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Herbicide Phytotoxicity and Resistance to Herbicides in Legume Plants

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1. Introduction

Active substances in herbicides, just like in other pesticides, are chemical compounds synthesized in order to kill organisms which are harmful for cultivated plants. Therefore, they are toxins introduced on purpose by man into the environment. From the perspective of environmental protection, it is very significant that herbicides are most often applied directly into the soil to manage weeds. Since DDT and chloro-organic herbicides such as 2,4,5-T were withdrawn (in the 1970s) and since the EU regulations were unified for all its member countries, plant protection techniques have advanced considerably. Yet, pesticides, thus herbicides as well, continue to be a big group of xenobiotics periodically occurring at high levels in agroecosystems. These compounds infiltrate into related biocenoses from air, soil, water and food (Allinson & Morita, 1995; Kolpin et al., 1998; Adomas at al., 2008). Soil may become a reservoir of various pollutants, including herbicides. Herbicides remain active in soil for different periods. Paraquat has a relatively long half-life in soil (estimated at about 1000 days). The half-life of glyphosate in soil is only 10 to 100 days, and according to Monsanto the average half-life of this herbicide is 32 days (Hornsby et al., 1996; Monsanto, 2005). Remainders of persistent herbicides (e.g. atrazine, metribusin, and trifluralin) can stay in soil and destroy subsequent plantations a year or more after herbicides had been used. Herbicides from soil leach into surface water and ground water. The assessment of herbicides content in the aquifers in Iowa shows that 75% of herbicides (Kolpin et al., 1998), despite degradation, are still detected. From soil, water or air, herbicides get into crops (Adomas et al., 2008). When pesticides are applied, acceptable remainders of active substances (MRL) can often be detected in cultivated plants. Depending on physicochemical properties of the active substances of pesticides and the ways of their detoxification, some of these pollutants tend to increase concentration while passing through organisms of higher trophic levels. It can lead to a significant bioaccumulation of toxins in the food chains (Allinson & Morita, 1995; Dinis-Oliveira et al., 2006). No doubt therefore, monitoring of herbicide (including desiccant) residues in cultivated plants is needed, so that people and environment can be safe. Moreover, application of herbicide desiccants modifies physiological properties of seeds and may thus lead to delayed problems, becoming evident long after the treatment.

2. Physiological changes after preharvest desiccation of plants

Preharvest application of herbicides (desiccants, e.g. glufosinate ammonium (Basta 200 SL) or diquat (Reglone Turbo 200 SL)), is aimed at removing excessive water from plant cells and balancing maturation. Dehydration is best tolerated by plant reproductive organs/cells: seeds, pollens, spores, aestive buds and somatic embryos. In the developmental cycle, the majority of seeds go through the stage of natural, physiological drying, yet not all the seeds dry. Based on their tolerance to desiccation, seeds have been divided into three groups: orthodox (which can be chemically desiccated, Fig. 1), recalcitrant and intermediate (Piotrowicz-Cieślak et al., 2007). Orthodox seeds tolerate dehydration relatively well and may retain their viability for a long time (Murthy et al., 2003).

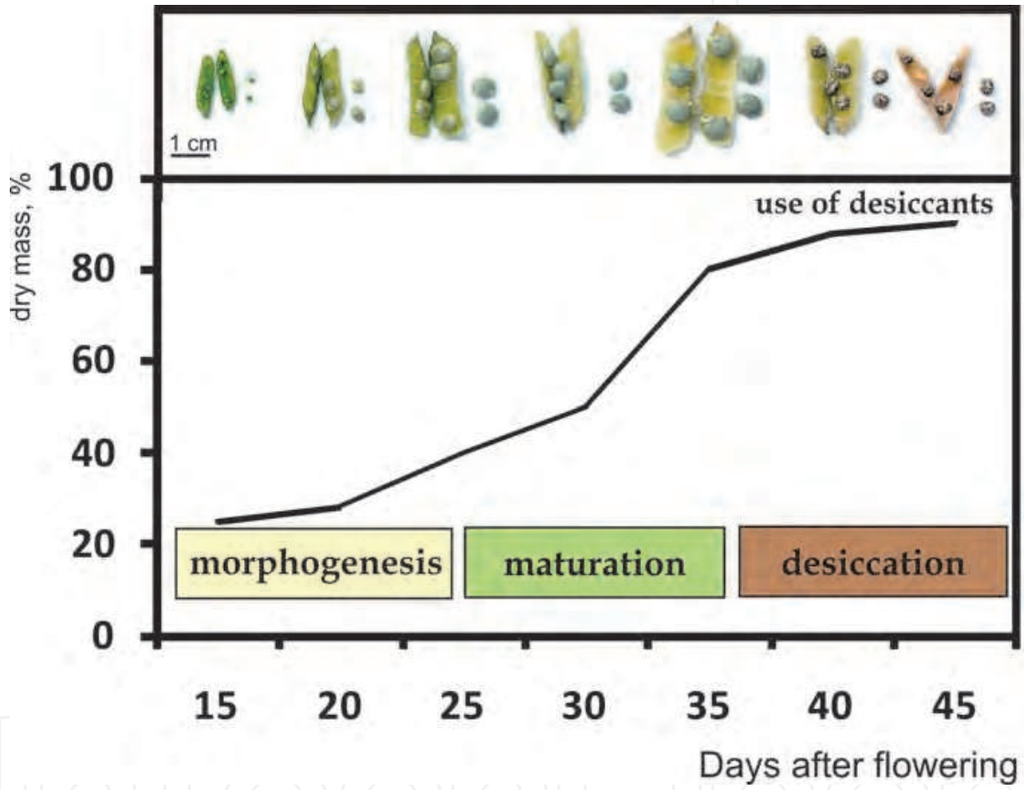

Fig. 1. Basic stages of seed ontogenesis and dry mass accumulation in yellow lupin seeds. Chemical desiccation is carried out during natural dehydration of seeds.

They are resistant to water loss below 5% of the initial water content. During desiccation, orthodox seeds can lose over 90% of water, and as a result slow down their metabolism and become dormant (Adams & Rinne, 1980). Seeds of this type are produced by most monocots and dicots of the temperate climate zone (Walters & Towill, 2004). Seed desiccation is a prerequisite for completion of their life cycle and it can be chemically forced. Recalcitrant seeds perish as a result of drying below a critical level of water, which is usually quite high and amounts to 40% (Roberts, 1973). Desiccation cannot be applied in this group of plants which includes tropical trees, e.g. avocado, mango, litchi, cocoa (Farrant et al., 1993) and some grasses (Probert & Longley, 1989). The third group – i.e. intermediate (suborthodox) seeds are tolerant to dehydration if it stops at seed water level 6-10% (Ellis et al., 1991). It

seems that chemical desiccation can be successfully applied in this group, however mild (with small dessicant doses). Moreover, intermediate seeds do not tolerate cold while being dehydrated (Hong & Ellis, 1996). Chemical desiccation of plants is performed during the period when orthodox seeds start their natural drying. Yet, precise determination of this period depends on the species, the pace of water loss and its expected final content. Many processes contribute to preservation of seed vigour and viability during both natural and accelerated (chemical) drying.

2.1 Biochemical changes in seeds resulting from desiccant application

The rate of water loss during seed desiccation has a profound effect on seed condition at cellular and subcellular levels. Organelles in the cytoplasm become compacted together, cytoplasm viscosity increases and a number of degenerative reactions intensify. In order for the cell to protect itself against dehydration, it has to accumulate protective compounds. A wide group of such substances was detected in seeds, for example proline, glutamine, fructans, poliols, trehalose, carnitine and others. A lot of plants respond to desiccation caused dehydration by accumulating so-called 'compatible solvents' (i.e. molecules not interfering with the structure and functions of the cell), including one kind or more of the above given substances. Unfortunately, concentration of these hydrophilic compounds is often not sufficient to bind water properly (Hare et al., 1998). Yet, probably it is one of the most important mechanisms protecting macromolecules under conditions of limited water loss. Molecules of compatible compounds form a protective layer around proteins, thus preventing protein deformations (Crowe et al., 1990). During desiccation, changes occur at all levels of plant cell functions. Maintaining the integrity of genetic material and keeping the DNA repair mechanisms functional during dehydration are the most important for seed survival. As a result of water loss during seeds desiccation the genetic material undergoes conformational changes, depending on its nucleotide sequence and interactions with specific DNA-binding proteins (Osborne & Boubriak, 1994). A higher stability of seed DNA structure probably indicates increased seed tolerance to desiccation. At the early stages of seed ontogenesis, desiccation leads to frequent chemical modifications of DNA bases, resulting in a modified DNA methylation level. The later chemical desiccation is applied in the ontogenetic development of seeds, the fewer epigenetic changes are thus caused.

2.1.1 Soluble carbohydrates and their derivatives content in seeds

Yellow lupin plants (*Lupinus luteus* L. cv. Taper and Mister) were grown in 10-L pots in greenhouse (Fig. 2) with a 12-h photoperiod at 20^{o}C day/18^{o}C night and 140 µmoles photons m^{-2} s^{-1} irradiace. Mixture of peat, garden soil and sand (1:1:1, v:v:v) was used as substrate for plant growth. On the day of flowering the plants were labelled and divided into three groups: a control and two treatments. Basta 200 SL (producer Bayer Poland, active substance (a.i.) glufosinate ammonium) was applied in the amount of 4,1 µg a.i. per pot (the preparation was diluted in 15 ml of distilled water). Roundup Ultra 360 SL (producer Monsanto Poland, a.i. glyphosate) was applied in the amount of 4,1 µg a.i. per pot (here the preparation was diluted in 15 ml of distilled water too). Herbicide levels applied in this experiment corresponded to the field doses 2.5 and 3 L/ha, respectively.

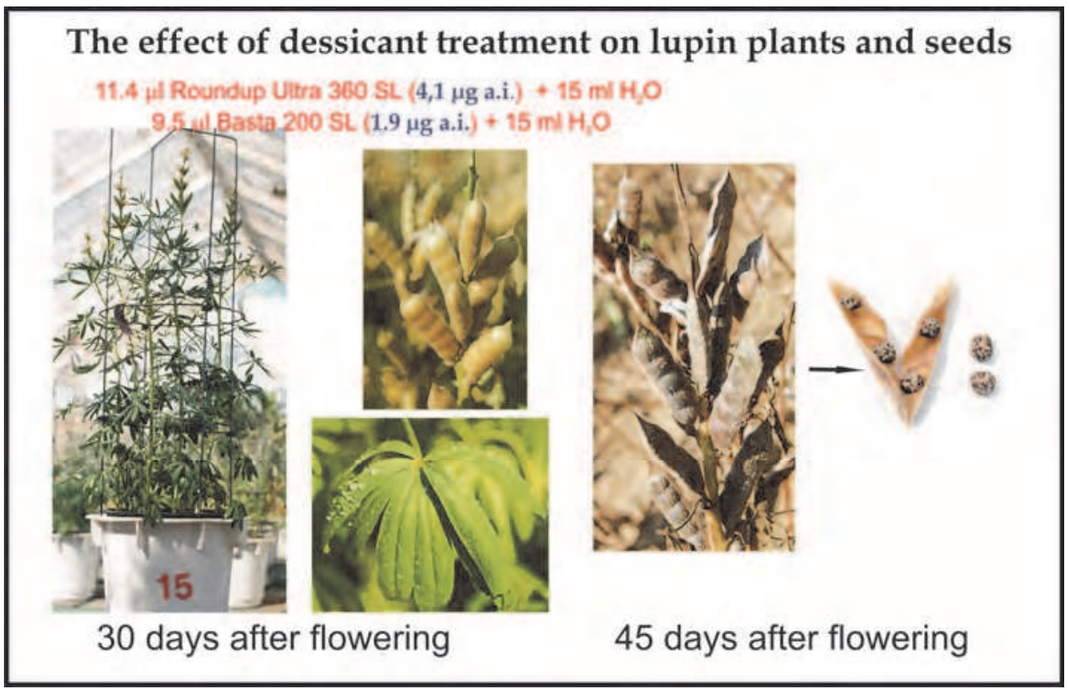

Fig. 2. Lupin plants and seeds during and two weeks after desiccation treatment.

Seeds were collected in five-day intervals, starting from 15 days after flowering (DAF) until full physiological seeds maturity. Soluble carbohydrates content in seeds were analysed by GC chromatography according to Piotrowicz-Cieślak (2005). Seeds (30 – 60 mg fresh mass) were homogenised in ethanol: water, 1:1 (v/v) containing 300 µg phenyl-α-D-glucose as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated at 75°C for 30 min to inactivate endogenous enzymes and centrifuged at 15 000 g for 20 min. The supernatant was passed through a 10 000 MW cut-off filter (Lida, Kenosha, WI USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness (Eppendorf Concentrator 5301). Dry residues were derived with 300 µl of silylation mixture (trimethylsilylimidazole : pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 70°C for 30 min, and then cooled at room temperature. One µl carbohydrate extract was injected into a split-mode injector of a Thermo Scientific gas chromatograph equipped with flame ionisation detector. Soluble carbohydrates were analysed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific). Soluble carbohydrates were identified with internal standards as available, and concentrations were calculated from the ratios of peak area, for each analysed carbohydrate, to the peak area of respective internal standard. Quantities of soluble carbohydrates were expressed as mean ± SD for 3-5 replications of each treatment.

2.1.2 The role and content of soluble carbohydrates in seed drying

The composition of carbohydrates, with particular regard to content of raffinose family oligosaccharides, has been measured in seeds of many species. Raffinose content exceeds 1 % d.m. in seeds of bean, soybean and pea. The high content of stachyose (over 4 % d.m.) is found in vetch and soybean seeds, whereas verbascose content over 2 % d.m. was measured in bean and pea (Horbowicz & Obendorf, 1994). Soluble carbohydrates content in lupin

seeds varies from 6 to 15.6 % d.m. (Piotrowicz-Cieślak et al., 1999; Piotrowicz-Cieślak, 2005). Raffinose family oligosaccharides (RFO) content in the overall content of soluble carbohydrates in lupin seeds amounts to 46 to 76 %, with the prevalence of stachyose. A common characteristic of lupin seeds is that the RFO content in embryonic axes is two times higher than in cotyledons.

This feature, connected with higher metabolic activity of embryonic axes than cotyledons, was also observed in all other legume seeds studied (Horbowicz & Obendorf, 1994; Górecki et al., 1997). Oligosaccharide contents in seeds depend on the rate of seed maturation (Piotrowicz-Cieślak et al., 2003). In maturing seeds of lupin, Taper and Mister varieties, accumulation of fresh and dry mass was at first slow, and then it rapidly increased between the 25th and 35th day after flowering. The first symptoms of seed viability were observed on the 20th day after flowering. In the period of natural seed desiccation, the seed mass increased rapidly. In both lupin varieties, dry mass rapidly increased after applying desiccants, which resulted in an decrease in fresh mass. Quick desiccation also increased the electrical conductivity of seed exudates. Yet, such accelerated maturation does not have any significant impact on seed germination (Fig. 3). A properly conducted desiccation is of key importance for the maturing seeds to acquire vigour (Sanhewe & Ellis, 1996). Both these phenomena are dependent on biosynthesis of specific stress-related proteins, induced by ABA (Late Embryogenesis Proteins, Blackman et al., 1992) and accumulation of a considerable level of soluble carbohydrates in seeds. The assessment of soluble carbohydrates accumulation has been carried out in maturing seeds of *L. luteus* cv. Taper and Mister. The length of maturation period, from flowering to full maturity of seeds, was 45 days. Seeds accumulated monosugars (glucose, fructose, galactose) sucrose, cyclitols, raffinose family oligosaccharides and galactosyl cyclitols.

In the initial phase of seeds development (15-20 days after flowering) the monosugars content (fructose, glucose and galactose) was high (Fig. 4) and gradually growing, reaching the maximum on the 30th day after flowering (DAF). At full seed maturity monosugars were present in negligible amounts. Upon chemical drying the content of monosugars decreased rapidly, particularly in the first five days after desiccants application. The rate of the decrease in monosugars content was similar when Basta and Roundup were applied (Fig. 4).

The chemical drying of seeds facilitated increase of cyclitols content, particularly *myo*-inositol. The content of sucrose in the initial phase of seeds development was high. At the beginning of desiccation the sucrose level decreased, reaching the minimum at full physiological seed maturity (45 DAF).

Chemical drying induced sucrose synthesis in seeds (Fig. 5). Among galactosyl cyclitols: galactinol, ciceritol and trigalactopinitol A, galactopinitol B and digalacto-*myo*-inositol were present in the highest amounts. In seeds which were not chemically dried the content of galactinol increased to reach the maximum at 30 DAF. Its maximum concentration in seeds desiccated with Basta and Roundup dropped five days after treatment. In the course of seeds drying up, the content of galactinol decresed, reaching the minimum level in the naturally growing, fully mature seeds. In the chemically dried seeds the content of galactinol was higher; also, more intense synthesis of ciceritol and trigalactopinitol A was found after chemical drying of seeds (Fig. 5).

The dominant reserve carbohydrates in lupin seeds were raffinose family oligosaccharides (Fig. 6). The level of these metabolites gradually increased in the course of seeds desiccation,

with particular intensity in chemically dried seeds. Stachyose was the member of raffinose family oligosaccharides that occurred in the highest concentration. In mature, non-desiccated seeds its level was 43.17 mg/g d.m. In the chemically dried seeds, after the application of both Basta and Roundup, the level of stachyose was higher and persisted until the end of the physiological maturity of seeds (Fig. 6).

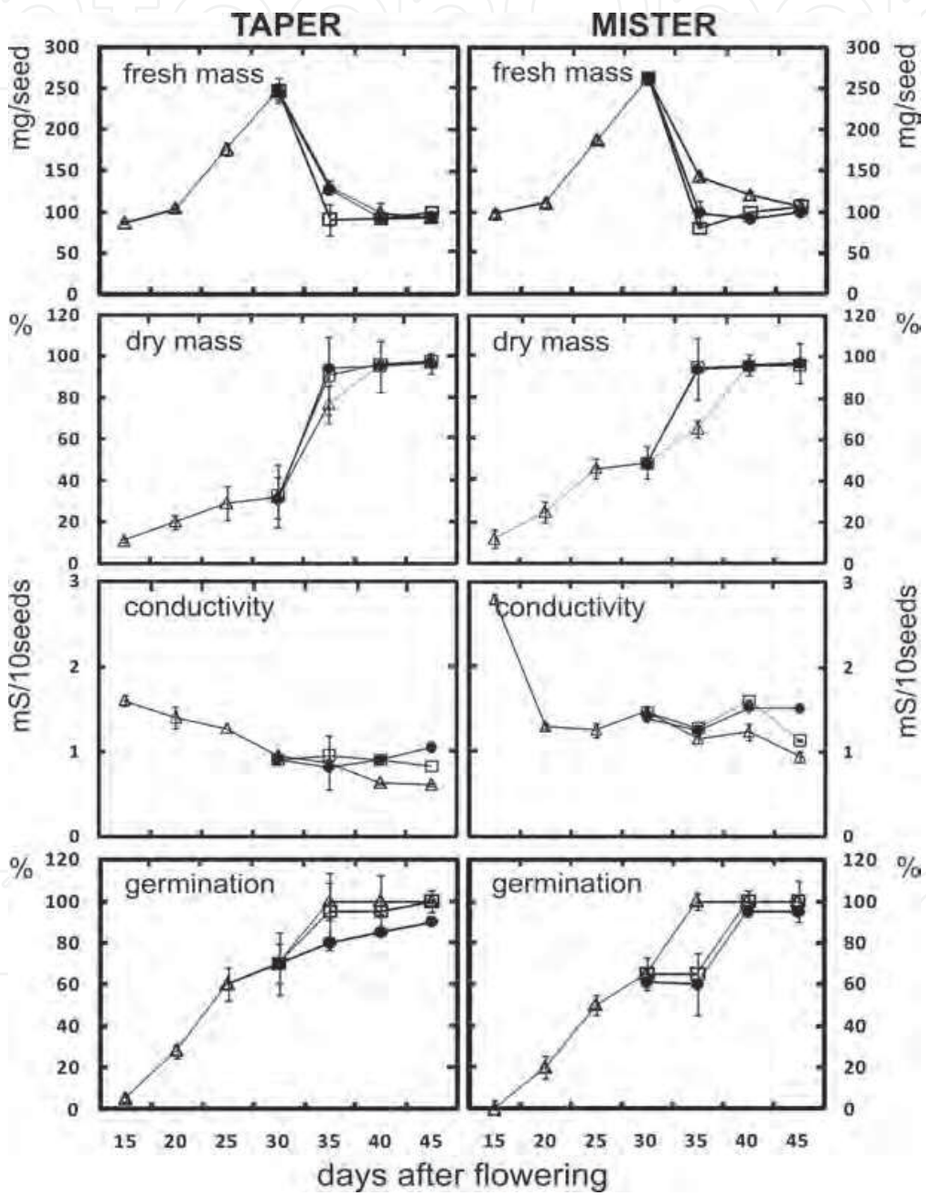

Fig. 3. Seed dry and fresh mass, conductivity and germination during maturation and after application of herbicides: Basta 200 SL and Roundup Ultra 360 SL. Data points represented the mean ± SD for fifteen replicate samples – control (Δ), Basta treatments (□), Roundup treatments (●).

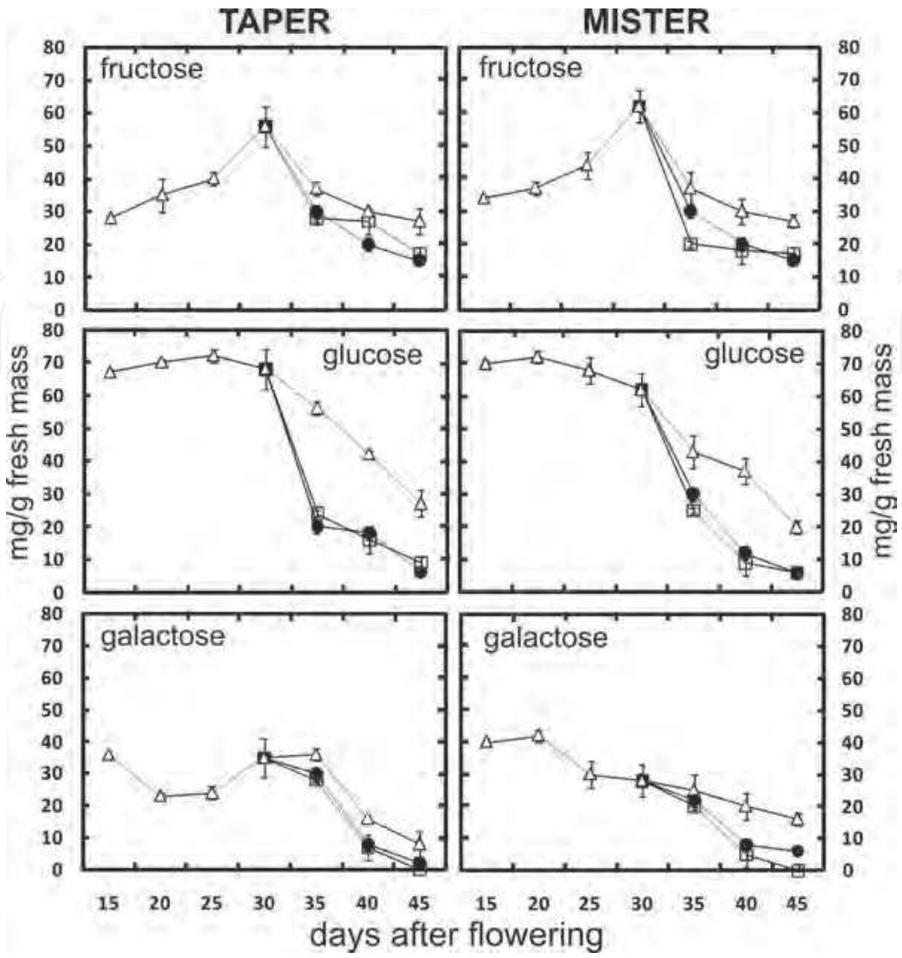

Fig. 4. Monosugars content in lupin seeds during maturation and after application of herbicides: Basta 200 SL and Roundup Ultra 360 SL. Data points represented the mean ± SD for fifteen replicate samples – control (Δ), Basta treatments (□), Roundup treatments (●).

The authors of the papers published so far on the accumulation of RFO in seeds come to unequivocal conclusion that the drying induces the accumulation of RFO in embryonic tissues. The increases in the RFO contents are paralleled by increasing seed resistance to desiccation (Obendorf, 1997). The protective action of RFOs results from their role in inducing the cytoplasm vitrification and stabilization of membranes and macromolecules in dehydrated cells (Bernal-Lugo &Leopold, 1985). In the course of seed drying (chemical or natural) accumulation of osmoprotective substances takes place, such as proline, betaine, oligosaccharides including RFO, cyclitols and their derivatives (Carpenter & Crowe, 1989; Ramanjulu & Bartels, 2002; Piotrowicz-Cieślak et al., 2007). Our research demonstrates that the dominant soluble carbohydrates in maturing embryos of *L. luteus* were raffinose family oligosaccharides and galactosyl cyclitols.

Seeds accumulated significant amounts of stachyose, verbascose, trigalactopinitol A and B. Soluble carbohydrates characterised by high molecular weight (stachyose, verbascose) belong to the main osmoprotectors in seeds of lupin. They also contain several hydroxyl groups (seven in the case of verbascose). Polyhydroxy compounds can substitute for water

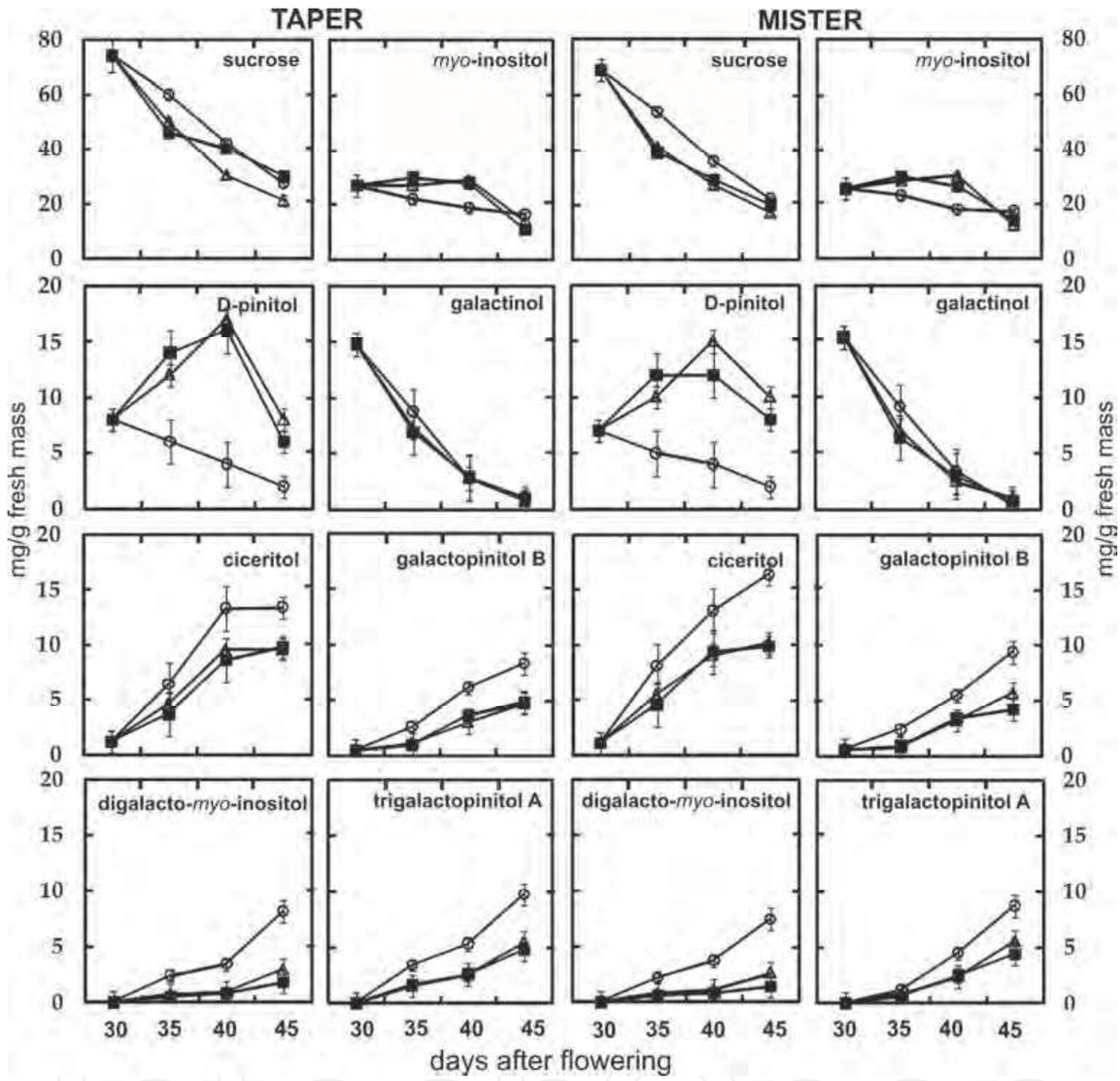

Fig. 5. Soluble carbohydrates content [mg/g fresh mass] in lupin seeds during maturation and after application of herbicides: Basta 200 SL and Roundup Ultra 360 SL. Data points represented the mean ± SD for ten replicate samples – control (o), Basta treatments (Δ), Roundup treatments (■).

in stabilizing membrane structure in the dry state. Trehalose is indicated as the optimal osmoprotector; it contains 8 hydroxyl groups and has molecular weight 2.5 times lower (Crowe & Crowe, 1984) than verbascose. Lupin seeds do not accumulate trehalose, but similar functions can be performed in them by other soluble carbohydrates. Bianchi et al. (1993) point out that tissues should contain from 10 to 15 % of sucrose or 5g sucrose for every gram of lipid to tolerate the drying well. Lupin embryos contain less than 3 % of sucrose, but they tolerate chemical drying relatively well. Therefore, it is the total content of all soluble carbohydrates, including sucrose, that likely becomes significant. Chemical drying of seeds enhances the increase of soluble carbohydrates content in lupin embryos –

raffinose family oligosaccharides and, to a smaller extent, galactosyl cyclitols. Thus, galactosyl cyclitols are probably important osmoprotective agents in lupin seeds.

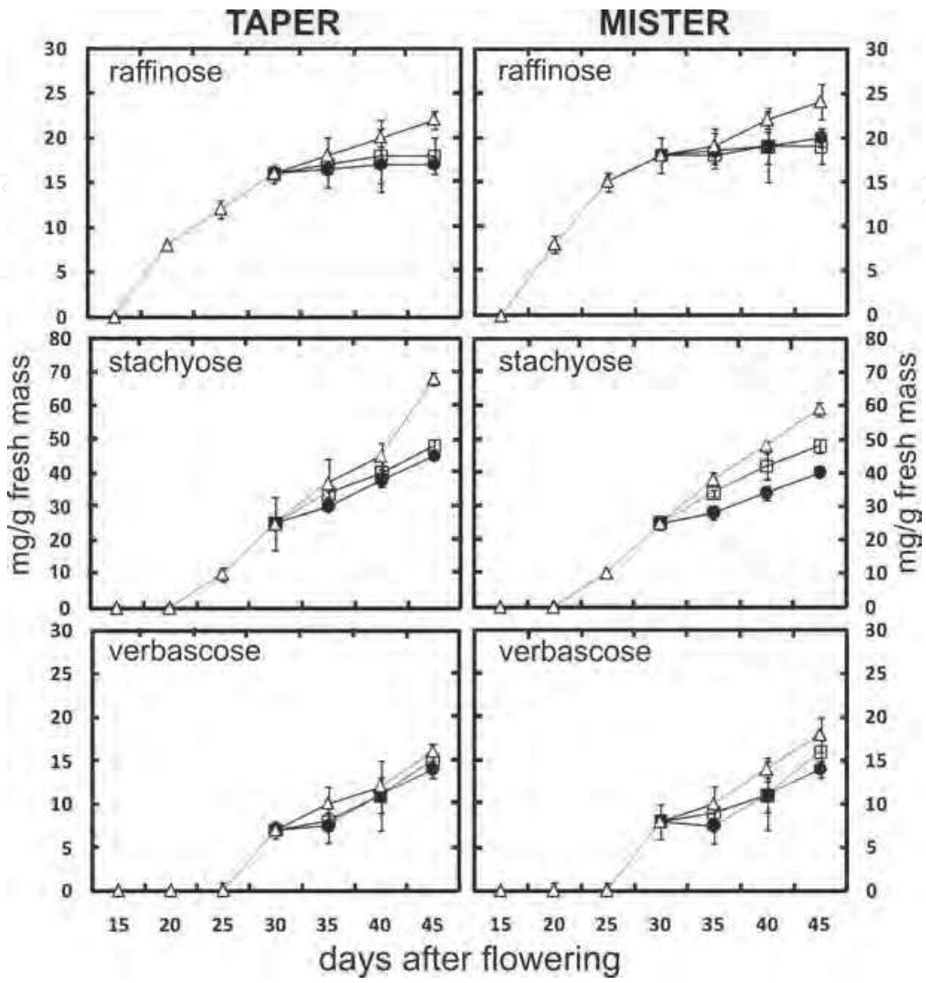

Fig. 6. Raffinose family oligosaccharides content in lupin seeds during maturation and after application of herbicides: Basta 200 SL and Roundup Ultra 360 SL. Data points represented the mean ± SD for fifteen replicate samples – control (Δ), Basta treatments (□), Roundup treatments (●).

This hypothesis is based on biochemical and physiological characteristics of galactosyl cyclitols, similar to those of RFO (Obendorf, 1997). It was shown that in buckwheat seeds galactosyl cyclitols indeed contribute to the development of seed resistance to desiccation (Horbowicz et al., 1998). These compounds are formed by attaching one or more galactosyl radicals to cyclitol (most commonly inositol). Galactose is attached to cyclitol by α-(1→2) or α-(1→3) bindings. Galactosyl cyclitols series based on the form of cyclitol which is an acceptor or galactosyl radical. So far the following structures of galactosyl cyclitols have been discovered and defined: galactinol, galactopinitol, fagopyritol, galactoononitol. The accumulation of galactosyl cyclitols was observed in seeds of different plants (Piotrowicz-Cieślak, 2004). Particularly rich in galactosyl cyclitols are the seeds of buckwheat, castor oil plant and lentil (Horbowicz & Obendorf, 1994). Lupin seeds accumulate up to 2 % d.s. of

galactosyl cyclitols (Piotrowicz-Cieślak et al., 2003). Under desiccation seeds of legume plants (soybean, yellow lupin) accumulate mainly RFO, despite the fact that during the natural drying these seeds also form galacto-D-pinitol and D-*chiro*-inositol (Górecki et al., 1997). Water stress, resulting from soil drought, low or high temperature, may have a significant influence on prompting the biosynthesis of galactinol and RFO in vegetative tissues and maturing seeds. In leguminous plants exposed to water stress an intense accumulation of α-D-galactosides is found (Streeter et al., 2001). Water stress resulting from soil drought and chill induces the accumulation of galactinol and raffinose also in vegetative tissues of alfalfa and *Arabidopsis* (Taji et al., 2002; Cunningham et al., 2003; Zuther et al., 2004) and tomato germinating seeds (Downie et al, 2003). RFO level depends on the activity of galactinol synthase. The activity of this enzyme is related to temperature (Panikulangara et al., 2004) and RFO total amount in leaves and seeds (Castillo et al, 1990). In the genome of *Arabidopsis* seven genes have been identified as responsible for the activity of galactinol synthase. Two of them are activated by drought and salinity, whereas one is activated by low temperature (Taji et al, 2002). The level of stachyose and verbascose depends on the level of initial substrates, including *myo*-inositol (Hitz et al., 2002) and sucrose. Herbicides modify the content of soluble carbohydrates in seeds and remain in soil after having been applied. Toxicological tests are a simple, inexpensive, and quick method to assess their impact on subsequent plants.

3. Toxicological tests in the environmental assessment

A wide range of analytical approaches are used to asses the effect of contaminants on environment at all levels of its complexity - from studying the biochemical changes in single cells to changes measured on ecosystem levels. The holistic, system approach so characteristic of modern science, combining efforts of experts in many fields, increasingly becomes applied also in environmental toxicology studies. Yet, it is not possible to precisely characterize all possible pollutants due to their big number, different concentrations, molecular weights, or high reactivity of widely spread low-molecular-weight substances (Kahru et al., 2008). The key task of toxicology and ecotoxicology is a direct evaluation of risks resulting from environmental contamination and refers to, among other issues, creating classification systems based on increasing levels of toxicity (Persoone et al., 2003). Until recently, physical-chemical methods were considered the basic way to diagnose the condition of environment and its specific elements. Although these analyses greatly facilitate elimination of some toxic substances (Wolska et al., 2007), they do not fully characterize the biological activity of any substance on affected organisms. They only inform us about the level of contamination, but do not predict its biological consequences. The precise analytical methodologies are mostly worked out for those compounds only which are subject to strict legal regulations. Moreover, such chemical analyses sometimes blur the real environmental threats (Manusadžianas et al., 2003; Persoone et al., 2003; Wolska et al., 2007). A valuable alternative supplementing this purely chemical approach was worked out, based on the following principle: measure the exposure and analyze the accumulation and metabolism of contaminant in living organisms. Relatively inexpensive and biologically founded biotests are being developed for this purpose.

A biotest is a standard procedure indicating the impact of contamination or their mixture on a biological system, i.e. a plant or animal organism, a system of organisms or a fragment of

an ecosystem (a mesosystem, a microsystem), at the same time defining make-up of substances and their possible interactions (Kratasyuk et al., 2001; Wang et al., 2003; Persoone et al., 2003). Biotests show an interdisciplinary approach to organisms' response to the ocurrence of particular chemical substances. In order to determine the impact of these chemical substances, biotests employ the methods of physiology and biochemistry. The obtained results unambiguously determine the toxicity of a given sample or lack of its toxicity, as compared to the response of an organism not exposed to the activity of all chemical substances present in the analysed sample (Simeonov et al., 2007). Methods used in toxicology and ecotoxicology based on morphological and/or physiological disturbances at the cell level or organism level, and sometimes as a consequence death of a given organism, aim at protecting the environment, which indirectly results in protecting the life and health of human population (Celik et al., 1996; Dinis-Oliveira et al., 2006). The current form and shape of these tests and recommendations for their proper usage are a result of more than twenty years of research. In consequence, conditions of conducting such tests became standardized, costs related to their application decreased, the test cultures became widely available (Kaza et al., 2007), and measurements unified and comparable.

3.1 Biotests and their classification

Interdisciplinary analytical methods based on living material illustrate potential threats posed by contaminants or their mixture in diversified environmental matrices. The multitude of methods causes many problems with classifying them (Persoone et al., 2003). Primary classifications referred to information about the level of contamination in a given element of the environment, considering the place of performed analysis. There are laboratory tests conducted, which are based on modelling samples in controlled conditions, results of which determine the level of toxicity for real samples. Moreover, samples retrieved from specific components of the environment (e.g. water, soil, wastewater) are analysed and compared with standard samples. In comparative studies of water environment, it is best to apply various methods and to test the same samples with various organisms in order to determine the level of test sensitivity and eliminate errors resulting from application of only one test (Kratasyuk et al., 2001). Another way to best account for the specific environmental conditions is to perform an *in situ* analysis, which utilises responses of organisms living in the natural environment (Anderson et al., 2004; Mc William & Baird, 2002). Such an analysis enables continuous replacement of the medium, mainly water, when studies are performed on fish or plants as indicator organisms, and it is classified to be a dynamic biotest (Sundt et al., 2009). If replacement takes place in set time periods, such biotest is defined as a half-static one (Blanck et al., 2003; De Liguoro et al., 2009). In a static test the medium is not replaced until the end of analysis. While analysing toxicity, the tested pollutant at a certain concentration is only once placed on the medium, i.e. water, sediment, or soil.

Studies based on the assessment of various substances' impact on morphological and physiological changes in the indicator organism pose many difficulties in classifying them to a specific group of toxicological tests due to a wide spectrum of methods they employ. The active element of the test – i.e. an organism used in the test – may be considered as a basic criterion in classification of toxicological and ecotoxicological biotests. Various plants, bacteria and animals are used as active elements (Nałęcz-Jawecki & Persoone, 2006; Adomas & Piotrowicz-Cieślak, 2008; Nałęcz –Jawecki et al., 2010). Nowadays, microbiotests called

'toxkits' are available. Sets of microbiotests – TOXKIT – used to determine acute and short-term chronic toxicity have been developed with the use of many test organisms which belong to many phylogenetic groups and trophic levels. They are used in order to assess land and fresh water, as well as coastal and sea environments. Among tests used to assess fresh water there are:

- ALGALTOXKIT F™ using *Selenastrum capriconrnutum*
- DAPHTOXKIT F ™ magna using *Daphnia magna*
- DAPHTOXKITF ™ pulex using *Daphnia pulex*
- CERIODAPHTOXKIT F ™ with the aid of *Ceriodaphnia dubia*
- THAMNOTOXKIT F ™ with *Thamnocephalus platyurus*
- ROTOXKIT F ™ acute using *Brachionus calyciflorus*
- ROTOXKIT F ™ short – chronic with *Brachionus calyciflorus*
- PROTOXKIT F ™ with the aid of *Tetrahymena thermophila*
- MARA biotest with 11 microorganisms individually lyophilized in microplate wells.

Tests used to assess brackish and saline waters include, among others:

- ROTOXKIT M™ rotifers *Brachionus plicatilis* are the test organism;
- ARTOXKIT M™ with sea crustacean *Artemia salina;*
- MARINE ALGALTOXKIT ™ uses *Phaeodactylum tricornutum.*

Sediments and soil are assessed with the use of:

- PHYTOTOXKIT – seeds of mono- and dicotyledonous plants are the phytobioindicators;
- OSTRACODTOXKIT F™ uses *Heterocypris incongruens;*
- PHYTOTOXKIT (Fig. 7) is a microbiotest of phytotoxicity of soil, sludge, compost sewage used for watering, chemical substances and biocides.

Biotests based on the analysis of changes within a plant organism and called phytotests provide information concerning organisms of key importance to a given ecosystem, thanks to which it is possible to determine its condition and disturbances in matter flow or substance circulation. Phytotoxic substances disturb absorbance and transport of essential micro- and macro-elements in plants, which results in delayed seed germination and plant sprouting as well as deformations and underdevelopment of certain plant elements (Jin et al., 2009). Thanks to phytotests, a solid knowledge of the impact of an environmental factor is acquired. Phytotests are used to assess soil (Piotrowicz-Cieślak et al., 2010 a) and water (Fernández-Alba et al., 2002; Drzewicz et al., 2004) contaminated with, among others, pesticides. Biotests used in biomonitoring (Holgado et al., 2004) are applied in order to assess the condition of ecosystems, to establish the capacity of ecosystems to absorb pesticides, including herbicides, as well as to assess interactions among pesticides, and between pesticides and the environment. In these studies many kinds of plants are used; algae from the class of *Chlorophyta: Selenastrum capricornutum, Scenedesmus quadricauda, S. subiscatus* are used most often to evaluate fresh waters (Küster et al., 2003; Simeonov et al., 2007; Wadhia & Thompson, 2007), while the class of *Bacillariophyta: Phadeodactylum tricornutum, Skeletonema constatum* is used to evaluate brackish and saline waters (Nendza, 2002; Wadhia & Thompson, 2007). Duckweed is widely used in laboratory studies of substances and their accumulation (OECD, 221), *Lemna minor* and *L. gibba* are the organisms most frequently applied as indicators in biotests (Lemna test). Phytobioindicators are chosen

also from among tracheophytes, among which *Sorghum saccharatum, Lepidium sativum* or *Sinapis alba* can be distinguished. They have very small seeds, and the test is performed for three days (OECD, 208). To evaluate soil contaminated with glyphosate yellow lupin was applied, with seeds 20 times bigger than the size recommended by the PHYTOTOXKIT™ producer (MicroBio Test Inc., Belgium) (Adomas & Piotrowicz-Cieślak, 2008).

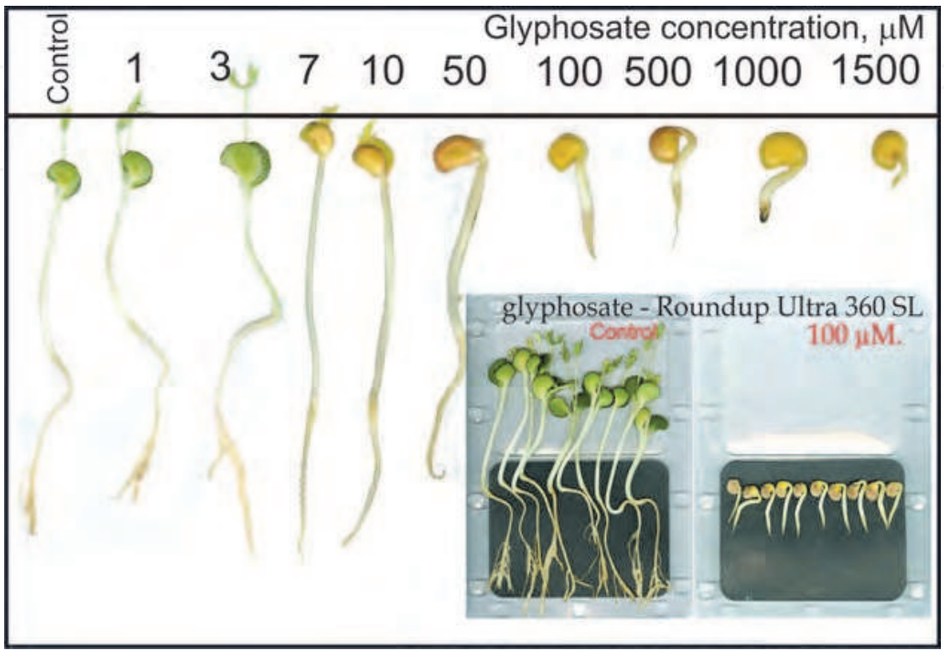

Fig. 7. PHYTOTOXKIT is used to determine phytotoxicity of soil contaminated different glyphosate concentration.

In soil environment assessment, apart from assessing the germination of grasses, crucifers, leguminous plants and grains, also macrophytic plants are taken into consideration. However, a complicated process of growing them as well as a long time of their growth and their requirements with reference to space precludes their common use in biotests.

The choice of plant organisms should characterize a given ecosystem. Plant species are chosen considering their availability, methods of growing, simplicity of conducting studies and biological sensitivity to a compound or a group of compounds, confirmed in a number of repeated tests (Eberius et al., 2002). In such kind of research, the opportunity to assess toxicity in a multigenerational cycle is extremely important. Thus the majority of applied phytotests are of chronic character, i.e. they last longer than 1/3 of pre-productive period. Nowadays, as a result of long-lasting studies in the field of environmental assessment, scientists tend to use available plant cultures in their experiments, which can be used in laboratories. They grow their own algae and tracheophytes or use commercially available selected cultures in the cryptobiotic or lyophilized form, available with microbiotests, e.g. Algaltoxkit F™, Phytotoxkit™ (Persoone et al., 2003; Wolska et al., 2007).These are kits equipped with accessories essential in determining the level of toxicity in samples. Every microbiotest contains an active element, i.e. a living organism, ready to be activated at any moment. Toxicological assessment with the use of such tests makes it possible to compare results between continents. Moreover, thanks to cultures attached to the test, there is no

need to grow any organisms. Assessments performed with the use of these tests are characterized by lower research costs than in the case of the conventional ones; the time of organism response is shortened, and the study can be performed for samples of lower volume. Additionally, a researcher can work on a few samples at the same time (Persoone et. al., 2003; Kaza et al., 2007; Wolska, et al., 2007), which enables planning the experiment as a battery of tests. A battery of tests is a toxicological study which encompasses more than just one species of indicator organisms. It is used in order to widen the scope of research conducted on a given chemical substance onto a bigger portion of the ecosystem, considering also other organisms which live there on different trophic levels, in the following order: producer-consumer-reducer (Kaza et al., 2007). Toxicological analyses as well as the obtained results are specifically diversified. They often aim at determining the very lethality or inhibition effects with reference to different bioindicators, after particular exposure times. Lethal Concentration (LC) or Lethal Dose (LD) is often determined taking into account Lowest Observed Effect Concentration (LOEC) or Observable Effect Concentration (NOEC). Typically, Effect Concentration (EC) is established for rotifers and crustaceans, and denotes inhibition of a particular physiological or biochemical activity expressed as a percentage in a given test group. In growth tests, inhibition of growth of plants, fungi as well as biomass of algae is considered. Apart from the above parameters, due to the development of biotechnology, also enzymatic tests (Budantsev, 2005) and tests of genotoxicity (Yamamoto et al., 2001; Küster et al., 2003; Jha, 2008) have become important in toxicological studies. The first group determines inhibition of activity of an enzyme or a group of enzymes which catalyze a given biochemical reaction, while the second group determines genetic changes brought about by a toxicant. Sometimes toxicologists analyze parameters relating to absorption and storage of certain substances in tissues and organs depending on the time of exposure. These tests are referred to as bioaccumulation tests (Kahle & Zauke, 2002). Sensitivity of leguminous plants to herbicides has been successfully used in our research.

3.2 Estimation of herbicites phytotoxicity to legumes

Lupinus luteus, *Glycine max, Pium sativum, Lens esculenta, Vigna angularis* and *Medicago sativa* were used to estimation soil contamination with glufosinate ammonium, glyphosate and diquat. Seeds of yellow lupin (*Lupinus luteus*), soybean (*Glycine max*), adzuki bean (*Vigna angularis*), pea (*Pium sativum*), lentil (*Lens esculenta*),) and alfalfa (*Medicago sativa*) were germinated for nine days using PHYTOTOXKIT™ (MicroBio Test Inc., Belgium). Germination was carried out in controlled climatic conditions, 25ºC and 90 % RH humidity, in darkness. Ninety ml of soil (sand, vermiculite, peat, 1:0.3 :1, v:v:v) were placed in plastic microbiotest plates. The soil was covered with Whatman No. 1 filter-paper and watered with 27 ml distilled water supplemented with different glyphosate (Roundup Ultra 360 SL), glufosinate ammonium (Basta 200 SL), and diquat (Reglone Turbo 200 SL) final concentrations: 1, 3, 7, 10, 50, 100, 500, 1000, 1500 µM. The control plants were watered with pure distilled water. The root length was estimated using Image Tool for Windows. The effective concentration causing a 50% response (EC_{50}) was calculated for inhibition of root growth and no observed effect concentration (NOEC) that did not affect germination was noted. Electroconductivity of seedlings soak water (4×10 seedlings, each in 100 ml deionised water for 24 h) was measured using Hanna conductivity meter HI 4321. Dry and fresh mass and *myo*–inositol content (see 2.1.1) were determined.

3.3 Phytotest used in herbicides study

Yellow lupin, soybean, adzuki bean, pea, lentil and alfalfa seedlings responded in a similar manner to herbicides (glyphosate, glufosinate ammonium and diquat). All seeds, irrespectively of the concentration of herbicides, germinated with frequency between 65% and 95% (Fig. 8). Seeds were considered germinated when the radicle penetrated the seed coat. With increasing concentration of herbicides an inhibition of root elongation was observed in all plants (Fig. 9).

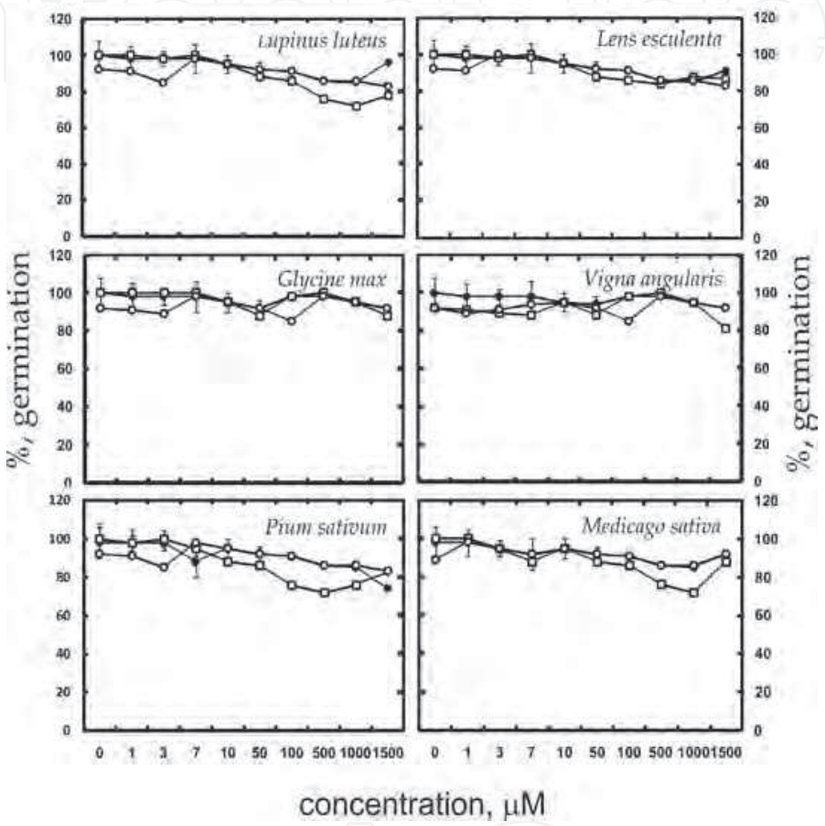

Fig. 8. Yellow lupin (*Lupinus luteus*), soybean (*Glycine max*), adzuki bean *(Vigna angularis)*, pea *(Pium sativum)*, lentil (*Lens esculenta*) and alfalfa (*Medicago sativa*) seedlings germination [%] after application of herbicides: glyphosate, glufosinate ammonium and diquat. Data points represented the mean ± SD for fifteen replicate samples – glyphosate (o), glufosinate ammonium (□) and diquat treatments (●)

Active substances of herbicides, depending on their chemical structure, remain in the soil solution and they are absorbed by roots of germinating seeds. When the weeds are removed, within one vegetation season these substances should decompose to compounds which are natural in the environment (e.g. nitrogen and carbon dioxide). Yet, among herbicides there are chemical compounds which have varying half-lives. For instance, the half-life of glyphosate in soil is only 10 to 100 days (47 days on average) according to Hornsby et al., (1996) and according to Monsanto, (2005) the average half-life is 32 days. Paraquat has a relatively long half-life in soil (estimated at about 1000 days). The residue of persistent herbicides (e.g. atrazine, metribuzin, trifluralin) may stay in soil and negatively

affect subsequent crop even more than a year after application. It pertains especially to active substances used year by year on the same field (e.g. atrazine) (Sheets & Shaw, 1963). Thus for both agriculture and environmental protection it is important to check what happens with the active substances of herbicides in soil (their translocation on different levels of soil and water, and their degradation) and how they are absorbed by plants (Beckie & McKercher, 1990).

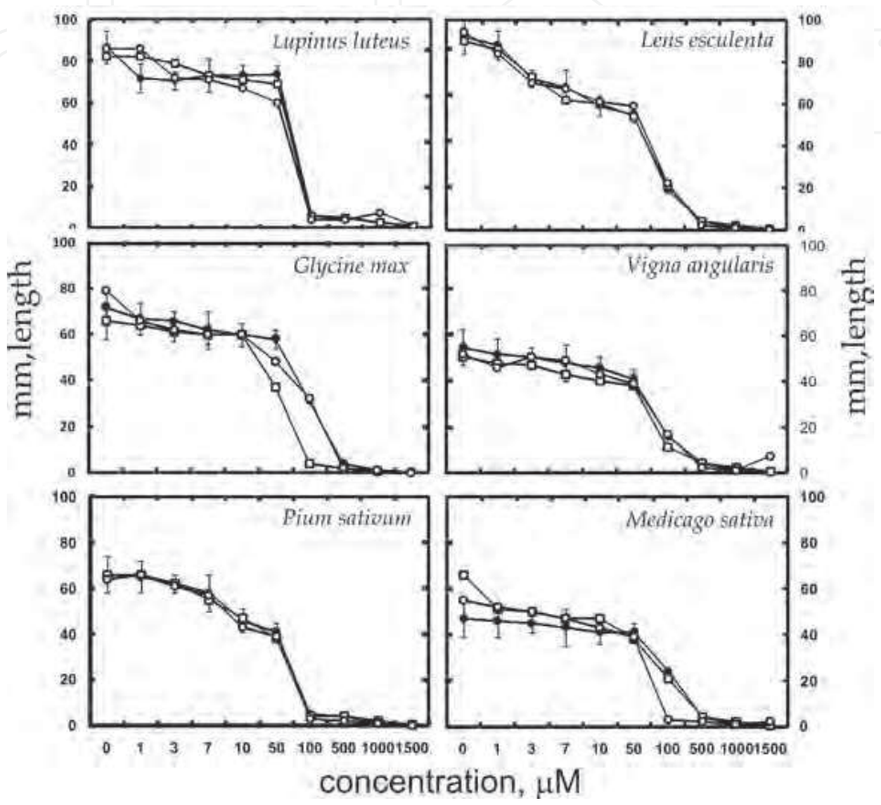

Fig. 9. Yellow lupin (*Lupinus luteus*), soybean (*Glycine max*), adzuki bean *(Vigna angularis)*, pea *(Pium sativum)*, lentil (*Lens esculenta*) and alfalfa (*Medicago sativa*) seedlings length [mm] after application of herbicides: glyphosate, glufosinate ammonium and diquat. Data points represented the mean ± SD for fifteen replicate samples – glyphosate (o), glufosinate ammonium (□) and diquat treatments (●).

In such research, phytotests in which different plants are phytobioindicators are successfully applied. Plant sensitivity to environment contamination is often used to estimate the degree of environment degradation. Plants respond in different manner to many kinds of toxic substances. The symptoms include morphological deformations (e.g. seed germination or the length of roots and shoots) and changes in plant biochemistry (osmoprotectors content) (Pardo, 2010; Piotrowicz-Cieślak et al., 2010a). The phytotoxic effect is a result of an interaction between the compound and the plant in given environmental conditions. Environmental toxicity is usually determined with the use of phytotests according to OECD norms (2006) (OECD, 221), mainly with reference to pesticide (Stork & Hannah, 1996) and veterinary medicine contamination (Piotrowicz-Cieślak et al., 2010b). Biotests, in contrast to instrumental (chemical) methods, enable simple and inexpensive estimation of very low

levels of active substances in soil which can be phytotoxic to crop plants (Wolska et al., 2007).

Phytotoxkit has been successfully applied to estimate phytotoxicity of glyphosate used all over the world to fight weeds and desiccate crop plants (leguminous plants and rape seed). Soil contaminated with increasing concentrations of glyphosate from 0 to 2000 μM was assessed with the use of leguminous plants (*Lupinus luteus*), crucifers (*Brassica napus*, *Sinapis alba*, *Lepidium sativum*), grains (*Avena sativa*) and a plant from the *Poaceae* family (*Sorghum saccharatum*) (Piotrowicz-Cieślak et al., 2010a). Glyphosate concentrations highter than 40 μm inhibited root growth.

In order to see a complete picture of herbicide phytotoxicity, one has to assess also key cell metabolites, among others *myo*-inositol, in seedlings which grow in contaminated soil. *myo*-Inositol is an aliphatic alcohol derived from glucose-6-phosphate, it has six OH groups on its ring. It is the most commonly spread cyclitol in the environment and a precursor of optical and methyl derivatives. *myo*-Inositol is localized in cytosol and plastids in small quantities (Paul & Cockbourn 1989), it is easily incorporated into cell metabolism and thus its (galactosyl) derivatives are said to have storage functions (Piotrowicz-Cieślak et al., 2008). *myo*-Inositol constitutes a key component of cell membranes (poli-phosphatidylinositols take part in receiving and transducing signals). This compound occurs in considerable quantities in the form of an ester of phytic acid, being an easily accessible form of phosphate ions (Loewus et al., 1990). In dehydration stress, *myo*-inositol plays the role of an osmoprotectant (Nelson et al., 1998; Piotrowicz-Cieślak et al., 2007), first of all limiting destructive changes in the biological membranes which were induced by the stress factor.

The level of *myo*-inositol in plants treated with glyphosate, glufosinate ammonium and diquat indeed increased when the herbicides were used at very high concentrations (Fig. 10).

Phytotoxkit was also used to assess the residue of herbicides applied to control weed in winter wheat: chlorsulfuron, nicosulfuron, 2,4 DP and dicamba within the range of 0.025–1.2 mg/kg of soil. Seeds of *Sinapis alba, Fagopyrum esculentum* and *Cucumis sativus* were phytobioindicators here. On the basis of root elongation in 5-day seedlings, it was shown that *Sinapis alba* was most effective in detecting chlorsulfuron and nicosulfuron residues in soil. *Cucumis sativus* seedlings were the most sensitive plants to 2,4 DP residue in soil, as the highest of the analysed concentrations reduced root growth by 99%. The roots of *Fagopyrum esculentum* were most suitable for the detection of dicamba residue. The research demonstrated that very sensitive plants are able to detect the residue of herbicides in soil at the level of 0.0015 mg/kg (Sekutowski & Sadowski, 2009).

While applying herbicides, e.g. in agrotechnical activities and to destroy plants in water reservoirs (e.g. glyphosate), xenobiotics are introduced into natural aquatic systems. The mechanism of toxic activity of herbicides inhibiting photosynthesis in plants in PI and PII system was used to assess phytotoxicity of paraquat, atrazine, metribuzinan and diuron towards *Scenedesmus obliquus* green algae. The F684/F735 chlorophyll fluorescence ratio in *S. obliquus* algae can be a quick and sensitive measurement method of contamination levels in water reservoirs when they were contaminated with active substances of herbicides chosen for the research (Eullaffroy & Vernet, 2003).

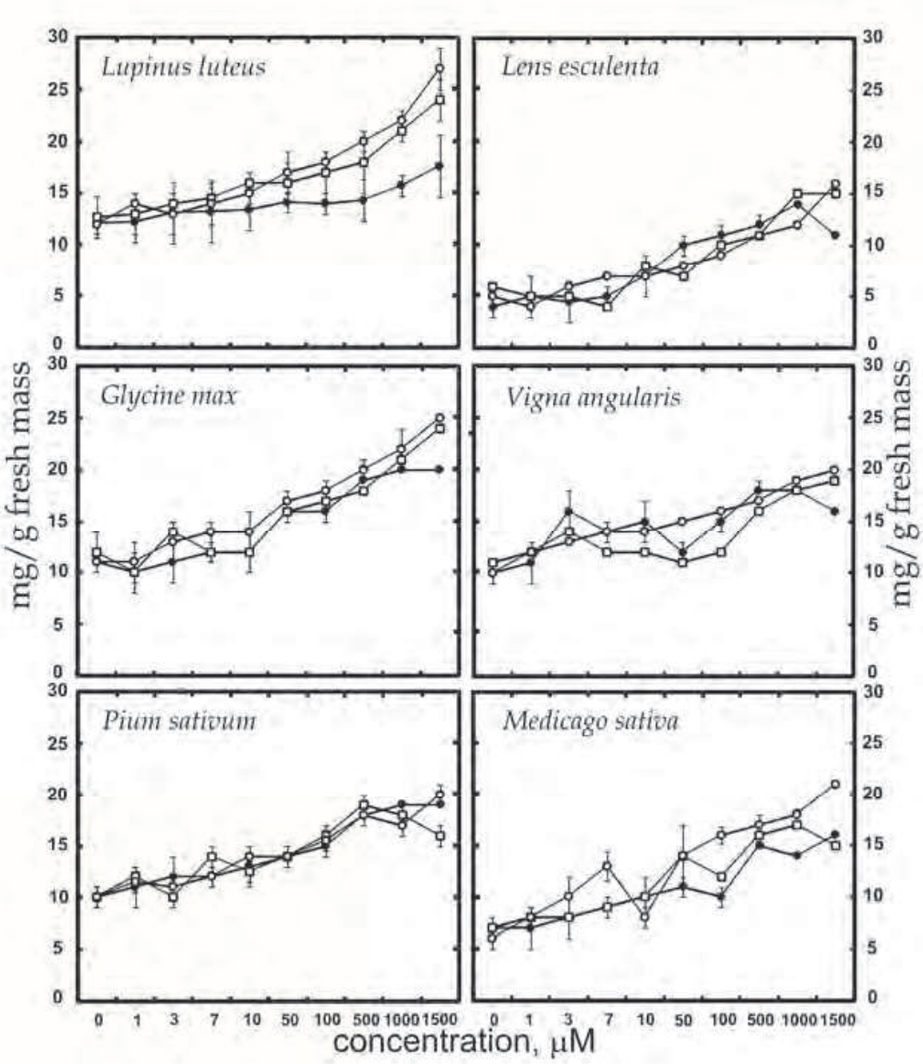

Fig. 10. *myo*-Inositol content [mg/g fresh mass] in yellow lupin (*Lupinus luteus*), soybean (*Glycine max*), adzuki bean *(Vigna angularis)*, pea *(Pium sativum)*, lentil (*Lens esculenta*) and alfalfa (*Medicago sativa*) seedlings after application of herbicides: glyphosate, glufosinate ammonium and diquat. Data points represented the mean ± SD for fifteen replicate samples – glyphosate (o), glufosinate ammonium (□) and diquat treatments (●).

The unicellular algae *Chlamydomonas reinhardtii* were used to detect 16 herbicides, belonging to 11 different chemical groups, in water. Different reactions (sensitive or no effect) of one species of algae were shown for acifluorfen, chlorpropham, diclofop-methyl (DFM), glyphosate, isoxaben, pinnacle, and trifluralin, dichlorobenzonitrile (DCB), 2,4-dichlorophenoxyacetic acid (2,4-D), metobromuron, 2-ethyl-4-chlorophenoxyacetic acid (MCPA), metribuzin, atrazine, hexazinone, norflurazon and terbacil – after 3 days from their application. The reaction of algae depended on the quantity of substances and the mechanism of toxic activity of particular chemical groups of herbicides. Such unicellular organisms can be used as phytobioindicators for a quick and easy detection of different active substances of herbicides in water (Li et al., 2008).

The results of toxicity assessment of active substances in herbicides may vary, depending on the sensitivity of the species used for biotests. These relations were demonstrated through the analysis of pesticides (42 insecticides and 45 herbicides) transported in 1985-2004 into the rivers in Mississippi River basin, which then were entering Chesapeake Bay. The U.S. EPA estimates that approximately 75% of all pesticide usage in the United States is agricultural. The other 25% is for home and garden use, industrial, commercial and government sectors. It has been found that of the 45 tested herbicides the ones most often utilized in 1985-2004 were atrazine, glyphosate, and metalachlor. Atrazine and metalachlor have been identified as more toxic to nontarget plant species than glyphosate. On the other hand, insecticides were more toxic to animals and plants used in the study (trout, bluegill, daphnia, selenastrum, skeletonema, lemna) than herbicides. In the years 1985 and 2004, 12 herbicides inhibiting weed roots were examined in Maryland. The lowest amounts of these herbicides (0.0054, 0.0145, 0.0345 mg/L) were phytotoxic for selenastrum, lemna, and skeletonema that had been used as bioindicators section (Hartwell, 2011).

Pea (*Pisum sativum* L.) and lupin (*Lupinus angustifolius* L.) roots were used seven days after sowing for assessment of soil contaminated with herbicide from sulfonylurea class (chlorsulfuron, triasulfuron and metsulfuron-methyl) and from the sulfonanylide class (flumetsulam and metosulam). The sensitivities of the species were similar in chlorsulfuron and flumetsulam trials and their response range varied with soil type and herbicide, e.g. between 0.75 and 6.0 ng triasulfuron g^{-1} in the Wimmera grey clay and between 0.125 and 8.0 ng chlorsulfuron g^{-1} soil in the Mallee sand (Stork & Hannah, 1996). Sensitivity of leguminous plants to herbicides has been successfully used in our research.

4. Conclusion

Research into pesticides toxicity to animal and plant organisms has been conducted for many years and has developed as production and registration of active substances of these xenobiotics have increased. Most toxicological studies concern animal organisms, yet embryophytes - and especially crop plants - have been used rather more rarely as bioindicators for the environment contaminated with pesticides. Over the recent years the ecotoxicological research has proved that active substances of herbicides present in water and soil are, even in very low quantities (examples), phytotoxic. Yet, these are very low quantities (e.g. over 10 μM of glyphosate) (Piotrowicz-Cieślak et al, 2010a) in soil and even lower in water - 0.08 mg/L of atrazine for *Lemna* (Harwell, 2011).

On the other hand, before it is registered, every active substance is analysed in order to determine its dosage phytotoxic to standardised mono- and dicot assay plants. Our studies have proved that glyphosate, glufosinate ammonium and diquat applied in doses recommended by producers are not phytotoxic to leguminous plants. Only if a 50 μM dose is exceeded, morphological and biochemical changes are apparent in these plants. Applying desiccants prior to harvest (to immature seeds) induces changes in their metabolism of carbohydrates. We also found that in order to determine phytotoxicity it is better to use a root elongation test than a test of seed germination in soil contaminated with herbicides. The need to conduct research into the natural environment assessment with the use of up to-date toxicity assessment methods has lead to many various legal modernisations in the field of environmental protection of many countries (European Commission, 76/464/EEC; European Commission, 2455/2001/EC). Thanks to this, a lot of concentrations of pollutants

so far believed to be safe have been found unsuitable, and appropriate regulations require their elimination or lowering their content in the environment. Biotests – as compared with conventional methods – constitute a relatively quick and inexpensive analysis method. Combining two methods (the biological and chemical ones) enables to provide an exhaustive and wide-ranging toxicological description. As a consequence, the natural environment protection, including human population, is becoming more and more effective and preventive steps are taken much earlier than they used to be.

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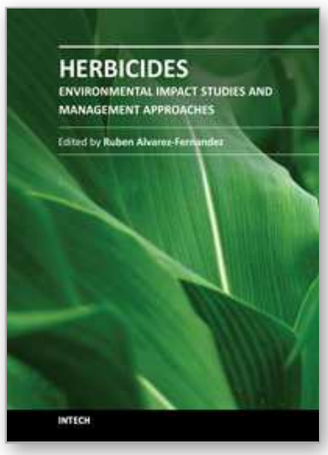

Herbicides - Environmental Impact Studies and Management Approaches

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Weeds severely affect crop quality and yield. Therefore, successful farming relies on their control by coordinated management approaches. Among these, chemical herbicides are of key importance. Their development and commercialization began in the 1940's and they allowed for a qualitative increase in crop yield and quality when it was most needed. This book blends review chapters with scientific studies, creating an overview of some the current trends in the field of herbicides. Included are environmental studies on their toxicity and impact on natural populations, methods to reduce herbicide inputs and therefore overall non-target toxicity, and the use of bioherbicides as natural alternatives.

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