20

The Bioassay Technique in the Study of the Herbicide Effects

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1. Introduction

Strategies for weed control are based primarily on chemical control, since the last decades the use of synthetic chemical products has been dramatically increased. The use of plant protection products is a source of concern for the society of developed countries, which has a growing interest in the environment, nature conservation and public health in general. This situation has led to deep changes in the objectives of the research on agriculture. The development and implementation of sustainable agriculture conduct to a rational use of plant protection products. The regulatory organisms (national and international) and the chemical industry of pesticides have taken steps to reduce the environmental impact of such organic compounds. In this context, there is now a great concern about the chemical nature of the products used in agriculture and its impact on adjacent ecosystems and the toxicity of these substances in ground and surface water.

The widespread use of herbicides create also concern about the possibilities of the risk of phytotoxicity on other species which are not direct object of the treatment. On the one hand, the risk involved in rotational crops due to of the accumulation in the field of herbicides that have a high persistence and are applied repeatedly each year, and on the other hand, the crops or plants adjacent to the treated crop may be affected by herbicide drift during the application of the product (Pestemer & Zwerger, 1999).

On the basis of these considerations, the risk assessment of the use of plant protection products on non-target plants should focus taking into account the agronomic use of the product. In this context, the bioassay technique is a useful tool that complements the analytical methods and provides information regarding herbicide bioavailability for the plant and its possible phytotoxicity (Kotoula-Syka et al., 1993; Stork & Hannah, 1996). Therefore, in the case of herbicides, we can define two groups according to good agricultural practices: the vegetation adjacent of agriculture areas and successive crops in the rotation.

2. The role and application of bioassay techniques on the impact assessment of herbicides

Bioassays or biological tests applied to the study of herbicides are based on the response of different species, chosen as controls, to the application of the herbicide under study

(Horowitz, 1976). They represent a valuable and necessary tool that provides an overview of soil-plant-herbicide relationships (Rahman et al., 1993; Hernández-Sevillano et al., 1999). Although there are chemical methods of analysis accurate and simple to use, bioassays have certain advantages in the study of herbicides:

- Phytotoxicity bioassays detect both the active substance and the possible degradation products of the herbicide.
- The biological assays provide practical information, being based on observation of the response of the plant to the herbicide (Blacklow & Pheloung, 1991).
- The methodology and materials necessary to carry out bioassays are generally simple and inexpensive.

The sensitivity, low cost and reproducibility of bioassays fulfil the criteria for a good technique (Günther et al., 1993). However, the bioassays by themselves cannot provide complete information on the environmental performance of these substances. Although reveal potential problems that residues of these products may present, as the effect on non-target species or in successive crops do not provide information needed to relate these effects with the chemical nature of the residue. Therefore, it is also necessary to study the nature of this residue by conventional analytical methods to identify potential causes of environmental problems and possible solutions. In this sense, it is very important to know whether any phytotoxic effects were detected in the bioassay due to active substance applied or to some of its metabolites or degradation products. In this case, it is necessary to know the route and rate of degradation not only for the active substance but also for the products of degradation (Parrish et al., 1995).

There is a need for evaluating and assessing the risk of the use of Plant Protection Products on non-target plants. The requirements for non target plants testing of pesticides vary among international agencies and their member countries. The risk assessment of the use of plant protection products on non-target terrestrial plants has been included until now as a generic assessment for registration of the plant protection product in the European Union (EU). However, generally state that there is a need to report all potentially adverse effects and undertake additional studies where there are indications of such effects.

The European Commission recommends the use of bioassays as an acceptable method to detect low levels of herbicide residues in soil. Such recommendation has been published in the European Commission Guidance Document Residue Analytical Methods (Anonymous, 2000) has accepted bioassays as suitable screening test that can be useful to exclude the occurrence of low levels of residues of phytotoxic compounds. Bioassays have become a necessary tool to detect herbicide soil residues and the results of these bioassays are now used to guarantee non-injury to the succeeding crop in crop rotation (Pestemer et al., 1980). Additionally, available pesticide phytotoxicity data on crop and non-crop species included in dossiers submitted to EU Member States for evaluation of active substances that could be commercialised in Europe according to the requirements of the Directive 91/414/EEC (Anonymous, 1991), concerning the placing of plant protection products within the European Union provides a harmonized procedure for the approval of these products. This directive requires that plant protection products marketed and used in the EU meet in their normal use, the following requirements: a) they do not produce harmful effects on human or animal health nor an unacceptable effect on the environment, and b) waste resulting from their application does not have harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment, and that can be measured by methods in general. It is not acceptable the use of products with toxic effects on its target

species, whether caused by themselves or their products applied waste. This directive has been extended by a set of guidelines detailing the information required in each of the sections, to be submitted by notifiers to justify the inclusion of active substances in Annex I to that directive. Therefore, the results of herbicide bioassays are essential input for many herbicide optimization programs, and for many studies of plant biomechanisms.

To assess the acute risk for terrestrial plants, the EU Guidance Document (SANCO, 2002) suggested starting with a first tier. If negative effects on terrestrial plants occur in the screening test or if results indicate a hazard potential for further risk assessment, then specific information on the toxicity of the substance to terrestrial plants should be requested. It is recommended to conduct dose-response test on 6-10 plant species representing as many taxonomic groups as possible. Germany Federal Environmental Agency propose phytotoxicity test with at least six plant species, three monocotyledonae, three dicotyledonae, including one leguminose and *Avena* and *Brassica,* (Füll et al., 2000). Other researchers (Gong et al., 1999) suggested four species of higher plants used for testing, two monocotyledons and two dicotyledons. We propose that selection of plant species would be specific for each situation, and it does depend of each herbicide. All selected species should present a useful tool in laboratory and are representative of important families in the agrarian ecosystem.

2.1 The use of Bioassays to detect herbicide phytotoxicity

The power of bioassays to detect bioavailable residues has led to success (Streibig et al., 1993). Bioassay methods have been developed to determine the residue level of many herbicides in soil and water. There are different types of bioassays, depending on the species, the type of herbicide used, its mode of action, substrate and other environmental conditions, as well as the measured parameter. Essentially, the biological test requires the choice of an indicator organism or specie that in the study of herbicides´ effects is very often a terrestrial plant. After the herbicide treatment one or more biological parameters of the plants that were affected will be assessed. At this point, visual assessment is recommended, but more rigorous measures are needed such as germination percentage, size or weight of the plants, or changes in physiological activities like photosynthesis or respiration (Horowitz, 1976).

The relationship between herbicide dose and plant response is of fundamental importance in understanding herbicide efficacy and mode of action. A thorough understanding of this relationship is essential for the design and interpretation of research in the field, greenhouse, or laboratory. The results of the measurements should be statistically analyzed to determine whether the observed effects are due to herbicide treatment or there is a response to increasing doses of the herbicide. The results of bioassays show the potential risk to sensitive crops after treatment, and provide information about the phytotoxicity of herbicide residue in the soil at sowing time. The classical bioassay, often used to quantify the amount of herbicide in soil, employs a single "standard" dose-response curve. This standard curve show the plant response to different herbicide concentrations and report information of different concepts related to herbicide efficacy, such as selectivity, tolerance and resistance.

A typical dose-response curve is sigmoid in shape. One example of such a curve is the log-logistic curve (Seefeldt et al., 1995). The mathematical expression relating the response y to de dose x is:

LOG-LOGISTIC: $Y=C+ ((D-C)/ (1+\exp (b.\ln(X)-\ln(EC_{50}+1)))))$

(*C*=lower limit, *D*= upper limit, *b*= slope, and EC_{50}= dose giving 50% response)

The log-logistic is the most common model used in bioassays to describe dose-response relations. Other relevant sigmoid curves might be the Gompertz (Streibig et al., 1993), is used sometimes, for instance, in cases where a log-logistic model did not fit well to the data.

GOMPERTZ: $Y= \exp[\ln(A). \exp(-rX)]$

(*A* is the upper asymptote and *r* the slope of the linearized function)

The methodology of the risk assessment consist in comparing the toxicity with the predicted exposure applying a safety factor to this ratio in order to cover the uncertainty of the extrapolation from laboratory data to the field. The toxicity factor used in the risk assessment is usually the EC_{50} (concentration required to give 50% reduction of the plant growth with respect to the control) and NOEC (No Observable Effect Concentration) of representative species assayed in laboratory.

In order to mitigate the adverse effects of the use of plant protection products have been developed in recent decades molecules effective at low doses in order to fulfill environmental requirements, water and soil pollution set by international legislation.

There are scientific references concerning the effect of low-dose herbicides belonging to the family of sulfonylureas, in susceptible crops in the rotation (Blacklow & Pheloung, 1991; Alonso-Prados et al., 2001). Sulfonylurea herbicides inhibit plant metabolism by inhibiting acetolactate synthase, a crucial enzyme for the biosynthesis of branched-chain amino acids. These herbicides have a high specific activity in cereal crops and can be used effectively to control a wide range of grass and broad-leaved weeds at low doses (between 4 and 20 g a.i./ha). Previous studies have shown that sugar beet, sorghum, barley, pea or oilseed rape were injured in field assays when they were grown after wheat treated with sulfonylurea herbicides during the preceding spring or even autumn (Günther et al., 1993; Szmigielska et al., 1998; Shinn et al., 1999). It has been described a 20% barley growth inhibition caused by 0.0015 mg/L sulfosulfuron in growth chamber bioassay and also some injury on this crop in field assay (Parrish et al., 1995); according to other authors, crops like lucerne, oilseed rape, flax and sugar beet respond to sulfonylurea herbicides residues similarly in the field and growth chamber experiments with soils (Moyer, 1995). Also, Hernández-Sevillano (2001) found that quantities between 0.008 and 0.003 mg/L of sulfosulfuron and triasulfuron reduced sunflower root length by 50% in soil bioassays carried out in growth chamber.

Several bioassay methods for sulfonylurea herbicides have been reported using lentil (*Lens culinaris* Med.), lettuce (*Lactuca sativa* L.), sunflower (*Helianthus annuus* L.), corn (*Zea mays* L.), pea (*Pisum sativum* L.) and lupin (*Lupinus angustifolius* L.). In some studies, plant height or dry or fresh weight has been found to be a sensitive response parameter to sulfonylurea exposure (Blacklow & Pheloung, 1991; Günther et al., 1993; Junnila et al. 1994; Stork and Hannah, 1996; Vicari et al., 1994; Walker & Welch, 1989). Root growth effects have also been assessed after sulfonylurea treatments due to improved precision and sensitive (Blacklow & Pheloung, 1991). Root responses to sulfonylurea exposure have been measured by root dry weight, but previous studies in our laboratory shown that the most sensitive biological parameter used in bioassay with sulfosulfuron was root length (Hernández-Sevillano et al., 2001); therefore we have used the inhibition of root growth as susceptible parameter that

indicate injuries in plants. Landi & Catizone (1989) found that response of corn cultivars to soil-applied chlorsulfuron in the field correlated better with root length than with root dry weight. Sunflower root dry weight was used by Kotoula-Syka et al. (1993) to study the persistence and phytotoxicity of several sulfonylureas in three different soils.

Also, some authors have found that plant response to the total herbicide residue in soil is site-specific; Stadler & Pestemer (1980) found that herbicide damage in crops is related to water-extractable (plant-available) residues. Thus, relationships between crop response and herbicide dose could be determined in a soil-free system bioassay to eliminate the confounding effects of soil adsorption and degradation of the herbicide (Ferris & Haigh, 1992; Jettner et al., 1999). Hydroponics' conditions promote herbicide activity because allow the maximum herbicide bioavailability to the plant, all the roots were confined within the solution with the plant at maximum water uptake. Plants are growing in most uniform conditions in the soil-free system bioassays, and the variability of the results due to environmental conditions was reduced with this method.

On the basis of these considerations, it has been studied the response of seven species (flax, corn, onion, vetch, lepidium, tomato and barley) to different doses of sulfosulfuron in hydroponic culture (Santín-Montanyá et al., 2006), in order to use this system as a rapid bioassay to detect phytotoxic levels of herbicide in crops and non-target plants and determine its effect in the most susceptible specie and also the most sensitive biological parameter for each species. We generated the dose-response curves of root growth 7 days after treatment for sulfosulfuron with the susceptible species, in order to estimate the EC_{50}. The most susceptible biological parameter was root growth for all species studied; this parameter permit us knowing the effect of sulfosulfuron on plants and it was used for obtain the dosis-response curves for each specie studied that have been treated with sulfosulfuron. The results showed that all species were susceptibles to sulfosulfuron, therefore the injuries caused in shoot fresh weight and shoot dry weight were growing with the doses of herbicide for all species. Additionally, root system control and less injured plants were increasingly deformed (main tap root twisted and lack of secondary roots); we could see how root growth was increasingly affected with increasing doses for all species, causing between 60 % and 98 % of root growth reduction with doses of 5.10^{-4} and 0.1 ppm a.i. of sulfosulfuron respectively applied on flax. These experiments with known concentrations of sulfosulfuron on the eight bioassays species showed that flax was the most susceptible specie to this herbicide.

Log-logistic and Gompertz model were tested for all species and the root length estimated by non-linear regression in the fitted model (Table 1). The Gompertz was considering the better model for the response in flax, corn, tomato and onion to sulfosulfuron; and Log-logistic regression model describe the data for lepidium, vetch and barley. The $EC_{10,30,50}$ were calculated in flax, maize, onion, vetch and *Lepidium sativum* according the estimated equations of each bioassay varied from 0.000053 mg/L to 0.0017 mg/L. Therefore, we could see that flax, corn, onion, vetch and lepidium root growth proved sensitive enough to detect very low phytotoxic level of sulfosulfuron; while tomato and barley were the less susceptible species.

Ciclohexanodione herbicides are also a family used at low-dose rate as they are biologically active at very low concentration (0.2-0.5 kg a.i./ha). These herbicides inhibit the activity of acetyl CoA carboxilase, a crucial enzyme in fatty acid synthesis. Furthermore, their polar character makes them easily to leach and potentially contaminate groundwater. However,

Specie	Upper asymptote (cm)	Lower asymptote (cm)	Slope [cm/(mg.m.L^{-1})]	EC_{10} (mg/L)	EC_{30} (mg/L)	EC_{50} (mg/L)	R^2 (%)
Flax [a]	21.69	-	629.52	0.000053	0.00019	0.0004	97
Maize [a]	10.83	-	526.40	0.000085	0.0003	0.00065	92
Onion [a]	9.48	-	271.46	0.00017	0.00063	0.0013	83
Tomato [a]	8.37	-	33.05	0.0015	0.0055	0.011	98
Vetch [a]	11.83	-	171.34	0.00025	0.009	0.0019	98
Lepidium sativum [a]	4.55	-	151.90	0.00047	0.0017	0.004	93
Barley [b]	5.86	0.92	0.99	0.042	0.16	0.39	93

[a & b] Regression Equations by Gompertz and Seefeldt models respectively

Table 1. Parameters of regression equations that describe the relationship between sulfosulfuron and root growth of different species and plant response for 10%, 30% and 50% inhibition of root growth (EC_{10}, EC_{30}, EC_{50})

due to high phytotoxicity, small amounts of residual herbicide in soil may affect sensitive succeeding crops. In this context, there is some information about the mobility, degradation and persistence in soil and water. These studies were performed with a variety of analytical techniques like gas chromatography, liquid chromatography, mass spectroscopy, photodegradation studies, studies with ^{14}C, immunoassays, etc. However, most studies have been made in water and soil, occasionally there is some bioassays in microalgae (Santín-Montanyá et al., 2007). The last results obtained confirm that could be a susceptible specie capable to detect the presence of some herbicides (Fig. 1 & Table 2).

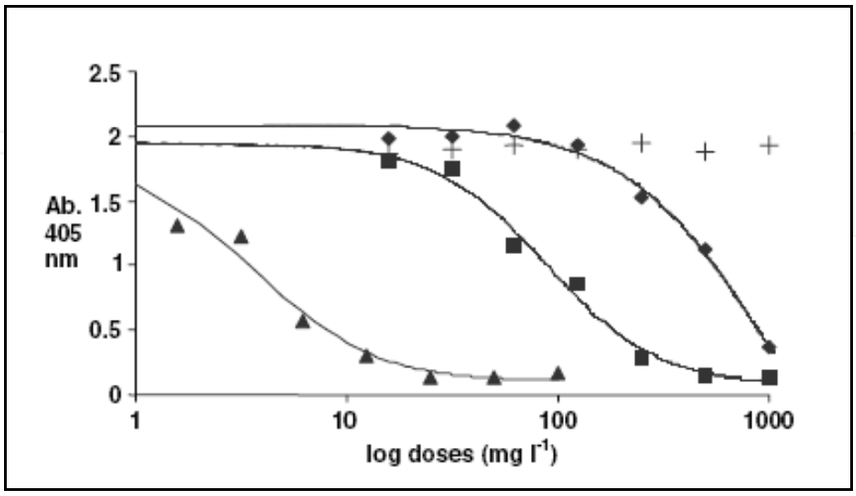


Fig. 1. Dose-response relationships of microalgae *Dunaliella primolecta* growth in the presence of different concentrations of alloxydim (♦), sethoxidim (■), metamitron (▲) and clopyralid (+)

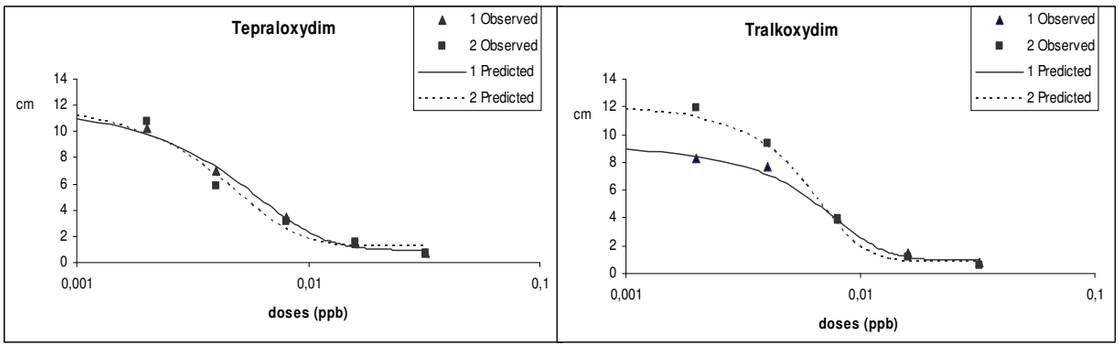
Herbicides	**D** (cm)	**C** (cm)	b [cm/(mg.m.L^{-1})]	**EC_{10}** (mg/L)	**EC_{50}** (mg/L)	**R^2** (%)
Alloxydim [a]	2.09	-1.28	1.29	177.20	973.20	87.1
Sethoxydim [a]	1.95	0.072	1.66	23.32	87.63	87.6
Metamitron [a]	2.12	0.103	1.67	0.76	2.87	89.6
Clopyralid	Not adjusted to regression equation. No inhibitory effect					

[a] Regression equation by Seefeldt model

Table 2. Parameters of regression equations that describe the relationships between increasing rates of herbicides and growth of *Dunaliella primolecta*

Previous bioassays have been developed to detect phytotoxic residues of herbicide sethoxidim (Hsiao & Smith, 1983). In our group, initial attempts to obtain a practical hydroponic bioassay that allowed us to quantify tepraloxydim were frustrated due to the lack of repeatability and random results. Therefore, an investigation was carried out to determine the fate of tepraloxydim under bioassay conditions in order to clarify the reason for poor bioassay repeatability. The presence of residual chlorine in water was identified as a key factor on the repeatability of the bioassay. Finally, an extensive research was conducted to develop and optimize a bioassay based on the high sensitivity of wheat (*Triticum aestivum* L) to tepraloxydim in hydroponic culture using chlorine free mineral water (Sandín-España et al., 2003). Afterwards, similar studies were carried out with tralkoxydim (Fig. 2).

It has been demonstrated that water chlorination with disinfection purposes degrades completely any possible residue of herbicide clethodim (Sandín-España et al., 2005a). This degradation is very rapid, giving rise to different degradation products.



	Bioassay	EC_{50} (µg/L)	R^2 (%)
Tepraloxydim	B1	4.6	99.8
	B2	3.8	97.2
Tralkoxydim	B1	6.8	98.4
	B2	7.0	99.6

Fig. 2. Dose-response curves and EC_{50} to ciclohexanodione herbicides in hydroponic culture of wheat

The foregoing results suggest that the use of low dose herbicides can produce damage on succeeding crops, neighbouring crops and on non target plants. Overall, there is no one species or endpoint that is consistently the most sensitive for all species or all chemicals in all soils, and differences in bioavailability among compounds may confound comparison of test results (Clark et al., 2004). Therefore, bioassays can provide additional information, with acceptable reproducibility (Nyffeler et al., 1982; Streibig et al., 1995) on herbicide uptake and translocation (Horowitz 1976; Best et al., 1975). Besides, bioassays are employed in studies of persistence and mobility of herbicide soil residues (Ragab, 1974).

3. Bioassays in selectivity and resistance to herbicides

The basis for much of the work done in crop-weed management is weed control. In areas of well-developed agriculture weed control is mainly based on chemical control by herbicides. The extensive and redundant use of herbicides could present problems both in agricultural systems and in the surrounding environment. To detect any possible effect of herbicides in the plant and to test herbicides efficacy, response assays and tests must be carried out at various levels in the laboratory, greenhouse and field. Field studies are the best way of studying herbicide effect but accurate and efficient greenhouse and laboratory tests could be of the up most importance. The quicker and more simple the testing is, the more effective it will be. Because most laboratory research work utilizes large numbers of plants, a simple and rapid method is desirable. This is the case in determining crop selectivity, herbicide resistance in weeds or selecting individuals resistant to a particular herbicide, as a part of an improvement, mutagenic and/or gene flow process.

3.1 Crop selectivity

Conventional breeding programs frequently don't consider the herbicide response of cultivars during the selection process, it is why some cultivars show problems when treated with herbicides in culture in field. This is particularly true for crop response to new herbicides or new use of an herbicide. Cultivars show wide differences in response to herbicides and in many cases the concentration of herbicide needed to control weeds, or a particular weed, is deleterious if not lethal to the crop. For example, the control of *Bromus diandrus* in cereals is of concern. Bromes are vigorous competitors in winter cereals in many parts of the world (Blacksshaw, 1993) and cultural methods are the basis for their control because the herbicides used for weed control in cereals are not effective in controlling brome grass. The development of a sulfonilurea herbicide allowed a good control of Bromus spp in wheat. Hydroponic *in vitro* herbicide treatments were carried out. In those assays, germinated seeds were disposed on a grid in a black beaker filled with nutrient solution at the grid level (Fig. 3). When plants were 10 days old they were placed during 24 hours in another vessel filled with herbicide solution.

Six days after herbicide treatment plants were weighted. The results obtained from hydroponic herbicide treatment of wheat, barley and *B.diandrus* besides glasshouse spread of plants allowed to confirm the varietal selectivity of *Triticum aestivum* L.and T*riticum turgidum* L. cultivars and the susceptibility of barley cultivars to the herbicide doses that controls *B.diandrus* (Villarroya et al., 1997).

There are herbicides as glyphosate that when applied on the plant leaf, damaged the plant but the effect is relatively slow; several days will elapse before symptoms of damage appear (Duke, 1988). For cereals four to six weeks will be necessary; during this time the seedling

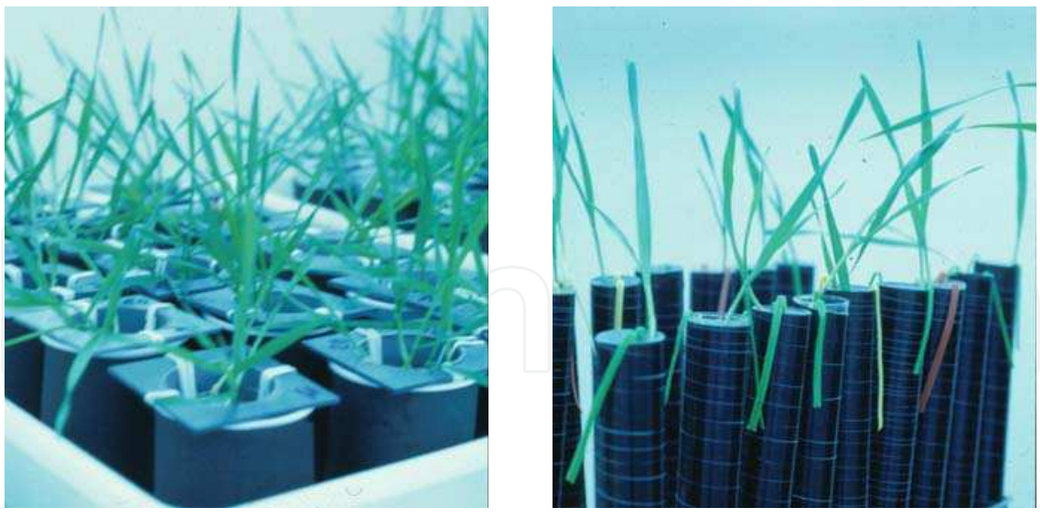


Fig. 3. Wheat plants in an *in vitro* herbicide response test

will need both soil or hydroponic support and space in a room or greenhouse. Quick methods to detect glyphosate's effect have been developed, (Harring et al., 1998; Madsen et al., 1995) including methods based on root absorption of glyphosate (Duke, 1978). Root application of the herbicide presents fewer problems than foliar application, especially with regard to interaction with ambient conditions (Hull et al., 1975). Trial assays in sorghum (Hensley et al., 1978), peas (Yenne et al., 1988) and corn (Racchi et al., 1995) have been carried out using root absorption. A method to evaluate the response of wheat and barley to glyphosate by measuring coleoptile length allows for the rapid detection of the more sensitive cereal lines and the selection of the more tolerant ones. Two barley cultivars (*Hordeum vulgare* L), "Jeff' and 'Amaji Nijo' (AN) as well as two wheat cultivars (*T.aestivum*), 'Chinese Spring' (CS) and 'Pavon' were used. Seeds were germinated in glyphosate solution in Petri dishes. After 24 hours, the dishes were opened and placed on a tray lined with water-moistened filter paper and covered with a transparent plastic film to maintain humidity. The tray containing the dishes was kept in a culture chamber under controlled conditions. The length of the coleoptile was measured four days after treatment (Fig. 4). The barley cultivars tolerated a higher dose of glyphosate than the wheat cultivars allows this method to evince differences in the responses of the cultivars as is shown by the log-logistic regression model applied. This method correlated with plant responses has provided an accurate model for describing the data with a good estimation of dose response (Fig. 5), equations for each cultivar by both methods.

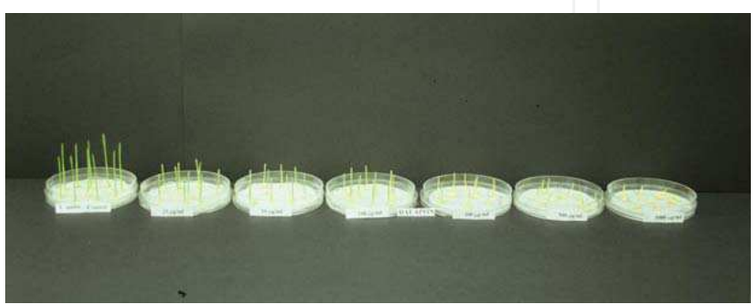


Fig. 4. Effect of herbicide dosage on wheat coleoptile length

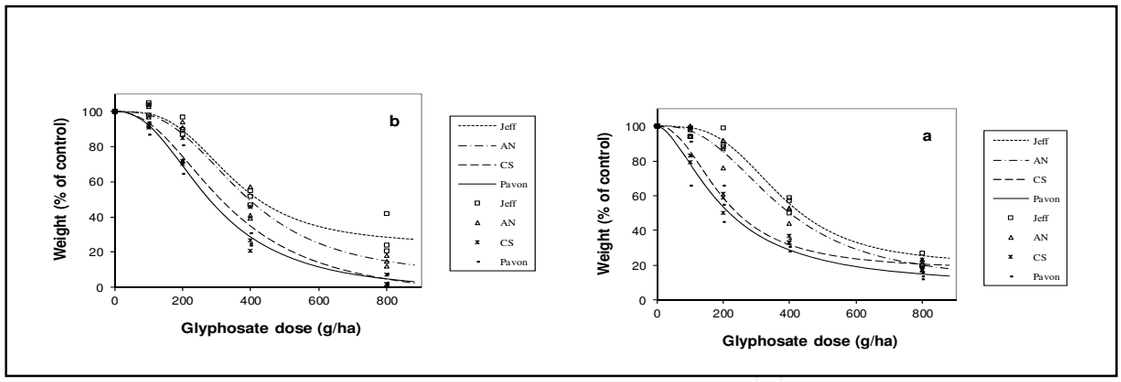


Fig. 5. Response in fresh weight of wheat (CS and Pavon) and barley (AN and Jeff) to glyphosate

The seed assay proved an accurate and rapid method to evaluate glyphosate efficacy. The seed assay can be completed in four to five days while the plant assay requires up to 30 to 45 days. The possible resistant plants detected by this method can be grown out after treatment in a greenhouse or in the field, where their resistance will be confirmed. This method is highly useful to detect tolerance to other herbicides as dalapon (Loureiro et al., 2001) and to detect populations of resistant weeds (Barroso et al., 2010) in the field as well as to initially select the lines obtained after mutagenic treatments or *in vitro* regeneration (Escorial et al., 2001).

Crop selectivity is related with the genetic control of herbicide response**.** Knowledge of the sources and genetic control of tolerance to herbicides should always be taken into account in the development of new improved crop varieties and in implementing a weed management system. Although genetic control of tolerance to herbicides was not largely been investigated in wheat, authors have already reported cytoplasmic, poligenic nuclear control as well as monogenic nuclear control of the response of different crops to the herbicides.

The bread wheat cultivars 'Castan' and 'Recital' are tolerant and susceptible respectively to chlorotoluron herbicide (Sixto & Garcia-Baudin, 1988). However, while the distribution of responses among wheat cultivars to chlorotoluron reported so far are discrete, some papers report only two classes, tolerant and susceptible (Tottman et al., 1975). A single seedling, non-destructive, easy to handle, cheap, fast and efficient assay was developed to score wheat responses to herbicides and to investigate the genetic control of the differences in response to chlortoluron between the cultivars 'Castan' and 'Recital' Its efficiency makes possible the detection not only of differences due to major genes but also to minor or modifier genes (Sixto et al., 1995). The results not only confirm the presence of a major tolerant allele controlling the differences in response between the two cultivars, but also, show the contributions of modifier genes present in 'Castan', 'Recital' and other related cultivars. This assay is applied nondestructively to single individuals plantlets that are scored in vitro in a herbicide solution of clortoluron (Fig. 6) and, if selected, can be transplanted, grown to maturity and cross-fertilized if desired. The test may save up to two generations in genetic schemes where scoring is done in large samples grown to maturity. This test was also used to conclude that in the inheritance of durum wheat (*T.turgidum* var *durum*) to metribuzin (Villarroya et al 2000) the tolerance is dominant and relatively few genes (around four) are involved in tolerance for this character. Heretability of this trait was very high with value of 0.60 in narrow sense and of 0.86 in broad sense. The results of this

work can help in the selection techniques employed to obtain durum wheat with increased tolerance to metribuzin, that could increase the margin of safety in Brome control in wheat.
If selectivity is not present for a herbicide-crop couple in vitro selection could be used to detect herbicide tolerance over mutations produced by somaclonal variation. *In vitro* culture has been used to select herbicide-tolerant plants of dicotyledonous (Aviv & Galum, 1977; Wersuhn et al., 1987) and of monocotyledoneous species between them *T.aestivum* L. tolerant to chlortoluron and to difenzoquat (Bozorgipour & Snape, 1991), and *H.vulgare* L. tolerant to chlorsulfuron (Baillie et al., 1993) and glyphosate (Escorial et al., 1996). In the last case, *in vitro* culture of barley calluses from immature embryos of barley (*H.vulgare* L. 'Jeff') were cultured for some months on medium with glyphosate. Plants were regenerated and the progeny of each regenerated plant was analyzed for response to glyphosate. An herbicide test was adapted to detect plants tolerant to glyphosate. Plants between 3 and 6 cm tall were treated with one drop of 1µl of glyphosate solution applied on the base of the third leaf. The length of the third leaf was measured at the time of herbicide treatment and seven and fourteen days after the treatment. Some progenies showed increased tolerance to glyphosate and show that glyphosate tolerance in barley can be increased by *in vitro* culture selection.

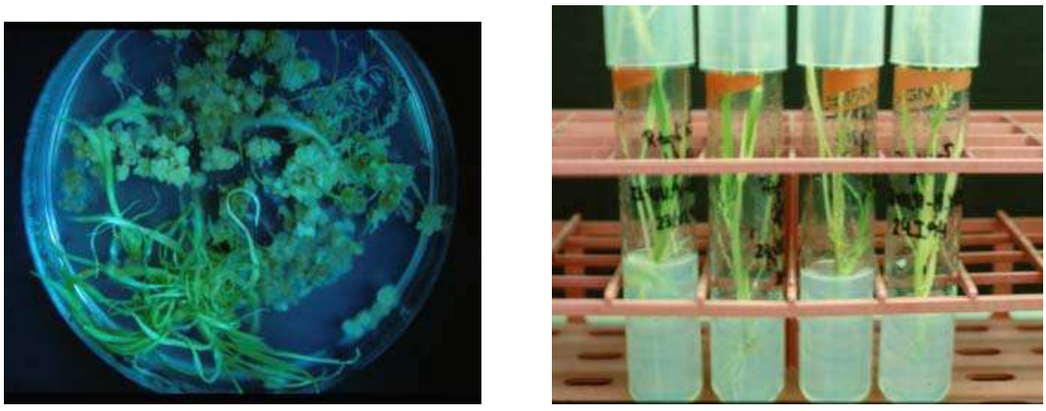


Fig. 6. Barley regenerants obtained after *in vitro* culture in medium with glyphosate herbicide

3.2 Herbicide resistance in weeds

The widespread use of herbicides for weed control over the past decades has exposed huge weed populations to strong selection pressures that lead to the appearance and proliferation of weeds resistant to different chemical classes of herbicides. The adoption of genetically modified crops will promote in the future a greater use of monoculture systems and generate a higher risk of possible appearance of resistance through the selection pressure produced by the continued use of a single herbicide (Powles, 2008). Thus, studies of weed resistance are important to stop or mitigate it. Herbicide resistance could be a field concern if it is spread in a field or in an area, or in a previous stage (not apparent in the field) in which an increase of proportion of resistant plants and/or a decrease of response in a given population.
To detect herbicide resistance, several authors have adjusted short, quick and cheap bioassays to evaluate herbicide effect, which have allowed to detect responses of biotypes to

diclofop-methyl, trifluralin, acetyl-coenzyme A carboxylase inhibitors, dalapon or glyphosate herbicides (Beckie et al., 2001; Barroso et al., 2010).

The above mentioned Petri dish bioassay cereal method adapted for weeds (*B.diandrus* and *L.rigidum*) is a proper method to detect weed resistant populations as well as to establish a baseline sensitivity, although caution is needed with the results obtained by this method if the resistance mechanisms are unknown. Baseline sensitivity gives information about the level of resistance to a particular plant protection product in a weed population and allow comparisons among different populations and between the same populations at different times, allowing the evaluation in sensitivity changes both between populations and along a period of time. The method was validated positively for dalapon and *B. diandrus and L. rigidum* (Barroso et al., 2010). This method once validated is much more practical than others methods used for herbicide resistance evaluation (Carrera de la et al., 1999; Carrera de la et al., 2000) as were methods based on mortality of the plant, number of leaves developed by the plant in a period of time and/or the length of the third or fourth leaf.

Before the molecular identification of resistant weeds (Sherwood & Jaseniuk, 2009) was largely used, this Petri dish bioassay could be of interest in the study of the structure of the populations in terms of evaluating the response of a population as the integrated response of each of the individuals that belongs to the population. By this way resistant plants can be detected and the inter and intra-population variability could be assessed. It is well known that the evolution of resistance will be much more rapid if a population carries resistant alleles before selection is imposed (Loureiro et al., 2010). The variation of the response of a population to herbicides is the result of the previous management of the fields and is the starting point for future weed-management strategies. It is likely that the frequency of resistant plants increase further if measures are not taken and control rely on herbicides with the same mode of action. It will thus be necessary to diversify the managements by rotating herbicides with different modes of action, by alternating crops and by implementing diversified cropping programs.

3.3 The importance of degradation products in the study of herbicides

The current tendency in agrochemical industry points to the development of herbicides more selective, less environmentally persistent, with less toxicity and bioaccumulation. In this regard, families of herbicides like sulfonilurea and cyclohexanedione oxime that belong to the third generation of pesticides, have appeared in the period 1970-1980 to fulfil these environmental requirements.

Although little attention has been paid to degradation products and metabolites in the past, by-products need to be considered to gain complete understanding of the environmental impact of these xenobiotics, otherwise herbicides' fate could be substantially underestimated. Determination of degradation products of organic compounds, such herbicides, is nowadays one of the major challenges in analytical chemistry of environmental pollutants.

In many cases, parent compound and transformation by-products possess different physico-chemical properties. The higher polarity and hence solubility in water of some degradation products increase the risk to contaminate the aquatic media. Data available show that concentration of degradation products presents in water is sometimes higher than those of the parent compound. Besides, degradation products are often more toxic and/or persistent in environmental matrices than their parent (Barceló & Hennion, 1997).

However, determination of transformation products is sometimes difficult to carry out. In many cases they have never been identified nor characterized before and the availability of analytical standards is scarce.
Therefore, to predict their fate in the natural environment and to assess their risk, it is necessary to improve our knowledge on the reactions under environmental conditions.

3.2.1 Processes of herbicide degradation

Degradation of herbicides can begin as soon as they are synthesized. Formulation processes, transport and/or storage can initiate degradation of the active substance. As well, once the herbicide is prepared in the tank mix, further transformation can take place because of the reactions with other substances present in the water or due to interactions with other herbicides.
Once applied to the field, most of the herbicides applied do not immediately enter the plant, but remains in soil, water, air and surface of the plant leaves where are subject to different agents capable of transforming by abiotic and/or biotic processes into one or more transformation products.
Most pesticides applied to the environment are ultimately degraded into universally present materials such as carbon dioxide, ammonia, water, mineral salts and humic substances. Different chemicals, however, are formed before the herbicides are completely degraded. If the products are results of biological degradation, they are referred to as metabolites.
Agents responsible for the transformation of herbicides in the field can be physical, chemical and biological. The influence of each agent in the herbicide depends on the physical properties and chemical structure of the herbicide molecule.
The two main physical agents involved in the degradation process are light and temperature. Solar radiation is responsible for the photolysis and thermal degradation of the herbicides in the surfaces of soil, plant and water. It is known that photodegradation is one of the main abiotic processes that take place for many herbicides (Dimou et al., 2004; Scrano et al., 1999; Saha & Kulshrestha 2002; Ibáñez et al. 2004). For this to occur in water, the emission spectrum of the sun needs to fit the adsorption spectrum of the pollutant. Cyclohexanedione oxime herbicides photodegrade rapidly when they are exposed to simulated or natural solar irradiation in different types of water showing a dependence both on the irradiation energy and on the composition of the water sample (Sevilla-Morán et al., 2010a).
The effect of temperature on degradation has been studied in tropical ecosystems (Sahid & Teoh, 1994). High temperatures encountered in the tropics will lead to enhance degradation of herbicide.
Chemical degradation can take place when the herbicide gets in contact with water that possesses substances that promote its degradation. It is known that the presence of substances employed for the disinfection of water such as hypochlorite and chloramines degrade herbicide to compounds more or less toxic that the active substance. Rapid degradation of herbicide tepraloxydim was observed in the presence of chlorine. In the same way, clethodim was degraded completely in a few minutes when is exposed to chlorinated water, giving rise to the formation of various oxidation by-products (Sandín-España et al., 2005a).
It is worth noting that natural substances present in aquatic systems (dissolved organic matter (DOM), nitrate and metal ions, …) may influence the photochemical behaviour of

organic compounds (Mazellier et al., 1997; Quivet et al., 2006; Sevilla-Morán et al., 2008). Diverse studies are available from literature where humic acids act enhancing (Santoro et al., 2000; Vialaton & Richard, 2002) or inhibiting (Dimou et al., 2005; Dimou et al., 2004) the degradation of herbicides. For instance the irradiation of sethoxydim (Sevilla-Morán et al., 2010a), alloxydim (Sevilla-Morán et al., 2008) and clethodim (Sevilla-Morán et al., 2010b) solutions containing humic acids slowed down the rate of the photodegradation, suggesting a strong "filter effect", while the presence of nitrate ions had no effect on the degradation.

In general, by increasing the organic-matter content ant the temperature, the degradation of herbicides in soils is enhanced. When the organic-matter content increases, the biomass of the active microbial population also increases and so does the degradation.

The role of organic matter in soils is very important. It has been shown that the most persistent complexes result from the direct covalent binding of pesticides to soil humic matter or clay. The pesticides most likely to bind covalently to the soil have chemical functionalities similar to the components of humus. The humic material is derived from the remains of decomposing plants, animals and microorganisms, and is composed primarily of humic and fulvic acids.

In order to investigate the soil degradation of pesticides, laboratory incubation studies with ^{14}C-labelled pesticides are required. These allow one to assess the likely rate of degradation of parent pesticides in soil, and provide information on the structure and likely degradability of metabolites.

In the same way, a variance of pH can accelerate the degradation of herbicides. The soil pH, for example, is an important parameter affecting the persistence of chemically unstable herbicides. The mobilities of acidic herbicides are related to pH, with higher mobility in soils with higher pH (Brown, 1990; Scrano et al., 1999; Boschin et al., 2007). Microorganisms are the most important group of biological agents present in the soil that degrade herbicides.

3.2.2 Biological activity of degradation products

All these degradations imply different reactions before the active substances are completely degraded or mineralized and one or two transformations are sometimes sufficient to alter the biological activity of the parent compound. For some herbicides a change that takes place in its molecular structure can change the physicochemical properties and also the toxicity to different species. Herbicides alachlor and metolachlor showed that the toxicity to the bacteria *V. Fisheri* was enhanced upon degradation (Osano et al., 2002). On the contrary, other studies showed that herbicides and its degradation products cannot be considered a risk for the environment. This is the case of some sulfonilureas herbicides, where neither the active substance nor the metabolites are toxic to *D. Magna* and *V. Fisheri*. (Martins et al., 2001; Vulliet et al., 2004).

Major degradation products of some herbicides also have herbicidal activity against target and/or non-target weeds. However, few studies have documented the level of herbicidal activity. Some pesticide degradation products are of significance in crop protection by being effective against the target weeds. It has been demonstrated that the formation of the sulfoxide by-product of thiocarbamate herbicides like butylate (Fig. 7), increased the herbicidal activity (Tuxhorn et al., 1986). On the contrary, some can be responsible for inadequate weed control by inducing rapid degradation of their parent compounds.

Evidence shows, however, that for some pesticides, the herbicidal activity attributed to parent compound is partly due to the products formed (Tuxhorn et al., 1986; Bresnahan et al., 2004). In some cases, herbicides are formed as degradation products of other herbicides for instance, chlorthiamid, a benzonitrile herbicide (Fig. 7), is the parent compound and the precursor of dichlobenil that is a degradation product formed in soil and also an herbicide.

Oxidation reactions occur frequently in the soil and are extremely important transformation pathway. S-containing herbicides are often rapidly oxidized to sulfoxide and afterwards more slowly to sulfones. Sulfoxidation can occur in soil and water mediated chemical or biologically (López et al., 1994; Hsieh et al., 1998; Ankumah et al., 1995).

This oxidation is so rapid and complete that sulfoxides are often the compounds found in soil shortly after application of the parent sulfide compound. Furthermore, in some cases, sulfoxides and sulfones are suspected to have the herbicidal activity (Campbell & Penner, 1985).

The herbicidal activity of carbamothiate herbicides sulfoxides has been previously reported. (Tuxhorn et al., 1986). In soils treated with butylate (Fig. 7), herbicide residues of the parent

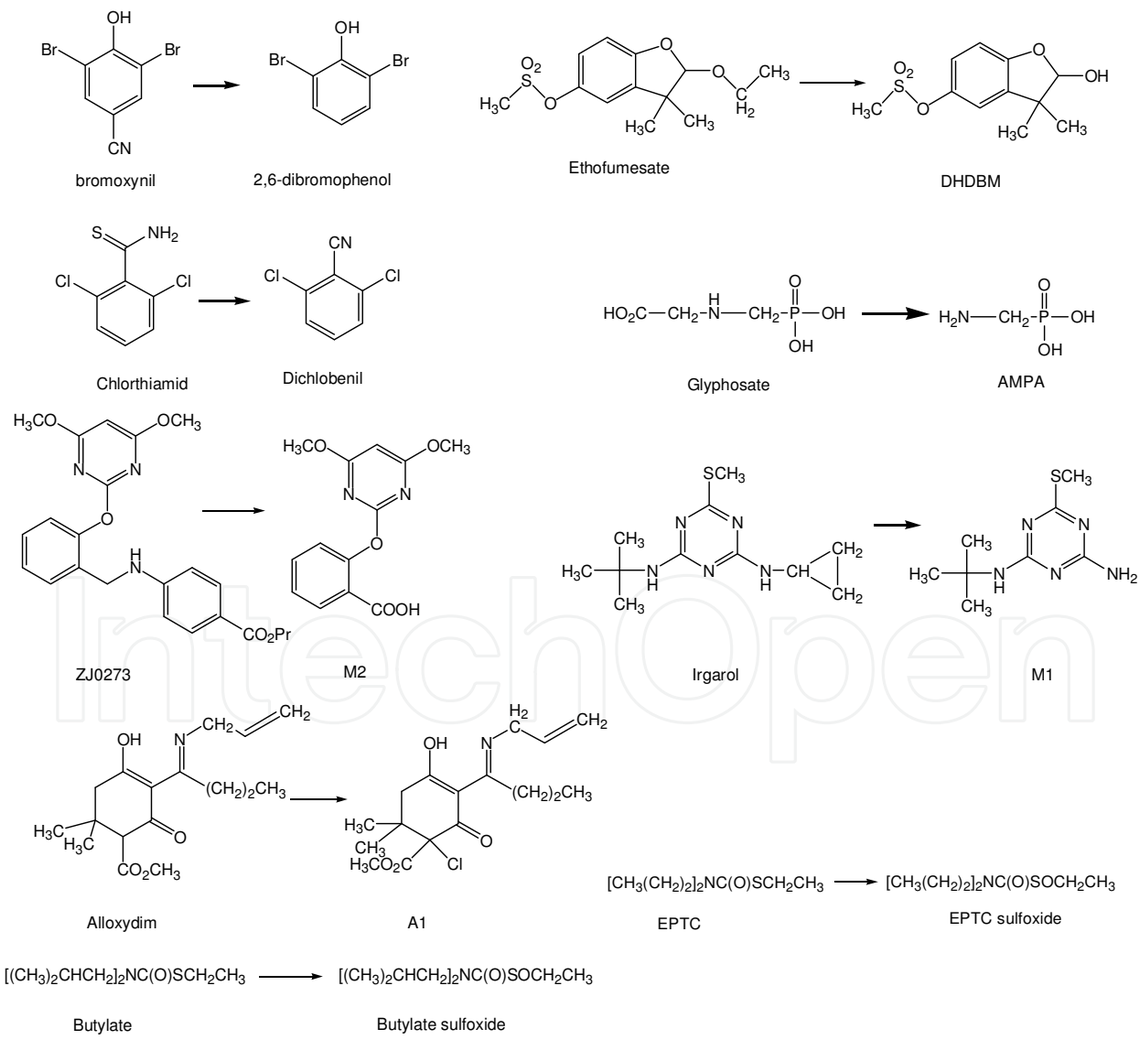


Fig. 7. Chemical structures of herbicides and degradation products discussed

compound were not detected in significant amounts within a few weeks after application. However, good control of weeds was observed in these fields. The good performance of this herbicide despite its lack of persistence was probably due to the by-products formed. Other degradation products effective on controlling target weeds are ETCP sulfoxide, which is the oxidation degradation product of the herbicide thiocarbamate EPTC (Fig. 7) (Somasundaram & Coats, 1991).

Relatively little is known of the potential phytotoxicity of degradation products and little literature exists on this topic.

Just as herbicides can be selective between plant species, metabolites can differ in their phytotoxicity pattern. Metabolites can have different mechanism of action and selectivities than the parent compound. For instance, bromoxynil (Fig. 7) is biological degraded in soil into 2,6-dibromophenol that is a potent growth regulates (Frear, 1976).

Kawahigashi et al., (2002) showed that the phytotoxicity of the de-ethylated metabolite of ethofumesate, DHDBM (Fig. 7), to rice plants was at least four times greater than that of the parent compound. Reddy et al., (2004) suggested that soybean injury to glyphosate-resistant soybean from glyphosate is due to its degradation product formed in plants, aminomethylphosphonic acid (AMPA). The degradation product of Irgarol 1051, M1 (Fig. 7) in the root elongation inhibition bioassay, showed a phytotoxicity at least 10 times greater than that of Irgarol and six other triazine herbicides (Okamura et al., 2000).

In many cases, degradation products are not phytotoxic as in the case of herbicide metsulfuron-methyl where the phytotoxicity of metsulfuron-methyl bound residues was mainly caused by the parent compound that became available during plant growth and no other metabolites detected (Ye et al., 2003).

As it has been explained before in this chapter, bioassays are important tools to screen herbicide residues and can be useful to exclude the occurrence of low levels of phytotoxic residues in soil (Hsiao & Smith, 1983; Sandín-España et al., 2003). In this sense, we have studied the phytotoxicity of alloxydim and its main metabolite with hydroponic bioassays on wheat (Sandín-España et al., 2005b).

Degradation product of alloxydim (Fig. 7) was the main product obtained in its degradation with chlorine, one of the most common disinfectant agents employed in water treatment.

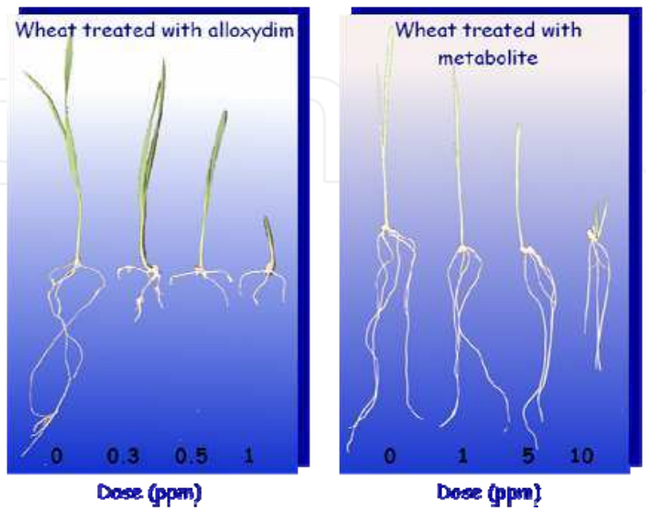


Fig. 8. Response of wheat plant to different doses of alloxydim and its metabolite

Results showed that after seven days of treatment the most sensitive biological parameter for alloxydim was root length, causing in the root growth of plants a 40% of significative reduction at the dose of 0.3 mg.l^{-1} and 94% of reduction at the highest dose. However, the effect of metabolite on root growth only occurred at the highest metabolite dose (10 mg.l^{-1}), causing a 32% of reduction in root growth. Root system control presented normal growth (main tap root plus secondary roots), while those from injured plants were increasingly deformed (main tap root twisted and lack of secondary roots). Root growth was increasingly affected with doses from 0.1 mg. l^{-1} to the highest dose (Fig. 8).

It is also important to highlight that a part of the degradation products formed in the soil from the herbicides remains as bound residues (Bresnahan et al., 2004; Albers et al., 2008; Rice et al., 2002). This non-extractable residue retained by organic matter in soil is bioavailable to plants. Therefore this portion of residue of degradation products and/or metabolites is underestimated if bound residues may be released from soil and absorbed by plants.

A study on phytotoxicity of soil bound residues of herbicide ZJ0273, a novel acetolactate synthase potential inhibitor, to rice and corn, revealed that one of his main metabolite (M2) (Fig. 7) played a dominant role in the inhibition effect on the growth of rice seedlings. In the extractable residues released from bound residues, the most biologically active M2 accounted for the largest fraction in all soils. Therefore, it was concluded that the main cause of phytotoxicity from exposure to soil bound residues of ZJ0273 is related to the release of ZJ0273 and its degradation products and the subsequent inhibition on ALS by M2 (Han et al., 2009).

In recent years it has been revealed the lack of data on the phytotoxic effects of herbicide residues. In this sense, it is necessary to study and develop simple methods for evaluating the environmental impact of these products based on hard scientific data. Besides, though most degradation products of herbicides are converted into less toxic or nontoxic compounds, some degradation products, because of their characteristics, may be biologically and/or environmentally active. Thus, major degradation products should be also considered in evaluating the potential bioactivity and environmental contamination of the parent compound. From these studies should be able to derive recommendations for agricultural practices for the use of these products to be environmentally friendly in general and in particular the agricultural environment capable of guaranteeing the future productivity of farms in the context of sustainable agriculture.

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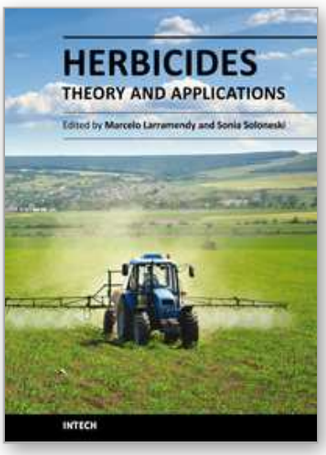

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The content selected in Herbicides, Theory and Applications is intended to provide researchers, producers and consumers of herbicides an overview of the latest scientific achievements. Although we are dealing with many diverse and different topics, we have tried to compile this "raw material" into three major sections in search of clarity and order - Weed Control and Crop Management, Analytical Techniques of Herbicide Detection and Herbicide Toxicity and Further Applications. The editors hope that this book will continue to meet the expectations and needs of all interested in the methodology of use of herbicides, weed control as well as problems related to its use, abuse and misuse.

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