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Application of Bioassays in Studies on Phytotoxic Herbicide Residues in the Soil Environment

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> Science is food for the mind
> *War and Peace*, L. Tolstoy 1869

1. Introduction

The primary aim of the application of herbicides is to protect plantations against the competitive action of many weed species found in the field of a given crop. Herbicides may be used both directly to the soil and in foliar applications. In relation to the type and method of application (single vs. split dose) a portion (in foliar applications) or the entire amount (in soil-applied agents) of herbicide reaches the soil (Praczyk & Skrzypczak, 2004; Woźnica, 2008). Each active ingredient in a herbicide which penetrates the soil medium, undergoes certain biophysical and biochemical processes. At the time the herbicide active ingredient enters the soil it is separated between the solid phase (soil particles) and the aqueous phase (soil solution). In the soil medium only this portion of the active ingredient is available to plants, which is found in the liquid phase. However, herbicide molecules adsorbed or chemically bound with the solid phase are not absorbed by plants. Under field conditions this balance is constantly disturbed as a result of the action of the edaphone and through changes in temperature and moisture content of soil, which affects the availability of herbicide to weeds and crops (Vicari et al., 1994; Sadowski, 2001).

Depending on the applied cultivation regime and climatic and soil conditions observed in a given vegetation season only a portion of herbicide active ingredient residue found in the soil is available to plants and under advantageous conditions may exhibit phytotoxic action. Thus the determination of the level of residue, degradation rate and translocation of herbicide active ingredients in the soil is so significant both for the agricultural practice and for the protection of the agricultural environment (Sadowski et al., 2002; Sadowski & Kucharski, 2004).

At the selection of a detection technique the most important criterion in the evaluation is the concentration, at which a given analyte may be found in the tested sample. Instrumental methods, such as gas chromatography (GC) or liquid high performance chromatography (HPLC), make it possible to determine the total content of active ingredients in the soil at the time of the application or several weeks after the application of herbicides (Ahmad & Crawford, 1990; Sadowski, 2001; Sadowski et al., 2001a; Kucharski & Sadowski, 2006). This

problem appears when the herbicide is used once or several times in the vegetation season in small doses of <50 g/ha, since already at the moment of application the level of herbicide active ingredients is slight and does not exceed 10^{-2} mg/kg (Sadowski et al., 2002). The evaluation of risk resulting from the occurrence of herbicide residue in the soil medium until recently was based only on the results of chemical analyses, which supplied information on the presence, content and type of the chemical substance, preventing an evaluation of harmful ecological effects of the herbicide residue. Thus the traditional, chemical approach to the assessment of the level of herbicide residue in the agrophytocenosis for well over a decade has been supplemented by ecotoxicological analyses. In such analyses the level of herbicide residue is evaluated on the basis of a specific, comprehensive response of standard indicator organisms to the active ingredient varying both chemically and in terms of its concentration, contained in the tested soil sample. In such analyses the biotest methods is used with the application of e.g. a plant biodetector. This method is to determine a biologically effective level of the herbicide active ingredient residue immediately after application, as well as to follow the dynamics of decline for this substance in the soil environment in the course of several months or even for more than a year. Biotests also facilitate an objective evaluation of the level of residue, due to the fact that all higher plants have a certain sensitivity to different xenobiotics (e.g. herbicides) found in the soil environment. The phytotoxic effect of active ingredients originating from herbicides may be observed on the basis of the reduction of dry or fresh weight of roots or aboveground parts (stems, leaves) of test plants (Günther et al., 1993; Stork & Hannah, 1996; Sarmah et al., 1999; Sadowski et al., 2002; Demczuk et al., 2004; Sekutowski & Sadowski, 2005; 2006; 2009). Thanks to the wide-scale application of the bioindication method using plants it is possible to evaluate the degree of contamination not only for the soil, but also for the entire agrophytocenosis (Dećkowska et al., 2008).

2. The behavior of herbicides in the soil environment

Active ingredients of herbicides, after penetrating to the soil, are separated between the solid phase (soil particles) and the liquid phase (soil solution). In the soil only this portion of the active ingredient is available to plants, which is found in the soil solution within the rhizosphere. In turn, herbicide molecules adsorbed or chemically bound with the solid phase are not available to plants. They may constitute a certain reserve, which under advantageous climatic conditions may become available to plants. Availability of these active ingredients in the soil fluctuates constantly, since they are removed from the soil solution as a result of immobilization, elution or diffusion. Bounding of these chemical substances by the soil sorption complex is a factor determining their occurrence and through accumulation may significantly alter their deposition time in the soil environment. Under field conditions this equilibrium is constantly disturbed by changes in temperature, moisture content, cultivation measures and the soil entomofauna, which has a crucial effect on the amount of the herbicide active ingredient which is available to weeds or crops within a specified period of time. On the one hand, the process of binding reduces mobility of these residues in the soil profile and their penetration to aquatic zones, while on the other hand, it reduces the possibility of their removal from soil using plants themselves and soil microorganisms (Sadowski et al., 2001a, 2002; Praczyk & Skrzypczak, 2004; Woźnica, 2008).
The process of degradation and translocation of herbicide active ingredients depends on many environmental and soil factors, which determine their adsorption and absorption in

the soil. Such factors include the type of soil, mechanical composition (particularly the content of clays and zeolites), temperature, moisture content, content of organic matter (humus), pH of soil as well as the content of soil entomofauna biomass (Walker & Welch, 1989; Vicari et al., 1994; James et al., 1999; Sarmah et al., 1999; Sadowski & Kucharski, 2004).

The sorption capacity of herbicides is defined by the index of soil sorption of the herbicide (K_d) and sorption of the herbicide to organic carbon (K_{oc}). Active ingredients of herbicides characterized by very high mobility in the soil environment have $K_{oc} < 100$ ml/g (clopyralid, nicosulfuron, sulfosulfuron, sulcotrione, dicamba), while herbicides with $K_{oc} > 2000$ ml/g exhibit poor mobility (trifluralin, diquat, pendimethalin, diclofop, fenoxaprop-P) (Praczyk & Skrzypczak, 2004; Woźnica, 2008).

The translocation of herbicides in the soil profile is frequently disturbed by crops themselves or by weeds, absorbing water from the soil solution. Since a portion of the root system of plants frequently reaches a depth of 50 - 60 cm and has a very big suction power we may often observe the process of leaching or even the movement of residue of certain herbicides (e.g. chlorsulfuron) from deeper soil layers towards the rhizosphere (Walker et al., 1989; Sadowski et al., 2001b). In the opinion of Sadowski & Kucharski (2004), elution of herbicide active ingredients being derivatives of sulfonylurea (chlorsulfuron, sulfosulfuron) and phenoxyacetic acid (2.4 D, MCPA) from the soil profiles is strongly dependent on the initial moisture content and the absorbing capacity of soil. They showed in their studies that with an increase in the initial moisture content of soil, the degree of leaching for these substances increased markedly, reaching a certain maximum. When soil reached the maximum water capacity (under field conditions this process is observed during heavy rains), then the percentage of leached active ingredient of a herbicide is markedly reduced. Also Beckie & McKercher (1990), Oppong & Sagar (1992) and Günter et al. (1993) were of an opinion that apart from moisture content, also absorbing capacity of soil has a decisive effect on herbicide mobility. In their studies Günter et al. (1993) showed that in soil with poor absorbing capacity metsulfuron and triasulfuron were subjected to elution much faster than in soil with a high absorbing capacity. Mobility was also dependent on the active ingredient itself, with metsulfuron being much more active than triasulfuron.

Also the depth to which herbicide active ingredients penetrate under field conditions is not specifically defined, since it depends on many factors (e.g. absorbing capacity, granulometric composition, cultivation measures). On the basis of studies concerning the translocation of herbicide active ingredients Helling & Turner (1968) determined the relative mobility index of herbicides (R_f), dividing them into five classes. In another study Walker & Welch (1989) showed that chlorsulfuron (ALS group) was capable of penetrating to a depth of 50 cm 63 days after application, despite the fact that a bigger part of its residue was detected in a layer up to 25 cm deep. In turn, another active ingredient from the same chemical group, i.e. triasulfuron, did not penetrate deeper than 10 cm, and its residue remained at that depth throughout the entire period of the experiment (125 days).

Stability of active ingredients of herbicides in the soil is also dependent on its physico-chemical properties and on the course of degradation dynamics. A very important indicator, which defines potential persistence of the herbicide active ingredient in the soil environment, is the half-life period (DT_{50}). It is a time period required for the degradation of the active ingredient to half its initial concentration in soil. The value of DT_{50} is a characteristic feature of individual active ingredients of herbicides and it may range from several days (e.g. quizalofop-P, mesotrione, MCPA) to as long as several months (e.g. trifluralin, ethofumesate, pendimethalin). Most active ingredients of herbicides used in

agricultural plantations has DT_{50} of less than 60 days (e.g. florasulam, clomazone, clopyralid, bentazone), while in vegetable growing it is below 20 days (e.g. clethodim, cycloxdim, metazachlor, pyridate) (Praczyk, 2004; Praczyk & Skrzypczak, 2004; Woźnica, 2008). Half-life (DT_{50}) is only a rough indication of the potential persistence of herbicide active ingredients in soil. Under field conditions degradation of a herbicide and its translocation may occur faster or much slower, since it is a result of interactions between chemical properties of the active ingredient itself and moisture content, temperature, absorbing capacity of soil, pH and soil microorganisms. Thus the risk of persistence and translocation of herbicide active ingredients in soil may not be considered only on the basis of one of the above mentioned parameters (e.g. DT_{50}, K_{oc}, R_f), as under field conditions the interactions of all these factors affect the rate of chemical and biological processes, which in turn determine the behavior of active ingredients of herbicides in the soil environment. Table 1 presents characteristics of selected active ingredients of herbicides, which have a decisive effect on their behavior in the soil environment.

Active ingredient	Group HRAC	Solubility in water [mg/l]	DT_{50} [days]	K_{OC} [ml/g]	R_f movement index in soil environment
quizalofop-P	A	0.4	<1	1024	small (R_f = 0.0-0.34)
florasulam	B	6360 (pH 7)	2-18	4-54	
trifluralina	K1	0.22	60-132	2500-13700	
diquat	D	700000	1000	>32000	
pendimethalin	K1	0.3	30-150	6700-29400	
amidosulfuron	B	9 (pH 5.8)	3-29	33.7	medium (R_f = 0.35-0.64)
clomazone	F3	1100	15-45	104-608	
ethofumesate	N	50	15-250	97-245	
alachlor	K3	242	15-30	170-200	
MCPA	O	734	5-6	25-157	
sulfosulfuron	B	1627 (pH 7)	11-47	5-89	large (R_f = 0.65-1.0)
metamitron	C1	1700	7-70	91-392	
bentazone	C3	570	12-45	13-176	
mesotrione	F2	2200	3-7	19-390	
clopyralid	O	143	14-56	4.6	

Source: Helling & Turner, (1968); Praczyk & Skrzypczak, (2004); Woźnica, (2008); modified

Table 1. Examples of active ingredient of herbicides and selected physico-chemical properties affecting their behavior in soil

Annually repeated application of herbicides in the same field may affect the dynamics of degradation and translocation, as well as the level of residue of their active ingredients. After penetrating into the soil the action of a herbicide on a crop or weeds is determined within the rhizosphere by the degree of availability and the sensitivity of the plant to the active ingredient. Strong vertical translocation of certain active ingredients of herbicides several days after application, particularly in lessive soils may be dangerous for the soil

environment due to the possible penetration into the ground waters causing their contamination (Beckie & McKercher, 1990; Sadowski & Kucharski, 2003). Thus studies are necessary which would facilitate an evaluation of a threat posed by the application of herbicides in relation to agrophytocenosis. In ecotoxicology the adopted methods for the determination of the levels of bioavailable phytotoxic residue of herbicide active ingredients in soil include biotests, due to their high efficiency, relatively very high sensitivity and limited testing costs in comparison to instrumental methods (Fahl et al., 1995; Hollaway et al., 1999; James et al., 1999; Sadowski et al., 2002; Sadowski & Kucharski, 2004; Sekutowski & Sadowski, 2006). Plant species exhibiting high sensitivity to the action of selected active ingredients of herbicide, such as *Sinapis alba, Fagopyrum esculentum, Sorghum saccharatum, Lepidium sativum, Helianthus annuus, Zea mays* or *Cucumis sativus* are used as detectors. In particular cases biotests may also provide information on transport and on the situation of applied active ingredients (Günther et al., 1993; Sadowski & Kucharski, 2004). We may find numerous examples in literature concerning applications of plant biodetectors in studies on herbicide active ingredient residue (Günther et al., 1993; Stork & Hannah, 1996; Sarmah et al., 1999; Sekutowski & Sadowski 2005; 2006; 2009).

3. Division of biological methods used in studies on the soil environment

Analytical methods using biological material are becoming promising alternatives for conventional analytical methods and in certain cases they may even replace them (Hollaway et al., 1999). They are commonly applied mainly due to their specificity and low unit costs. In toxicological analyses we may distinguish two groups of applications for biological methods in the assessment of the effect of xenobiotics (e.g. herbicides) on the soil environment:

a. bioanalytical tests, which are connected with the use of biological organisms as receptors of specific chemical substances, e.g. herbicides. Due to the method of the utilization of the biological component we distinguish:

-   biosensors, in which the biological component is the active element (e.g. an enzyme, antibodies – ELISA test),
-   biotests, in which a whole plant organism or its part (e.g. seeds, roots) are the control and measuring element (Hollaway et al., 1999; van Wyk & Reinhardt, 2001).

b. biomonitoring, which may be conducted in two ways:

-   through the formation of passive accumulation samplers based on typical analytical tests of biological samples,
-   through observation of plant or animal bioindicators (Fahl et al., 1995; Alonso-Prados et al., 2002).

4. Bioassay

Bioassay or biotest (Greek *bios* – life + Latin *testari* - indicate) may be defined as an experimental biological sample (the whole organism or its part), which aim is to detect a toxic substance found in the environment or to identify its harmful action, by quantitative determination of the effect of the tested substance in relation to the control object.

In studies conducted using biotests three methods are typically applied, with the first two being conducted under controlled (laboratory) conditions, while the third being run using a population of organisms living under natural conditions (*in situ*).

a. phytotoxicity tests conducted in a laboratory, during which the substance exhibiting phytotoxic action is artificially introduced to the tested object (e.g. soil). Next the test is performed with an appropriately selected indicator organism e.g. a plant (a phytotest). Thus collected results are a source of information on toxicity of a given substance under controlled conditions. The main aim of such a test is to calibrate the biotest, which will next be used to estimate phytotoxicity of tested samples (e.g. collected from contaminated areas).

b. phytotoxicity tests conducted at a laboratory on the basis of respective samples (e.g. soil) collected from contaminated areas. Phytotoxicity of such samples is compared with the phytotoxicity of reference samples (biotests). On this basis the interval is determined, within which residue e.g. of herbicides may have an adverse effect on crops (e.g. residual effect).

c. phytotoxicity tests conducted on the site in which a population of sensitive organisms is living (conditions of their natural occurrence) (Kuczyńska et al., 2005, Namieśnik & Szefer 2009).

Moreover, biotests may be classified in terms of the used organism (e.g. bacteria, plants, animals), which constitute the active element of the test. In ecotoxicology in studies on the residue of different xenobiotics (e.g. herbicides) the most frequently applied include plants and their seeds, due to the specific action of the tested preparations and in view of the humane, economic and practical aspects.

On the basis of the dose ↔ final effect dependence, which may be expressed e.g. by the reduction of fresh or dry weight of the test plant in comparison to the control object, we may determine values of indicators being a quantitative measure of phytotoxicity of the tested substance. Phytotoxic action of active ingredients contained in herbicides may be determined using such indicators as ED_{10}, ED_{50} or ED_{90} (effective dose), i.e. determining the concentration of the active ingredient causing a specific biological effect at 10%, 50% or 90% its maximum value. Another applied indicator is index IC_{50} or IC_{90} (inhibition concentration), i.e. the concentration of e.g. herbicide in the soil environment, which causes a reduction of fresh or dry weight of the test plant (roots, stems, leaves) by 50% or 90% in comparison to the control (not treated with this herbicide).

The dose ↔ final effect dependence may also be used to predict risk, i.e. to determine the dose and persistence of herbicide residue, at which the probability of phytotoxic effects is high or small. An example exhibiting this dependence may be here a study conducted by Sadowski et al. (2007) or Sadowski & Sekutowski (2008), referring to the phytotoxic action of herbicide active ingredient residue on successive crops. Those authors using biotests showed that herbicide residue in soil may be hazardous for successive crops at two critical moments. The first refers to resowing, i.e. situations when for different reasons, most frequently independent of the farmer, the plantation is eliminated. In turn, the other moment refers to residue persisting in soil and exhibiting phytotoxic action immediately after the crop is harvested or even for the next several months. A similar phenomenon on fields in which chlorsulfuron and metsulfuron were applied, observed in the form of extensive damage to sugar beet or rape plantations found in the period of 2 successive years, was reported by Walker & Welch (1989) and Walker et al. (1989). The above mentioned effect is manifested only because crops (e.g. beet, rape) exhibit very high sensitivity to herbicides from the ALS group. Plant species with a narrow range of tolerance (stenobionts) characterized by high sensitivity to specific chemical groups or active ingredients of herbicides are referred to as indicator species or bioindicators. Thus biotests are very often

used in biomonitoring, to evaluate the consequences potentially caused by herbicides on individual elements of agrophytocenosis (e.g. crops, soil or water).

4.1 Criteria for the selection of a bioindicator

Species of indicator plants should be characterized by a narrow range of responses and exhibit high sensitivity to specific chemical substances, with their response being specific and adequate to the concentration of the chemical substance and easily observable (e.g. strong inhibition of root growth).

Bioindicators should meet the following requirements:

- common occurrence,
- a wide range of distribution,
- a long life cycle or several generations within a year,
- being easily recognizable,
- genetic homogeneity,
- high sensitivity to specific chemical substances,
- stability and repeatability of responses,
- low unit costs and easy laboratory culture.

In turn, plant bioindicators used in phytotests should have the following characteristics:

- small and even seeds,
- uniform germination power and energy of seeds,
- a short emergence period (1-2 days),
- a short vegetation period,
- high biomass of stems, leaves or roots,
- high sensitivity in relation to one chemical group (e.g. phenoxy acids, sulfonylurea).

When selecting a bioindicator for a test it is also necessary to take into consideration the age and sensitivity of individual tissues to the tested herbicide. A similar opinion was also expressed by Shim et al. (2003) and Demczuk et al. (2004), who in their studies conducted using different weed species and *Cucumis sativus* plants observed a diverse response of individual plant tissues to tested active ingredients of herbicides. They showed that sensitivity to residue of sulfonylurea herbicide depended to a considerable degree on the age of tissues and their location. The youngest roots and leaves of test plants turned out to be most sensitive.

Thus one of the basic guarantees of an appropriately conducted biotest is the selection of an appropriate test plant. An example of a dependence between the phytoindicator and the response to the herbicide active ingredient is presented in Fig. 1-2. In the analyses 3 test plants were used, i.e. *Sinapis alba, Fagopyrum esculentum* and *Cucumis sativus*, as well as 2 active ingredients of herbicides belonging to different chemical groups (phenoxy acids – 2.4 D and sulfonylurea – nicosulfuron). For the detection of 2.4 D residue *Cucumis sativus* proved to be most suitable, since root growth inhibition by 50% (IC_{50}) occurred already at a concentration of 0.18 mg/kg. For the two other species, i.e. *Sinapis alba* and *Fagopyrum esculentum,* IC_{50} ranged from 0.4 to 0.5 mg/kg (Fig. 1).

In turn, in the detection of nicosulfuron residue the highest sensitivity was found for the test with the use of *Sinapis alba*. Root length reduction by 50% (IC_{50}) occurred already at a concentration of 0.125 mg/kg. Sensitivity of the test (IC_{50}) with the use of *Fagopyrum esculentum* and *Cucumis sativus* was markedly lower and amounted to 0.25 mg/kg for *Fagopyrum esculentum* and 0.55 mg/kg for *Cucumis sativus*, respectively (Fig. 2).
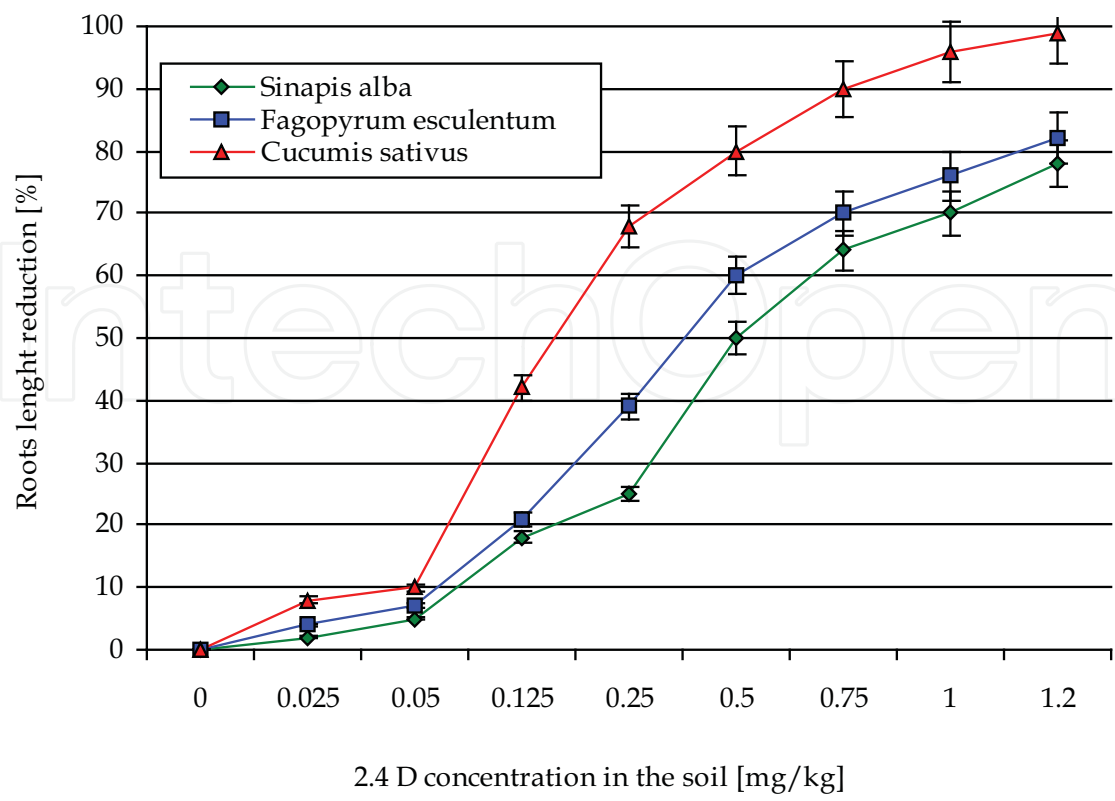
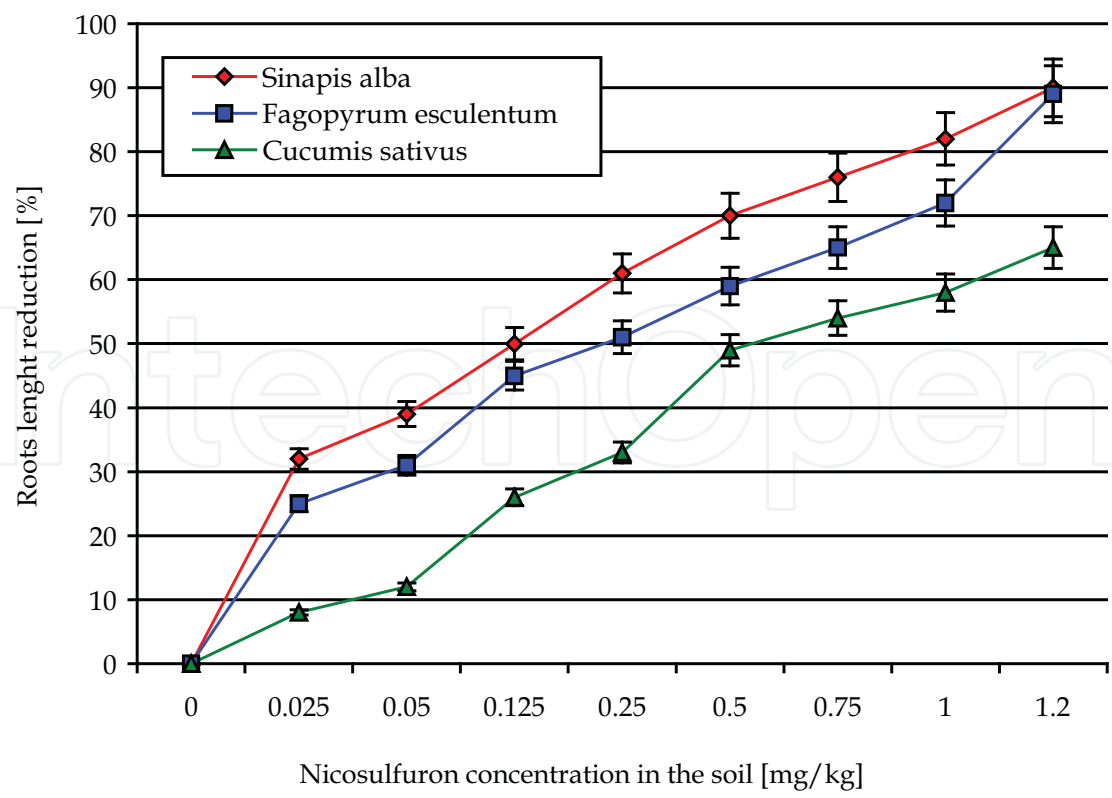Fig. 1. 2.4 D effect on the tested plant in terms of roots lenght reduction

Fig. 2. Nicosulfuron effect on the tested plant in terms of roots lenght reduction

4.2. Conventional bioassays

A conventional bioassay, used in the detection of herbicide active ingredients in soil, consists in the sowing of seeds of a test plant (adequately sensitive to the tested substance or chemical group) into the soil sample containing the residue. Examples of procedures required for the establishment of such a bioassay are presented in a diagram in Fig. 3.
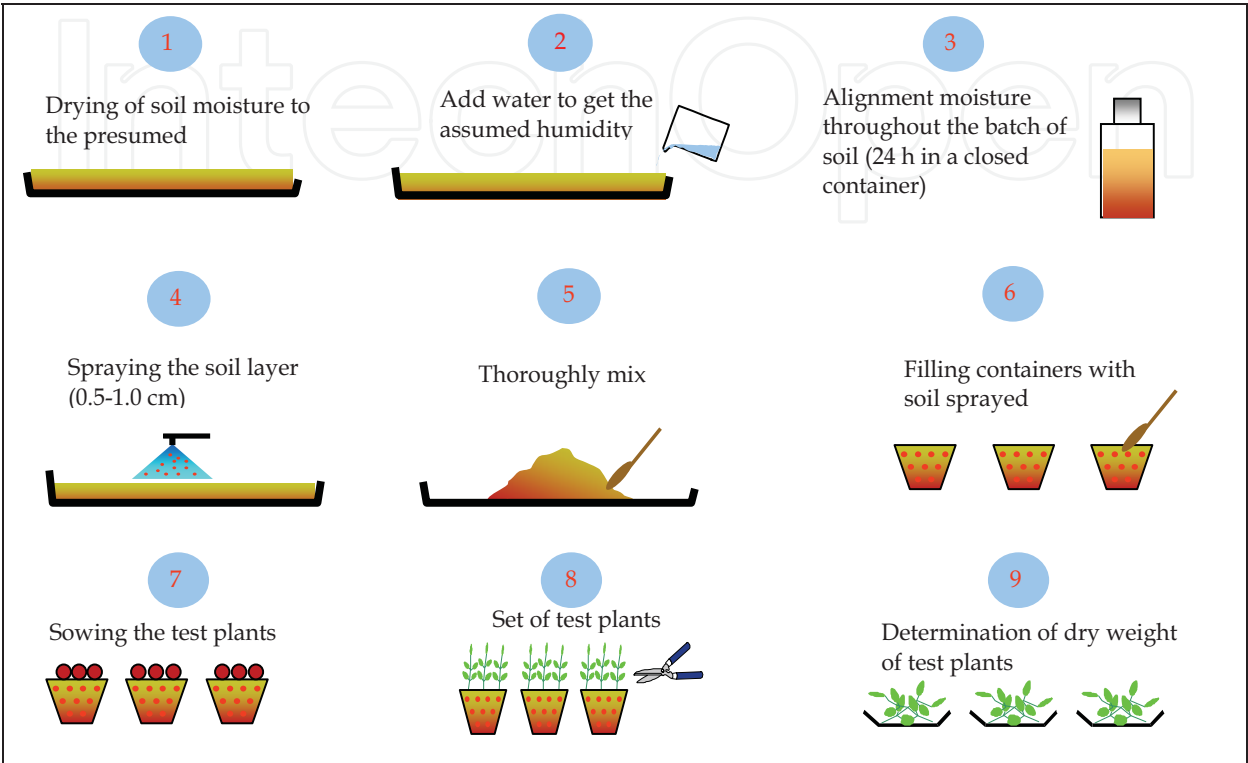
Source: Sadowski et al., (2002); modified

Fig. 3. Example diagram of a conventional bioassay setting

4.2.1 Availability of herbicide active ingredients to plants

The bioassay method is also used in the determination of values of ED_{50}, ED_{90} or IC_{50}, IC_{90}. The duration of a conventional bioassay depends to a considerable degree on the test plant, or rather on the tested part of the bioindicator (roots, leaves) and the active ingredient of a given herbicide, and it may range from 7 days (roots) to 14 days (leaves, stems). After a period of 7 or 14 days from the establishment of the test fresh and then dry weight of roots or leaves and stems is determined (by cutting and drying at a temperature of 105^0C, and weighing on an analytical scale). Next the percentage loss of fresh and dry weight is calculated in relation to the control plants (sown into the soil containing no herbicide), while thus collected results for the dependence between weight loss in the phytotest and the concentration of the herbicide active ingredient in the soil are used in the graphic presentation of this dependence (Fig. 4).

Figure 5 presents an example of a phytotest using *Cucumis sativus* established in soil containing different concentrations of chlorsulfuron. Results recorded from the bioassay constitute a source of information on the toxicity of chlorsulfuron under controlled conditions. The theoretical objective of such a test is to determine IC_{50} for chlorsulfuron and to calibrate the phytotest, which will next be used in the estimation of phytotoxicity of
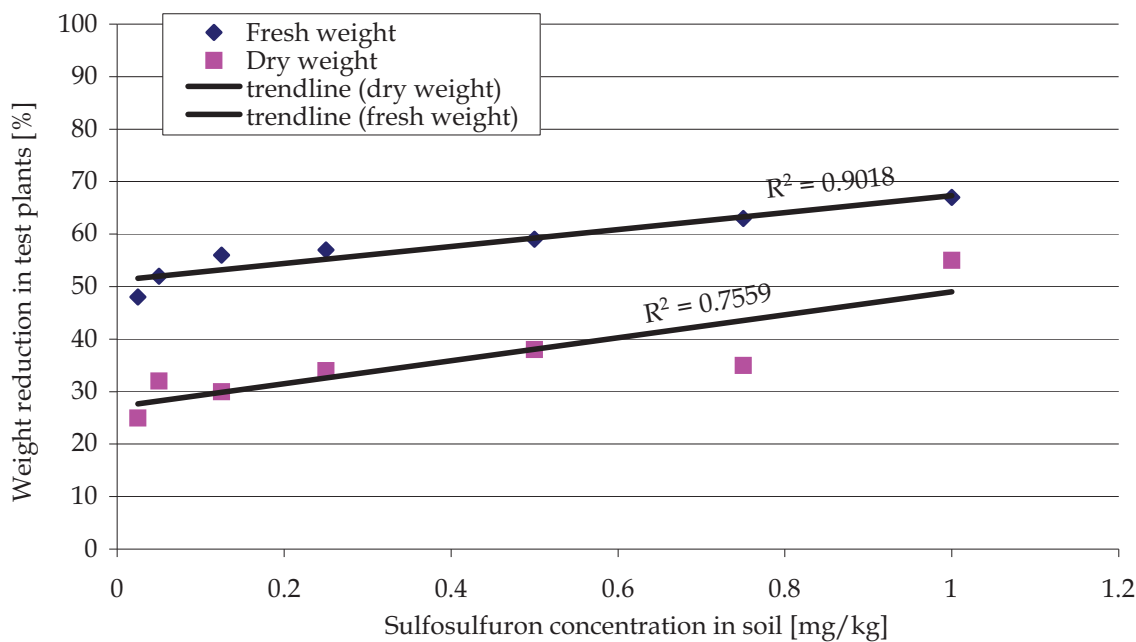
Fig. 4. Changes fresh and dry weight of *Sinapis alba* under the influence of different sulfosulfuron concentrations in soil

Fig. 5. Te effect of chlorsulfuron on fresh weight reduction of *Cucumis sativus* (determination of IC$_{50}$)

samples of soil collected from a field containing residue of chlorsulfuron (the practical objective). Thanks to this test it will al be possible to determine whether in that field plants from family *Cucurbitaceae* will be exposed to the phytotoxic action of chlorsulfuron residue.

4.2.2 Distribution of herbicide active ingredients in the soil profile
Knowledge on the translocation and distribution of active ingredients in the soil profile and factors affecting this process is required both for the protection of the soil environment and a more efficient use of herbicides. Most studies in this field have been conducted mainly

using lysimeters. Unfortunately, the primary drawback of the lysimeter model is connected with the high cost of one assay and limitations related with the collection of soil samples, resulting from the disruption of the soil profile in the lysimeter column. After several samplings the lysimeter column has to be refilled with a new undisturbed soil profile. In turn, analyses conducted under laboratory conditions using bioassays do not have such limitations. Moreover, they are more efficient and provide the experimenter with more flexibility and control over a much bigger number of parameters observed during the process of herbicide translocation. Soil collected from such a model is used as a substrate for bioassays and the filtrate may be used in chemical analyses. This method makes it possible to determine in a very precise way the distribution of phytotoxic residue of herbicide active ingredients in the soil profile. Another advantage of this model is the possibility of arbitrary modeling of irrigation in the soil profile, which facilitates a comprehensive evaluation of the residue balance in the soil – water system. Figure 6 presents an example diagram of such a model in action, in which the bioassay method was used to determine the distribution of herbicide residue.

In the opinion of Sadowski & Kucharski (2004) the degree of leaching and as a consequence the distribution of a portion of herbicide active ingredients depends on the initial soil moisture content. Figure 7-8 presents the distribution of certain active ingredients of
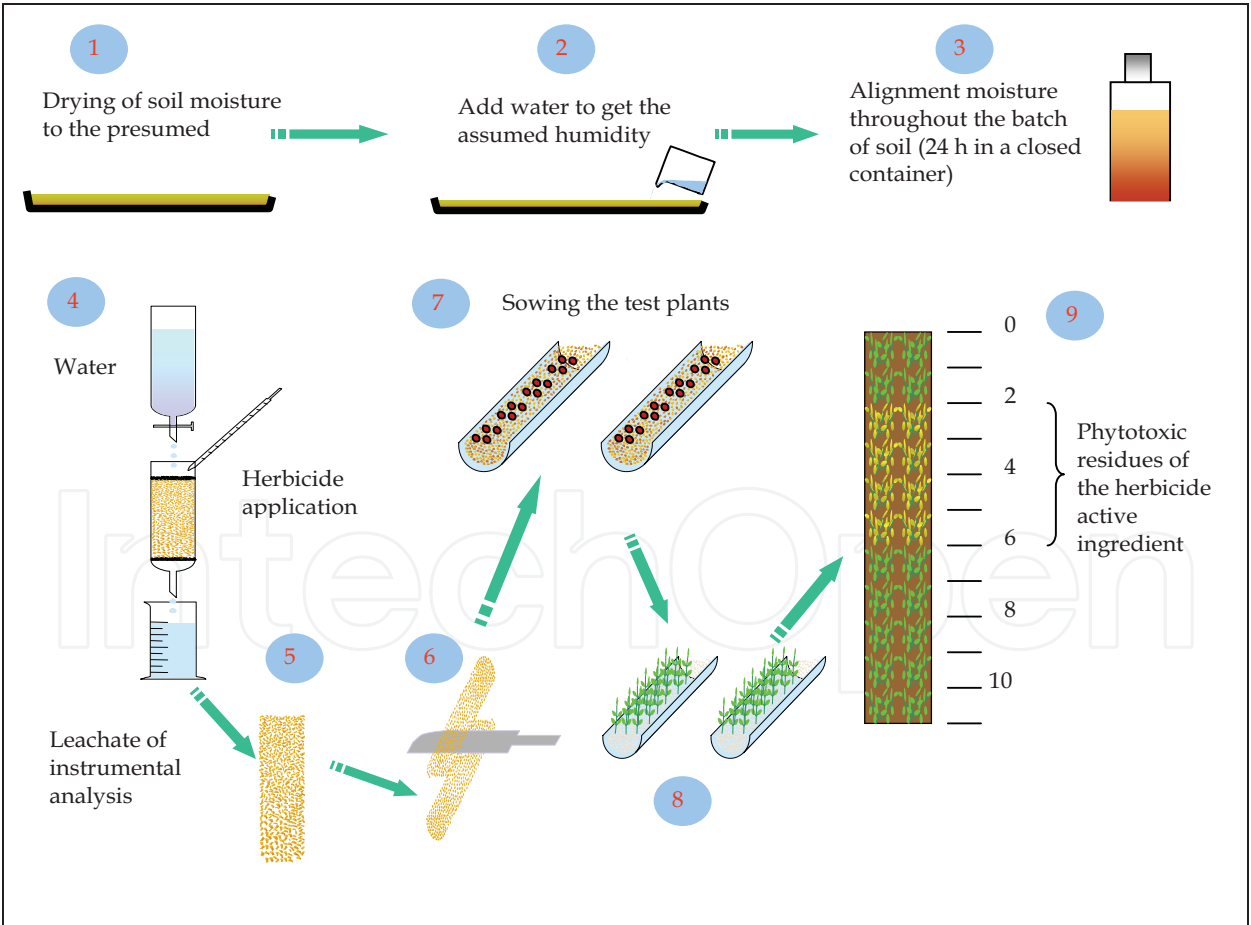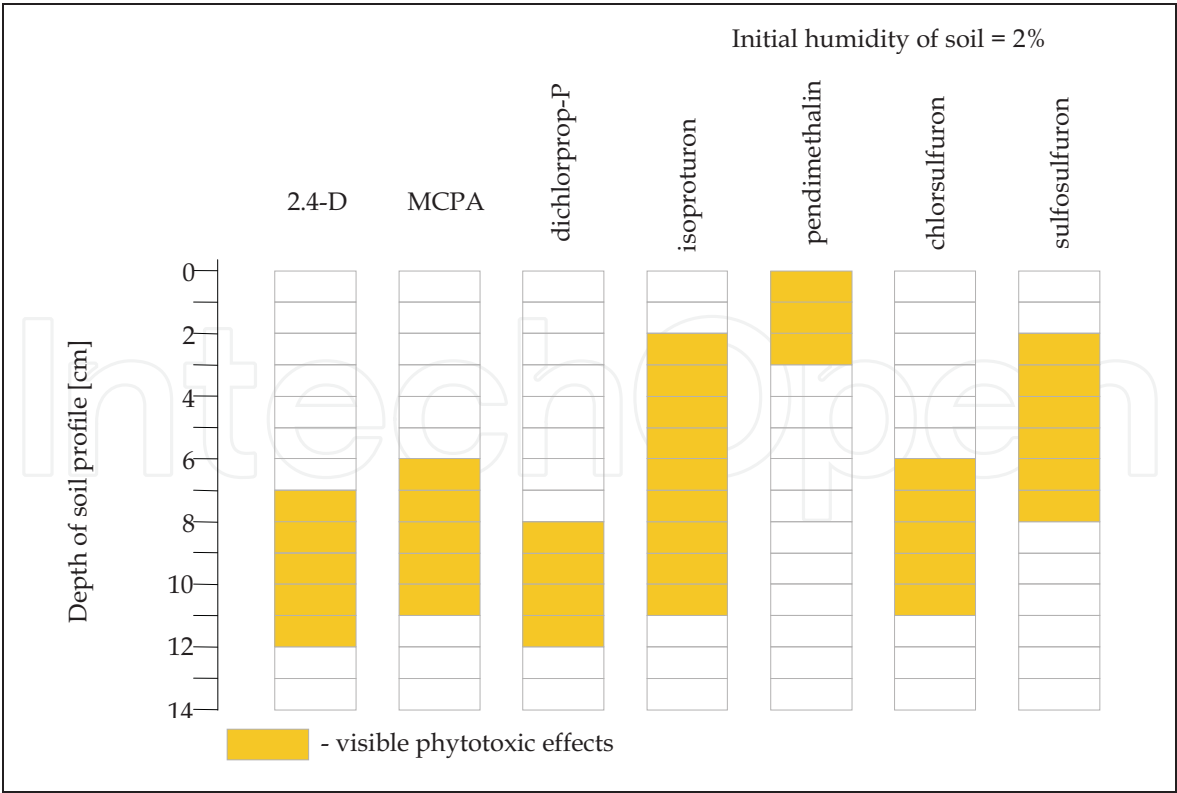

Source: Günther et al., (1993); Sadowski & Kucharski, (2004); modified

Fig. 6. The application of the phytotest method to determine residue of herbicide active ingredients in the soil profile
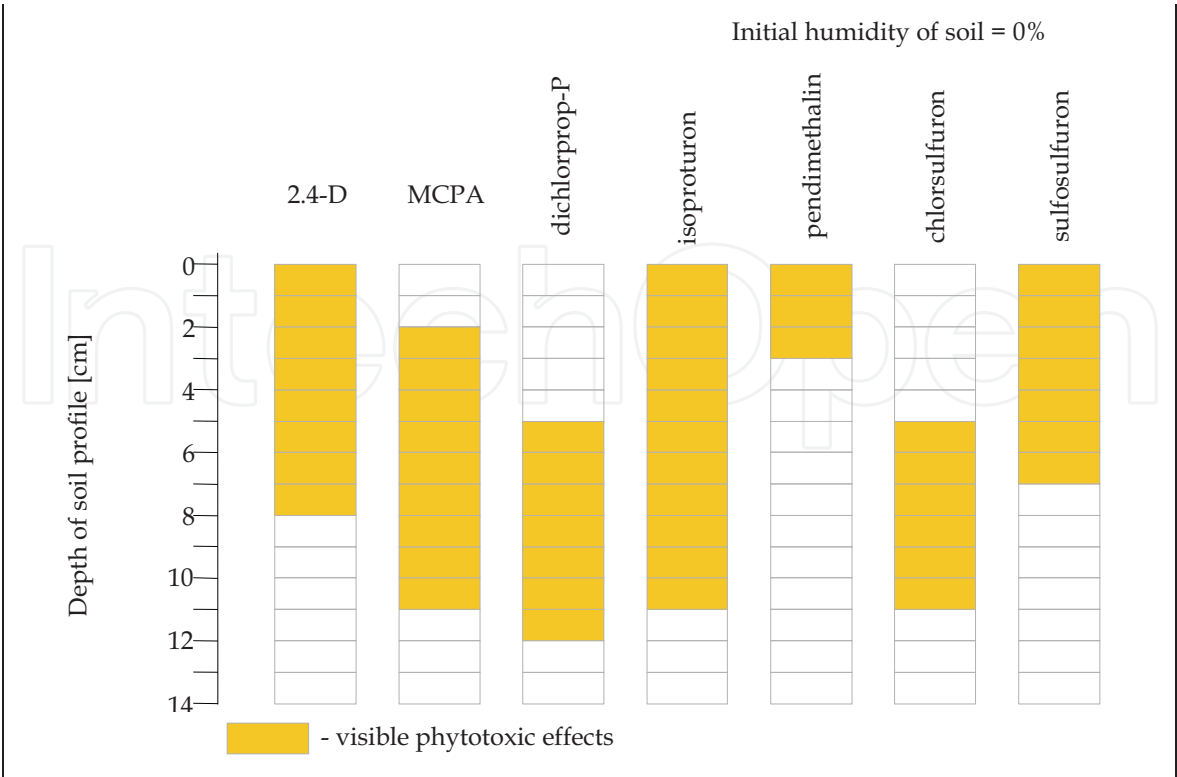
Source: Sadowski & Kucharski, (2004); Sekutowski & Sadowski, (2006); modified

Fig. 7. Rate of translocation of active ingredients depending on initial soil moisture content

Source: Sadowski & Kucharski, (2004); Sekutowski & Sadowski, (2006); modified

Fig. 8. Rate of translocation of active ingredients depending on initial soil moisture content

herbicides depending on changes in the initial soil moisture content. Most active ingredients, which were transferred on air dry soil (0% moisture content) were detected by test plants (*Sinapis alba*) mainly in the surface soil layer (Fig. 7). The highest leaching level was found for isoproturon (0-11 cm), while the least leached substance turned out to be pendimethalin (0-3 cm). An increase in the initial soil moisture content by 2% caused a marked shift of residue deeper within the soil profile practically for all the tested active ingredients. Only pendimethalin residue remained at the same level (Fig. 8).

From the practical point of view the distribution of the main portion of herbicide active ingredients, as well as the degree of their leaching to deeper soil layers are highly significant, since they determine the effectiveness of herbicides (particularly those soil-applied). Moreover, they also determine the degree of herbicide translocation outside the root zone, which may increase the risk of their being transferred to ground waters (Sadowski & Kucharski, 2004).

4.2.3 Dynamics of degradation of herbicide active ingredients in soil

Dynamics of degradation occurs most intensively in the surface soil layer (0-20 cm) and it is closely related with the processes of degradation and translocation of herbicide active ingredients. In this layer the intensity of biological and chemical processes is dependent to a high degree on the temperature and soil moisture content, as well as the tillage systems (Sadowski, 2001; Sadowski & Kucharski, 2004; Rola & Sekutowski, 2005). In order to determine the dynamics of degradation and translocation of herbicide active ingredients in the soil, at specified time intervals samples are collected, onto which test plants (phytoindicators) are sown. An example of a phytotest for different herbicide active ingredients is presented in Fig. 9.

An example given here presents an experiment conducted using the bioassay method (with *Sinapis alba* as a phytodetector) under field conditions referring to the dynamics of translocation and degradation of rimsulfuron depending on the tillage method applied. In the first 6 weeks after application no marked differences were observed in the course of the dynamics of rimsulfuron degradation depending on the tillage method used. Accelerated

Test plant – *Sinapis alba*

1 = Control
2 = foramsulfuron + iodosulfuron (45 g/ha + 1.5 g/ha)
3 = nicosulfuron (60 g/ha)
4 = rimsulfuron (12 g/ha)
5 = rimsulfuron (15 g/ha)
6 = tifensulfuron (11.5 g/ha)
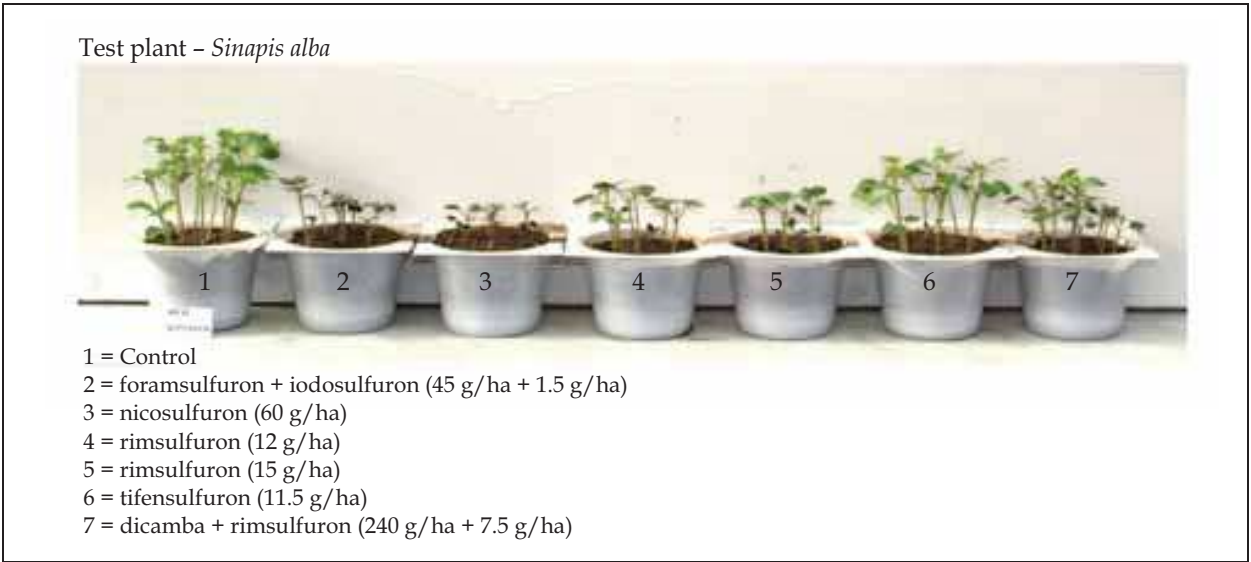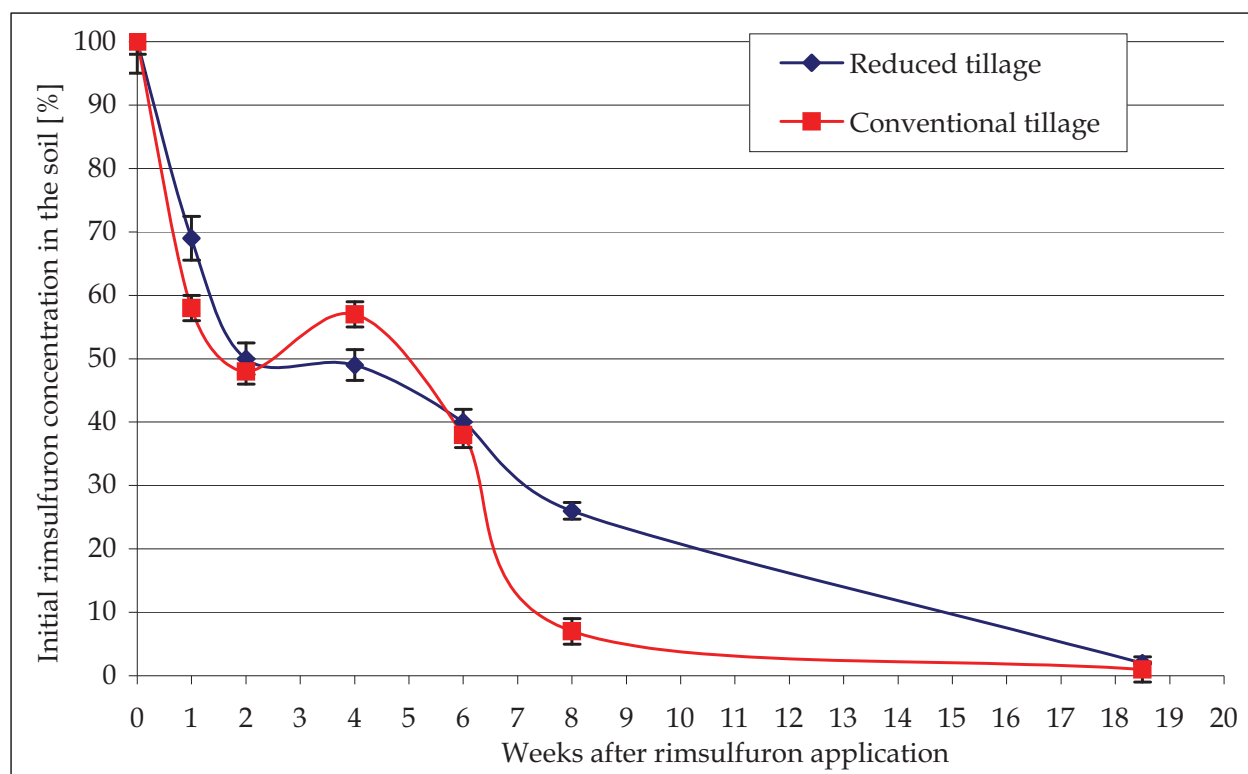7 = dicamba + rimsulfuron (240 g/ha + 7.5 g/ha)

Fig. 9. Degradation of different active ingredients of herbicides in the 0-20 cm soil layer (biotest method)

Source: Sekutowski, (2009)

Fig. 10. Rimsulfuron degradation rates in the 0-20 cm soil layer (mean in the years 2005-2007)

translocation and dynamics of degradation was found as late as 7 weeks after application and it was markedly diversified depending on the tillage systems (Fig. 10).

The presented examples of the application of plants as phytodetectors in the bioassay method more precisely illustrate the phytotoxic action of herbicide active ingredients (even those found in trace amounts) for agrophytocenosis than their concentration in soil determined using chemical analyses. A similar opinion was presented by Hollaway et al. (1999), who in their studies concerning the detection of sulfonylurea herbicide residue in soil using three methods, i.e. bioassay, ELISA and HPLC, stated that a bioassay using *Pisum sativum* and *Lens culinaris* plants as bioindicators was most sensitive. Biotests detected residue of sulfonylurea herbicides at 0.1 – 1.0 mg/ha soil, ELISA at 0.1 – 10 mg/ha, while HPLC at 3 – 10 mg/ha, respectively.

Depending on soil and climatic conditions only a portion of residue contained in soil is available to plants. Biotests used in biomonitoring make it possible to evaluate whether this part of residue may exhibit phytotoxicity towards agrophytocenosis.

The presented examples of conventional phytotests using different plants and their seeds as phytodetectors, conducted according to standardized national procedures, frequently happen to be complicated, they require considerable laboratory space and are time-consuming (BN-83 9180-25, 1983; BN-83 9180-27, 1983; BN-84 9180-30, 1984; PN-ISO 17616, 2010). For several years now ready-to-use tests (toxkits) have been commercially available, sold in the form of packages, allowing the evaluation of phytotoxicity of tested samples within a short time (1-3 days). They contain cryptobiotic forms of bioindicators (e.g. seeds of plants – Phytotoxkit™), coming from standard breeding, which may be stored for 6 months and when needed prepared for the test within a very brief time (Phytotoxkit, 2004).

4.3 Phytotoxkit microbiotest

The necessity to conduct analyses of many soil samples within a relatively short time has led to the introduction of miniature phytotoxicity tests, called microbiotests or second generation tests, as alternatives for conventional phytotests. An example of such a microbiotest is a rapid (72 h) test - Phytotoxkit™ (Phytotoxkit, 2004). Professor Guido Persoone (with a team of co-workers) from the University of Ghent in Belgium was the creator of the toxkit tests (Persoone, 2005). The principle of such a phytotest is based on germinating seeds of *Sorghum saccharatum, Lepidium sativum* and *Sinapis alba,* which as a result of contact with the tested herbicide active ingredient found in soil exhibit a specific reaction (a lack of germination or reduced root length). The use of standard seeds facilitates test standardization and maintenance of reproducible results irrespective of the laboratory, at which analyses are being conducted. The specific nature of Phytotoxkit™ results in the omission of all labor-consuming activities connected with conventional biotests, thus considerably reducing the time required to obtain the reading (from 14 to 3-5 days). Moreover, this test makes it possible to obtain a direct measurement of root length using image tools, thanks to which a graphic presentation of the dependence between root length reduction in phytodetectors and the phytotoxic concentration of tested herbicide active ingredients is faster and much easier in comparison to a conventional biotest. This test makes it also possible to more comprehensively estimate the phytotoxic effect of herbicide residue not only on the soil environment, but also on the entire agrophytocenosis. An example of a Phytotoxkit™ tst conducted using a standard set of plants is presented in Fig. 11.

The diverse chemical character of herbicides prevents the use of only one type of a Phytotoxkit™ containing standard phytodetectors supplied in the kit. Due to the specific response of different plant species to the presence of herbicide active ingredients belonging to different chemical groups it is necessary to supplement knowledge on the applicability of other plants. Thus the test is very often modified, which consists in the replacement of standard test plants with other plant species, such as e.g. *Helianthus annuus, Cucumis sativus* or *Fagopyrum esculentum.* Thanks to the modification of Phytotoxkit™ it was possible to extend the collection of plants potentially applicable in the determination of herbicide residue, e.g. derivatives of benzoic acid, phenoxy acids and sulfonylourea (Fig. 12).

Similarly as in case of conventional biotests, Phytotoxkit™ may be used in the determination of values of ED_{50} and IC_{50} and the determination of the level of residue, rates of degradation and translocation of herbicide active ingredients in soil. An example in this respect may be an experiment conducted using a modified Phytotoxkit™ under laboratory conditions, consisting in the determination of ED_{10} and ED_{50} for dicamba. The run biotest

| Test containers - Phytotoxkit™ | *Sorghum saccharatum* | *Sinapis alba* |

Fig. 11. Phytotoxkit™ with standard test plants.

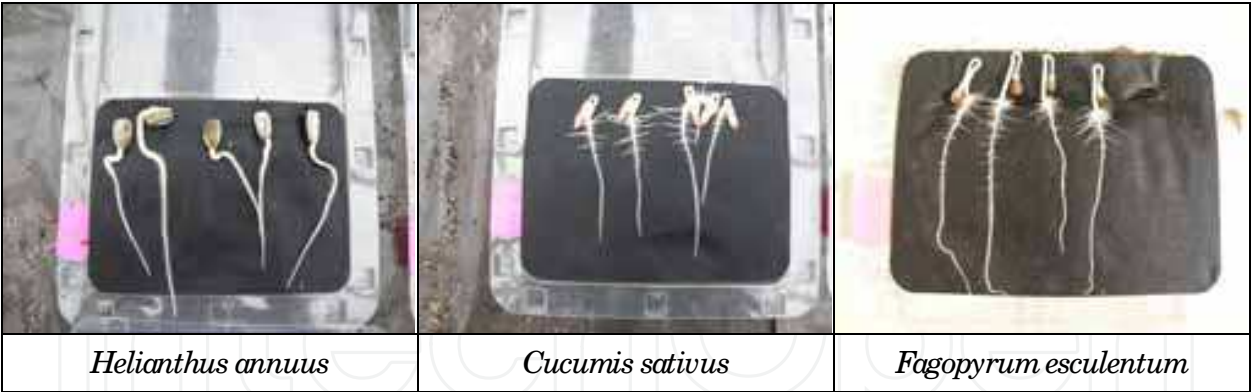| *Helianthus annuus* | *Cucumis sativus* | *Fagopyrum esculentum* |

Fig. 12. Phytotoxkit™ with alternative test plants.

showed that significant differences in the reduction of root length in *Fagopyrum esculentum* and *Cucumis sativus* were obtained for concentrations ranging from 0.025 mg/kg to 0.25 mg/kg. The strongest response to the tested substance was recorded for *Fagopyrum esculentum,* while it was weakest in case of *Sinapis alba*. The detoxication capacity in relation to dicamba in *Fagopyrum esculentum* (ED_{50}) was eliminated already at a concentration of 0.125 mg/kg, while a further increase in the concentration of the tested substance in soil (1.2 mg/kg) resulted in root length reduction by 99%. In turn, ED_{50} for the other two species, i.e. *Cucumis sativus* and *Sinapis alba* fell within the range of 0.25 – 0.5 mg/kg soil (Fig. 13).
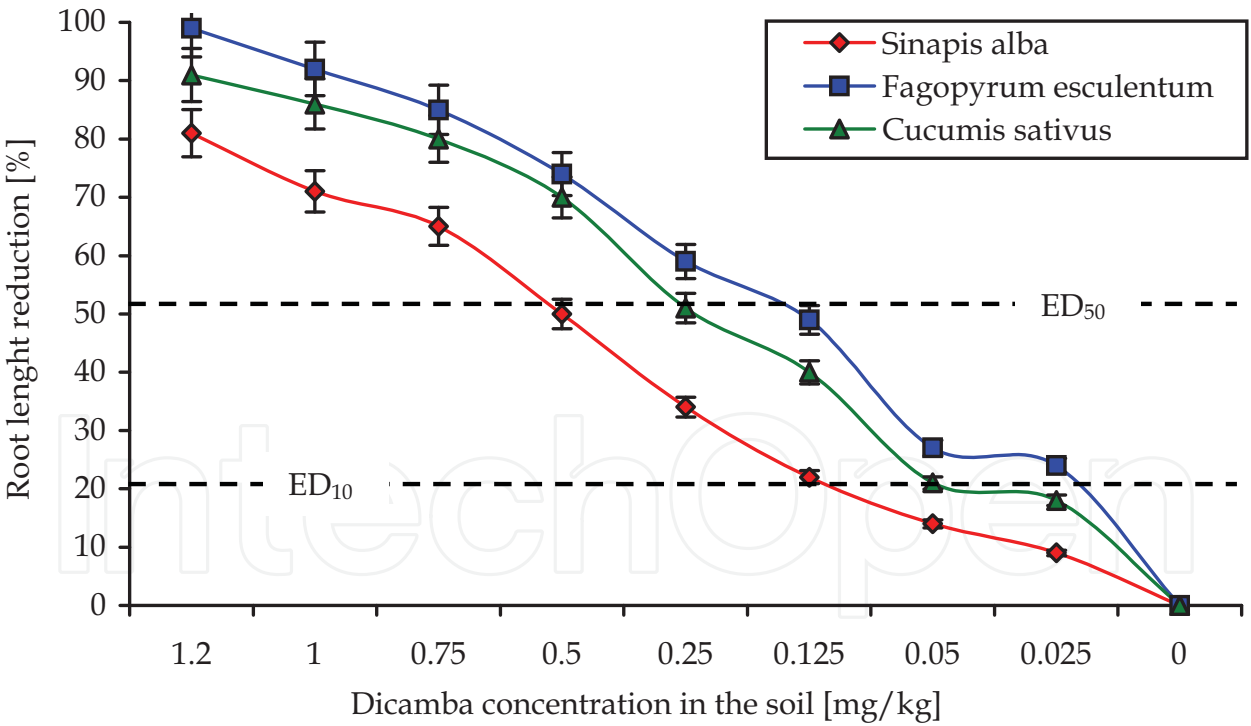
Source: Sekutowski & Sadowski, (2009)

Fig. 13. The effect of dicamba on root length reduction in tested plant

The above example very well shows the response (sensitivity) of the phytodetector to the tested active ingredient of the herbicide. In analyses using plants as detectors, it is crucial to select an appropriate plant for the tested herbicide active ingredient. A sufficiently sensitive plant detector makes it possible to conduct tests on microresidue of 0.01 mg/kg soil.

4.4 Sets of biotests (batteries)

The selection of an appropriate biotest in studies on agrophytocenosis depends on the type of required information, the concentration of herbicide active ingredient residue in the analyzed sample of soil (water), as well as the species-specific sensitivity of the tested plant. In case of the use of only one phytodetector species the estimated phytotoxicity reflects the sensitivity of only this one tested species. Such a procedure may result in an error connected with an underestimation of phytotoxicity of the analyzed herbicide active ingredient in relation to the entire agrophytocenosis. This risk may be minimized thanks to the application of a battery of biotests, which action is based on the use of plant species of different sensitivities to active ingredients of herbicides belonging to one chemical group. Batteries of tests may be formed within one test (e.g. Phytotoxkit™), which may include several species of test plants exhibiting different sensitivity to a given chemical group. Moreover, sets of batteries may be established within several tests using different biodetectors of varying sensitivity to the same chemical group, e.g. Phytotoxkit™ → ELISA → HPLC (Hollaway et al., 1999).

5. Conclusion

Bioassays are methods commonly applied in ecotoxicology in the determination of the levels of bioavailable phytotoxic residue of herbicide active ingredients in soil. Tests with the use of rapidly germinating seeds have several very important advantages, as they are cheap and easy to perform, they do not require expensive laboratory equipment and they yield reproducible results. The phytotoxic effect of herbicide active ingredient may be stated on the basis of the dynamics of germination, seedling growth, reduction of dry or fresh weight of roots or aboveground parts (stems, leaves) of test plants. On the basis of selected parameters, such as the reduction of root length, the toxic effect of herbicide active ingredients may be determined already after approx. 24 h, while the dynamics of root growth - after 3-5 days from the onset of the test (Phytotoxkit™). In turn, the reduction in fresh or dry weight of aboveground parts of plants may be established after approx. 10-14 days (a conventional biotest).

Unfortunately, drawbacks of such a method include first of all the fact that it is impossible to identify the tested active ingredient. This problem may be solved by using different biological factors forming a set of biotests (Phytotoxkit™ → ELISA → HPLC), which will make it possible to precisely determine the herbicide active ingredient. It also needs to be stressed that biotests with the application of rapidly germinating seeds of selected plant species may be a good supplementation or even an alternative to classical instrumental measurements, used in the detection of phytotoxic residue of herbicide active ingredients in soil.

Probably the scope of bioassay application within the next few years will be increasing and thus collected information will constitute the basis for the initiation of analyses using classical analytical methods.

6. Acknowledgement

7. References

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Herbicides and Environment

Edited by Dr Andreas Kortekamp

Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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