THE PRESENT STATE OF HERBICIDE RESISTANCE OF WEED POPULATIONS IN THE CZECH REPUBLIC

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Abstract: In 1985–2002 thirteen weeds resistant to atrazine were selected by a repeated application of triazine herbicides on arable land, in orchards, non-agricultural land and at railways in the Czech Republic. Recently *Digitaria sanguinalis* biotypes resistant to atrazine have been found at three railway junctions. Long-lasting application of the active ingredient imazapyr at railways caused selection of resistant *Kochia scoparia* biotypes. High resistance to chlorsulfuron has been discovered in five *Apera spica-venti* biotypes originating in winter cereals fields. The molecular basis of resistance to atrazine has been identified in the following weeds: *Kochia scoparia*, *Solanum nigrum*, *Senecio vulgaris*, *Conyza canadensis*, *Digitaria sanguinalis*, *Amaranthus retroflexus* and *Chenopodium album*. The resistance was conferred by a glycine for serine substitution at residue 264 of the D1 protein in all of those weeds. The resistance to imazapyr in Czech *Kochia scoparia* biotypes was conferred by a mutation at codon 574 of the ALS gene. Analysis of the results of DNA sequencing indicated, that the mutation induced a leucine for tryptophane substitution. There was excellent correspondence between the phenotypic resistance to herbicides of individual plants and the presence of mutations.

Key words: triazines, ALS inhibitors, *psb*A gene, ALS gene, mutation, PCR, sequencing

INTRODUCTION

   Thirteen weed species resistant to atrazine have been developed in the Czech Republic (Mikulka and Chodová 2002) as a result of long-term application of triazine herbicides. Most of resistant weeds were found at railways. Triazine herbicides were used at the Czech railways in the 1980s and 1990s and then from 1997 to 1999. Long-term use of imazapyr at the Czech railways led to selection of 4 kochia [*Kochia scoparia* (L.) Schrad.] biotypes resistant to acetolactate synthase (ALS)-inhibiting herbicides

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(Mikulka and Chodová 2002; Salava *et al.* 2004; Chodová and Salava 2004b). Recently, resistance to chlorsulfuron has been detected in silky bent-grass [*Apera spica-venti* (L.) P. Beauv] by Nováková *et al.* (2006) and a biotype of large crabgrass (*Digitaria sanguinalis*) resistant to atrazine has been detected by Salava *et al.* (2006a, 2006b).

The aim of the work presented is to summarize information on the occurrence and research on the resistance of weeds to herbicides in the Czech Republic.

MATERIALS AND METHODS

A number of methods are used for identification of weeds resistant to herbicides in the Czech Republic.

Whole-plant response assay

The plants arisen from seeds of unknown susceptibility to the herbicide are grown to the stage of 1–4 right leaves and they are treated with graduated doses of the herbicide. Phytotoxic symptoms are rated individually according to the European Weed Research Society (EWRS) classification scheme for plant tolerance (Anonymous 1992).

Chlorophyll fluorescence assay

The method is based on the fact that the function of photosystem 2 in weeds susceptible to triazine herbicides is disturbed in the presence of triazines and fluorescence parameters change (Chodová *et al.* 1995; Nováková *et al.* 2005; Salava *et al.* 2006a, 2006b, 2006c). Fully expanded leaves are incubated in atrazine solutions, fluorescence induction curves as well as the fluorescence parameters are recorded with a chlorophyll fluorometer (Ahrens *et al.* 1981).

Photochemical activity of isolated chloroplasts

The photochemical activity of chloroplasts of susceptible plants is disturbed in the presence of triazines (Kočová *et al.* 1988; Körnerová *et al.* 1998). The photochemical activity is measured polarographically as the Hill reaction activity and characterized as the amount of oxygen formed by the chloroplast suspension in defined conditions after the white light irradiation and addition of the electron acceptor (Holá *et al.* 2004).

In vivo acetolactate synthase activity

The specific activity of ALS is measured by *in vivo* ALS assays (Sprague *et al.* 1997). This assay is based on detection of acetoin accumulation in plant tissue treated with either a KARI (ketolacid reductoisomerase) inhibitor alone or a KARI inhibitor (CPCA – 1,1-cyclopropanedicarboxylic acid) plus an ALS inhibitor. The accumulated acetolactate is converted to acetoin, which can be quantified colorimetrically.

Molecular analysis

DNA is extracted from the apical portion of individual plants using the DNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions. The herbicide binding region of the *psb*A gene and two regions of the ALS gene (Domain A and B), respectively, are PCR-amplified and sequenced for detection of mutations. Sequencing is performed directly on PCR products. Sequence editing and analysis is done

using the program BLAST (Altschul *et al.* 1997). The ExPASy translate tool (Gasteiger *et al.* 2003) is used to determine the peptide sequences.

RESULTS AND DISCUSSION

Fourteen weeds resistant to atrazine were selected by a long-term application of triazine herbicides on arable land, in orchards, non-agriculture land and at railways in the Czech Republic: *Amaranthus retroflexus, Chenopodium album, Amaranthus powellii, Polygonum lapathifolium, Conyza canadensis, Senecio vulgaris, Echinochloa crus-galli, Polygonum persicaria, Setaria viridis, Chenopodium strictum, Chenopodium pedunculare, Poa annua, Solanum nigrum* (Mikulka and Chodová 2002; Chodová *et al*. 2004) and *Digitaria sanguinalis* (Salava *et al.* 2006 a, 2006b).

The effect of atrazine on susceptible and resistant plants is presented in Table 1. All tested plants from the resistant biotypes remained green and undamaged after the treatment with atrazine. The resistance was verified by measuring the Hill reaction activity in the presence of atrazine. Susceptible plants showed 0–17% of the photochemical activity of chloroplasts of the control, resistant plants did 62–89%.

Table l.   Comparison of Czech atrazine resistant and susceptible biotypes of seven weed species with respect to results of whole-plant response assay, Hill reaction activity, chlorophyll fluorescence assay and the presence of mutation in *psb*A gene

Species	Whole-plant response assay[a]		Hill reaction activity[b]		Chlorophyll fluorescence assay[c]		Mutation at codon 264 of *psb*A gene[d]		
	S	R	S	R	R	S	R	S	literature cited
Solanum nigrum	9	1	not tested		–	+	+	–	[1]
Kochia scoparia	9	1	16.9	88.8	–	+	+	–	[2]
Senecio vulgaris	9	1	8.1	81.3	–	+	+	–	[3]
Conyza canadensis	9	1	0	65.2	–	+	+	–	[4]
Amaranthus retroflexus	9	1	0	80.3	–	+	+	–	[5]
Digitaria sanguinalis	9	1	0	61.7	–	+	+	–	[6]
Chenopodium album	9	1	0	62.1	–	+	+	–	[7]

S – susceptible biotype, R – resistant biotype
[a] EWRS classification scheme for plant tolerance (1–9): 1 = resistant plants (showing no symptoms/healthy plants), 9 = susceptible plants (heavy damage to total kill)
[b] % of control
[c] after application of atrazine: changes compared to control (+), not changed (–)
[d] mutation: detected (+), not detected (–)
[1] Salava *et al*. 2004a
[2] Chodová and Salava 2004a
[3] Nováková *et al*. 2005
[4] Salava and Chodová 2006b
[5] Salava *et al*. 2006c
[6] Salava *et al*. 2006a, 2006b
[7] Salava *et al*. (not published)

Triazine resistance of the weed biotypes was also confirmed by *in vivo* measurement of chlorophyll fluorescence emitted by leaves treated with atrazine (Table 1). The results obtained for *Digitaria sanguinalis* are presented in Figure 1.
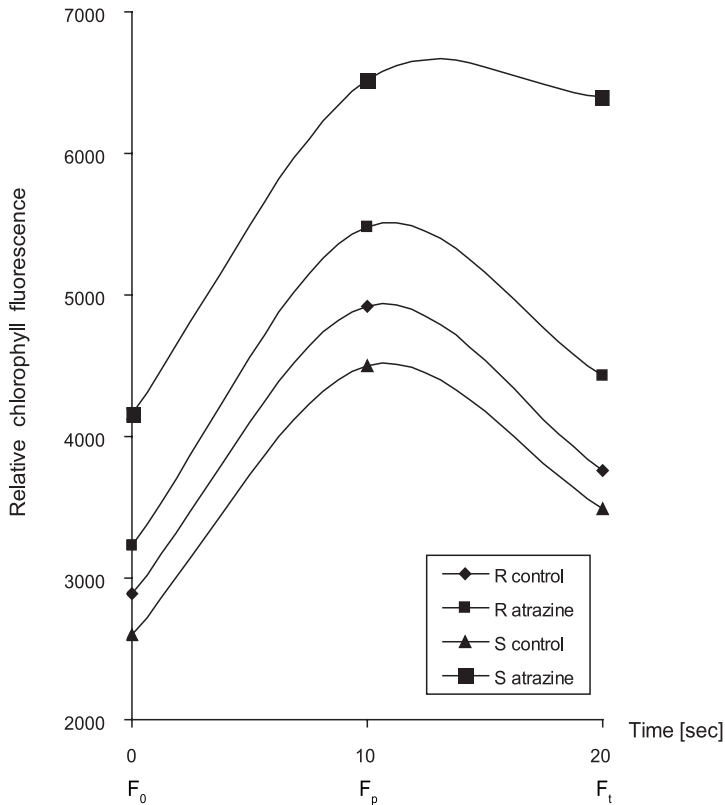

Fig. 1. Curves of slow chlorophyll fluorescence induction of *Digitaria sanguinalis* atrazine susceptible biotype (F_0 = 2597, F_p = 4503, F_t = 3492) and resistant biotype (F_0 = 2886, F_p = 4921, F_t = 3756) in the untreated control and after application of 10^{-2}M atrazine (susceptible biotype F_0 = 4158, F_p = 6520, F_t = 6396) and resistant biotype (F_0 = 3227, F_p = 5480, F_t = 4434). The value F_0 was recorded in 0 sec, F_p in 10 sec and F_t in 20 sec. [F_0-origin (basic) relative fluorescence, F_p-peak fluorescence, Ft-terminal fluorescence]

After atrazine treatment, leaves from the resistant biotypes did not exhibit any changes in the fluorescence curve pattern, when compared with untreated control leaves, and points of the peak and terminal fluorescence could be clearly distinguished. Leaves from the susceptible biotypes showed typically changed fluorescence curve patterns without distinguishable points of the peak and terminal fluorescence.

In almost all cases, a Ser_{264} to Gly mutation in the D1 protein is responsible for conferring resistance to photosystem 2 (PS 2) inhibitors in weed biotypes (Gronwald 1994). Therefore a region of the *psb*A gene that encodes for amino acids 163 to 329 of the D1 protein was sequenced. Sequences from atrazine susceptible and resistant biotypes differed by a single nucleotide substitution at the variable Ser codon (AGT

to GGT) at position 264, predicting a Ser in the susceptible but a Gly in the resistant biotype. This amino acid change causes a decreased binding affinity of triazine herbicides to the D1 protein of PS 2, and thus assures an uninterrupted electron transport from PS 2 to the quinone pool in the presence of the herbicide. A Ser to Gly substitution has previously been shown to encode a resistant D1 protein in many species (Hirschberg and McIntosh 1983; Goloubinoff and Edelman 1984; Blyden and Gray 1986; Foes *et al.* 1998, 1999). The DNA sequence and the deduced amino acid sequence of the *psb*A gene fragment from *Digitaria sanguinalis* atrazine susceptible biotype are shown in Figure 2.

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S   1   D   G   M   P   L   G   I   S   G   T   F   N   F   M   I   V   F   Q   A   E
S   1   GATGGTATGCCTCTAGGAATCTCTGGTACTTTCAACTTTATGATCGTATTCCAGGCTGAG
R       ....................................................

S  21   H   N   I   L   M   H   P   F   H   M   L   G   V   A   G   V   F   G   G   S
S  61   CACAACATCCTTATGCACCCATTTCACATGTTAGGTGTAGCTGGTGTATTCGGCGGCTCC
R       ....................................................

S  41   L   F   S   A   M   H   G   S   L   V   T   S   S   L   I   R   E   T   T   E
S 121   CTATTTAGTGCTATGCATGGTTCCTTGGTAACTTCTAGTTTGATCAGGGAAACCACAGAA
R       ....................................................

S  61   N   E   S   A   N   E   G   Y   R   F   G   Q   E   E   E   T   Y   N   I   V
S 181   AATGAATCTGCTAACGAAGGTTACAGATTCGGTCAAGAGGAAGAAACTTATAACATCGTA
R       ....................................................

S  81   A   A   H   G   Y   F   G   R   L   I   F   Q   Y   A   [S]   F   N   N   S   R
S 241   GCTGCTCATGGTTATTTTGGTCGATTGATCTTCCAATATGCT[AGT]TTCAACAACTCTCGT
R       ........................................[G.].................

S 101   S   L   H   F   F   L   A   A   W   P   V   I   G   I   W   F   T   A   L   G
S 301   TCTTTACACTTCTTCTTAGCTGCTTGGCCTGTAATCGGTATTTGGTTTACTGCTTTGGGT
R       ....................................................

S 121   I   S   T   M   A   F   N   L   N   G   F   N   F   N   Q   S   V   V   D   S
S 361   ATTAGTACTATGGCTTTCAACCTAAACGGTTTCAACTTCAACCAATCTGTAGTTGATAGT
R       ....................................................

S 141   Q   G   R   V   I   N   T   W   A   D   I   I   N
S 421   CAAGGTCGTGTAATTAACACCTGGGCTGATATCATTAAC
R       ......................................
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Fig. 2. Comparison of nucleotide sequence of the 459 bp fragment from resistant and susceptible biotypes of *Digitaria sanguinalis* using susceptible (S) as a reference. Dots in the resistant (R) sequence indicate matches to the reference sequence; differences indicated by A, C, G or T. Bold print in the amino acid sequence indicates the site where the mutation confers atrazine resistance. The boxed codon is the location of the single nucleotide substitution (GGT) encoding Gly in the resistant sequence and is the only amino acid difference predicted between the two sequences

Resistance of kochia to imazapyr was proved by biological, *in vivo* ALS assays and by a mutation in the ALS gene (Table 2). The two Czech kochia biotypes (Praha-Bubny and Praha-Libeň) displayed a high level of resistance to ALS-inhibiting herbicides in *in vivo* ALS enzyme assays, indicating that resistance to these herbicides was site-of-action mediated. Two regions of the ALS gene (Domain A and B) were PCR-amplified

and sequenced for detection of mutations. There was one nucleotide polymorphism between the alleles from the susceptible and resistant biotypes. The polymorphism conferred a substitution of leucine in the resistant biotype for tryptophan in the susceptible biotype at position 574. This mutation has been shown previously to confer resistance to ALS inhibitors in kochia (Foes *et al.* 1999) and in other species (Woodworth *et al.* 1996; Foes *et al.* 1998; Boutsalis *et al.* 1999; Patzoldt *et al.* 2002). Restriction enzyme analysis of the polymerase chain reaction products was used to confirm results of the sequence analysis (data not shown). All kochia biotypes involved in this study had excellent correspondence between the presence of the mutation and herbicide resistance (Table 2). Rapid and reliable assays based on restriction analysis of PCR products for the detection of mutations in the *psb*A and ALS genes in populations of weeds were developed. These assays are less labour-intensive and much faster than conventional field or greenhouse testing.

Table 2. Comparison of Czech imazapyr resistant and susceptible biotypes of kochia with respect to results of whole-plant response assay and presence of mutation in ALS gene

Locality	Whole-plant response assay[a]	Mutation at codon 574 of ALS gene[b 1]
Prague-Bubny	1	+
Prague-Libeň	1	+
Prague-Vršovice	1	+
Olbramovice	9	–
Prague-Invalidovna	9	–
Prague-Karlín	9	–
Prague-Žižkov	9	–
Jihlava	1	+

[a] EWRS classification scheme for plant tolerance
(1–9): 1 – resistant plants (showing no symptoms/healthy plants)
     9 – susceptible plants (heavy damage to total kill)
[b] mutation: detected (+), not detected (–)
[1] Salava *et al*. 2004

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POLISH SUMMARY

OBECNY STAN ODPORNOŚCI NA HERBICYDY POPULACJI CHWASTÓW W REPUBLICE CZECH

W latach 1985–2002 wyselekcjonowano w Republice Czech 13 gatunków chwastów odpornych na atrazynę stosując powtarzające się zabiegi herbicydami triazynowymi na polach uprawnych, w sadach, na terenach nierolniczych i torach kolejowych. Ostatnio wykryto biotypy *Digitaria sanguinalis* odporne na atrazynę na trzech rozjazdach kolejowych. Długotrwałe stosowanie składnika aktywnego imazapyr na torach kolejowych spowodowało wyselekcjonowanie się odpornych biotypów *Kochia scoparia*. W pięciu biotypach *Apera spica-venti* pochodzących z upraw pszenicy wykryto wysoką odporność na chlorsulfuron. Molekularną bazę odporności na atrazynę określono u następujących gatunków chwastów: *Kochia scoparia*, *Solanum nigrum*, *Senecio vulgaris*, *Conyza canadensis*, *Digitaria sanguinalis*, *Amaranthus retroflexus* i *Chenopodium album*. Odporność była uwarunkowana zamianą seryny na glicynę w szczątkowym elemencie 264 białka D1 wszystkich wymienionych chwastów. Odporność na imazapyr w czeskich biotypach *Kochia scoparia* była uwarunkowana mutacją w kodonie 574 genu ALS. Analiza wyników sekwencjonowania DNA wskazywała, że w wyniku mutacji nastąpiło zastąpienie tryptofanu leucyną. W ramach fenotypowej odporności na herbicydy stwierdzono bardzo dobrą zgodność odporności u wszystkich gatunków roślin a obecnością mutacji.