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Weed Resistance to Herbicides in the Czech Republic: History, Occurrence, Detection and Management

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1. Introduction

Herbicide resistance results from the repeated selection for resistant individuals in field populations. Herbicide resistance has evolved to many important groups of herbicides, encompassing almost all of the major herbicide mode-of-action groups (Heap, 2000).

In the Czech Republic, herbicide resistance has been reported in 16 weed species, mostly found on non-arable land (railways) and in orchards with a history of intensive herbicide use. In maize and sugar beet, *Echinochloa cruss-galli*, *Chenopodium album*, *Chenopodium rigidum* and *Solanum nigrum* have been identified with resistance to photosystem 2 inhibitors. Seven *Kochia scoparia* biotypes resistant to chlorsulfuron have been found on railways, roadsides and car parks in the Czech Republic since 1996 (Salava *et al*., 2004). *Alopecurus myosuroides* is the weed with local importance in the Czech Republic. However, the poor weed control had been reported in several fields and the tested populations showed sulfonylurea resistant phenotype (Slavíková *et al*., 2011). On arable land, the most important seems to be ALS-resistant *Apera spica-venti* (Hamouzová *et al*., 2011). ALS-resistant *A. spica-venti* was first documented in 2005, in populations collected from fields in western and southern part of the Czech Republic that had continuous chlorsulfuron treatment for at least 5 years (Hamouzová *et al*., 2011). To date *A. spica-venti* resistant to ALS herbicides has been reported in several countries in Europe (Heap, 2011).

In this chapter, history and present state of weed resistance to herbicides in the Czech Republic is reviewed, with emphasis on origin, development, level of resistance, methods of testing for herbicide resistance, mechanisms, molecular basis, and effective management of herbicide resistance.

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2. Weed resistance to photosystem 2-inhibiting herbicides

Triazine herbicides were repeatedly applied on railways in the Czech Republic for about 20 years. The use of atrazine herbicides was terminated in 1992 as a consequence of their long persistence or tendency to move off site, damage of surrounding vegetation, enter watercourses and detection of resistant biotypes of ten weed species (Anonymous, 2010). Atrazines were replaced with terbuthylazine. Meanwhile, plants of *Digitaria sanquinalis* that are not susceptible to terbuthylazine are found on the Czech railways.

A biotype *of Digitaria sanquinalis* resistant to atrazine was first described by Gasquez in 1983 (Heap, 2011). Resistance to triazine herbicides can be conferred by a modified target site (mutation in the *psb*A gene which codes for the D1 protein of PS2) or an enhanced detoxification of herbicide (Robinson and Greene, 1976; Gawronski *et al.*, 1992; Monteiro and Rocha, 1992; De Prado *et al.*, 1999 and Chodová *et al.*, 2004).

3. Materials and methods

Seed source

Seeds of *Digitaria sanquinalis* were originally collected from plants growing on the railway tracks and in the vicinity of the railway station Prague-Libeň in 2005. Plants were grown in a greenhouse under controlled conditions. The presence of individuals resistant to atrazine in the population was confirmed by the whole-plant response assay. Seeds of susceptible biotypes were collected from the fields, private gardens or places without application of herbicides.

Whole-plant response assay

Seeds were sown into plastic pots 10x10 cm in size, 0.2 g seeds per pot, and plants were thinned to 5-10 individuals per pot after their emergence. The pots were placed in a greenhouse (temperature 20-24°C, relative air humidity 70-90%, no supplemental lighting).

Herbicides were applied at the stage of 2-4 right leaves of plants with a hand sprayer using 2 000 $g.ha^{-1}$ of atrazine and simazine (Gesaprim 500 g a.i. l^{-1}, Novartis Crop Protection AB, Basel, Switzerland; Gesatop 900 g a.i. kg^{-1}, Ciba-Geigy AB, Basel, Switzerland). The effect of the herbicide was assessed 15 days after treatment. Phytotoxic symptoms were ranked individually according to the European Weed Research Society classification scheme for plant tolerance (Frey *et al.*, 1999).

Chlorophyll fluorescence parameters

Chlorophyll fluorescence for *Digitaria sanquinalis* was measured in 10 plants using a chlorophyll fluorometer FMS2 (Hansatech Instruments, UK). The leaf discs were cut from the middle part of leaf blade and soaked at 1, 2, 3 and 4 hour-intervals in either 1% atrazine solution or water. Measurements were done on 30-min dark-adapted leaves. Maximum quantum efficiency of PS 2 photochemistry, i.e. F_v/F_m [defined as $[(F_m - F_o)/F_m]$, where F_o is the minimum, and F_m is the maximum chlorophyll fluorescence yield in the dark-adapted state], was calculated.

Biotype	Hours after treatment	F_0		F_m		F_v/F_m		Differences	
		1% atrazine	*water*	*1% atrazine*	*water*	*1% atrazine*	*water*	*A*	*B*
Susceptible	1	383	241	1107	1200	0.654	0.799	*	*
	2	*391*	*239*	*1247*	1243	0.686	0.808	*	*
	3	*323*	*242*	*1055*	1250	0.694	0.806	*	*
	4	*494*	*241*	*939*	1220	0.474	0.802	**	**
Resistant	1	341	267	1099	1118	0.690	0.761	**	*
	2	*311*	*295*	*1101*	1253	0.718	0.765	*	*
	3	*328*	*281*	*1165*	1152	0.718	0.756	ND	*
	4	*313*	*313*	*1147*	1159	0.727	0.730	ND	**

A - *significant differences at 0.05 probability level, **significant differences at 0.01 probability level in F_v/F_m values of susceptible (or resistant) plants treated with atrazine compared with relevant control sample (water), NDsignificance of difference not determined
B - *significant differences at 0.05 probability level, **significant difference at 0.01 probability level in F_v/F_m values between resistant and susceptible biotype after 1 to 4 hours exposure to the herbicide

Table 1. Chlorophyll fluorescence measurements on dark-adapted leaves of *Digitaria sanquinalis*.

Photochemical activity of isolated chloroplasts

Isolation of chloroplasts from leaves and measurement of their activity was performed as described in Holá *et al.* (1999). Photochemical activity was determined polarographically as the Hill reaction activity (HRA), characterized as the amount of oxygen formed by the chloroplast suspensions in defined conditions after white light irradiation and addition of an artificial electron acceptor.

Atrazine concentration	Susceptible biotype	Resistant biotype
0 M	46.07 ± 4.77	51.22 ± 2.30
0.0000001 M	37.34 ± 4.83	47.06 ± 3.73
0.000001 M	6.55 ± 0.73**	43.37 ± 0.92
0.00001 M	0 ND	30.92 ± 1.39*
0.0001 M	0 ND	22.61 ± 0.46*
0.001 M	0 ND	7.39 ± 0.93**

*difference (compared to the relevant sample measured without the addition of atrazine) significant at 0.05 probability level, ** difference significant at 0.01 probability level, ND significance of difference not determined

Table 2. Hill reaction activity (mean values in mmol O_2 kg^{-1} of chlorophyll s^{-1} ± standard error) of mean in suspensions of chloroplasts isolated from leaves of four atrazine -resistant or -susceptible plants of *Digitaria sanguinalis* after the addition of various concentrations of atrazine.

Gene sequencing

Plants of which susceptibility or resistance was confirmed using the whole-plant response assay (for details see Nováková *et al.*, 2005), Hill reaction activity and chlorophyll fluorescence assay were chosen for molecular analysis. DNA of individual plants was

extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer´s instructions. Polymerase chain reaction (PCR) and sequencing of the *psb*A gene were conducted using standard methods as described previously (Nováková *et al.* 2005). Sequence editing and analysis were done using the BLAST program (Altschul *et al.* 1997). The ExPASy translate tool (Gasteiger *et al.* 2003) was used to determine the peptide sequences.

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                                  264
R                                 Gly
S   Gly Arg Leu Ile Phe Gln Tyr Ala Ser Phe Asn Asn Ser Arg Ser Leu His Phe
S   GGT CGA TTA ATC TTC CAA TAT GCT AGT TTC AAC AAC TCT CGT TCT TTA CAC TTC
R   ..............................G.....................................
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Fig. 1. Alignment of cpDNA encompassing the atrazine resistance-conferring region of the *psb*A gene of *Digitaria sanguinalis* using susceptible (S) as a reference. Dots in the resistant (R) sequence indicate matches to the reference sequence; differences are indicated by A, C, G or T. Bold print in the amino acid sequence indicates the site where the mutation confers atrazine resistance.

4. Results

The screening of large crabgrass plants obtained from the railway station Prague-Libeň revealed the presence of individual plants resistant to atrazine in this population. The resistance to atrazine was confirmed by the *in vivo* measurement of chlorophyll fluorescence (Table 1) emitted by leaves treated with this active ingredient, as well as by the measurements of the Hill reaction activity in suspensions of isolated chloroplasts (Table 2) after the addition of atrazine solution.

After atrazine treatment, leaves from the resistant biotype did not show any changes in the fluorescence curve pattern when compared with the control leaves treated with water. On the other hand, leaves from the susceptible biotype showed changed fluorescence and the F_v/F_m ration was considerably reduced.

The Hill reaction activity in suspensions of chloroplasts isolated from large crabgrass plants susceptible to atrazine distinctively decreased with the addition of atrazine to these suspensions and its values were zero after the treatment with 0.01 mM (or higher) atrazine solutions. Contrary to this, chloroplasts isolated from leaves of the resistant biotype showed certain Hill reaction activity even with the 1 mM atrazine treatment and the decline with the increasing atrazine concentration was not as pronounced as in the previous case (Table 2). The ED_{50} value for the susceptible biotype was 0.17 μM, for the resistant biotype 29.21 μM, and RF_{50} was calculated as 166.

A region of the chloroplast *psb*A gene that encodes for amino acids 163 to 329 of the D1 protein of PS2 was selectively amplified using PCR. Sequence analysis of the fragment from the herbicide-resistant biotype of large crabgrass exhibited a substitution from serine to glycine at position 264 of the D1 protein of PS2 (Figure l).

5. Discussion

Recent screening of large crabgrass in the Czech Republic (particularly along railways and other transport corridors) has revealed the presence of atrazine-resistant plants of this weed

species that tolerated the dose of 3 000 g atrazine.ha^{-1} at the railway station Prague-Libeň. Since the active ingredient atrazine was irregularly used along the Czech railways only till 1992 (and again for a short period 1997-1999), these resistant individuals had to grow up from seeds that retained their germinability in the specific railway conditions for fairly long time.

Besides the basic screening tests commonly used for the determination of plant susceptibility or resistance to atrazine, evaluation of photosynthetic parameters can be with advantage used for the evaluation of resistance in individual plants. The mutation in the 264th codon of the *psb*A gene, usually found in atrazine-resistant plants (Hischberg and McIntosh, 1983) reduces binding capacity of triazine herbicides to the D1 protein, thus enabling the electron transfer in PS2 even in the presence of atrazine. Our measurements of chlorophyll fluorescence and/or photochemical activity of isolated chloroplasts confirmed this, as we found that PS2 in atrazine-resistant plants of large crabgrass from the railway station Prague-Libeň indeed retains its activity after treatment of leaf discs or chloroplasts with atrazine, though to a lesser degree than in the untreated plants. There were some differences in chlorophyll fluorescence curves as well, similarly to the findings of De Prado *et al.* (1999).

The photochemical activity of chloroplasts isolated from atrazine-resistant plants, measured without the addition of atrazine to chloroplast suspensions, did not significantly differ from the activity of chloroplasts isolated from atrazine-susceptible plants, contrary to the previous findings for other weed species (e.g. Ort *et al.*, 1983; Sundby *et al.*, 1993; Chodová *et al.*, 1994; Körnerová *et al.*, 1998; Devine and Shukla, 2000). This was rather interesting, as the mutation in D1 protein usually decreases the efficiency of electron transport in PS2 under normal conditions. Gadamski *et al.* (1996), who characterized atrazine-resistant biotypes of large crabgrass in several Polish populations, did not find the target-site mutation at codon 264 of the *psb*A gene and suggested the possibility of different mechanism of resistance (*e.g.* non-target site). We have analysed the herbicide-binding region of the *psbA* gene and found out that in our case the molecular basis of resistance to atrazine in large crabgrass is the „classical" G264S substitution, similarly to other atrazine-resistant biotypes of various weed species (*e.g. Kochia scoparia, Solanum nigrum, Senecio vulgaris*) found in the Czech Republic previously (Chodová and Salava, 2004; Salava *et al.*, 2004; Nováková *et al.*, 2005). Moreover, there was an excellent correspondence between the presence of the mutation and phenotypic resistance to atrazine of individual plants, as determined by the assays of chlorophyll fluorescence or photochemical activity of chloroplasts. It thus seems that the Czech biotype of atrazine-resistant large crabgrass is not related to the Polish ones.

6. Weed resistance to acetolactate synthase-inhibiting herbicides

Acetolactatesynthase (ALS), also known as acetohydroxyacid synthase (AHAS) is required for the production of amino acids valine, leucine and isoleucine (Duggleby and Pang, 2000). The branched-chain amino acids are synthesized by plants, algae, fungi, bacteria and archaea, but not by animals, therefore are potential targets for the development of herbicides (McCourt and Duggleby, 2006). Worldwide, herbicides inhibiting acetolactatesynthase (ALS) are the most used ones at present. ALS is the primary target site of action for five structurally distinct classes of herbicides including pyrimidinylthiobenzoates, sulfonylureas,

imidazolinones, triazolopyrimidine sulfonamides and sulfonylaminocarbonyltriazolinones (Shimizu *et al*., 2002).

In 1987, *Lactuca serriola* was the first resistant weed species reported to be selected by ALS-inhibiting herbicides (Mallory-Smith *et al*., 1990).

Since then, rapid emergence of resistance to ALS-inhibitors has been identified in 110 weed species around the world (Heap, 2011).

Currently, five mechanisms of herbicide resistance have been identified in weeds: 1) altered target site due to a mutation at the site of herbicide action, 2) metabolic deactivation the active ingredient is transformed to nonphytotoxic metabolites, 3) reduce absorption and/or translocation of herbicide, 4) sequestration/compartmentation herbicide is located in vacuoles or cell walls and 5) gene amplification/over-expression of the target site, respectively (Nandula *et al.*, 2010).

In Europe, to the most important herbicide-resistant weeds belong blackgrass (*Alopecurus myosuroides*), *Lolium* species, *Papaver rhoeas* and wild oat (*Avena* spp.) (Moss *et al*., 2007). As seen from the list of resistant species, the grass weeds are on the top of farmers and scientific concern.

The occurrence of grass weeds is strongly influenced by geographical factors. In the areas with predominate ocean climate (such as the Great Britain, north France, western part of Germany, Denmark), the most harmful grass weed is *Alopecurus myosuroides* and *Lolium multiflorum*, in the Mediterranean Europe to the most detrimental weed belongs *Avena fatua*, *Avena sterilis* and *Lolium rigidum*. In the central and north-west Europe, *Apera spica-venti* is the biggest threat; *Bromus sterilis* and *Avena fatua* propagate quickly in the recent years. Generally, the protection against certain weed grasses in cereals is expensive and the spectrum of herbicides is not broad. Interaction of cultural methods and plant protection methods supports the occurrence of weeds and in many cases the herbicide-resistance. At present, populations with resistance to herbicides with different modes of action can be found across the world, whilst the information on extent of herbicide-resistant species has been best reported in the North America and Europe (Moss, 2002).

7. Reasons of origin of resistance in *Kochia scoparia* and *Apera spica-venti*

Crop rotation dominated by winter cereals and often autumn sown crops, together with reduced soil tillage, favour the spread of *A. spica-venti,* encouraging its life cycle (winter annual weed) reproducing exclusively generatively (Soukup *et al*., 2006). In recent years, *A. spica-venti* is progressively occurring in spring cereals.

Repetitive use of the same mode of action herbicides resulted in the frequent appearance of ALS-resistant *A. spica-venti*. It was proven that ALS-inhibitors are the most resistance-prone herbicide group (Délye and Boucansaud, 2008) and has occurred with only three applications of these herbicides (Preston *et al*., 1999). Major factors influence the evolution of herbicide resistance including the intensity of selection by herbicide and the initial frequency of herbicide resistant individuals in the populations (Preston and Powles, 2002).

8. Material and methods for identification of resistant weeds

Reliable diagnostic testing methods for determining weed population on resistance to ALS inhibitor are an important aspect. Herbicide resistance can be studied by different methods, their selection depends on the purpose of the scientific research (confirmation and characterization of resistance, examination of the biological and physiological traits of resistance, weed management, etc.). Various methods have been developed for confirming ALS resistance in grass weeds, these ranged from field experiments over whole-plant pot trials carried out under controlled conditions. To date, the whole-plant test remains the most commonly employed method for confirming resistance (Beckie *et al.*, 2000). However, there are many aspects significantly affecting the response to herbicides in whole-plant assays. Among the most important variables are formulation, adjuvant, spray volume, time of application as well as growth stage.

In those biotypes, where resistance was confirmed, more sophisticated laboratory based enzyme and DNA/RNA assays have been used and optimized for *A. spica-venti* (Hamouzová *et al.*, 2011; Massa *et al.*, 2011). For the quick detection of amino acid substitutions cleaved amplified polymorphic sequences markers can be used and partial *als* gene sequencing (Kaundun and Windass, 2006; Délye and Boucansaud, 2008). Most applicable to testing for ALS resistance are quantitative/RT-PCR and SNP analysis. Most recently, pyrosequencing technology is an established method for genotyping of SNPs, enabling rapid real-time sequence determination (Wagner *et al.*, 2008). The specificity of molecular techniques is a major advantage for research purposes. There is a need for the routine resistance screening which will be cheap and available for the general public. One of the approaches could be Syngenta RISQ method based on screening the survivorship of the seedlings in Petri dishes exposed to the different doses of herbicides (Kaundun *et al.*, 2011).

To assess the mechanism of resistance the HPLC methods (Chodová *et al.*, 2009; Massa *et al.*, 2011) and uptake and translocation of radiolabelled herbicides (Everman *et al.*, 2009) are used.

The interpretation of the results cannot be missed and is dependent on methods used, the mechanism of resistance, and proportion of plants within the population. The comparison of results deriving from different methods is not straightforward as the data on genetic, enzyme level and whole plant level cannot be compared e.g., herbicide efficacy under controlled conditions is usually significantly higher than in fields. The proper statistical evaluation of the data obtained from different datasets is crucial to make a right decision if the population is/is not resistant to herbicide.

Seed sources

The susceptible population of *A. spica-venti* had never been exposed to ALS-inhibiting herbicides and was sampled on the organic farm in the Czech Republic. The other susceptible population was obtained from Syngenta (Hamouzová *et al.*, 2011).

Seeds of *A. spica-venti* plants surviving applications of the registered field dose of ALS inhibiting herbicides were collected from winter wheat fields in July 2004-2010. Seed samples were obtained from fields with poor weed control and were not derived from the random sampling, thus extrapolation of results was not possible. Site selection was based on farmers' suspicion and past herbicide use. There were tested more than 100 populations using 12 different herbicides (Table 3) in total.

	Chlorsulfruon (15g a.i. ha^{-1})	Sulfosulfuron (19.5g a.i. ha^{-1})	Iodosulfuron (7.5g a.i. ha^{-1})	Mesosulfuron-methyl (4.5g a.i. ha^{-1}) + Iodosulfuron (0.9g a.i. ha^{-1})	Sulfometuron methyl (52.5g a.i. ha^{-1})	Pinoxaden (30g a.i. ha^{-1})
Number of samples tested	158	40	15	16	15	53
Samples showing resistance	118	22	13	16	13	0
	Isoproturon (1000g a.i. ha^{-1})	Fenoxaprop –P- ethyl (69g a.i. ha^{-1})	Prosulfocarb (1600g a.i. ha^{-1})	Propoxycarbazone (42g a.i. ha^{-1})	Pyroxsulam (18.75g a.i. ha^{-1})	Imazetapyr-NH_4 (50g a.i. ha^{-1})
Number of samples tested	72	26	6	5	70	3
Samples showing resistance	4	0	0	5	55	1

Note: In the table, populations in which herbicide efficacy was lower than 50% as compared to untreated are mentioned.

Table 3. Overview of *A. spica-venti* populations tested in whole-plant assays to herbicides with different mode of action at recommended doses.

Whole-plant response assay

Whole-plant response assays are the most widely used methods, while mimic the real field conditions the best. To characterise the susceptibility of individual populations of *A. spica-venti* to herbicides and calculate the resistance factor (RF), dose-response assays were performed.

Seed were sown in plastic pots (25 cm^2) filled with a chernozem soil [clay content 46% (loamy soil), soil pH (KCl) 7.5, sorption capacity of soil: 209 mmol (+), 87 mg kg^{-1} P, 203 mg kg^{-1} K, 197 mg kg^{-1} Mg, 8073 mg kg^{-1} Ca] and placed in a greenhouse. For screening resistance to commercially formulated herbicides applied post-emergently, the herbicide was applied to grass weed at the two- to three-leaf stage using a laboratory chamber sprayer equipped with a nozzle Lurmark 015F80 delivering 250 l ha^{-1}. The recommended doses of herbicides were included and the doses ranged from 0.01 to 150-fold of recommended one were applied. The herbicide dose ranges for the S biotypes differed from those of R biotypes, to provide more accurate models. Pot experiments were conducted in a randomized block design containing four replicates. Herbicide susceptibility was assessed 4 weeks after

treatment, when plants from the susceptible population were all clearly controlled. Visible symptoms on surviving plants were expressed as a percentage (0% no survival, 100% plants without any visible damage) of the untreated pots for the same population. Plants were cut at the soil surface, oven dried for 48 h at 105°C and weighed. Data were fitted to the non-linear regression using the Weibull four-parameter function where parameters are as follows: the dependent variable (efficacy or enzyme activity expressed as percentage of the untreated control, dry biomass weight in grams), the coefficients corresponding to the lower and upper limits, the herbicide dose at the point of inflection halfway between the upper and lower asymptotes (GR_{50}, I_{50}), and the slope around inflection point, independent variable is the herbicide dose (Nielsen *et al.*, 2004; Ritz *et al.*, 2006) The statistical software R 2.8.0 was used (R Development Core Team, 2008).

Figure 2 illustrates the resistance pattern as found in 3 *A. spica-venti* biotypes from the Czech Republic compared to susceptible standard. Growth reduction of 50% could not be achieved for some resistant biotypes even at very high application doses of some herbicides (more than 100-times higher than the recommended dose).

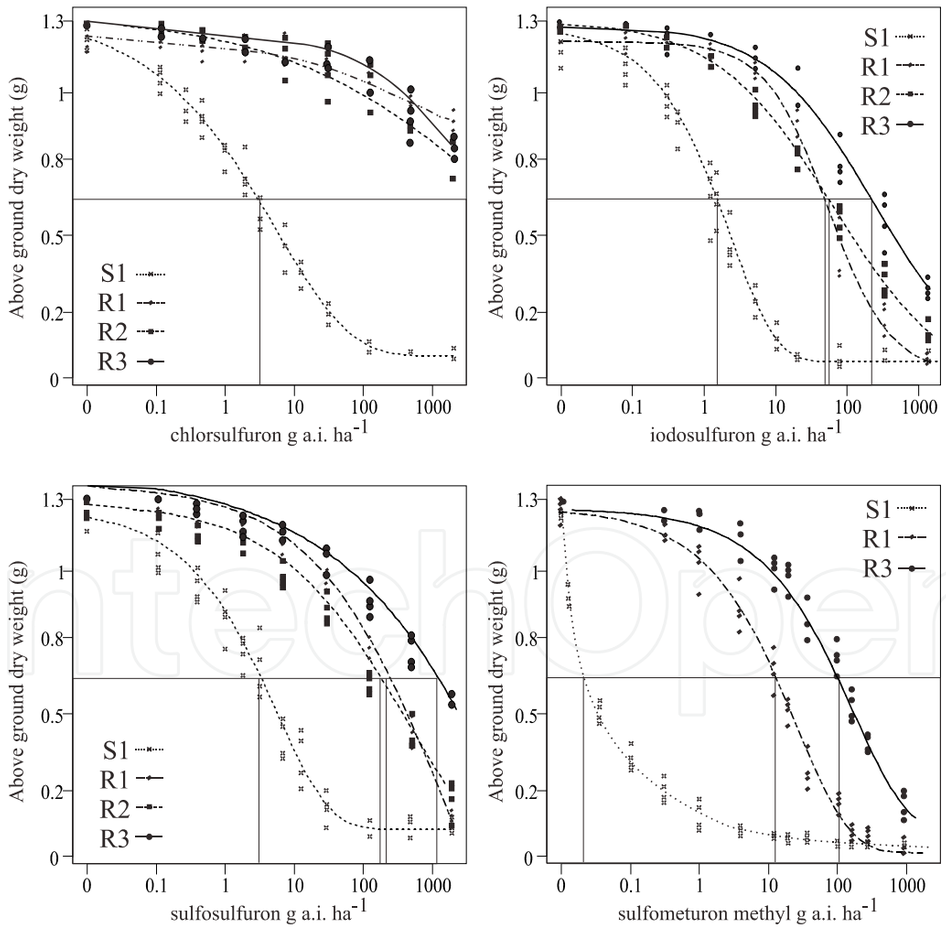

Fig. 2. Observed and fitted values of chlorsulfuron, iodosulfuron, sulfosulfuron and sulfometuron-methyl above ground dry biomass weight (g) with 3 resistant and 1 susceptible biotype of *A. spica-venti* (adapted from the paper of Hamouzová *et al.*, 2011).

***In vitro* acetolactate synthase activity**

The use of ALS *in vitro* assay is effective for determining the involvement of target-site insensitivity in conferring resistance to the herbicide studied. ALS activity was measured by estimation of the product, α-acetolactate, after its conversion to acetoin by decarboxylation in the presence of acid (Ray, 1984). The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. *A. spica-venti* seedlings were grown in pots. Seedlings were harvested 28 d after sowing when at the tree- to four-leaf stage. Two grams of leaf tissue from *A. spica-venti* were harvested and homogenised in liquid nitrogen and mixed with extraction buffer [5 mM $MgCl_2$, 50mM thiamine pyrophosphate (TPP), 1 mM flavine adenine dinucleotide (FAD), 10 mM sodium pyruvate and 126 g L^{-1} glycerol]. The mixture was homogenized during 10 min using magnetic stirrer. The homogenate was filtered through six layers of cheesecloth and centrifuged 10,000g for 15 min. The ALS was precipitated from the supernatant at 50% of $(NH_4)_2SO_4$ saturation. The mixture was stirred during 30 min after $(NH_4)_2SO_4$ addition and then centrifuged 20,000g for 20 min. The final pellet was resuspended in extraction buffer, and the enzyme was desalted on Sephadex G-25 PD-10 columns equilibrated with elution buffer (1 M potassium phosphate (KH_2PO_4 /K_2HPO_4), pH 7.5; 50 mM sodium pyruvate; 0.1 M $MgCl_2$). Acetolactate synthase activity was assayed by adding enzyme extract to freshly prepared assay buffer (1 M KH_2PO_4 /K_2H-PO_4), pH 7.5; 0.5 M sodium pyruvate; 0.1 M $MgCl_2$, 50 mM TPP; 1 mM FAD), with concentrations of herbicides increasing from 10^{-2} to 10^8 nM active ingredient. The assay mixture was incubated at 37°C for 2 h, and the reaction was terminated by the addition of 6 M H_2SO_4. Acetoin was detected as a coloured complex ALS activity was formed after addition of creatine and 1-naphtol and absorbance was measured at wavelength of 540 nm. Background was subtracted using control tubes where the reaction was stopped before the incubation. Specific enzyme activity was calculated from the standard curve and was expressed as µmol acetoin mg^{-1} protein h^{-1}. Data from experiment were fitted to the log–logistic model described above. The equations obtained were used to calculate the herbicide concentrations that reduced enzyme activity by 50% (I_{50}) of the untreated control. R/S ratios were calculated dividing the I_{50} of the R population by the I_{50} of the S population.

The data in Table 4 provide different I_{50} values for 5 putative resistant biotypes of *A. spica-venti*. Statistically significantly higher values of I_{50} proved that resistant plants have an altered form of the ALS enzyme. The level of resistance represented by RF varied significantly between the biotypes. The highest resistant ALS to chlorsulfuron was observed for the biotypes R3 and R4, while the biotypes R1 and R5 were less resistant. The isolation of ALS was similar among biotypes examined on the basis of the extractable protein, however all resistant biotypes had higher amounts of extracted leaf protein.

Molecular analysis of *ALS* gene in *Kochia scoparia* and *Apera spica-venti*

The modifications identified in naturally occurring plant populations occur in one of seven amino acids in the ALS enzyme where numbering was standardised in accordance with the *Arabidopsis thaliana* L. Heynh. sequence: Ala122, Pro197, Ala205, Asp376, Trp574, and Ser653 and Gly654 (Tranel and Wright, 2002; Powles and Yu, 2010). To date, twenty-two amino acid substitutions at all domains have been identified (Yu *et al.*, 2010). Other mutation sites that give resistance have been created in the laboratory but are not known to occur naturally. The most commonly encountered mutations in weed grasses involve the residues of proline

Biotype	B	C	D	I_{50} (nM)	RF	ALS specific activity (µmol acetoin mg^{-1} protein h^{-1})	Protein concentration (µg protein mg^{-1} fresh weight)
S1	0.441	0	1	30.39	-	33.09	1.29 ± 0.13
S2	0.515	0	1	68.51	2.25	27.80	1.16 ± 0.19
R1	0.280	0	1	1318.23	43.37	38.34	1.45 ± 0.37
R2	0.366	0	1	3815.71	125.55	38.89	1.39 ± 0.22
R3	0.245	0	1	119514.82	3932.70	30.20	1.34 ± 0.18
R4	0.380	0	1	5160.17	169.79	38.95	1.46 ± 0.38
R5	0.420	0	1	1830.42	60.21	44.77	1.52 ± 0.20

S – susceptible standard, R1-R5 – resistant biotypes, B – slope of the curve around I_{50}, C – minimum ALS activity, D – maximum ALS activity, I_{50} – concentration of chlorsulfuron that causes 50 % inhibition of ALS activity related to the maximal activity reached, RF – resistance factor = I_{50} R/ I_{50} S,

Table 4. Parameters of the log-logistic dose-response model describing the ALS-activity in *Apera spica-venti* as affected by chlorsulfuron (adopted from the paper of Nováková *et al.*, 2006).

at position 197 and tryptophan at position 574 (Yu *et al.*, 2010). So far, three mutations have been described in *A. spica-venti* (Thr, Ala and Ser) at Pro197 and one mutation (Leu) at Trp574 (Balgheim *et al.*, 2007; Hamouzová *et al.*, 2011) and His substitution at Arg377 (Massa *et al.*, 2011).

Mutations occurring at these sites confer different resistance patterns. Substitutions at Ala122 and Ala205 mostly result in resistance to imidazolinone herbicides. The mutation at P197 as well as a different amino acid change has been reported in sulfonylurea-resistant biotypes, Asp376 is responsible for resistance to all classes of ALS inhibitors. It was first shown that the mutation in position 574 confers resistance to both sulfonylureas and imidazolinones using the mutated gene generated by site-directed mutagenesis (Hand *et al.*, 1992). On the other hand, a mutation of Ser653 was first found in imidazolinones-resistant *Arabidopsis* (Haughn and Somerville, 1990), with evolving resistance to pyrimidinylthiobenzoates as well and Gly654 are associated with resistance to imidazolinones solely (Tranel *et al.*, 2010).

It was found that higher plants have a variable number of ALS genes varies from one in *Arabidopsis* (Mazur *et al.*, 1987) to five in *Brassica napus* (Rutledge *et al.*, 1991; Oullet *et al.*, 1992), mainly depending on the level of ploidy: diploid *Arabidopsis thaliana* has one constitutive copy; allotetraploid *Nicotiana tabacum* has two unlinked ALS loci (Keeler *et al.*, 1993); *Zea mays* has two very similar ALS isozymes (Fang *et al.*, 1992); *Brassica napus*, an amphidiploid of *B. campestris* and *B. oleracea*, has five ALS genes. No introns have yet been found in ALS genes.

To determine the molecular basis of resistance, a highly conserved region of the ALS gene containing potential mutation sites was amplified, sequenced and compared between the R and S biotypes. For better targeting of resistant plants for DNA analysis, shoot material of survivors from the R biotypes, following 500 g ha^{-1} sulfomethuron-methyl treatment, were used for DNA extraction. In *A. spica-venti*, it was conferred substitution of alanine in the R biotype for proline in the susceptible biotype at amino acid position 197 (Figure 3). Mutation was found in 10% of tested biotypes. The existence of resistant plants without a mutation at the domains A, B, and D suggests that other mutation(s) can be involved in resistance to

sulfonylureas. In some biotypes, mechanism of resistance is probably enhanced metabolism. The mechanism of resistance in the tested populations is not fully clear at present. The complete sequence of the *als* gene of a resistant biotype was gained (GenBank accession number JN646110).

Domain A

	191												203
D36-S	Ala	Ile	Thr	Gly	Glu	Val	**Pro**	Arg	Arg	Met	Ile	Gly	Thr
D36-S	GCC	ATC	ACG	GGG	CAG	GTT	**CCC**	CGC	CGC	ATG	ATC	GGC	ACG
D75-R	-	-	-	-	-C	-	**GCC**	-T	-	-	-A	-	-C
D75-R	-	-	-	-	-	-	**Ala**	-	-	-	-	-	-

Domain B

	593			596
D36-S	Glu	Trp	Glu	Asp
D36-S	CAG	**TGG**	GAG	GAC
D75-R	-	-	-	-

Domain D

	205					210
D36-S	Ala	Phe	Gln	Glu	Thr	Pro
D36-S	GCC	TTC	CAA	GAG	ACG	CCC
D75-R	-	-	-G	-	-	-G

Fig. 3. Nucleotide sequence and inferred amino acid sequences of ALS gene Domain A, B and D from chlorsulforon-resistant (R) and -susceptible (S) *A. spica-venti* biotypes. The codon for amino acid 197 is indicated in bold type. – indicates identical codon to the consensus sequence.

9. Cross resistance

The cross resistance is usually defined as the mechanism (target-site based or non-target-site based) that endows the ability to withstand herbicides from the same of different chemical classes with similar mode of action. We are talking about multiple resistance when a population is resistant to more than one herbicides from different chemical classes (Hall *et al.*, 1994).

As mentioned above, not all ALS-resistant weed biotypes are cross-resistant to all classes of ALS-inhibiting herbicides (Tranel and Wright, 2002) and variable patterns of cross-resistance between ALS-inhibitor classes occur depending on the ALS amino acid position affected and the specific substitution (Shaner, 1999). Some populations of blackgrass and wild oat found in the United Kingdom showed multiple resistance to chlorotoluron, chlorsulfuron, diclofop-methyl, fluazifop-butyl, imazamethabenz-methyl, pendimethalin, simazine, tribenuron and triallate herbicides (Moss, 1990; De Prado and Franco, 2004).

Varying, but very high levels of resistance to chlorsulfuron, sulfosulfuron and iodosulfuron were found, and cross-resistance to all three herbicides was confirmed in *A. spica-venti* from the Czech Republic (Figure 2, Table 5). The resistance to other sulfonylureas was confirmed in the same biotypes. The cross-resistance pattern was obtained by sulfonylurea and triazolopyrimidine herbicides (pyroxsulam), but not by imazethapyr-NH_4 while

imidazolinones controlled *A. spica-venti* completely. The only population resistant to imidazolinones did not show resistance to other sulfonylureas. All the biotypes tested showed high susceptibility to the ACCase herbicides and PS 2 inhibitors. These results suggested the potential use of the ACCase inhibiting herbicides, as well as isoproturon, in control of ALS resistant biotypes of *A. spica-venti*.

Biotype	Active ingredient and dose							
	Chlorsulfuron 15 g a.i. ha^{-1}	Iodosulfuron 10 g a.i. ha^{-1}	Sulfosulfuron 20 g a.i. ha^{-1}	Mesosulfuron + iodosulfuron 18 g a.i. ha^{-1}	Pyroxsulam 10 g a.i. ha^{-1}	Pinoxaden 30 g a.i. ha^{-1}	Fenoxaprop-P-ethyl 69 g a.i. ha^{-1}	Isoproturon 1000 g a.i. ha^{-1}
1	85	97	90	98	97	100	99	100
2	70	98	99	98	98	99	98	100
3	30	50	55	70	60	100	100	100
4	10	100	91	100	95	100	100	100
5	90	100	100	100	100	100	100	100
6	0	40	20	80	25	100	99	100
7	0	40	25	60	20	100	98	100
8	40	60	40	92	20	100	100	100
9	10	90	5	99	40	100	100	100
10	0	100	98	100	60	100	100	100
S standard	90	95	91	99	98	100	98	100
R standard	40	25	40	70	40	100	94	100

Table 5. Multiple-resistance pattern of *A. spica-venti* tested in whole plant experiment expressed as efficacy (% biomass reduction vs. untreated) 30 days after the treatment.

10. Relative fitness of the resistant and susceptible biotypes

The fitness is often described as an ability of biotype to survive and reproduce in an environment that may or may not include herbicide treatment (Nandula, 2010). Several studies have been taken to assess the differences in resistant and susceptible biotypes to ALS inhibitor with no clear results. Some of them identify significant differences in biomass production, seed production and competitiveness (Christoffoleti *et al.*, 1997), others did not find such differences (Eberlein *et al.*, 1999). The interpretation of the plant fitness comparison is very difficult and the differences may be caused by genetic polymorphism of the biotype rather than by resistance mutation.

Vila-Aiub *et al.* (2009) reviewed that certain gene mutations endowing target site-based herbicide resistance have adverse pleiotropic effects on plant growth and fitness. It is likely that some mutations would impair ALS functionality, and was proven that resistant mutations resulted in higher extracted ALS activity, slightly increased enzyme kinetics and

markedly increased sensitivity to feedback inhibition by branched chain amino acids (Yu *et al.*, 2010).

Park *et al.* (2004) observed no differences in competitive ability between resistant and susceptible biotypes of *Bromus tectorum* and it appears that ALS-resistance trait is not associated with growth reductions.

Soukup *et al.* (2006) observed that *Apera* seeds germinated well after maturation, but during after-ripening the germination increased linearly to mid-October (60 %). No differences in germination were found between the resistant and sensitive populations during after-ripening, later the resistant biotype showed slightly lower germination than the susceptible one (Figure 4). Primary dormancy was short and the seeds germinated soon after harvest. Buried seeds are persistent in the soil for only a short time (1 - 4 years) *A. spica-venti* emerges mainly in autumn, best from a depth of a few millimetres, especially under conditions of warm and rainy weather.

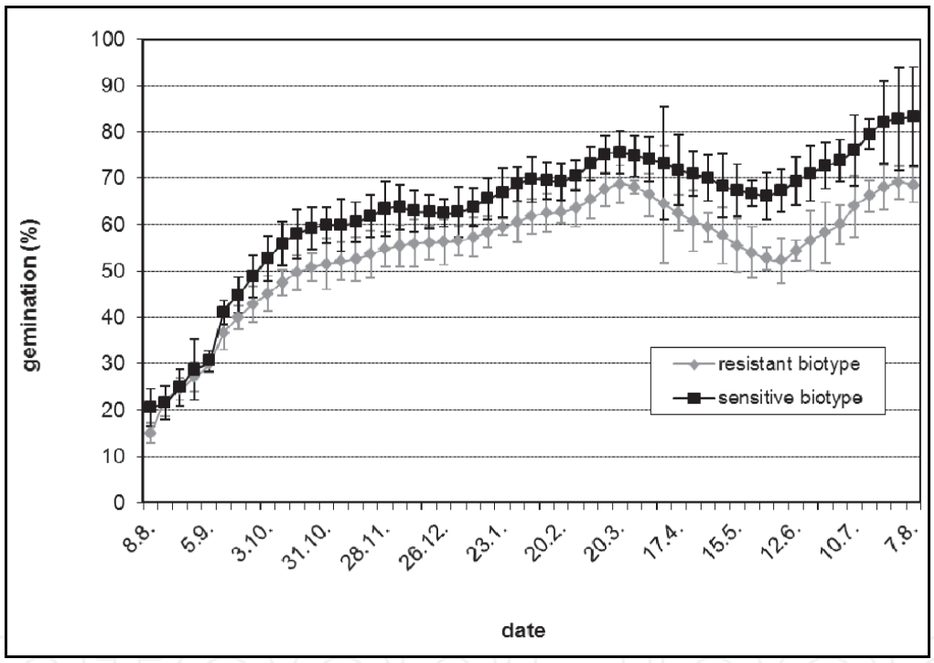

Fig. 4. Germination and loss of primary dormancy of the ALS-resistant and -susceptible biotype of *A. spica-venti* seeds under controlled conditions (20°C, 12 h light) during one year (adopted from the Ph.D. thesis of Náměstek, 2008).

11. Localities with occurrence of resistant biotypes

A. spica-venti occurs frequently in the west and central part of the Czech Republic. Soukup *et al.* (2006) referred that *A. spica-venti* occurs regularly in 80 % of all observed localities and increasing harmfulness was declared by more than 30 % of the inquired farmers and problems with lower efficacy was noticed on 20 % of farms (150 respondents in total). There is no clear pattern of resistance in *Apera* throughout the country (Figure 5); the rapid emergence of the resistant *A. spica-venti* populations was attributed to the lack of herbicide rotation.

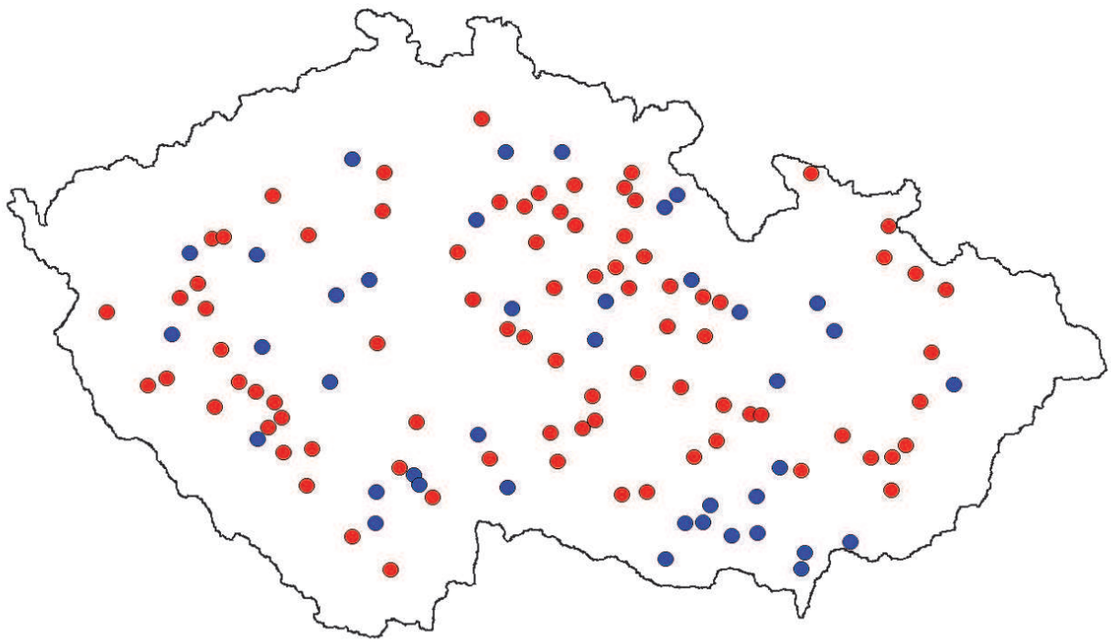

Fig. 5. Map of the areas infested with chlorsulfuron-resistant *A. spica-venti* biotypes (in red), data are not derived from the random sampling but based on poor weed control observed by farmers.

12. Forecast of occurrence and level of resistance

The continuous spread of *A. spica-venti* sulfonylurea resistance has been observed since 2003, it can be also attributed to the increasing interest in this grass weed. As seen from Figure 5, the problem is widespread throughout the whole country, but the question how rapidly resistance is spreading was not satisfactorily discussed up to now. During the first years, herbicide resistant plants are present at small numbers and can be incorrectly recognized as escapes. There are suggestions that 30% of a population must be resistant before it is recognized at the field level (Nandula, 2010). At this time, the soil seed bank is established and the control against the resistant weed must be managed integrally. Nevertheless, it may be slowed by using alternative herbicides with more effective modes of action.

Detailed studies on the biochemical basis of resistance in populations of *A. spica-venti* were not undertaken. The recorded actual levels of resistance differed significantly from relatively low (1-fold) and extremely high levels (>200-fold) in terms of resistance factors. These showed that enhanced metabolism existed in some populations but ALS target site resistance occurred in others. From the series of studies, enhanced metabolism could be concluded as the major mechanism of resistance, but target site resistance or mixed resistance (exhibiting both mechanisms) is also present (Hamouzová *et al*., 2011).

13. Means of control of resistant biotypes

Modern herbicides must provide effective control of *A. spica-venti*, as this is one of the most competitive weeds present in winter cereals in Central and North-west Europe and, if not appropriately controlled, causes serious yield losses in cereal crops. Unfortunately, one of the newest and favourite herbicide groups, the sulfonylureas, is losing its effect due to frequent use. The evolution of resistance against sulfonylureas cannot be underestimated. Their market share is still increasing, and eight new active ingredients have been introduced in the last seven years. Sulfonylureas (chlorsulfuron, iodosulfuron, sulfosulfuron), and ureas (isoproturon, chlortoluron) which create together 80 % of the total usage of herbicides are the most and long-term used herbicides against *A. spica-venti*. On many farms with product failures, there is a tendency to replace sulfonylureas with other herbicides. Early post-emergence herbicides include diflufenican, flufenacet, isoproturon, pendimethalin and prosulfocarb. For later post-emergence application, preferred in integrated weed management, there are only a few products available with different modes of action. ACCase inhibiting herbicides at the moment provide an alternative for resistant biotypes control in winter wheat, but these must be combined with active ingredients against dicotyledonous weeds. The PS2 inhibiting herbicides seem to be effective as well. It is predicted that isoproturon-based products will be restricted soon. It is unlikely that any herbicides based on completely new modes of action will become available for grass weed control within the next few years. The reliance on one herbicide and a shift to another one cannot fully solve the resistance problem.

Crop rotation is considered an effective strategy to manage weed species, in long-term management of particular field, can create unfavourable conditions for a specific weed species and sustain the weed control.

The development of strategies to prevent and manage herbicide resistance should be approached by integrating knowledge from population and evolutionary biology into weed science. Reduction of selection pressure is the main goal of resistance management strategies that rely on herbicide rotations, mixtures and sequences (Neve, 2007).

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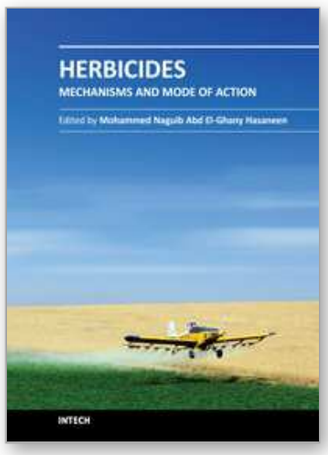

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This volume contains two sections: Mechanisms of herbicidal action (chapters 1-4) and Mode of action of selected herbicides on controlling diseased, weed growth and productivity and/or growth and development of field crops (chapters 5-10). Topics by chapters are: molecular mechanism of action, immunosensors , laboratory studies, molecular modeling, weed resistance, community response, use of herbicides in biotech culture, gene flow, herbicides and risk, herbicides persistence. These recurring themes reinforce my view, held over a very long time, that experience with one crop or problem can sometimes be relevant, often to an unexpected extent, to an apparently dissimilar situation in a different crop. I hope that readers interested in herbicides and pesticides will be satisfied with all the chapters in the book as its content might be of interest and value to them in the future.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

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