential of *Dactylaria higginsii* as a substitute to methyl bromide fumigation in an integrated approach to *Cyperus rotundus* L. control in a tomato production system was examined by Rosskopf *et al*. [80]. The results obtained showed that weed seedlings between 3 and 5 weeks of age were the most susceptible to the disease. Besides the use of fungi as bioherbicides, several strains of soil bacteria as pre-emergent biological control agents against annual grassy weeds have been identified and field-tested. Up to 85–90% control of green foxtail (*Setaria viridis* (L.) P. Beauv.) and wild oat (*Avena fatua* L.) was achieved using a granular formulation called "pesta" [81–83]. The leading bacterial candidate for biological control of the grass weeds is a *Pseudomonas fluorescens*, strain BRG100, which delays the emergence of the weeds and significantly inhibits root growth. Charudattan *et al*. [84] reported on potential virus-based bioherbicide tobacco mild green mosaic virus (TMGMV), which caused 83–97% mortality of *Solanum viarum* plants of different sizes and ages.

5. Interaction between bioherbicides and synthetic herbicides

The idea of combining bioherbicides with synthetic herbicides or adjuvants has been the issue of substantial research work. Moreover, it has been revealed that mixtures of some bioherbicides and synthetic herbicides can be synergistic [85,86], culminating from reduced weed defence reactions caused by the herbicides, consequently making the weeds more sensitive to pathogen attack [87,88]. Christy *et al*. [86] reported a synergy between trimethylsulfonium salt of glyphosate and *Xanthomonas campestris* against several weed species. Other synergistic interactions involving chemical herbicides and bioherbicides have been discovered and some were granted patents in the United States [85,89]. According to Caulder and Stowell [85,89], acifluorfen and bentazon were the most effective synergists and provided significant control in several weed/pathogen combinations: (*Senna obtusifolia*, formerly *Cassia obtusifolia* [L.] Irwin & Barneby) and *Alternaria cassiae* Jurair & Khan; *Aeschynomene virginica* [L.] Britton, Sterns & Poggenb. and *Colletotrichum gloesporioides*; *Sesbania exaltata* (Raf.) Cory and *Colletotrichum*

truncatum; and *Desmodium tortuosum* [SW.] DC. and *Fusarium lateritium* Nees. A sublethal dose of glyphosate (50 mmol L^{-1}) suppressed the biosynthesis of a phytoalexin derived from the shikimate pathway in Senna obtusifolia (L.) H. S. Irwin & Barneby, infected by *Alternaria cassiae* Jurair & Khan, reducing the resistance of the weed to fungal infection and disease development [90]. Similarly, 12 DAT, *Brunnichia ovata* [Walt.] Shinners and *Campsis radicans* [L.] Seem. ex Bureau were controlled by 88 and 90%, respectively, through a synergistic interaction between the fungus *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar: Fr. and the herbicide glyphosate. Neither glyphosate nor *M. verrucaria* controlled these weeds at commercially acceptable levels (≥80%) [73]. According to Boyette *et al*. [91], timing of glyphosate application in relation to combined treatment with the bioherbicide *M. verrucaria* can improve the control of *Pueraria lobata* (Willd.) Ohwi, *Brunnichia ovata* [Walt.] Shinners and *Campsis radicans* [L.] Seem. ex Bureau. Heiny [92] revealed that *Phoma proboscis* Heiny at 1 x 10^7 spores mL^{-1} mixured with reduced rates of 2,4-D plus MCPP controlled field bindweed (*Convolvulus arvensis* L.) more effectively than the herbicide mixture alone and as effectively as the pathogen at a 10-fold higher rate. Application of various crop oils [68,93–97] and invert emulsions [15,98–100] improved efficacy and performance of many bioherbicides and biocontrol fungi. For instance, according to Hoagland *et al*. [10] treatment of fungus *Myrothecium verrucaria* (MV) strain originally isolated from sicklepod (*Senna obtusifolia* L.) mixture with the surfactant Silwet L-77 caused 100% mortality of *Pueraria lobata* (Willd.) Ohwi seedlings under greenhouse conditions, and 90–100% control of older *Pueraria lobata* (Willd.) Ohwi plants in naturally infested and experimental plots, respectively.

6. Bioherbicide limitations

In spite of considerable research in bioherbicides, there are only a few commercially available products worldwide. This lack of availability is mainly due to limitations in bioherbicide development, which need to be overcome to ensure the future commercial success of bioherbicides [22, 101]. Limitations in bioherbicide development can be classified as either environmental (temperature and, particularly, humidity as major factors influencing the efficacy of bioherbicides), biological (mainly host variability and resistance), or technological–commercial (mass production and formulation, which often blocked bioherbicide development) [17,22,102].

7. Environmental limitations

Environmental limitations are a constraint to the effective use of many biological agents, including bioherbicides. Environmental factors influence formulation performance of bioherbicides as inoculum production is dependent on sporulation of the formulation. This process, although rapid, might continue over several weeks subsequent to applications and might encounter variable environmental conditions [18,21,22]. In the application of bioherbicides, environmental conditions prevailing in the phyllosphere of plants are frequently hostile for

biological control agents [103,104]. A requirement of more than 12 h of dew period for severe infection by a pathogen has been reported for several potential bioherbicides [105–108] and this may limit the efficacy of the bioherbicide in the field. Temperature generally has not been considered to be as critical as moisture for mycoherbicide [109], although field efficacy of *Colletotrichum orbiculare* in controlling *Xanthium spinosum* L. is reduced by high-temperature conditions after inoculation of plants [110]. However, dew period length requirement and temperature typically interact [111]. Low temperatures may greatly extend dew period length requirements for bioherbicides developed for use in crops, such as winter wheat.

Nutrient balance can play an important part in sporulation of fungi. Studies with *Colletotrichum truncatum* have shown how carbon concentration and carbon to nitrogen (C:N) ratio influence propagule production [112]. Moreover, a defined amino acid composition of the N source improved the production of conidia [113]. In addition, spore fitness in terms of germination and appressoria formation rate and subsequent disease production [114] was influenced by C:N ratios.

Soil environment, moisture and the nutrient status of the soil can influence the physiology of target plants and, therefore, their interaction with aerial applied bioherbicides [21]. Pre-emergence application has been considered as an alternative approach to overcome some of the environmental stresses imposed upon propagules applied onto the foliage or soil surface [115]. Bioherbicides consisting of propagules of soil-borne pathogens, which normally infect at or below the soil surface, appear to be more protected from environmental extremes and may persist and give residual control [116,117]. In this context, Jackson et al. [113] reported for 95% control of the emerging *Sesbania exaltata* (Raf.) Rydb. ex A. W. Hill seedlings when *Colletotrichum truncatum* (Schw.) Andrus and Moore was incorporated into the soil.

There are many environmental limitations to applying bioherbicides and maintaining their efficacy in water as well [118]. Auld and McRae [4] stated that for control of aquatic weeds a biocontrol agent would need to possess a high ecological capability to contend with varying conditions between surface and bottom, as well as across even small bodies of water. Oxygen concentration, temperature, light intensity and salinity are just four of the variables to contend with.

8. Biological limitations

From a biological viewpoint, a good bioherbicide acts relatively quickly and has acceptable efficacy in control of weeds. Unfortunately, Charudattan [8] stated that many of the discovered weed pathogens may provide partial control of only one weed species, even under ideal conditions. This host particularity is related to the fundamental bio-physiology of the pathogen and to host changeability [119,120] and resistance as well [17]. In other words, within a population of weed species there will usually be a range of genetically diverse biotypes [121] that may include some resistant biotypes, just as there may be a range of biotypes of microorganisms [122], for instance within fungal species, with slightly different host ranges [14,123,124], so that there is potential to mix and vary the biotypes of a species used as a

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bioherbicide. Non-target plant protection in relation to the potential use of *Chondrostereum purpureum* (Pers ex Fr.) Pouzar (silverleaf disease) to control black cherry (*Prunus serotina* Erhr.) in coniferous forests by modelling the dispersal of spores and therefore quantitatively assessing the risks to susceptible fruit trees outside the forest was noted by De Jong *et al*. [125]. Concerns have been raised regarding the potential for sexual or asexual gene exchange between bioherbicide strains and strains attacking distantly related crop plants [109,126,127].

9. Technological–commercial limitations

Several technological limitations have been identified that could prevent the widespread use of bioherbicides [21]. Pathogenic strains, formulation method and the interaction of these two parameters significantly affect the shelf life of the formulations at room temperature [21,128]. High concentrations and the alteration of formulations are needed to increase bioherbicide activity [129]. Compatibility testing of formulation components that range from registered agricultural products to novel substances, such as sunscreens, humectants and starches, can consume a great deal of time and resources [130].

The most challenging aspect of formulating bioherbicides is to overcome the dew requirement that exists for several of them. Attempts to overcome this limitation have included developing various water-retaining materials; invert and vegetable oil emulsion formulations [15,94,131] and granular pre-emergence formulations [132] are considered as a promising approach to make pathogens less dependent on available water for initial infections to occur [133,134]. In addition, appropriate formulations can also reduce the dosage of inoculum required to kill weeds [135], thus potentially reducing the cost of bioherbicides.

Experiments conducted with a number of potential bioherbicides have demonstrated that an invert emulsion allowed infection to occur in the absence of available water [15,133,136] and reduced the need to apply high dosages of inoculum [135]. Invert emulsions consist of a continuous oil phase that contains water droplets. Connick and Boyette [137] have developed an invert emulsion formulation exhibiting lower viscosity and greater water-retention properties. Auld [93] reported that application of low concentrations of vegetable oils with an emulsifying adjuvant enhances efficacy of *Colletotrichum orbiculare* in inciting disease on *Xanthium spinosum* L. in the absence of dew in greenhouse conditions. However, according to the same author, oil emulsions were not effective in the field conditions. An invert emulsion has been shown to overcome dew requirements and reduce the spore concentrations required [15]. But, unfavourable characteristic is containing of more than 30% oil which makes these formulations expensive and very viscous, typically requiring special spraying equipment such as air-assist nozzles, and because of the high oil content it is likely to produce phytotoxic effects on non-target plants [135,138]. Invert emulsions have been shown to cause phytotoxicity in some cases and to predispose a variety of plants to opportunistic pathogens as well [99].

From the other side, the main restriction in the application of solid (dry) forms of bioherbicide is that they must await suitable, moist conditions for fungal growth and infection [139].

Moreover, during this waiting period the living active ingredients must survive in the field. In addition, ant theft has been a problem with some formulations [140].

The simplest liquid formulations of bioherbicides are water suspensions of spores often with a small amount of wetting agent. These are generally used as standards against which to compare more complex formulations. However, under ideal conditions for fungal infection, simple aqueous suspensions can be successful in the field [110]. Pathogenicity of an aqueous mycelial inoculum of *Alternaria eichhorneae* Nag Raj & Ponnapa in a controlled environment experiment was improved with hydrophilic polymers such as gellan gum, alginates and the polyacrylamide [141]. Although several polymers retained considerably more water after 6–8 h than the water-suspension controls, no increase in efficacy of the fungus *Colletotrichum orbiculare* was found [142]. Vegetable oil emulsions that contain 10% oil and 1% of an emulsifying agent reduced dew dependence in controlled environment studies using *C. orbiculare* in control of *Xanthium spinosum* L. [93]. Unfortunately, in the field conditions, the efficacy of these formulations was variable [143].

A novel bioherbicide formulation uses a complex emulsion – water-in-oil-in-water (WOW) emulsion [144]. It contains at least one lipophilic surfactant, at least one hydrophilic surfactant, oil and water. Although used in the pharmaceutical [145], cosmetic [146] and food industries [147], WOW emulsions do not appear to have been widely used in agricultural or horticultural technology. Although numerous improvements of liquid formulations of bioherbicides have been made, genetic manipulation of fungi offers a broad extent of opportunities to adjust formulations and to ameliorate bioherbicide characteristics [148].

Taking into account the above-mentioned restrictions, the production of bioherbicides by profit-oriented companies would involve additional expenditure without guaranteed income. The amount of abundant development and production of phytopathogenic microorganisms or their phytotoxins for bioherbicides in immerse or in solid-state systems, which would alter from one bioherbicide to another, is relatively high [149]. In addition, the small market capacity of considerable competent bioherbicide aspirants reveals that market capacity could be a restraint for developing such herbicides. Because of that, firms are suspicious that development and registration expenditures will be paid back [21,22].

10. Conclusion

The bioherbicide access to weed control is attaining impetus. New bioherbicides will be applicable in inundate lands, badlands as well as in control of parasite weeds or HR weeds. Research on synergism between pathogens and herbicides for their incorporation in effective weed management, applied science, fungal metabolites and biotechnology utilization, principally genetic engineering is needed. Bioherbicides will not deal with all of the environmental and weed control issues related with synthetic herbicides, nor will they alter the present or future depository of synthetic herbicides. To a certain degree, their appearance will presumably be complementary components in lucrative weed management systems, and in the revelation of different phytotoxins with new performances and new molecular sites of

action. Advanced research on this field is imperative in order to entirely find out mutual interactions of phytopathogenic microorganisms, crops and weeds, and to identify new plant pathogens or their phytotoxins promising effective for the new-generation bioherbicides.

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Chapter 12

Determining the Selectivity of Herbicides and Assessing Their Effect on Plant Roots - A Case Study with Indaziflam and Glyphosate Herbicides

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Additional information is available at the end of the chapter

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Abstract

This chapter explores the general aspects of herbicide selectivity on plants, describing the various aspects of the topic, especially the action of herbicides on root crops and presenting a case study with the suggestion of a methodology to evaluate herbicide action on roots in perennial culture and thus determine their selectivity. This study was carried out under field conditions, over a period of four years, where the effect of indaziflam and glyphosate herbicides on roots of Coffee and Citrus plants was evaluated. The results demonstrate that the methodology used to assess the effect of herbicides on the roots was important to validate and qualify safe herbicide selectivity towards crops. Thus, this analysis should be indicated as a routine method for studies to assess the selectivity of herbicides to crops.

Keywords: Herbicide, roots, selectivity, coffee, citrus, indaziflam, glyphosate

1. Introduction

In the proto-Neolithic era, between 7500 and 5000 BCE, early in the development of agriculture by human communities, a psychic change is observed, resulting in an *increased awareness*, diagnosed by the many myths attached to these communities. One such myth is of the young Wunzh (Native American folktale, the Father of Indian Corn) entering adolescence, fighting and killing a divine being, burying it and fulfilling his order of "keeping the area free for plants," thus allowing the "friend" to be able to be reborn, now in the form of a beautiful corn plant, being able to grow, generating new seeds in the ears and food for the people [1].

Instinctively, man realized that the plants that lived together with the ones he was growing were harming them; therefore, since then he has sought ways to reduce the labor of controlling these plants. English agricultural pioneer Jethro Tull's book, *Horse Hoeing Husbandry* (1731), stands out among the first to indicate the use of horsepower for weed control.

In the 1950s, the relationship between cultivated plants with those that were vegetating together began to be analyzed by scientific experimental methods—observation of regular events that can be repeated and assessing assumptions—and it was found that in the implementation of an agricultural area by means of a cultivation system there are serious and significant changes in the geomorphic, edaphic, and biological subsystems, making them simpler (agroecosystem) compared with the ecosystem, this one being more complex. This transformation resulted in a drastic impairment of the system's self-regulation capacity, thus making it more unstable and susceptible to power inputs.

One of the main consequences of this transformation was the excessive increase of the populations of certain species of insects, microorganisms, nematodes, and plants in such a way as to significantly compromise crop production, which are therefore called agricultural pests and in the case of plants are named "weeds" [2].

2. Weeds [3]

Among the various definitions found in textbooks involving weeds, I highlight one that I think is the simplest and most direct: "plants that are born outside of the desired place," adding that they are always present in the agricultural ecosystems, are difficult to control, and damage agricultural crops [3].

For a long time, agricultural research has been showing that weed control in the most diverse agricultural ecosystems is essential for successful crop production. All the technological development of the crop in nutritional, phytotechnical, or improvement aspects may be compromised if weeds are not controlled.

The control of these plants is performed by combining various methods, such as preventative, cultural, weeding, and the chemical ones with the use of herbicides.

Herbicides are chemicals used to eliminate plants. They are applied in suitable doses directly on the vegetation for foliar absorption (postemergence treatment) or to the soil for absorption by tissues formed after seed germination before the emergence of the plant on the surface (preemergence treatment). They are generally used to control weeds that infest the various agricultural ecosystems, or any other ecological niches favorable to these organisms: vacant lots, edges of roads, railways beds, parking lots, and aquatic environments.

Herbicide use should be made in a technical and discerning way, always seeking to maximize its benefits of use and minimizing toxicological and environmental risks.

Its use is not without risks, among which the selectivity of herbicides stands out, particularly those applied continuously to perennial crops.

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3. Selectivity of herbicides ([4] modified)

Herbicides have the selectivity characteristic when, in contact with plants of different species, they kill or slow the growth of some, while not affecting others. Therefore, in agronomic terms, a selective herbicide is the one that kills or slows the growth of weeds while others (in this case crops) are tolerant to the same treatment.

A herbicide is selective for a particular crop within certain limits, governed by complex interactions and covering factors such as the plant itself, the herbicide, and the weather.

4. The plant

Seven factors related to a particular plant influence the selectivity of herbicides: development stage (age), growth rate, morphology, physiology, and biophysical, biochemical, and genetic processes.

4.1. Development stage (age)

When the plant is young, it presents its meristems in a clear biological activity, and this influences the herbicide response. New plants are generally more susceptible to herbicides than the older ones; therefore, treatment with preemergence herbicides acts on seedlings which are germinated and not on the ones already established.

4.2. Growth rate

Probably, likewise, when the plant has an outstanding growth, this favors the reaction with the herbicide. In general, fast-growing plants are more sensitive to herbicides than those growing more slowly.

4.3. Morphology

The morphology of weeds is very important in determining the death of the plant for a given herbicide. It is differentiated in roots, in growth meristems and leaves.

4.4. Morphology of the roots

The roots are structures that may be alive or dead. They are the first structure emerging from a seed during germination. Its main purposes are to fix the plant in the soil and absorb water and minerals. Its whole, with the total of its branches, is called a root system [5].

The fine roots of the plants are a major means for removing soil resources, and their length and number are indicative of the nutrient absorption capacity [6]. The higher the roots of a plant, the greater their ability to exploit the soil and absorb available nutrients and water [7].

Perennial weeds have roots that are deeper than the annual ones. Likewise, dicotyledons present roots that plunge into the ground without too many branches, deeper in relation to monocotyledons, which are hair-like and more superficial. This difference in position is significant to the susceptibility of the plant's absorption processes of herbicides by the root.

4.5. Growth meristems

In Poaceae (also called Gramineae or true grasses) and Cyperaceae, the growth meristem is situated in the base or beneath the soil surface; when the herbicide is not systemic and is applied as postemergence, these meristems are protected and can sprout again. In other species, broad leaves (dicotyledons), the growth meristems are located at the epigeal part: in the apex of the growth points and in the leaf axils, directly exposed to herbicide application; if there is death of the meristems, the plant dies.

4.5.1. Leaves

Some properties of the leaves protect the crops treated with herbicides. For example, vertical leaves hinder the attachment of the spray solution, as well as the layer and the type of waxes present in the leaves when the spray droplets to reach these kinds of leaves, that tend to bounce or wet the surfaces only on small points, thereby reducing the effect of the herbicide.

The leaf form also interferes with the selectivity of herbicides. Broadleaf trees usually have broadleaf and smooth surfaces, horizontally extending from the stem. Therefore, they easily intercept the spray droplets and these are less likely to bounce off the leaf.

Thus, when broadleaf weeds are sprayed with contact herbicides, the spray solution tends to spread as a thin film and the droplets are scattered to wet a large portion of the sheet, thus facilitating the absorption of the herbicide, unlike when the same herbicides are applied on cereals (Poaceae) or on crops of onions, when there is the rebound of drops, avoiding the absorption and effect of the herbicide, and preserving the plant.

4.6. Physiology

The plant physiology determines the mode and amount of herbicide which enter the plant (absorption) and its movement (translocation).

4.6.1. Absorption

Plants that have favorable cuticles and many large stomata favor the entry of the herbicides into the plant.

4.6.2. Translocation

After the entry of the herbicide into the plant, it may move within the plant (translocation). This movement may be downward by Liberian vessels (phloem) or ascending by timber vessels (xylem).

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The movement in the conducting vessels of the plants by the herbicides is usually in one direction or another and governed by their chemical affinity: lipophilic, movement via phloem or hydrophilic, movement via the xylem, but in some cases, such as 2,4-D (2,4-Dichlorophenoxyacetic acid, weak acid), they can move in both directions.

4.7. Biochemical processes

Biochemical processes are responsible for the protection or activation of herbicides in plants.

4.7.1. Enzymatic inactivation

The biochemical reactions involve enzymes and are responsible for the activation or inactivation of the herbicides.

In this process, selectivity is expressed in the differential inactivation of the activated herbicides decreasing the enzymatic activity in a particular plant species, and another one that does not interfere with the metabolic processes of the plant, for example, in photosynthesis. This can kill certain plants and leave the others unharmed.

Biochemical reactions can inactivate the herbicides, and thus be selective, preserving the crop and killing the weeds. For example, compound 2,4-D is biochemically metabolized in plants of the Poaceae family in 2,4-DB (or 4-(2,4-dichlorophenoxy)butyric acid) and does not affect these plants.

4.8. Genetic heritage

Genetic characteristics intrinsic of plants define their morphological, physiological, and biochemical characteristics, and these, being influenced by weather conditions, determine the intensity of herbicide effect response.

Currently, science uses biotechnology tools that modify the genetic heritage of plants, obtaining genetically modified organisms (GMOs), significantly changing their physiology and turning cultivated plants, once sensitive, intolerant to a certain herbicide.

5. Herbicides

5.1. Molecular configuration

Size, shape, and chemical characteristics (molecular, acid, base) influence herbicide entry and consequently herbicide effect on the plant. Brigs [8, 9] has demonstrated that lipophilicity is related to the herbicide ability to systematically move by conducting vessels of the plant when they present Log K_{ow}[1] with values close to 2 that show this characteristic.

1 Kow: octanol–water partition coefficient, measures chemical affinity of herbicides: hydrophilic (< 0) and lipophilic (> 0).

5.2. Toxicity

The herbicide can have two types of effect or toxicity: acute or chronic. Sharp toxicity is characterized as intense and usually quickly kills the plant; contact herbicides have this feature and this type of toxicity.

Chronic toxicity is characterized by the effect of the herbicide along time and generally has a slow action. They are herbicides that are usually applied to the soil as preemergence, have biological persistence with effect on plants, and in the case of weeds can take up to 10 weeks to eradicate them.

5.3. Concentration and formulation of herbicides

The concentration and the commercial formulation are important factors characterizing the selectivity of herbicides on plants.

The concentration of the herbicide determines whether or not it is selective for a given crop. For example, 2,4-D in low concentrations induces the increase of cell transpiration and cell division of plants, but in larger doses it reduces this process and can kill cells.

Formulation is the vehicle that, along with water, forms the spray solution application and leads the herbicide to contact the soil and plants and is preponderant to determine the selectivity of herbicides in relation to a particular species, depending on various factors: granulometry of the formulations, surfactants, adjuvants and other additives which stabilize the formulations.

5.4. Root system

Plant roots are used for fixation in the soil and uptake of water and ions. They are also the site of synthesis of various compounds, for example, hormones and substances of allelopathic effects, and are also used as storage organs.

Morphologically, we highlight the meristems of the roots of higher plants, which at their apex are wrapped by a hoodlike structure (calyptra) whose cells produce mucilage to protect the meristematic cells from mechanical damage, and the cell elongation zone, which turn into differentiation areas and of root hairs. Theoretically, plants as a whole are capable of absorbing water, but the leaves and the stem are covered by the cuticle, which, depending on its thickness, prevents significant water absorption compared with the roots, for these have no cuticle and have absorbent hairs that increase much of the total area for absorption of water and may reach very high values; for example, a single rye plant may have roots with an absorbing area of 400 m^2.

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ing on its thickness, prevents a significant water absorption compared with the roots, for these have no cuticle and have absorbent hairs that increase much of the total area for absorption of water and may reach very high values; for example, a single rye plant may have roots with an absorbing area of 400 m^2.

Soil is composed of several fractions: sand, silt, clay, and organic matter, plus air and water. Clay and organic matter are constituents of the colloidal fraction of the soil, having charges that adsorb on the surface: ion attraction, anion–cation, molecules, hydrogen bonds, or van der Waals forces (or interactions). Herbicides are examples of molecules adsorbed by the soil.

On condition with higher soil moisture, there is competition for the adsorption site in the colloids by water, leading to the release of herbicide molecules, moving them to the soil solution and making them available for absorption by the roots.

5.5. Assessment of selectivity of herbicides on the roots

Soil is an opaque and solid environment of high density that creates resistance to root growth and thus makes it difficult to observe and assess root development "in loco" [10]. This shows that to analyze the performance of herbicides on the roots and assess their selectivity, the researcher is constantly working "in the dark" [11].

In fact, roots grow naturally in the soil porous volume, distributing in this volume according to non-uniform directions (anisotropy), dictated by the tropisms of each type of roots (e.g. ortho-geotropism in primary axes), and by the endogenous branching patterns [11].

Therefore, the root systems are complex branched structures that vary in space and time [11], and this has direct implications on the methodological aspects of the studies. Moreover, soil has its own patterns of spatial variability, expressed as different gradients of physical and chemical properties, which, superimposed to the endogenous variability of the roots, strengthen the anisotropy of the root system [11].

Generally, the assessment of the selectivity of the herbicides on the roots is done indirectly by assessing the epigeal parts of the plant, assuming that the mass of the roots is similar. For Hooker et al. [12], this comparison is not valid because significant variations may occur, both spatial and temporal, between the epigeal and hypogeal (roots) parts.

Thus, assay to qualify the selectivity of herbicides, when the object of study are the roots, are complex, all have advantages and limitations [11].

5.6. Methods of herbicide effect assessment

This method is based on separation by washing and/or sieving of the soil to distinguish the roots; depending on the plant stage of development, the methodology is different. For example, assessing the selectivity of herbicides acting on seedlings can be a direct and comprehensive approach to the root system, carefully removing this one to assess the mass, length, and visual harm. However, for annual crops established for many years in the full production stage, the assessment of the root system has to be carried out by an appropriate sampling of the roots,

and thus estimate the depth of rooting, biomass, and complex root demographics measurements in cycling studies of roots.

The measurement of these growth and development parameters of the root system is very difficult due to the difficulty in obtaining reliable data to assess the null hypothesis (H^0) among treatments. In general, the test tends to present a type II error,[2] mainly if, to carry out the assessments of the root system, the plant is removed by means of direct uprooting of the trees or shrubs. In this case, they are exposed only to assess the primary roots, for the remaining, secondary and mostly root hairs, remain attached to the ground and are not considered in the assessment.

In this case, an adequate sampling of the roots is advisable to assess the root system [11, 12, 13, 14].

In this regard, appropriate methodologies for adequate root system assessments are studied by means of roots sampling methods and strategies under field conditions by direct and indirect sampling, by means of destructive techniques and extraction of soil root.

Among these techniques are the use of cylindrical auger (volumetric ring), digging the roots system, and opening trenches.

The major problem in using this method, including selectivity studies, is the minimum number of samples. This should be determined by the statistical criteria used, thus making it possible to detect statistical effects by the analysis of variance of the data that must be representative. For each test, a prior sampling and analysis of the data is suggested, using the coefficient of variation ≤ 30% as an indication of the minimum number of samples sufficient to obtain reliable results.

Another factor which complicates the use of the destructive method is the necessary time and labor. For example, in soil removal of a volume of 1.0 m^3, with the soil with a real density of 1.2 $g.cm^3$, 1.2 tons of soil is removed.

Another way is to use trenches with a lattice framework on the soil profile wall for roots direct counting, assessing their thickness and thus estimating the root system of the plants.

Another way of assessing the roots is by using images: rhizotron, a system that uses glass to observe the roots; with the placement of this one in the subsoil, to observe the roots *in vivo*.

Another way of assessing the roots is by using images: rhizotron, a system that uses glass to observe the roots, with the placement of this one in the subsoil, to observe the roots *in vivo*, not interfering in the shoot, which is exposed to ambient conditions.

Due to the lack of resistance on the glass surface, the roots have a tendency to grow on its surface. Thus, this method is more suitable for phenological studies of the roots and not for analyzing the selectivity of the herbicides on them [15].

Besides the assessment of roots being done in two ways—directly in the soil profile or by means of washed roots—there are also methods that analyze digital images, a progress in the

2 This occurs when one accepts the H0 null hypothesis as true when this one is false, due to a β probability.

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techniques for studying the root system. In practice, in the herbicide selectivity studies, when the roots are assessed, this technique is interesting to check its effect on seedlings in trials conducted in a greenhouse or phytotrons, where the root system can be entirely removed in orchards or coffee plantations with large and deep root systems. It is necessary to build trenches to obtain many images and repetitions, thus obtaining data on which the statistics by the *F* (5%) test has the power to find significant differences.

Thus, this chapter describes an original research, in order to contribute to the research aimed to assess the selectivity of herbicides on perennial crops, having as a parameter the assessment of the effect of these on the root system.

6. Case study

Determining the selectivity of indaziflam and glyphosate herbicides, assessing their effect on the roots when applied for four years in a row in Coffee and Citrus crops [16, 17, 18].

Herbicides assessed:

Indaziflam: ($C^{16}H^{20}FN^5$), name IUPAC: N-[(1R,2S)-2,3-dihydro-2,6-dimethyl-1H-inden-1-yl]-6-[(1RS)-1fluoroethyl]-1,3,5-triazine-2,4-diamine, belongs to the group of alkylazinas and has the following chemical characteristics: vapor pressure (25º) 5.1 x 10^{-10} mm Hg, Log K^{ow} $^{(pH\ 7)}$ = 2.8; solubility $^{(pH\ 6.8)}$ and 2.8 mg.L^{-1} and dissociation constant (pKa) = 3.5. Its mode of action is the inhibition of cellulose biosynthesis and is suitable for preemergence applications for a broad spectrum of weed control, monocotyledons and dicotyledons by means of the the cell wall biosynthesis inhibition, acting on the growth of the meristematic cells and affecting the germination of sensitive weed seeds.

Glyphosate: ($C^{16}H^{20}FN^5$), name IUPAC: N- (phosphonomethyl) glycine, belongs to the group of substituted Glycine and has the following chemical characteristics: vapor pressure (45º) 2.45 x 10^{-8} mm Hg, Log K^{ow} = -3.22 to -2.76; solubility $^{(pH\ 7)}$ 15,700 mg.L^{-1} and the dissociation constant (pKa) = 2.6; 5.6; 10.3. Its mode of action is the inhibition of the enzyme enol-pyruvyl-shikimate-phosphate synthase (EPSPS), applied as postemergence for controlling a broad spectrum of weeds, monocotyledons and dicotyledons by inhibiting the biosynthesis of amino acids, such as phenylalanine, tyrosine and tryptophan.

6.1. Treatments

In crops with more than five years in the full production stage, tests were installed in the crops of Coffee cv. Catuaí Vermelho and Citrus cv. Valência (Table 1). The study commenced in December 2008.

The tests were performed on soil of medium texture with 28 g/dm^3 organic matter.

Treatments		Dose		
		Grams – a.i. ha^{-1} (1)	Liters – pc ha^{-1} (2)	*n* (3)
1	Weeded control	--	--	--
2	Indaziflam	75	0.15	1
3	Indaziflam	100	0.20	1
4	Indaziflam	150	0.30	1
5	Glyphosate	960	2.00	3

1. AI: active ingredient
2. CP: commercial product
3. Number of annual applications

Table 1. Treatments applied to the crops of Coffee and Citrus

6.2. Periods of application

Herbicide applications were done in the following times: indaziflam, only one time at the beginning of the rainy season (spring); glyphosate in three seasons: spring, summer, and fall.

6.3. Unit and experimental design

In the Coffee crop, the plots consisted of two rows, containing in total 16 plants, measuring 8 m x 7 m and in the Citrus test, 4 x 16 m covering four plants. Experimental design, randomized block design with four replications.

Each year and during the next four years, the same treatments were repeated on the same plots, and these treatments and their frequencies are described in Table 1.

6.4. Assessments of the roots

In the winter of the following year, after the first treatment application with indaziflam and the series of three glyphosate applications, before the new series of applications, the effect of the treatments on the root system of the Citrus and Coffee crops was assessed in each plot.

For opening the trenches, the methodology cited in Ref. [19] was used, in a modified manner. For each plot, the excavation of the trenches was in the longitudinal direction of the crop planting row, close to the root collar of the plant (10–15 cm), measuring 1.20 x 2.00 x 1.20 m (width, length, and depth).

After this procedure, the profile surface was adequately prepared following the methodology of the lattice framework for the assessment of the roots: cutting the exposed roots in the profile, profile scarification for new exposure of roots, profile painting with white paint, and washing with water for a highlighted exposure of the painted white roots, where a framework was placed for the assessments, number, and percentage of roots (Photos 1–5).

Photo captions by Flavio Martins Garcia Blanco.

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Figure 1. Backhoe for construction of the trenches.

Figure 2. Exposure of roots.

Figure 3. Fixation of the picture and profile painting.

Figure 4. Washing profile, soil removal highlighting the colorful roots of white.

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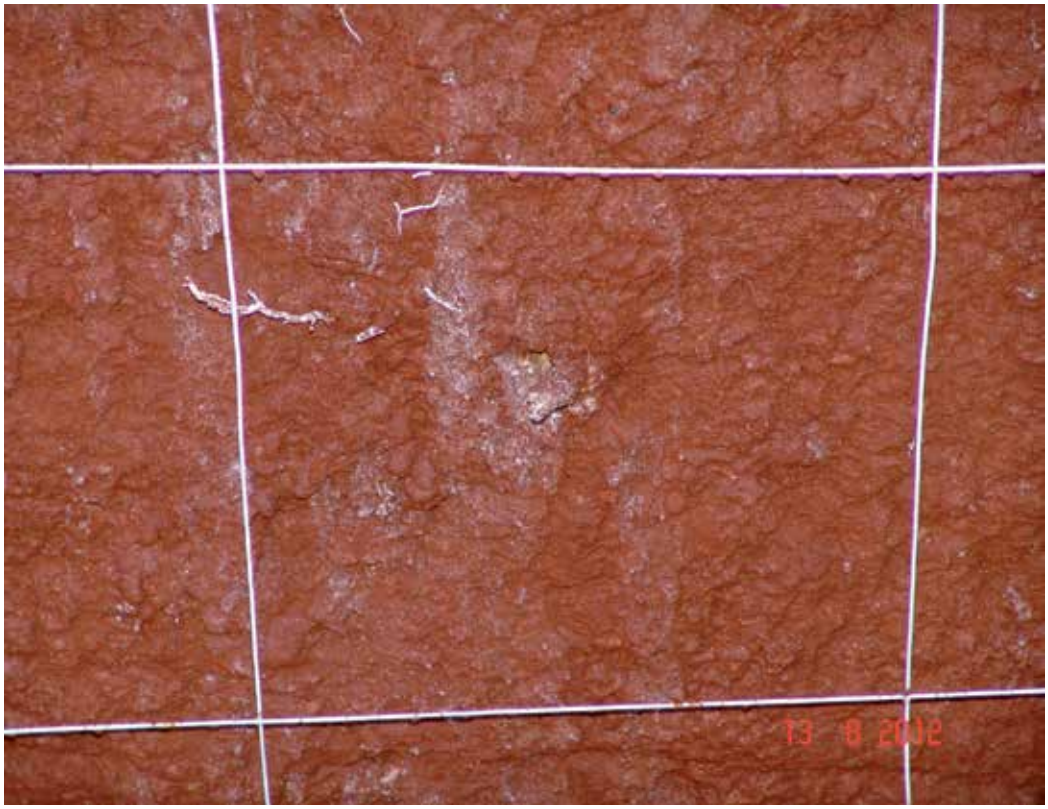

Figure 5. Roots highlighted facilitating reviews.

The wood frame 1 x 1 m (1 m^2) had 16 subdivisions, grids of 0.25 x 0.25 m (0.0625 m^2), and it was fixed in the soil profile after a preparation which outlined the roots by the white coloring. Each grid was assessed by counting the number of roots and visually estimating the percentage of area occupied by these grids.

Data were assessed at three levels: (1) spanning all the profile (1 m^2), (2) upper position of the profile (0–50 cm), and (3) lower position (50–100 cm), each of 0.5 m^2. It was thus possible to determine whether there was any phytotoxic action of herbicides in the development of the roots due to the root position in relation to the soil profile.

6.5. Statistical assessment

Data were subjected to analysis of variance, indicating the coefficient of variation. When the analysis of variance was significant at 5% probability, the *t*-test (5%) means were performed, individually comparing the null hypothesis between the means of the treatments with herbicides and the means of control weeded treatment.

For the standardization of data, these were transformed in $\sqrt{x+1}$ and arcsine $\sqrt{x/100}$ for the count and percentage of the roots, respectively.

7. Results

The results are shown in tables, separated into crops.

7.1. Citrus crop

Tables 2–9 describe the analyses of the annual assessments in the Citrus cv. Valência crops. It can be seen that the tables were separated by characteristics of counting the number of roots, Tables 2, 4, 6, and 8, and their respective cover was estimated and expressed in cover percentage, Tables 3, 5, 7, and 9, showing the assessment in the 0–100-cm overall shape profile and the two subdivisions, 0–50 cm and 50–100 cm.

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	16.42	11.10	12.14
2	Indaziflam	75	15.79	12.19	10.07
3	Indaziflam	100	16.55	12.58	10.73
4	Indaziflam	150	15.39	10.07	11.61
5	Glyphosate	3 x 960	15.84	10.69	11.57
	F		0.17 ns(1)	1.71 ns	0.57 ns
	CV %		14.4	14.0	19.3

1. Non-significant.

Table 2. Number of roots due to the treatments: first year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g. a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	0.18	0.21	0.15
2	Indaziflam	75	0.18	0.21	0.14
3	Indaziflam	100	0.19	0.24	0.13
4	Indaziflam	150	0.17	0.19	0.14
5	Glyphosate	3 x 960	0.16	0.18	0.13
	F		0.82 ns(2)	0.89 ns	0.56 ns
	CV %		17.4	24.5	19.1

1. Non-significant.

Table 3. Percentage of roots cover due to the treatments: first year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications

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	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	10.15	7.32	6.98
2	Indaziflam	75	9.29	7.07	5.90
3	Indaziflam	100	9.97	7.44	6.70
4	Indaziflam	150	9.54	6.96	6.57
5	Glyphosate	3 x 960	9.19	7.02	5.98
	F		0.36 ns(1)	0.12 ns	0.47 ns
	CV %		9.6	7.1	21.1

1. Non-significant.

Table 4. Number of roots due to the treatments: second year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	0.20	0.25	0.14
2	Indaziflam	75	0.19	0.24	0.12
3	Indaziflam	100	0.18	0.23	0.12
4	Indaziflam	150	0.20	0.25	0.12
5	Glyphosate	3 x 960	0.18	0.23	0.12
	F		0.49 ns(2)	0.74 ns	0.73 ns
	CV %		14.5	16.6	20.8

1. Non-significant.

Table 5. Root cover percentage: second year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	14.32	12.04	12.04
2	Indaziflam	75	12.96	10.22	10.22
3	Indaziflam	100	11.53	9.26	9.26
4	Indaziflam	150	13.68	10.45	10.45
5	Glyphosate	3 x 960	11.80	9.87	9.87
	F		2.49 ns(1)	3.05 ns	1.05 ns
	CV %		11.7	11.9	27.7

1. Non-significant.

Table 6. Number of roots due to the treatments: third year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the Profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	0.19	0.24	0.12
2	Indaziflam	75	0.20	0.24	0.13
3	Indaziflam	100	0.18	0.22	0.12
4	Indaziflam	150	0.19	0.22	0.14
5	Glyphosate	3 x 960	0.18	0.22	0.12
	F		0.40 ns(2)	0.28 ns	0.55 ns
	CV %		13.6	16.3	18.6

1. Non-significant.

Table 7. Root cover percentage: third year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	11.23	8.97	6.57
2	Indaziflam	75	11.56	8.82	7.49
3	Indaziflam	100	11.97	9.34	7.50
4	Indaziflam	150	12.23	8.46	8.82
5	Glyphosate	3 x 960	12.37	8.43	9.10
	F		2.49 ns(1)	3.05 ns	1.05 ns
	CV %		11.7	11.9	27.7

1. Non-significant.

Table 8. Number of roots due to the treatments: fourth year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0-100 cm	0-50 cm	50-100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	0.23	0.28	0.15
2	Indaziflam	75	0.22	0.28	0.14
3	Indaziflam	100	0.22	0.27	0.15
4	Indaziflam	150	0.23	0.28	0.15
5	Glyphosate	3 x 960	0.24	0.31	0.15
	F		0.78 ns(2)	0.74 ns	0.22 ns
	CV %		8.7	11.0	17.3

1. Non-significant.

Table 9. Root cover percentage: fourth year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications

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In all samples, analyses of variance of the treatments were performed, calculating the value of *F*, indicating its significance and its coefficient of variation.

Covering all depth ranges, the assessments of the parameters, score, and percentage of root cover, the values of the coefficient of variation had a range of 9.6% to 27.7% and 8.7% to 24.6%, respectively. These values are compatible for testing using this methodology, collaborating with the indication that technically the conduct of the tests was adequate. It is also observed that for the probability level (5%), used in the methodology to determine the significance of the analyses of variance, the value of *F* was always non-significant (ns).

This demonstrates that the methodology was not able to find significant differences among treatments by the assessed parameters, thus indicating that the treatments with the herbicides did not affect root development, therefore characterizing them as selective for growing Citrus cv. Valência.

The same format for reporting the results in the assessments in the coffee crop are described below.

7.2. Coffee crop

Following the same form of presentation of the previous crop, Tables 10–17 describe the analyses of the annual assessments on the crop Coffee cv. Catuaí Vermelho. The tables were also separated according to the parameters assessed, score of the number of roots, Tables 10, 12, 14, and 16, and their percentage of cover, Tables 11, 13, 15, and 17, all showing the assessment of the overall shape profile and in two subdivisions 0–50 cm and 50–100 cm. For each year, analyses of variance of the treatments were performed, calculating the value of *F*, indicating its significance, and also the coefficient of variation for each analysis.

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	14.18	11.51	8.33
2	Indaziflam	75	13.63	11.45	7.42
3	Indaziflam	100	14.47	11.82	8.36
4	Indaziflam	150	12.65	9.66	8.16
5	Glyphosate	3 x 960	12.04	9.42*(2)	7.52
	F		1.46 ns(1)	3.04*(3)	0.33 ns
	CV %		12.6	10.7	19.5

1. Non-significant.

2. Significant in relation to the weeded control by the $t_{(5\%)}$ test of means.

3. Significant by the analysis of variance, $F_{(5\%)}$ test.

Table 10. Number of roots due to the treatments: first year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control	--	0.14	0.16	0.12
2	Indaziflam	75	0.12	0.14	0.08
3	Indaziflam	100	0.15	0.18	0.11
4	Indaziflam	150	0.11	0.12	0.10
5	Glyphosate	3 x 960	0.12	0.14	0.10
	F		1.70 ns(1)	1.70 ns	3.00 ns
	CV %		20.29	25.2	19.16

1. Non-significant.

Table 11. Percentage of roots cover due to the treatments: first year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		10.05	8.87	4.76
2	Indaziflam	75	11.26*(2)	9.61	5.86
3	Indaziflam	100	11.74*	10.14*	5.96
4	Indaziflam	150	10.67	9.33	5.26
5	Glyphosate	3 x 960	10.05	8.23	5.68
	F		3.77(3)	6.09(3)	0.74 ns(1)
	CV %		7.7	16.1	20.8

1. Non-significant.
2. Significant in relation to the weeded control by the $t_{(5\%)}$ test of means.
3. Significant by the analysis of variance, $F_{(5\%)}$ test.

Table 12. Number of roots due to the treatments: second year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		0.22	0.30	0.08
2	Indaziflam	75	0.19	0.25	0.09
3	Indaziflam	100	0.21	0.28	0.11
4	Indaziflam	150	0.20	0.27	0.10
5	Glyphosate	3 x 960	0.18*(2)	0.24	0.09
	F		2.94 (3)	2.39 ns(1)	1.59 ns
	CV %		14.5	12.3	21.2

1. Non-significant.
2. Significant in relation to the weeded control by the $t_{(5\%)}$ test of means.
3. Significant by the analysis of variance, $F_{(5\%)}$ test.

Table 13. Root cover percentage: second year. Data transformed in arcsin $\sqrt{x/100}$ Average of four replications

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	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		15.48	13.02	8.39
2	Indaziflam	75	16.35	13.49	9.16
3	Indaziflam	100	16.57	13.97	8.91
4	Indaziflam	150	16.32	13.59	9.00
5	Glyphosate	3 x 960	13.61	11.69	6.98
	F		1.51 ns(1)	1.02 ns	1.58 ns
	CV %		15.6	13.3	16.1

1. Non-significant.

Table 14. Number of roots due to the treatments: third year. Data were transformed into $\sqrt{x+1}$. Average of four replications

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		0.15	0.19	0.11
2	Indaziflam	75	0.17	0.21	0.12
3	Indaziflam	100	0.21*(2)	0.26*(2)	0.13
4	Indaziflam	150	0.19	0.23*(2)	0.13
5	Glyphosate	3 x 960	0.13	0.16*(2)	0.10
	F		12.56* (3)	10.50* (3)	4.54* (3)
	CV %		9.3	11.9	5.5

1. Non-significant.
2. Significant in relation to the weeded control by the $t_{(5\%)}$ test of means.
3. Significant by the analysis of variance, $F_{(5\%)}$ test.

Table 15. Root cover percentage: third year. Data transformed in arcsin $\sqrt{x/100}$ Average of four replications.

	Treatments	g a.i. ha^{-1}	Parameters: number of roots in the profile layer		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		16.58	14.54	8.03
2	Indaziflam	75	16.10	13.73	8.27
3	Indaziflam	100	15.66	13.74	7.49
4	Indaziflam	150	16.01	14.05	7.72
5	Glyphosate	3 x 960	14.36	12.42	7.17
	F		0.92 ns(1)	1.23 ns	0.23 ns
	CV %		12.6	10.3	23.1

1. Non-significant.

Table 16. Number of roots due to the treatments: fourth year. Data were transformed into $\sqrt{x+1}$ Averages of four replicates.

	Treatments	g a.i. ha^{-1}	Parameters: percentage of cover in the profile		
			0–100 cm	0–50 cm	50–100 cm
			1 m^2	0.5 m^2	0.5 m^2
1	Weeded control		0.19	0.24	0.13
2	Indaziflam	75	0.19	0.23	0.15
3	Indaziflam	100	0.20	0.26	0.13
4	Indaziflam	150	0.19	0.23	0.13
5	Glyphosate	3 x 960	0.16	0.19	0.11
	F		2.10 ns(2)	2.01 ns	1.90 ns
	CV %		12.8	14.3	16.0

1. Non-significant.

Table 17. Root cover percentage: fourth year. Data transformed in arcsin $\sqrt{x/100}$. Average of four replications.

Regarding the coefficient of variation, in all the depth ranges the assessments of the parameters, scores, and percentage of cover had a range of 7.7% to 23.1% and 5.5% to 25.2%, respectively. These ranges were compatible for testing using this methodology, indicating that these were technically well conducted.

Analyzing the tables, it is observed that the probability level (5%) used as a threshold to determine the significance of the analysis of variance, the *F* value, was significant in some cases, thus demonstrating that the methodology was able to find differences.

This occurred in the statistical analyses indicated in the tables and their parameters: Table 10: first year, number of roots; Tables 12 and 13: second year, number of roots and percentage of cover; and Table 15: third year, percentage of cover.

In these tables, testing of the *t* (5%) means comparing the weeded control treatments with the treatments using herbicides (null hypothesis) was performed. It was observed in Table 16 in the assessment of the parameter percentage of roots cover in the soil profile in the 50–100-cm layer that the null hypothesis was accepted in all comparisons between each treatment with herbicides against the weeded control. Therefore, even if the *F* value is significant, because of the research objective, that is, to assess the effect of herbicides compared to the weeded control, the null hypothesis was accepted in all comparisons.

This situation did not occur in the other analyses where *F* was significant since the comparisons among treatments with herbicides and weeded control were significant by the rejection of the null hypothesis.

The treatment with herbicide glyphosate was the one with the largest number of rejected null hypotheses when compared with the weeded control treatment. In the first year, the number of roots in the upper layer was significantly lower (Table 10). This also occurred for the percentage of root cover that was lower in the second year throughout the soil profile assessed 0–100 cm (Table 13) and in the third year in the most superficial layer of the profile, 0–50 cm (Table 15).

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This can be explained by two factors: first, possible drift that hit the lower leaves of the coffee crop. Due to the downward movement of glyphosate by means of phloem vessels, it was transported to the roots, damaging them. The second factor is the exudation of this herbicide by the roots of weeds that received its application. Therefore, the coffee roots could absorb the herbicide expressing harm [18, 19, 20, 21, 22, 23].

The indaziflam herbicide also differs from the control in the parameter percentage of roots cover in the third year of assessment of the roots (Table 15), in treatments 100 g a.i. ha^{-1}, in all of the assessed profile range (0–100 cm) and also in the upper layer (0–50 cm). The same was found for this range of the profile for treatment 150 g a.i. ha^{-1} (Table 15). Only the parameter percentage of cover for this herbicide, regardless of the dose, was rejected by the null hypothesis and always with higher values than those obtained in the weeded control.

Research carried out by Blanco [24] has determined that indaziflam had long persistence in soil under conditions similar to the ones in the testing described herein. Thus, it can be inferred that indaziflam remaining in the soil solution can act on the secondary metabolism of the coffee roots and interfere in their secondary growth, which would explain their thickening.

In the fourth year of the test, assessing the number and percentage of roots, it was found that the analyses of variance were not able to find differences among treatments [25].

Thus, in the test scenario it can be concluded that:

- The methodology to determine the selectivity of herbicides by assessing the roots by means of trench and grid framework was appropriate.
- Herbicide glyphosate applied for four years, in three annual applications, can affect the development of crops of Coffee cv. Catuaí Vermelho.
- Herbicide indaziflam applied once for four years in crops of Coffee cv. Catuaí Vermelho and Citrus cv. Valência is selective for these crops.

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Chapter 13

Safety Measures for Handlers/Workers against Herbicide Intoxication Risk

Joaquim G. Machado-Neto

Additional information is available at the end of the chapter

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Abstract

With the use of herbicides, there is a certain risk of intoxication to directly exposed workers, which depends on several factors. Risk factors can be grouped in the toxicity of herbicides and exposure provided by the specific working conditions. From the assessment of the risk of intoxication, working conditions can be classified as safe or unsafe. The safety of working conditions is based on the chronic toxicity of the pesticide and the absorbable amount of dermal and respiratory exposure. Safety can be determined by calculation of the margin of safety calculation (MOS). If the value of MOS ≥1, the working condition is classified as safe, but if the MOS <1, the condition and work is classified as unsafe. For working conditions classified as unsafe, workers should adopt safety measures to become safe. Safety measures at work are grouped into preventive and protection. The preventive safety measures are grouped into the selection of workers/personnel: psychological measures; administrative: legislation, standards, and procedures; and hygiene, cleaning, maintenance, and safety of the environment. The protection safety measures are grouped into collectives and individual. The Brazilian labor law mandates the use of preventive measures and protection, according to the pesticide manufacturers' recommendations on the labels.

Keywords: Pesticides, herbicides, occupational exposure, risk of intoxication, safety measure

1. Introduction

The convention 012 (C012) of the International Labour Organization (ILO) on the Compensation for Work Accidents (Agriculture), 1921, became internationally effective on 02.26.1923. Convention 012 extends the benefit of the laws to agricultural workers and regulates mandatory indemnification of victims of work accidents [4].

Work accidents occur due to the existence of risks in the working environment. Work risk is any possibility that some element or condition in a given process can cause damage or be destructive to the physical and/or psychological health of the worker. The risk outcome can be an accident, illness or suffering, or exposition to environmental pollution.

The ILO Convention 184 on safety and health in agriculture, 2001, states that member states should define, implement, and revise a coherent national policy on agriculture safety and health periodically. The purpose is to prevent accidents and health injury related to or as a consequence of work and to eliminate, mitigate, or control the risks in the agricultural working environment [9].

In Brazil, the NR 31 approved in 2005, and amended in 2011 and 2013, is still in force [6]. It is related to safety and health in agriculture, livestock, forestry, and aquaculture, meeting the obligations of signatory member countries of the ILO Convention 184.

Pesticides are chemical compounds used to control organisms harmful to crops. Pesticides are carefully selected to intoxicate the target organisms. Toxicity is the ability to cause harm and death of the target organisms [21]. However, nontarget organisms that come into direct contact with pesticides can be intoxicated, especially if they have the points of the toxic action. Therefore, working with pesticides poses a chemical poisoning risk to exposed workers [1].

The overall Brazilian legislation on health safety (NR 9) in the workplace provides that companies must implement the environmental risk prevention program (ERPP). The ERPP consists of the following steps: anticipation, recognition, quantitative assessment, and control of risks according to specific action levels [7].

This chapter informs the reader about the safety procedures required while working with pesticides. It aims at motivating the worker to make a habit of preventing herbicide poisoning. Moreover, it also serves to motivate the businessmen to implement safety measures in the working environment, to keep the risks at acceptable levels, while increasing business productivity and competitiveness.

2. Risk of intoxication with herbicides

The risk of worker intoxication with pesticides, especially herbicides, depends on several elements that can be grouped into two major factors: the toxicity of the handled herbicides and the exposure under the specific working conditions [1]. Therefore, the safety and health management during work with herbicides should be addressed using the steps set out by the industrial hygiene and the ERPP [7]. The steps are the ability to anticipate, identify, recognize, evaluate, and control the risks in the workplace.

2.1. Anticipation and identification of the intoxication risks associated with herbicides

A safety and health management plan for workers exposed to herbicides must anticipate and identify potential risks and health hazards before a given production process is implemented or modified, or new risk agents are introduced in the workplace.

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The risks can be anticipated during the planning phase of the productive activity such as acquiring new equipment and/or materials (including pesticides), determining the work process, and preventive maintenance plans.

The identification of pesticide poisoning risks starts with the specific agricultural production system. The production systems can be organic, when the use of synthetic toxic products is not allowed, or conventional, when the use of new agricultural technologies is allowed, including the use of pesticides. Therefore, the risk of occupational intoxication with pesticides exists only in conventional farming systems.

The use of pesticides in conventional crops depends on a wide variety of interrelated factors that can be grouped according to the direct relation to the crop, crop environment, and target organisms.

During the planning of an agricultural crop, the professional must be able to predict the occurrence of harmful organisms and control methods. The pesticides recommended for chemical control are legally required to inform the formulation registered in the Ministry of Agriculture, Livestock and Supply, MAPA, in the package leaflet [10].

2.2. Recognizing the intoxication risks of pesticides

In this step, the work environment is observed and carefully analyzed to identify the agents and their potential risks. The priority assessment and control of the risk involved must be established. Risk analysis should answer the following questions: What? Where? andHow? A deep knowledge of the products involved in the process, working methods, process flow, and facilities layout is necessary to answer these questions [27].

It is necessary to analyze the information contained in the package leaflet regarding the product, the environment and protection of human health to recognize the poisoning risks of pesticides [10]. The leaflet also contains information about application methods and calibration settings of the application equipment. The handler is exposed to the product by drift, that is, the portion of the product that does not reach the target and drifts away. The extent of drift and exposure depends on several factors, predominantly the type of application and the equipment used. Therefore, the type of formulation is an important information contained in the leaflet regarding the handler's safety since it determines the type of application equipment to be used. The leaflet must also contain mandatory information on poisoning, acute and chronic toxicity of pesticides, the first aid procedures should an intoxication occur, and preventive measures to protect handlers.

The identification of the working conditions and the job are critical to determining the risk of poisoning with pesticides. Handler exposure is determined by the working conditions while working conditions are identified by the activities performed. The legislation on pesticide working safety (NR 31) defines workers in direct exposure as those handling pesticides, additives, and related products in any of the stages of storage, transportation, packaging opening, pesticides preparation and application, disposal and decontamination of equipment, and clothing [6].

Thus, it is necessary to identify homogeneous work groups (HWG). The HWG consists of workers performing the same activity and exposed to the same risks. Therefore, safety measures will be similar for all workers of the same HWG since the working conditions are the same [14]. The exposure assessment for any worker of the HWG is representative of the exposure of all workers of the group [29].

2.3. Quantitative assessment of pesticide poisoning risks

The procedure to perform risk assessment of intoxication with pesticides has been established in a document of the American National Academy of Sciences (USA) in 1983 as a four-step process [33]. The four steps are outlined in the diagram shown in Figure 1 [18].

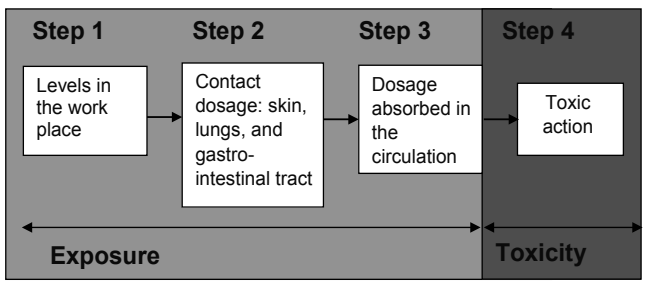

Figure 1. The steps of the intoxication risk assessment when working with pesticides [18].

Occupational exposure to pesticides is influenced by the type of work or activity performed, the way it is conducted, and the product formulation [20]. Toxicity is an intrinsic property of pesticides that is determined following national regulations, even before they are placed on the market.

2.3.1. Occupational exposure to pesticides

The worker's exposure to pesticides can be real or potential. The actual exposure refers to the absolute amount of pesticide that comes into contact with the body during a determined working period and is readily available to be absorbed via dermal, respiratory, or oral routes [1].

Potential exposure refers to the amount of the pesticide that could be absorbed via dermal, respiratory, and oral routes if the worker had not worn personal protective equipment during the operation [32].

The potential exposure results from the interaction of the dominant risk factors in specific working conditions. It has been determined that during the application of herbicides in field conditions, 99% or more of total exposure occurs via dermal and only 1% or less, via respiratory [35, 34, 26].

Therefore, knowledge about pesticide exposure and the relative importance of each pathway to the total exposure is essential to select the most effective, comfortable, economical, and applicable safety measures given the specific conditions.

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2.3.1.1. Exposure routes

The potential routes of exposure to pesticides in the workplace are dermal and, to a lesser extent, respiratory. The relative importance of each exposure route is directly related to the specific working conditions or determinants of exposure. To tractor operators spraying eleven pesticides in a citrus orchard using a turbo type sprayer, 99.7–99.9% of the total exposure occurs via dermal, and only 0.1–0.3%, via respiratory route, on average [35]. This finding is greatly important for adopting safety procedures, especially regarding personal protective equipment (PPE).

The predominance of the dermal exposure under field conditions is explained by the spray droplets or mist, which drifts away towards the handler reaching the skin. The lower respiratory exposure is explained by the low contamination of the air that the handler breathes. The low contamination of breathable air is due to the fantastic dispersion of the spray droplets in the atmospheric air. Wolfe et al. reported that it is due to the distance between the tractor driver and the nozzles of the spray jet, the area with the greatest concentration of drops [35].

The potential reach of the bloodstream and the absorption of pesticides on the exposure routes should be also considered. After contact, the first entry stage of the toxic compound is absorption by the body. The absorption of a pesticide that reaches the lungs via respiratory route is fast and complete [16]. On the contrary, the absorption of herbicides via the dermal route is slower, partly because the skin is a natural, efficient barrier.

Respiratory exposure

Respiratory exposure consists of exposure to spray droplets containing the toxic compounds and possible toxic vapors present in the air that the worker breathes. The drops are particles suspended in the air, called liquid aerosols. Liquid aerosols are liquid particles produced by mechanical disruption of liquid, called mist [28]. The pesticide spraying is a mist of water droplets. The aerosols are classified by the diameter (Ø), as shown in Table 1 [27].

Type of particulate	Size (μm)
Sedimentable	$10 < Ø < 150$
Inhalable	$Ø < 10$
Breathable	$Ø < 5$
Visible	$Ø > 40$

Table 1. Classification of aerosols according to the type and size of particulates, for respiratory exposure [27].

The inhaled and breathable particles are the most harmful. Inhalable particles are usually deposited in the respiratory tract. However, the breathable particles enter the upper and lower respiratory tracts, reaching the lungs and the pulmonary alveoli. In the alveoli, the toxic compounds permeate the cellular membranes reaching the bloodstream and the toxic action site in the body.

The droplet size is one of the most important parameters of pesticide application technology. The spray droplets typically have a mean volume diameter (MVD) in the range of sedimentable and visible particulates (Table 1), or higher, from 100 to 300 μm. The droplet size can be one of the factors that explain lower respiratory exposure under certain field conditions [35, 34, 26].

Dermal exposure

The skin consists of the outermost and innermost layers, the epidermis and dermis, respectively. The dermis consists of connective tissue, blood vessels, nerves, hair follicles, sebaceous, and sweat glands. Direct contact with the external environment occurs through the follicles and the dermal glands. Thus, chemicals can be absorbed mainly by the epidermal cells and hair follicles.

The absorption speed of chemicals into the skin is mainly limited by the continuous stratum corneum. The fat-soluble substances penetrate through the lipids existing between the keratin filaments by passive diffusion. The rate of absorption of oily substances is indirectly proportional to their viscosity and volatility. For polar substances of low molecular weight, the absorption occurs through the outer surface of the keratin filament, the hydrated extract. The transepidermal absorption is the most frequent due to the high number of epidermal cells, although not very easy for toxicants.

The transfollicular absorption is less significant than the transepidermal. Some chemicals can penetrate the hair follicles and quickly reach the dermis. Penetration is easier for chemicals because they do not need to go through the stratum corneum. Fat-soluble or water-soluble, ionized or nonionized, gas or vapor, and acidic or basic substances can penetrate the follicles. The penetration of toxic compounds in the skin depends on its absorption pharmacokinetic model [25] and can vary from 0.8% to 6.0% [19] up to 80% [11].

2.3.2. Quantification of dermal and respiratory exposures

The whole-body method is the most suitable for assessing dermal exposure. In this method, the worker dresses in a cotton coverall, according to the protocol VBC 82.1 [36]. This method evaluates the potential and actual dermal exposures directly on the coverall used by the worker over the safety equipment, under normal working conditions for a certain evaluation period (Figure 2A and B).

The coveralls are long-sleeved with a hood made of white denim material. They are used to quantify the dermal exposures of the head + neck, trunk (back and front), arms, and legs (back and front). Cotton gloves are used to quantify the dermal exposure of the hands. The face and feet exposure is evaluated using Carefree™ female sanitary napkins that are attached to the semidisposable facial masks and rubber boots [22, 23].

Respiratory exposure is evaluated using personal continuous air flow pumps (Figure 2A and B) with specific respirator filters, cartridges, or cassettes for collecting particulates, gasses, and vapors [27].

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Figure 2. Workers applying pesticide in the field during assessment of potential dermal exposure: A—quantified in coverall and unprotected, exposure real: B—quantified in coverall worn under the personal protective equipment during the evaluation period, according VBC 82.1 [36].

2.4. Controlling the risk of intoxication with pesticides

Safety, health, and environment management is regulated by a specific labor legislation for pesticides, the regulatory standard no. 31, known as NR 31 [6]. The NR 31 prioritizes implementation of safety measures to eliminate the risks by replacing or adjusting the production processes, machines, and equipment; by adopting collective protective measures to control the risk at the source; and by introducing personal protection. Safety procedures should be implemented based on risk assessment and risk acceptability criteria. Working conditions can be classified as safe or unsafe according to the adopted risk acceptability criterion. The acceptability criterion of poisoning risk with pesticides is based on toxicity and handler exposure determined by working conditions.

Safety evaluation is fundamental to classify working conditions and select the appropriate safety procedures needed to reduce the risk of poisoning to acceptable levels.

2.4.1. Acceptability criterion of intoxication risk with pesticides

The acceptability criterion of pesticide risk is based on the no observable effect level (NOEL) in mg/day/kg body weight. The NOEL is determined by assessment tests of the chronic toxicity of pesticides using mice in laboratory conditions. The studies follow international standard procedures.

The acceptability criterion of poisoning risk with pesticides can be quantified by calculating the margin of safety (MOS) proposed by Severn [30] and adapted by Machado-Neto [23]. MOS is determined dividing the safe dose (calculated by multiplying the NOEL value and the worker body weight) by the absorbable quantity of exposure (AQE) via the dermal and respiratory tract, under working conditions:

$$MOS = \frac{NOEL \text{ x } W}{AQE \text{ X } SF}$$

where NOEL is the no observed effect level (mg/kg/day); W is the mean body weight of the worker, considered to be 70 kg; AQE is the absorbable quantity of exposure (mg/day); and SF is the safety factor.

AQE via the dermal route is determined for each molecule in the dermal exposure studies with laboratory animals or human skin tissue grown in the laboratory. For molecules wherin dermal absorption has been determined, it is considered to be 10% of the evaluated dermal exposure for each working condition, according to Byers et al. [13].

The AQE in the respiratory tract can be considered as 100% of respiratory exposure evaluated for each working condition [23].

The SF, which multiplies AQE, is used to compensate for the extrapolation of the NOEL results obtained in laboratory animals, to humans [12]. The used safety factor may be 10 [12], to compensate for interspecies extrapolation, or 100; 10 for interspecies times 10 for intraspecies extrapolation.

The MOS is calculated for the activities and daily exposure times according to working conditions. For formulations with two or more active ingredients, the commercial product safety rating is based on the smallest value among the MOS calculated for the active ingredients.

The risk acceptability criterion depends on the toxicity of the pesticide, while the intensity of risk depends directly on the intensity of worker exposure to pesticides.

The working condition is classified as safe if MOS ≥1; exposure as tolerable and intoxication risk as acceptable.

The working condition is classified as unsafe if MOS <1; exposure as intolerable and intoxication risk as unacceptable.

2.4.2. Control action level of intoxication risk

According to NR 9, the action level is a value above which preventive measures should be initiated to minimize the likelihood that exposures to environmental agents exceed the exposure limits [7].

For pesticides, the action level to implement the risk control measures is when the working condition is classified as unsafe (MOS <1). For unsafe conditions, preventive and protective

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measures should be intensified enough to make the working conditions safe (MOS ≥ 1). The preventive safety and protection measures should control toxicity and/or exposure to make unsafe working conditions safe.

3. Safety measures while working with pesticides

Controlling the intoxication risk with safety measures is a key step to planning health and safety management while working with pesticides. The specific labor legislation for pesticides, the NR 31 [6], states that the rural employer or similar should conduct risk assessments to ensure the safety and health of workers. Based on the results, the employer must adopt preventive and protection procedures to ensure that all activities, workplaces, machinery, equipment, tools, and processes are safe and in compliance with health and safety standards. The legislation also determines the prioritization of measures to prevent accidents and occupational diseases [6].

3.1. Preventive safety measures

The preventive safety measures may be applied early in the planning of agricultural activities, during the previously described steps of anticipation, identification, and recognition of the risks. Preventive safety measures target the risk factors and can be divided into two groups, to eliminate and to reduce poisoning risk. Preventive measures aim to ensure a safe work environment and maintain workers skilled and motivated and in good health conditions.

Most safety measures determined in the specific legislation to work with pesticides, NR 31, are preventive [6]. The administrative measures are highlighted among the preventive safety measures determined in the legislation on safety and health at work. Administrative preventive measures are adequate policies and procedures to the specific working conditions.

In the planning of agricultural activities with the use of pesticides, prevention measures will act on risk factors, to reduce the toxicity of the pesticide and/or exposure caused to the workers by the working conditions.

3.1.1. Selection of workers/personnel

The personnel selection process is critical to prevent the occurrence of accidents. To work with risky activities, employees must have a physical and psychological profile suitable to the working conditions.

Every worker acquires personal characteristics that form the personality resulting from physical and mental growth over the years in the work environment in which he/she lived. The personality traits can be strong and striking, positive and negative, displaying strengths and weaknesses as irresponsibility, stubbornness, irritability, etc. [3].

The worker's personality, whether positive or not, is carried into the working environment and can cause unsafe acts or unsafe working conditions. Due to negative traits, the worker can

act unsafely ignoring safety rules and make mistakes when performing his or her work that may result in work accidents. Unsafe acts are the way workers expose themselves, consciously or unconsciously, to accident risks (e.g., using machines without a license or permission, not using the required PPE, etc. [3]).

Therefore, the selection process should take into account the physical and psychological profile of the employee required to work in risky activities. Thus, the occurrence of accidents can be prevented. Once selected properly, the worker must be trained to perform especially risky activities.

3.1.2. Psychological measures

When the worker interacts in the workplace as a whole (physical or abstract space), he/she is influenced positively or negatively, thus changing his or her physical, mental, and social state. On the other hand, it is natural that the worker brings his or her personal problems to the workplace [2]. Therefore, it is impossible to pretend that all is well when, in fact, the worker is experiencing personal problems. The only one able to understand and evaluate the anguish of losing a loved one, the disappointment of a treacherous love, among others is the person itself. These factors are, for the most part, largely responsible for the increase in the indices that measure delays, work absences, illnesses, and accidents [2].

In working activities, there is a personal unsafety factor when the employee works grudgingly, under abnormal physical conditions (illness, physical, or mental disability), without experience, knowledge, and proper training. Due to personal unsafety factors, the worker may cause accidents and/or occupational illnesses that result from negligence, recklessness, or malpractice [2].

Companies must implement measures that value the worker, by enabling professional growth and personal development to control the human factor effects on the risk of poisoning with pesticides. Such actions elevate the workers' self-esteem and improve performance and commitment, thus making the working environment more pleasant. There are other positive actions such as dynamics between employees, awards campaigns, and the willingness to hear suggestions to improve the workplace.

The work environment should be a harmonious and welcoming place for all to engage in their activities with satisfaction and enthusiasm. The standards and rules set by the company are also important to promote good communication. The basic behavioral rules that are desirable in the workplace are as follows: mutual respect, call people by name, be calm, participate actively in the activities that are delegated to you, be willing to help others, do not judge people, and do the best you can. Dialogue is always important to avoid conflicts, to respect people differences, and to avoid intrigues, gossip, and side conversations.

Companies, more than ever, are looking for professionals who can work in groups, be proactive, and capable of leading. The website of a Brazilian company recruiting employees states, "For all opportunities, Eldorado Brazil seeks professionals with good communication and interpersonal skills and 'owner' attitude" [17].

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3.1.3. Administrative: Legislation, standards, and procedures

The companies should implement security measures to meet specific labor laws and local regulations to prevent workplace accidents. The preventive security administrative measures are mandatorily applied at all stages, starting at the registration of pesticides up to before labor legislation. The legal commercialization of pesticides in the country is regulated by the legislation on pesticide registration, Decree Law no. 4074 [10].

According to the Decree Law no. 4074 [10], pesticides can only be produced, handled, imported, exported, commercialized, and used in national territory after being registered with the appropriate federal agency. They are required to meet the guidelines and requirements of federal agencies responsible for the sectors of agriculture, health, and environment.

The Ministry of Agriculture, Livestock and Supply (MAPA) is the federal agency responsible for registering pesticides. The Ministry of Health (MS) is responsible for evaluating and classifying toxicologically the pesticides; performing preliminary toxicological evaluation of pesticides, technical products, premixes, and the like, used for research and experimentation; and establishing the reentry interval in a treated environment, among others, as set out in Decree Law no. 4074 [10].

The main administrative preventive measure prohibits the registration of pesticides that can cause harm. The Decree Law no. 4074 [10] prohibits pesticide registration for which there are no available methods for disabling its components in Brazil. There is no antidote or effective treatment.

The teratogenic and carcinogenic compounds present enough evidence as such from observations in humans or studies in experimental animals.

The mutagenic compounds might induce mutations observed in at least two tests, one for detecting genetic mutations, performed using even metabolic activation and the other to detect chromosomal mutations.

Some compounds cause hormonal disorders and harm the reproductive system. They are more dangerous to humans than animals based on tests, according to updated technical and scientific criteria, and some compounds harm the environment.

Tests, trials, and studies about mutagenesis, carcinogenesis, and teratogenesis, conducted in at least two animal species should be performed according to criteria accepted by national or international technical and scientific institutions.

The legislation on working with pesticides (NR 31) requires a management plan regarding health, environment, and safety for rural works [6]. The management plan must include preventive measures for the workers' health (medical examinations) and other actions catering to specific needs, SESTR—Specialized Service on Safety and Health for Rural Work and CIPATR—Internal Commission for Rural Work Accident Prevention.

According to the risks in the workplace, the risk management program should also contemplate the Hearing Conservation Program (HCP), the Respiratory Protection Program (RPP), the Dermatoses Occupational Program (DOP), and the Ergonomic Action Program (EAP) [14].

As internal and specific standard, each company must have in written form the operating procedure (OP) or operating instructions (OI), based on the manuals for machines and pesticide application equipment (NR 31), and the service order (SO), as set out in NR 1 [5] for each OP.

Companies should also have other internal rules to regulate the health and safety of workers, such as hygiene, cleaning, and maintenance of machine and equipment. To meet the requirements of NR 31, the preventive safety procedures also include training workers on prevention of accidents with pesticides, ergonomic measures, safety of machinery, and equipment. The workers should also be granted access roads to workplaces, transportation of workers and cargo, living areas for meals in the field and toilets, and collective and personal protection measures [6].

3.1.3.1. Pesticide packaging

The Decree Law no. 4074 [10] states that pesticide containers shall be designed and constructed to prevent leakage, evaporation, loss, or alteration of its content, and to facilitate the cleaning, sorting, reuse, recycling, and proper disposal. The packages should be resistant to the contents and not able to form harmful or dangerous compounds. Packages must be resistant everywhere and adequately meet the conservation requirements.

Packages must have sealing wax or another external device to ensure visually the package has not been violated. When stacking of rigid packaging is allowed, the maximum number of units that can be stacked should be stated.

The rigid packaging containing formulations miscible or dispersible in water must be submitted to triple washing operation, or equivalent technology, by the user according to the instructions on their labels, leaflets, or brochure supplement.

Users should return the empty containers of water miscible pesticides and their lids, triple washed, to the shop of purchase at the latest 1 year from the date of purchase. Users should make available to the supervisory authorities the empty containers return vouchers, provided by the shops, receiving stations or gathering centers for at least 1 year after the packaging has been returned.

Packaging containing products unfit for use or unused should be disposed of following the guidelines informed in the package leaflet. It is the responsibility of the company that registered, produced, and commercialized the product to collect and dispose of the products.

3.1.3.2. Pesticide labels and package leaflets

The pesticide registration legislation, Decree Law no. 4074 [10], requires that warnings regarding the danger of pesticides should be placed on the label and package leaflet. The pesticide labels should have on the bottom a distinctly colored band separated from the rest of the label, with a height equivalent to 15% of the label/package height. The colors of the bands correspond to different toxicological classes established by the Ministry of Health, in Ordi-

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nance no. 03 [8], for pesticides: red, extremely toxic products (Class I); yellow, highly toxic (Class II); blue, moderately toxic (Class III); and green, slightly toxic (Class IV).

A white circle with a diameter equal to the band height containing the skull and crossbones in black on white background should be included on the front panel of the label, with the words "caution poison." Specific pictograms, internationally accepted, should be placed along the colored band from the center to the edge occupying 50% of the band height (Figure 3).

The label central column shall contain the manufacturing and expiration dates, indications whether the content is explosive, flammable, oxidizing, corrosive, irritant, or subject to applied sales. The following warnings should also be on the label: "The use of personal protective equipment is required. Protect yourself" and "The return of the empty package is required," along with the toxicological and potential environmental hazard classification.

Figure 3. Warning band required at the bottom of the label, colored according to the toxicological class and pictograms about environmental and worker safety.

The labor legislation NR 31 [6] prohibits the handling of any pesticide not registered and not authorized by the relevant government agencies, complementing the preventive safety measures established in the legislation for pesticide registration. It prohibits the handling by persons under 18 and over 60 years old and pregnant women. It prohibits the handling of any pesticides in the workplace at odds with the indications and the instructions on the label and package leaflet.

It prohibits working on newly treated areas before the end of the reentry interval set on the label, except with the use of recommended protective equipment. It also prohibits any person to enter and stay, during aerial spraying, in the area to be treated.

3.1.3.3. Mandatory precaution measures in the package leaflet of pesticides

Decree Law no. 4074 [10] requires that the precaution measures against the poisoning risk of workers should be on the labels and in the package leaflet. Sample data related to the protection

of human health are stated on the label and in the package leaflet of the herbicide Front™ follows [15].

The herbicide Front™ is registered by the company Du Pont of Brazil SA, in the Ministry of Agriculture, Livestock and Supply—MAPA, under number 10110, containing the following herbicides: diuron (603 g/kg), hexazinone (170 g/kg) and sulfometurom methyl (14.5 g/kg), and other inert ingredients (212.5 g/kg). To prevent spraying drift and reducing the occurrence of worker exposure, the herbicide package leaflet recommends the following:

Winds: Windspeed higher than 10 km/h or situations when the absence of winds causes a thermal inversion increases the potential for drift. The wind conditions and many factors, such as droplet diameter and type of equipment determine the potential spray drift. Do not apply if there are gusts of wind. Do not perform aerial application when there is no wind.

Reentry interval for people in the treated crops: People are not allowed to enter the area where the product was applied before it is completely dried (at least 24 hours after application). If it is necessary to enter during this period, the personal protective equipment (PPE) recommended for use during application should be used as well.

Instructions for product storage, conservation, and accident prevention: Keep the product always closed in its original packaging. The site should store exclusively toxic products, away from food, drink, feedstock, or other materials. The construction must be masonry or noncombustible material. The location must be ventilated, covered, and have waterproof floors. Warning signs such as "CAUTION: POISON" should be placed in the area. The place should be locked to prevent access of unauthorized persons, especially children. There must always be adequate packaging available to wrap broken packaging or to collect leaked product. In case of warehouses, the instructions of ISO 9843, ABNT—Brazilian Technical Standards Association should be followed. Observe the provisions of state and local legislation.

Data related to the protection of human health: Read the instructions carefully before use. Dangerous product. Use personal protective equipment as indicated.

General precautions: Product exclusively for agricultural use. Do not eat, drink, or smoke while handling and applying the product. Do not handle or apply the product without the recommended PPE. Recommended PPE must be worn in the following order: overalls, boots, apron, respirator, goggles, hood, and gloves. Do not use damaged PPE. Do not use equipment with leaks or defects. Do not unclog spray tips, nozzles, and valves with your mouth. Do not carry the product together with food, medicine, feed, animals and people.

Precautions when preparing the product for application: Product extremely irritating to the eyes. In the event of accidental contact with the product, follow the guidelines outlined in first aid procedures and quickly seek emergency medical service. Open the package carefully to avoid dispersion of dust. Use PPE: water-repellent cotton overalls with long sleeves going over the gloves and pants legs over the boots, rubber boots, waterproof apron, respirator with mechanical filter class P2, safety glasses with side shields, hood, and nitrile gloves. Handle the product in open and ventilated area.

Precautions during application: Avoid as much as possible contact with the treated area. Do not apply the product in the presence of strong winds and the hottest hours of the day. Do not

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apply product against the wind, when using a backpack herbicide applicator. When using a tractor (or plane), apply product against the wind. Apply the product only at recommended doses and observe the safety interval (time interval between the last application and harvest). Use personal protective equipment (PPE): water-repellent cotton overalls with long sleeves going over the gloves and trouser legs over the boots, rubber boots, respirator with mechanical filter class P2, safety glasses with side shields, hood, and nitrile gloves.

Precautions after application: Flag the treated area with the words "Entry prohibited. Treated area." Keep the warnings until the end of the reentry period. If you need to enter the area treated during this period, use the PPE recommended during application. Keep the remaining product properly closed in the original packaging and locked away from children and animals. Before removing PPE, wash the gloves while still dressed to avoid contamination. Recommended PPE must be removed in the following order: hood, goggles, aprons, boots, overalls, gloves, and respirator. Take a bath immediately after applying the product. Change and wash protective clothing separately from regular family laundry. When washing the clothes, use gloves and waterproof apron. Maintain and wash protective equipment after each product application. Follow correctly the manufacturer's specifications regarding filter usage time. Do not reuse the empty package. Use the PPE during package disposal: water-repellent cotton overalls with long sleeves, nitrile gloves, and rubber boots.

First aid: Go to an emergency medical center and take the packaging, label, package leaflet and agronomic prescription for the product. *Ingestion*: If the product has been swallowed, do not induce vomiting. If vomiting occurs naturally, lay the person sideways. Do not give anything to drink or eat. *Eyes*: In case of contact, wash with plenty of running water longer than 15 minutes. Prevent the wash water from entering the other eye. *Skin*: In case of contact, remove contaminated clothing and wash the skin with running water and mild soap. *Inhalation*: If the product has been inhaled, take the person to an open and ventilated place.

3.1.3.4. Training of workers

Even with technical training, the worker can make mistakes that result from carelessness, inattention, overconfidence, lack of technical expertise, and mechanical and emotional factors [3]. The employee must be trained, in detail, about the correct storage, transportation, and use of pesticides as described on the label and in the package leaflet.

The employee must also be trained on proper equipment maintenance of both backpack and mechanized applicators. Workers must be able to apply the pesticide with the least possible contact. On the other hand, the employer must select the least toxic products and create conditions to mechanize and automate operations to minimize contact between worker and pesticide.

The training on pesticide handling and application in field conditions is essential to reduce risks and prevent poisoning. Labor legislation NR 31 [6] states that the machinery and implements must be used according to the manufacturer's technical specification, within the operational limits and restrictions indicated. Therefore, equipment must be operated by qualified and adequately trained workers.

The NR 31 [6] also requires that rural employers, or equivalent, must provide training on how to prevent accidents with pesticides to all workers directly exposed to them. The NR 31 requires that training must be provided to workers with direct exposure, and the program should consist of a minimum twenty hours distributed in a maximum of eight hours during normal working hours. This training should provide minimum knowledge about direct and indirect exposure to pesticides; signs and symptoms of intoxication and first aid procedures; labeling and safety signs; hygienic procedures during and after work; use of clothing and personal protective equipment; and cleaning and maintenance of clothing and personal protective equipment.

The NR 31 also provides that the rural employer must inform all employees about the use of pesticides in the establishment, such as the treated area: description of the general characteristics of the location area, type of application, including equipment to be used; trade name of the product used; toxicological classification; date and time of application; reentry interval; withdrawal period/grace period; protection measures required for workers in direct and indirect exposure; and measures to be taken in case of poisoning [6].

3.1.3.5. Emergency medical procedures

Labor legislation NR 31 [6] states that the rural employer must preserve the occupational health of workers, prevent, and control the injuries resulting from work accidents. The actions should be planned and implemented based on the identification of risks and paid by rural employers.

The rural or similar employer must ensure medical examinations and meet the deadlines and the schedule required. The required medical examinations are as follows: admission, held during the hiring process, before the worker takes on his responsibilities; periodic medical examination, held annually; medical examination upon return to work, held on the first day when the employee returns to work after being absent for longer than 30 days due to any illness or accident; medical examination upon changing function, provided that the worker is going to be exposed to a specific risk different than the previous; and dismissal medical examination, performed at the end of the employment contract [6].

Medical tests include clinical evaluation and complementary exams when necessary and depending on the risks. After each medical examination, the Occupational Health Certificate (OHC) must be issued in duplicate, containing the following data: full name of the worker, worker ID and function; exposure to occupational risks; indication of medical procedures that the worker underwent and when they were performed; and whether the worker is fit or unfit for the specific function to be performed. The OHC should have the doctor's registration number issued by the Medicine Regional Council and be dated and signed by the doctor who performed the examination. The first part of the OHC should be filed and be available for inspection while a copy is compulsorily given to the employee, upon receipt of signing the first via [6].

Other health actions at work must be planned and executed according to the needs and peculiarities. All rural establishments must be equipped with the necessary first aid material, according to the characteristics of the activity to be performed.

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The employer should ensure the removal of the injured in an emergency, without cost to the employee. The employer must also allow workers access to health agencies for the purpose of prevention and prophylaxis of endemic diseases and the application of tetanus vaccine.

3.1.4. Hygiene, cleaning, maintenance, and safety of the environment

Occupational hygiene measures consist of actions and procedures to prevent, or minimize, environment and worker contamination with pesticides. These actions should be applied at all working stages.

The hygiene and cleaning measures are applied to machinery, pesticide application equipment, handling of pesticides, materials, and clothing. The occupational hygiene is also applied to workers, who must turn them into habits to reduce exposures and prevent contamination of the materials, equipment, and tools with pesticides.

Workers should be instructed to clean immediately after contamination occurs and to keep all components sanitized and clean. There are actions that should be performed before and after working with pesticides. For example, gloves should be decontaminated immediately after getting contaminated with pesticides during work.

Preventive health and environment safety after use of pesticides is also present in the Brazilian legislation for pesticide registration and labor. In the pesticide registration legislation, Decree Law no. 4074 [10] states that as a preventive safety measure, the agronomic prescription must have the diagnosis of plant health problem, the recommendation to read carefully the label, and the package leaflet of the recommended pesticides. The packaging and labeling of pesticides cannot be confused with toiletries, pharmaceuticals, food, diet, beverage, cosmetics, and perfumes. The package leaflet must be present in single packs and contain all the information on the label and instructions for use, poisoning signs, symptoms, and treatment.

The labor legislation NR 31 [6] states that all workers should be informed regarding the use of pesticides about the treated area, description of the general characteristics of the area and the location; type of application, including the equipment used; trade name of pesticide; toxicological classification; date and time of application; reentry interval; protective measures required for workers in direct and indirect exposure; and the measures to be taken in case of intoxication.

The preservation, maintenance, cleaning, and use of the equipment may only be performed by previously trained and protected persons. Equipment should be cleaned without contaminating wells, rivers, streams, and any other water collections NR 31 [6].

3.2. Protective measures

If the working conditions remained unsafe (MOS < 1) after the pesticide registration and labor legislation requirements were met and the preventive measures were put in place, the protective measures should be implemented intensively enough to make the working environment safe (MOS ≥ 1).

Protective measures aim to isolate or neutralize the risks. They are grouped into collective that control the risk at the source (generation) and on the path (propagation or trajectory) and individual, which controls the risk directly on the exposure routes in the worker's body (receiver).

Unsafe working conditions are compromised by defects, failures, technical irregularities, and lack of safety devices, therefore exposing to danger the physical integrity and/or health of the workers, facilities, and equipment. For example, lack of adequate protection when operating machinery and equipment [3].

For unsafe working conditions (MOS < 1), the need for exposure control (NEC) can be calculated using the formula proposed by Machado-Neto [23]:

$$\text{NEC} = \left(1 - \text{MOS} < 1\right) \times 100 \ (\%).$$

NR 31 requires that the employer must adopt collective protection measures to control the risks at the source, followed by personal protective measures at no cost to the worker, to complement when risk factors persist temporarily [6].

3.2.1. Collective protection

Collective protection measures can act on toxicity and/or exposure, considering the two main risk factors of poisoning with pesticides.

3.2.1.1. Controlling herbicide toxicity

The pesticide toxicity is controlled by replacing the pesticide with a less toxic one, with higher NOEL value (Table 2). The higher the NOEL value, the greater the safe dose and greater MOS value.

Pesticide formulation	**Common name**	**g i.a. /L**	**NOEL, mg/kg/day**	**Registration holder**
Gesapax 500 SC	Ametryn	500.0	2.0	Syngenta Proteção De Cultivos Ltda.
Aurora 400 EC	Cafentrazona-ethyl	400.0	3.0	FMC Química Do Brasil Ltda
Gamit Star EC	Clomazone	800.0	14.0	FMC Química Do Brasil Ltda
Roundup Original SL	Glyphosate	480.0	30.0	Monsanto Do Brasil Ltda
Dual Gold EC	Metolachlor	960.0	7.5	Syngenta Proteção De Cultivos Ltda.
Boral 500 SC	Sulfentrazone	500.0	12.0	FMC Química Do Brasil Ltda
Butiron SC	Tebutiurom	500.0	7.0	Milenia Agrociências S.A.
Combine 500 SC	Tebutiurom	500.0	7.0	Dow Agrosciences Industrial Ltda
DMA 806 BR SL	2.4D	806.0	1.0	Dow Agrosciences Industrial Ltda.
Callisto SC	Mesotrione	480.0	1.8	Syngenta Proteção De Cultivos Ltda.

Table 2. Commercial formulations of herbicides, active ingredients, and their concentrations in the formulations, NOEL values TGA [31], and registration holders in Brazil.

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3.2.1.2. Controlling worker exposure to herbicides

The collective protective measures that control exposure are applied to the components of the working environment. The first may be the automation of potentially contaminating operations performed by the worker; however, very rarely this action can be applied. For example, closed systems for preparing and supplying the mixture of pesticide with the carrier.

The main measure of collective protection, when working with pesticides in field conditions, is to isolate the risk areas or the worker doing the job. The isolation of the risk area can be achieved by using a bar protection adapted to the sprayer bar pulled by the tractor used for applying nonselective herbicide in eucalyptus culture (Figure 4). The spray bar was reduced to 2.5 m in length and covered with sturdy plastic blades at the front, sides, and rear (Figure 4). The bar has been adapted to apply nonselective pesticide over a 2.0-m-wide area on the weeds between the rows in areas planted with 1.0-m-tall young eucalyptus plants [24].

The total exposure of the tractor driver without individual protection, but with protected spray bar, was 838.88 mg/day of glyphosate and MOS of 8.62 [24]. Therefore, the protection of the spray bar made the tractor driver work safe, with acceptable poisoning risk and tolerable exposure levels.

Figure 4. Sprayer bar protected with plastic blades attached to the bar used to apply nonselective herbicide between plant rows in eucalyptus plantation areas [24].

Although affected by several factors, occupational exposure to pesticides is directly related to the concentration of active ingredient in the sprayed mixture and the effective exposure time

during the work day [1]. Therefore, another way of changing an unsafe working condition to safe is to limit the working hours to the number of hours during which it is safe to perform the activity (TST). This parameter can be calculated using the following formula proposed by Machado-Neto [23]:

$$SWT = MOS \times eet,$$

where SWT indicates safe working time (h); MOS, margin of safety; and eet, effective exposure time (h).

The calculated SWT values lead to two situations:

1. If MOS ≥ 1, the SWT is greater than the considered exposure time and reaffirms the safety of working conditions under study. For safe conditions, the SWT expresses their safety levels.
2. If MOS < 1, the SWT is less than the exposure time of the workday. Therefore, the calculation enables to restrict daily exposure to the SWT, using it as a collective safety measure.

3.2.1.3. Individual protection

The personal protective measures only control the exposure and consist of using PPE to complement the protection of collective and preventive measures. PPE should be used when proven by the employer that is technically unfeasible to adopt collective protection measures or when they are insufficient [6].

Labor legislation NR 31 provides that the rural employer must adopt at least the following personal protective measures: supply the PPE and work clothing appropriate to the risks undertaken without causing thermal discomfort to the worker. Provide PPE and work clothing in perfect working order and properly cleaned, replacing them as necessary. It should be made clear that their decontamination at the end of each workday is the responsibility of the rural employer. Instruct on the correct use of protective devices. Provide a suitable place for safekeeping of personal clothing. Provide water, soap, and towels for personal hygiene. Ensure that any protection device or contaminated clothing be taken out of the workplace. Ensure that no device or protective clothing is reused before due decontamination. Forbid the use of personal clothing when applying pesticides [6].

The same legislation further states that it is mandatory to supply PPE to the workers for free in the following circumstances: when the collective protection measures are technically proven unfeasible or when they do not offer complete protection against the risks resulting from work and while the collective protection measures are being implemented and to meet emergency situations. PPE should be appropriate to the risks and kept in perfect condition and operation. The employer must require and train the employee to use PPE [6].

PPE control exposures on the workers' body surface via dermal and respiratory routes. Respiratory exposure is controlled with respirators equipped with filters for particles or

particles and vapors, with activated carbon. Dermal exposure is controlled with PPE made of porous and nonporous materials, or impervious. Waterproof materials are plastic coated, laminated, and rubberized; for hand protection, waterproof gloves; feet protection, waterproof rubber boots; leg protection, aprons; and plastic goggles with side shield. The porous materials are various types of fabric impregnated with carbon-fluorine compounds, which repel the water droplets of the spray mist. These porous materials are used to make the hood, long-sleeved shirts, and pants.

There is no need to use any PPE when working conditions are classified as safe (MOS ≥ 1); however, PPE could always be recommended as accident preventive measure. The noncontrolled dermal exposure was reduced by 99%, from 838.88 to 8.62 mg/day of glyphosate in the case of the tractor driver applying glyphosate in eucalyptus plantations with the protective plastic cover over the sprayer bar (Figure 4) and wearing PPE consisting of a set of water-repellent parts (hood, long-sleeved shirt and pants), gloves, and waterproof boots. MOS increased from 8.62 to 103.24 [24]. The use of PPE made the working condition of the tractor driver even more secure.

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Chapter 14

Assessment of Wild Mustard (*Sinapis arvensis* L.) Resistance to ALS-inhibiting Herbicides

Anna M. Szmigielski, Jeff J. Schoenau and Hugh J. Beckie

Additional information is available at the end of the chapter

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Abstract

There is an urgent need for rapid, accurate, and economical screening tests that can determine if weeds surviving a herbicide application are resistant. This chapter describes development and application of a simple root length bioassay technique for detection of wild mustard (*Sinapis arvensis* L.) resistance to ALS-inhibiting herbicides. This bioassay was performed in 2-oz WhirlPak® bags filled with 50 g of soil wetted to 100% moisture content at field capacity. Wild mustard seeds were pre-germinated in darkness in Petri dishes lined with moist filter paper for 2 days. Six seeds with well-developed radicles were planted in the non-treated soil and in soil with added herbicide, and plants were grown in a laboratory under fluorescent lights. After 4 days of growth, WhirlPak® bags were cut open, soil was washed away, intact plants were removed, and root length was measured with a ruler. The concentration of each herbicide in soil at which a significant root inhibition of susceptible biotype, but no root inhibition of a resistant biotype occurred was selected. Susceptibility/resistance of wild mustard populations was estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil as compared to the plants grown in the non-treated soil.

Keywords: Herbicide resistance, wild mustard, ALS-inhibiting herbicides, bioassay

1. Introduction

Repeated applications of herbicides with the same mode of action have resulted in weeds developing resistance. Herbicide resistance in weeds refers to the inherited ability of a weed biotype to survive a herbicide application to which the original population was susceptible. It is not a genetic change caused by herbicides that allows resistance to develop. The resistant biotype is present in low numbers in natural populations, and when a herbicide is applied, most of the susceptible weeds die but the few resistant weeds survive, mature, and produce

seed. If the same herbicide continues to be applied and the resistant weeds reproduce, the percentage of the weed population that is resistant increases [1]. The risk of weeds developing resistance is particularly high in production of herbicide-resistant crops where only one or two herbicide modes of action can be applied, or are applied due to economic and convenience factors, for weed control. Development of resistance may lead to economic losses because of the lack of alternative herbicide choices [2].

Acetolactate synthase (ALS)-inhibiting herbicides have been used extensively in agricultural production mainly because of their remarkable efficacy at very low application rates. However, it has been recognized that the ALS-inhibitors are the most resistance-selective herbicide group. ALS-herbicides were first introduced in the early 1980s, and since then, rapid increase in incidence of resistance to these herbicides has been reported; more weeds have become resistant to ALS-inhibiting herbicides than to any other herbicide mode of action [1-3].

There is an urgent need for tests that can determine if weeds surviving a herbicide application are resistant. However, before assuming that weeds are resistant because they were not controlled, other factors that might affect herbicide performance, such as misapplication, unfavorable weather conditions, improper timing of herbicide application, and weed flushes after application of a nonresidual herbicide need to be considered. If resistance is occurring, the problem needs to be identified as early as possible as losses of herbicide options could have important economic and environmental consequences to agricultural production especially if herbicide cross-resistance or multiple resistance occur [1].

Various techniques have been proposed for confirming ALS-inhibitor resistance in weed populations. The whole-plant pot (soil) assay conducted in a greenhouse (ca. 4–6 week duration) is the most frequently used method for identifying herbicide-resistant weeds, as results are considered most relevant to field conditions [4]. Nevertheless, a number of rapid, soil-less (dish) bioassays have been developed over the past 25 years to reliably discriminate herbicide-resistant from herbicide-susceptible weeds, such as various 7-day acetyl CoA carboxylase inhibitor-resistant bioassays [4, 5]. However, dish assays have not been successful to date in reliably discriminating between ALS inhibitor-resistant and susceptible weeds. Validated rapid tests for resistance to herbicides with this mode of action would be less expensive than pot assays and allow for a quicker turnaround time to clients, thereby facilitating proactive and timely implementation of resistance management by producers. Additionally, molecular techniques are increasingly being used in testing laboratories to confirm ALS-inhibitor resistance, as target-site (*ALS*) mutation is the most common mechanism of herbicide resistance in broadleaf weeds [6]. However, cost of equipment and testing (multiple mutations can confer *ALS* resistance) may be prohibitive, and negative results cannot exclude the existence of a different possible mechanism of resistance.

Wild mustard (*Sinapis arvensis* L.) is a common weed in field crops in the Canadian prairies. It ranked 11th of 101 weeds in a 2002 Manitoba survey of cereal and oilseed crops; and 15th of 124 weeds in a 2003 Saskatchewan survey of cereal, oilseed, and pulse crops [7]. Herbicide resistance to ALS-inhibiting herbicides has been reported for a number of populations of wild mustard in Canada [8]. Herbicide-resistant populations were first reported in Manitoba in 1992 [9], Alberta in 1993 [10], and Saskatchewan in 2002 [11]. Based

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on samples submitted by growers between 2007 and 2011, 16 wild mustard populations from Saskatchewan were confirmed as ALS inhibitor-resistant, compared with 12 populations between 2002 and 2006 [5].

This chapter describes development and application of a rapid and simple root length bioassay technique for assessment of wild mustard susceptibility/resistance to selected ALS-inhibiting herbicides.

2. Development of a wild mustard bioassay

ALS-herbicides inhibit biosynthesis of branched-chain amino acids and affect primarily root growth of susceptible plants through inhibition of cell division at the root tips. Therefore, measuring root length reduction of sensitive plant species is the most common detection approach used in bioassays for ALS-inhibiting herbicides [12-18].

2.1. Wild mustard biotypes

Seeds of 15 wild mustard biotypes that were collected in western Canada and characterized as ALS herbicide-susceptible and herbicide-resistant based on pot assays were obtained from Agriculture and Agri-Food Canada (AAFC) in Saskatoon, SK (Table 1).

Seed germination was tested in Petri dishes lined with moist filter paper in darkness. After 1 day, only a few seeds germinated; after 2 days, the germination rates varied among biotypes from approximately 2 to 55% (Table 1). Two biotypes that had the highest germination rate, i.e., BT1 and BT7, were selected for further testing for the bioassay development.

2.2. Soil used for the bioassay

Soil used for the bioassay was collected from the lower slope position in a farm field (legal location SW36-20-4-3) near Central Butte, SK, Canada. The soil had the following selected properties: 2.2% organic carbon content, soil pH 6.9, 38% clay content, and 18% moisture content at field capacity.

2.3. Bioassay technique

Bioassay was performed in 57-g (2-oz) WhirlPak® bags [15]. A quantity of 50 g of soil was wetted to 100% moisture content at field capacity by adding 9 mL of water to soil; then soil was hand-mixed in a plastic dish and transferred to a WhirlPak® bag. Soil in the bag was gently packed to form a layer approximately 8 cm high, 6 cm long, and 1 cm wide. Six seeds were planted at a 2-mm depth and the soil surface was covered with a 5-mm layer of plastic beads to reduce soil drying (Figure 1). Plants were grown in the laboratory under fluorescent lights that had photosynthetic photon flux density of approximately 16 $\mu mol/m^2/s$ at the plant level, and plants were watered daily to 100% field capacity by adding water to a predetermined weight. At harvest time, intact plants were recovered

Wild mustard biotype	Susceptibility/resistance to selected ALS-inhibiting herbicides	Approximate germination rate (%)
BT1	Susceptible to tribenuron/thifensulfuron	55
BT2	Susceptible to tribenuron/thifensulfuron	24
BT3	Susceptible to tribenuron/thifensulfuron	3
BT4	Susceptible to imazethapyr	4
BT5	Susceptible to imazethapyr Susceptible to imazethapyr/imazamox	15
BT6	Susceptible to tribenuron/thifensulfuron	4
BT7	Strong resistance to ethametsulfuron Strong resistance to tribenuron/thifensulfuron	50
BT8	89% resistant to imazethapyr	8
BT9	100% resistant to imazethapyr/imazamox 100% resistant to tribenuron/thifensulfuron	23
BT10	100% resistant to imazethapyr/imazamox 100% resistant to tribenuron/thifensulfuron	15
BT11	90% resistant to imazethapyr/imazamox 50% resistant to tribenuron/thifensulfuron	9
BT12	100% resistant to imazethapyr/imazamox 100% resistant to imazethapyr 100% susceptible to tribenuron/thifensulfuron	16
BT13	100% resistant to imazamox 100% resistant to imazamox	7
BT14	100% resistant to imazethapyr/imazamox	2
BT15	98% resistant to imazethapyr 8% resistant to tribenuron/thifensulfuron	15

Table 1. Susceptibility/resistance of wild mustard biotypes to selected ALS-inhibiting herbicides evaluated in pot assays, and approximate seed germination rates.

from soil after the WhirlPak® bag was opened, and soil was washed away with water. After removal of plants, root length was measured with a ruler (Figure 2). This bioassay technique has been shown to be very useful for detecting ALS-inhibiting herbicides in soil with oriental mustard (*Brassica juncea* L.), primarily because plants with intact roots can be easily retrieved from soil and then measured [15, 16].

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Figure 1. Mustard bioassay performed in WhirlPak® bags.

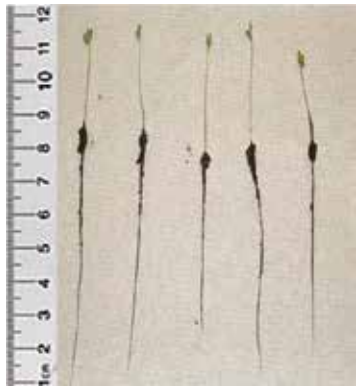

Figure 2. Intact mustard plants after removal from untreated soil.

2.4. Establishing conditions for growing wild mustard

The ideal root length of plants grown in a 57-g (2-oz) WhirlPak® bag is around 8 cm because of the 8-cm approximate height of soil in the bag. Root development beyond this height is obstructed as roots grow along the bottom of the bag and typically smaller increases in root elongation are observed at this point. In a root length bioassay, it is important that the measured root length reduction is in response to the herbicide of interest and not to other factors.

To establish the optimal duration of growth for wild mustard, two biotypes, i.e., BT1 and BT7, were grown from 2 to 6 days after seeding. Because of the low germination rates for most of the wild mustard populations, seeds were pregerminated for 2 days, and six seeds with well-developed radicles were transferred to soil. Root length increased with the duration of plant growth and was the highest after approximately 4–5 days (Figure 3). Growing plants longer did not increase root length, and a 4-day plant growth was selected for the wild mustard root length bioassay.

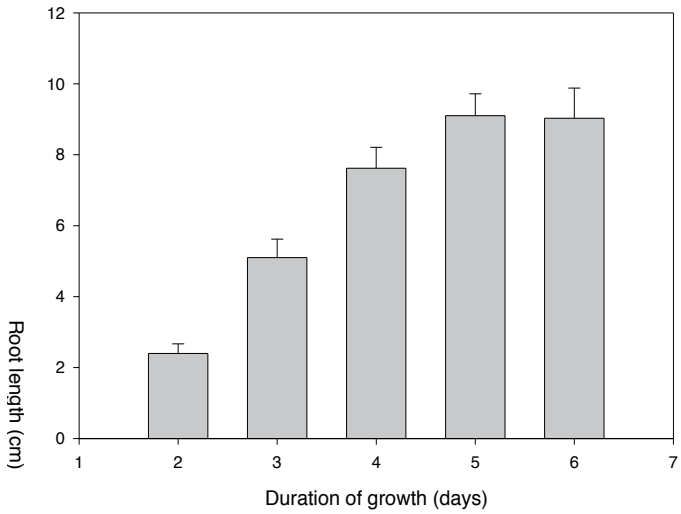

Figure 3. Root length of wild mustard grown from 2 to 6 days after seeding (each data point represents mean ± standard deviation).

3. Wild mustard response to selected ALS-inhibiting herbicides

Wild mustard response to four ALS-inhibiting herbicides was assessed. The selected ALS-herbicides were: flucarbazone – a sulfonylaminocarbonyltriazolinone (SCT) herbicide, pyroxsulam – a triazolopyrimidine (TP) herbicide, imazamox/imazethapyr – an imidazolinone (IMI) herbicide, and metsulfuron – a sulfonylurea (SU) herbicide.

3.1. Solution preparation and soil spiking

Technical-grade flucarbazone (99.1% pure) (from Bayer Co.), commercial formulation Simplicity containing pyroxsulam at a concentration of 30 g ai/L (from Dow AgroSciences Co.), commercial formulation Odyssey containing 35% imazamox and 35% imazethapyr (from BASF Co.), and technical-grade metsulfuron (93% pure) (from du Pont Inc.) were used for preparing stock solutions of each herbicide. The weighed quantity of a herbicide was transferred to a 1-L flask with 100 mL of methanol or acetone, and the flask was filled with water. A series of standard solutions in a concentration range from 0 to 1.5 ppm flucarbazone, 0 to 0.345 ppm pyroxsulam, 0 to 2.24 ppm imazamox/imazethapyr, and 0 to 0.32 ppm metsulfuron were prepared.

Soil was spiked with an ALS-inhibiting herbicide by first combining a 0.5-mL volume of a herbicide standard solution with 8.5-mL volume of water (for a total volume of 9 mL) and then transferring this solution to 50 g of soil yielding herbicide concentration from 0 to 15 ppb

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flucarbazone, 0 to 3.45 ppb pyroxsulam, 0 to 22.4 ppb imazamox/imazethapyr, and 0 to 3.2 ppb metsulfuron. These concentration ranges were equivalent to field application rates from 0 to approximately 1X. Soil was then mixed, transferred to a WhirlPak® bag, and bioassay was performed as described above. After removing plants from soil, root length was measured, and root length inhibition (RLI %) was calculated using the formula [17]:

$$RLI(\%) = \left(1 - \frac{L_t}{L_0}\right) \times 100\%$$

where L_t is the root length in the herbicide-treated soil and L_0 is the root length in the untreated (control) soil.

3.2. Dose–response curves

To select a concentration of each herbicide in soil at which a significant root reduction of susceptible biotype, but no root reduction of a resistant biotype is observed, dose–response curves were constructed for representative susceptible and resistant seed samples. Based on the available susceptibility/resistance data (Table 1), biotypes were selected for assessment of wild mustard response to the four ALS-inhibiting herbicides. For the wild mustard biotypes that showed root length inhibition to a herbicide in a selected concentration range, dose–response curves were constructed by graphing root length inhibition data versus herbicide concentration in soil using a log-logistic model [19]:

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^b}$$

where C is the lower limit of the curve, D is the upper limit of the curve, b is the slope of the curve around GR_{50} value, and GR_{50} is the concentration corresponding to 50% inhibition. For the wild mustard samples that showed zero or near-zero root length inhibition to a herbicide in a selected concentration range, linear regression was used. A WhirlPak® bag seeded with plants was a replicate and each measurement was replicated four times.

As can be seen from Fig. 4, the selected susceptible biotypes showed root length inhibition to flucarbazone, pyroxsulam, imazamox/imazethapyr, and metsulfuron. Resistant biotypes did not exhibit sensitivity to these herbicides and root length inhibition was zero or near-zero in the concentration ranges tested. Root length inhibition of susceptible biotypes was approximately 40% in response to 15 ppb flucarbazone, 70% in response to 3.45 ppb pyroxsulam, 60% in response to 22.4 ppb imazamox/imazethapyr, and 70% in response to 3.2 ppb metsulfuron. Thus, testing susceptibility/resistance of wild mustard populations to the ALS-inhibiting herbicides can be accomplished by growing mustard plants in the herbicide-treated soil at the above concentrations. If the root length reduction is observed at these herbicide concentrations

as compared to the root length in the untreated soil, the wild mustard biotype is susceptible, while no root length reduction indicates resistance.

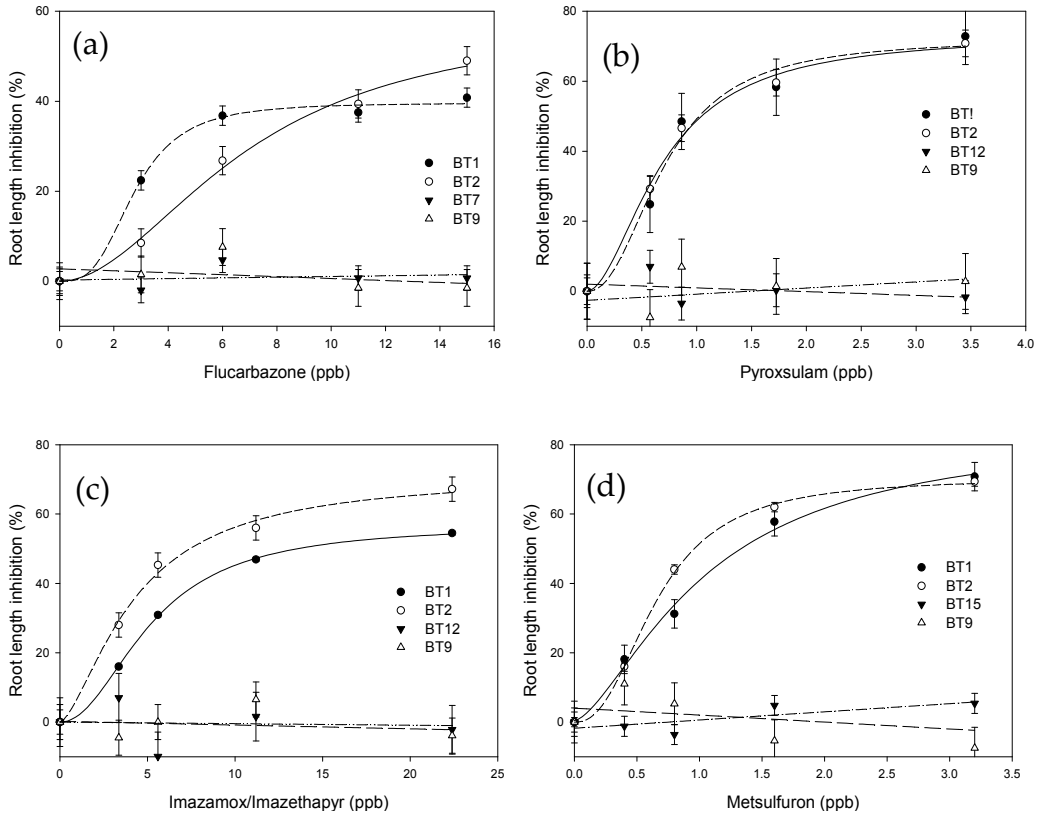

Figure 4. Dose–response of wild mustard to (a) flucarbazone, (b) pyroxsulam, (c) imazamox/imazethapyr, and (d) metsulfuron determined by the root length inhibition bioassay.

Percent resistance in the wild mustard populations was estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil (Table 2). This approach is particularly useful for biotypes that have variable root length in the untreated soil so that the percentage of uninhibited roots in the treated soil can be corrected. Typically, in the nontreated soil most of the wild mustard biotypes had roots that were approximately 7.5 ± 2.5 cm long and some short roots that had length less than 5 cm. Therefore, it is important that a wild mustard biotype being tested for susceptibility/resistance by this bioassay technique is grown both in the nontreated soil and in the herbicide-treated soil so that short roots obtained in the herbicide-treated soil would not be interpreted as herbicide-susceptible. Estimated percentages of resistant plants (Table 2) are in a very good agreement with the susceptibility/resistance data from the pot assays conducted in the greenhouse by AAFC Saskatoon (Table 1). Thus, these results show that the simple and rapid (6-day) root length bioassay performed

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in a laboratory can be used in place of the whole-plant pot assay that requires ca. 4-6 weeks for the assessment of wild mustard resistance to ALS-inhibiting herbicides [4].

Wild mustard biotype	% resistant plants[a]			
	Flucarbazone	Pyroxsulam	Imazamox/ imazethapyr	Metsulfuron
BT1	0	0	0	0
BT2	0	0	0	0
BT3	60	0	0	0
BT4	100	10	15	15
BT5	0	0	0	0
BT6	0	0	0	0
BT7	100	-		-
BT8	100	100	100	0
BT9	100	100	100	100
BT10	100	100	100	100
BT11	100	100	100	100
BT12	100	100	100	0
BT13	100	100	100	0
BT14	100	100	100	100
BT15	100	100	100	70

[a] % resistant plants = number of uninhibited roots/total number of roots × 100 % in response to flucarbazone at 15 ppb, pyroxsulam at 3.45 ppb, imazamox/imazetapyr at 22.4 ppb, and metsulfuron at 3.2 ppb.

Table 2. Estimated percentage of resistant plants in wild mustard populations in response to ALS-inhibiting herbicides.

4. Conclusions

- The root length bioassay is suitable for assessment of susceptibility/resistance of wild mustard populations to ALS-inhibiting herbicides.
- To perform this bioassay, no specialized equipment is required and the bioassay is completed in 6 days. Seeds are pregerminated for 2 days, and plants are grown for 4 days in a laboratory under fluorescent light in plastic bags filled with untreated soil and herbicide-treated soil (15 ppb flucarbazone, 3.45 ppb pyroxsulam, 22.4 ppb imazamox/imazethapyr, or 3.2 ppb metsulfuron). Removal of plants from soil with water allows for recovery of intact roots that can be easily measured.

- Due to variability in root growth, a minimum of four replications of plants grown in the untreated and in herbicide-treated soil, i.e., a total of eight WhirlPak® bags seeded with plants are recommended.
- Based on root length in the untreated soil and in the herbicide-treated soil, susceptible and resistant wild mustard populations can be identified. Typically, susceptible wild mustard biotypes have RLI (%) of approximately 40% to 15 ppb flucarbazone, 70% to 3.45 ppb pyroxsulam, 60% to 22.4 ppb imazamox/imezathapyr, and 70% to 3.2 ppb metsulfuron, while RLI (%) for resistant wild mustard is near-zero.
- Alternatively, susceptibility/resistance may be estimated by calculating the percentage of uninhibited roots of plants grown in the herbicide-treated soil as compared to the plants grown in the untreated soil.
- Testing susceptibility/resistance to herbicides from each class of the ALS-inhibitors is required as wild mustard biotypes may be resistant to one class but susceptible to another.

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Herbicides are one of the most widely used groups of pesticides worldwide for controlling weedy species in agricultural and non-crop settings. Due to the extensive use of herbicides and their value in weed management, herbicide research remains crucial for ensuring continued effective use of herbicides while minimizing detrimental effects to ecosystems. Presently, a wide range of research continues to focus on the physiology of herbicide action, the environmental impact of herbicides, and safety. The authors of Herbicides, Physiology of Action, and Safety cover multiple topics concerning current valuable herbicide research.

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