Tolerance of Blackgrass (*Alopecurus myosuroides*) to Sulfonylurea Herbicides in the Czech Republic

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Abstract

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Seeds of three blackgrass populations from Southern Bohemia were collected in 2007–2008. Biological tests with chlorsulfuron were performed at the 2–3 leaf stage. Some plants survived after treatment with the highest dose 37.5 g/ha. Biological tests showed a resistant phenotype to chlorsulfuron. Leaves of these plants were analysed by dCAPS assay. Two domains of ALS gene: domain A – P197 and domain B – W574 were targeted by PCR with regenerated primers P197 containing BamHI site and W574 containing site BstXI. PCR products of all tested samples were cleaved by BamHI in the codon P197. No mutation of proline in P197 was found out. The codon W574 PCR product of the samples was not cleaved by BstXI.

Keywords: *Alopecurus myosuroides*; ALS; resistance; sulfonylurea; dCAPS

Herbicide resistance can be defined as the inherited ability of a weed to survive a rate of herbicide which would normally result in effective control (Moss 2002). Acetolactate synthase (ALS), also referred to as acetohydroxyacid synthase (AHAS), is the first enzyme in the biosynthesis of the branched chain amino acids isoleucine, valine and leucine (Sibony *et al*. 2001). Herbicides that target the acetolactate synthase (ALS) enzyme are among the most widely used in the world. The ALS-inhibiting herbicides are classified into four distinct classes at least: sulfonylureas (SU), imidazolinones (IMI), pyrimidinylsalicates (PC), and triazolopyrimidines (TP) (Shimizu *et al*. 2002). Herbicide resistance to ALS inhibitors is caused by the substitution of an amino acid on codon A122, P197, A205, W574 or S653 (Tranel & Wright 2002). Substitutions of Ala122 or Ser653 result in IMI but not SU resistance, whereas the substitution of Pro197 usually results in SU but not IMI resistance. The substitution of Trp574 results in a high level of resistance to both IMI and SU (Tranel & Wright 2002). Whaley *et al*. (2006) detected a new mutation conferring resistance at position 376 (Asp to Glu) on *Amaranthus hybridus* L. Herewith herbicide resistance could be caused by another metabolism-based mechanism (Menchari *et al*. 2007).

The blackgrass (*Alopecurus myosuroides* Huds.) is a noxious, widespread grass weed of winter cereal crops in Europe (Delye & Boucansaud 2008). Selective herbicides are the main method of its control (Brown *et al*. 2002). Herbicide resistant populations to ALS inhibitors were first recorded in the UK in 1984. Over 750 cases of resistance widely distributed within 30 counties of England were confirmed. Resistant populations have also been identified in other six European countries: Belgium, France, Germany, Netherlands, Spain, and Switzerland (Moss *et al*. 2003). Up to now

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biotypes of blackgrass have been found to be resistant to ALS in France, United Kingdom, Germany, and Belgium. Blackgrass is also resistant to other herbicide groups, such as ACC inhibitors, PSII inhibitors, ureas and amides and dinitroanilines (Heap 2009).

Blackgrass resistance to chlorsulfuron has been observed in the fields in Southern Bohemia since late eighties. In this paper we describe the biological and molecular tests to confirm the resistance status of Blackgrass population from this region.

MATERIALS AND METHODS

Seeds of *Alopecurus myosuroides* Huds. were collected in 2007-2008 in localities in Southern Bohemia, namely in Žimutice (49°12'11.96"N; 14°30'37.6"E) – population Z and Olešník (49°6'25.74''N; 14°21'55.03''E) – populations O9 and O11. An experiment was carried out in containers 60 × 60 × 60 mm in size, filled with substrate consisting of 1/3 of sand and 2/3 of brown soil. Seeds sown at a 5-mm depth were grown in climate chambers (Sanyo MLR-HT 350) during a 14 h light period at 16°C and a 10 h dark period at 8°C.

Biological tests were performed in three experiments at 12–13 BBCH stage (2–3 leaves) by treatment with chlorsulfuron (content of chlorsulfuron active ingredient 75%) at 3.0, 6.0, 7.5, 12.0, 15.0, 22.5, 30.0, and 37.5 g/ha. In the first bioassay plants were treated with chlorsulfuron at 7.5, 15.0,and 22.5 g/ha. Plants were numerically evaluated weekly after treatment. Samplings were performed at 52 BBCH stage to determine fresh foliage weight (FW in g), dry weight of biomass (DW in g), variation of foliage weight and variation of dry weight. As a comparison a non-treated control was used. In the second bioassay, plants were treated with 7.5, 15.0, and 30.0 g/ha of chlorsulfuron. Samplings were performed three times (firstly, three weeks after treatment and then the second and the third sampling were performed at 10-day intervals). Three weeks after treatment the first sampling was performed and foliage weight (g), dry weight (g), reduction of foliage weight and reduction of dry weight (compared to the non-treated control) were analysed. The same characteristics were used also in the next two samplings.

In the third bioassay, plants were treated with the following doses of chlorsulfuron: 3.0, 6.0, 12.0 and 37.5 g/ha. These plants were evaluated only numerically and visually.

Molecular tests were performed by the derived cleaved polymorphic amplified sequence (dCAPS) method (Kaundun & Windass 2006). Leaf samples were collected from each individual plant and frozen at –20°C until used for DNA isolation. 100-mg leaves were ground in liquate nitrogen and total DNA was isolated using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two ALS domains A: P197 and B: W574 were targeted by dCAPS assay according to Delye & Boucansaud (2008). P197 and W574 were amplified using the primer pair P197F/P197R and W574F/WSR, respectively. A proofreading Ex*Taq* polymerase (TaKaRa, Japan) was used to limit the introduction of artificial mutations. The PCR reaction was carried out in 25 µl final volume containing 5 µl of 10× Ex*Taq* buffer (15mM $MgCl_2$), 0.2 µl of Ex*Taq* polymerase (5U/µl), 0.6 µl of forward and reverse primers (10µM), 2.5 µl of dNTPs mix (2.5mM) and 1 µl of total DNA. The PCR reaction was carried out in a thermocycler (iCycler, Biorad) as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 3 steps: 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min, the final elongation was performed at 72°C for 10 minutes. Thus, amplified DNA fragments of P197 and W574 were subjected to digestion

Table 1. dCAPS primers and restriction enzymes (modified according to Delye & Boucansaud 2008)

Target codon	Primers	Sequence (5'-3') from wild type	Tm (PCR)	Restriction enzyme	Recognition site
P197	P197F	TTCTCGACTCCATCCCGATGGTCGCTATCACGGGACAGGAT	60°C	BamHI	GGATCC
	P197R	ATCTGCTGCTGGATGTCCTTGGG			
W574	W574F	GGTGATGATACTGAACAATCAACATCTGGGAATGCCAGTGCAG	60°C	BstXI	$CCAN_6TGG$
	WSR	ATACACCAGCATCATGCTGATCAGG			

Table 2. The first bioassay – reduction or increase in foliage weight, results of three localities in Southern Bohemia, plants collected at stage 52 by BBCH scale

Locality	Dose (g/ha)	FW (g)	DW (g)	FW variation (%)	DW variation (%)
Olešník 9	CH 7.5	0.556	0.162	**+10**	**+29**
	CH 15	0.499	0.138	–1	**+10**
	CH 22.5	0.377	0.0967	–25	–23
	control	0.505	0.1253	0	0
Olešník 11	CH 7.5	0.687	0.186	-15	**+3**
	CH 15	0.57	0.16427	–30	–9
	CH 22.5	0.276	0.072	-66	–60
	control	0.81	0.1801	0	0
Žimutice	CH 7.5	0.656	0.179	**+7**	–3
	CH 15	0.482	0.17	–21	–8
	CH 22.5	0.557	0.148	–9	–20
	control	0.612	0.185	0	0

by BamHI (GGATCC) and BstXI (CCAN6TGG), respectively. Digestion was carried out at 37°C for 3 hours. dCAPS patterns were visualised by electrophoresis on 2% agarose gel. Sequences of these primers and recognition site of enzymes are shown in Table 1.

RESULTS

First bioassays

Biological tests showed that all plants survived after treatment with the highest dose (22.5 g/ha) of chlorsulfuron. Although plants were shortened, all of them were able to produce viable seeds. These plants were evaluated at stage 52 by BBCH-scale (beginning of heading). In the blackgrass population from Olešník 9, the reduction of foliage weight was 1% and 25% at the dose of chlorsulfuron 15.0 g/ha and 22.5 g /ha, respectively. The foliage weight increased by 10% at 7.5 g/ha chlorsulfuron compared with the non-treated control. In the population from Olešník 11 the foliage weight was reduced at all doses, by 15% to 66%. In the population of the third locality (Žimutice) the reduction of foliage weight was the lowest at each treatment dose (Table 2 and Figure 1).

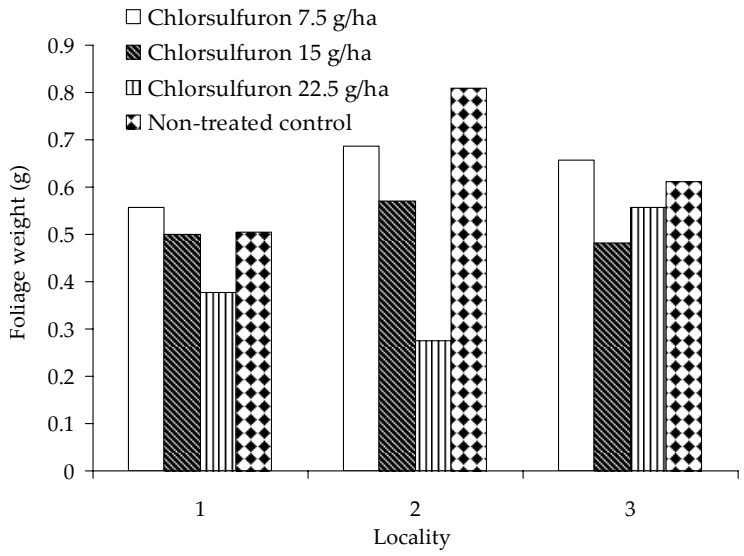


Figure 1. Foliage weight from several localities: 1– Olešník 9, 2 – Olešník 11, 3 – Žimutice

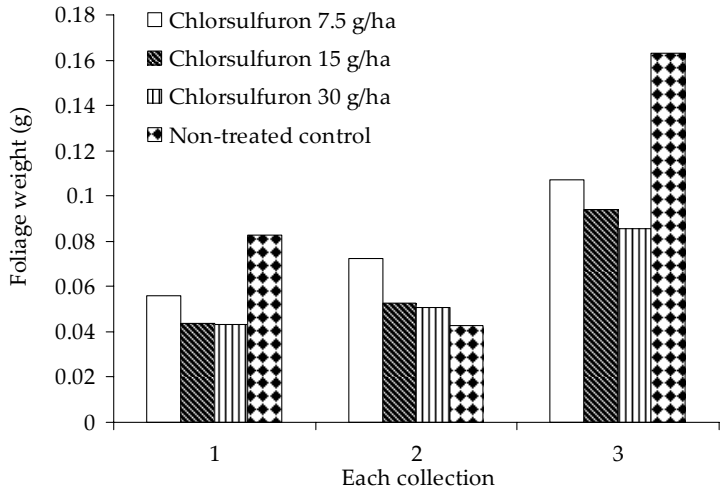


Figure 2. Foliage weight from several collection: 1– 3 weeks after treatment, 2 – 30 days after treatment, 3 – 40 days after treatment

Second bioassay

All plants from each tested blackgrass population survived the treatment with 7.5 g, 15.0 g and 30.0 g chlorsulfuron/ha. Similar results were obtained in the 1st and 3rd harvest. The reduction of foliage weight was observed for all doses. In the second harvest, the production of foliage weight was higher than that of the control (Table 3 and Figures 2 and 3).

dCAPS. The plants from all three locations surviving the highest doses of chlorsulfuron (e.g. 12.0 g, 22.5 g and 37.5 g) in each bioassay were tested for resistance. DNA of the plant samples was analysed by dCAPS. Two codons P197 and W574 were amplified from all tested samples by PCR in 238 bp-long fragments and 411 bp-long fragments, respectively. The amplified products of the codon P197 were cleaved by BamHI in expected dCAPS patterns 38 bp and 200 bp (Figure 4A). The amplified products of the codon W574 remained uncleaved by BstXI (Figure 4B).

DISCUSSION

Alopecurus myosuroides is considered to be an archeophyte in the Czech Republic. The chlorsul-

Table 3. Foliage and dry weight of *Alopecurus myosuroides* after the second bioassay, 1st collection 3 weeks after treatment, 2nd collection 30 days after treatment, 3rd collection 40 days after treatment, compared with untreated controls

Collection	Dose	FW (g)	DW (g)	FW var. (%)	DW var. (%)
1st	CH 7.5	0.0558	0.0152	–32	–20
	CH 15	0.0437	0.0137	–47	–28
	CH 30	0.0431	0.0133	–48	–30
	control	0.0825	0.0189	0	0
2nd	CH 7.5	0.0725	0.0202	**+69**	**+68**
	CH 15	0.0527	0.0169	**+23**	**+41**
	CH 30	0.0507	0.0153	**+18**	**+28**
	control	0.0428	0.012	0	0
3rd	CH 7.5	0.1072	0.0334	–34	–2
	CH 15	0.09406	0.0297	–42	–12
	CH 30	0.0857	0.02249	–47	–33
	control	0.1629	0.0339	0	0

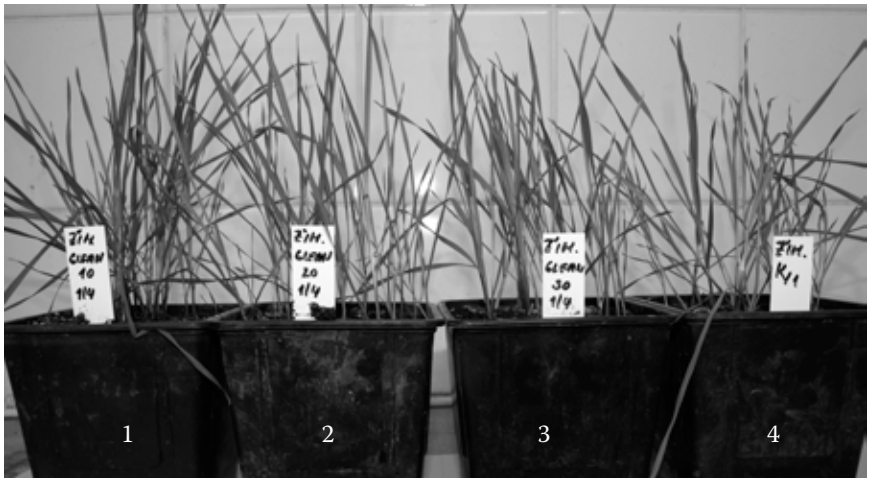
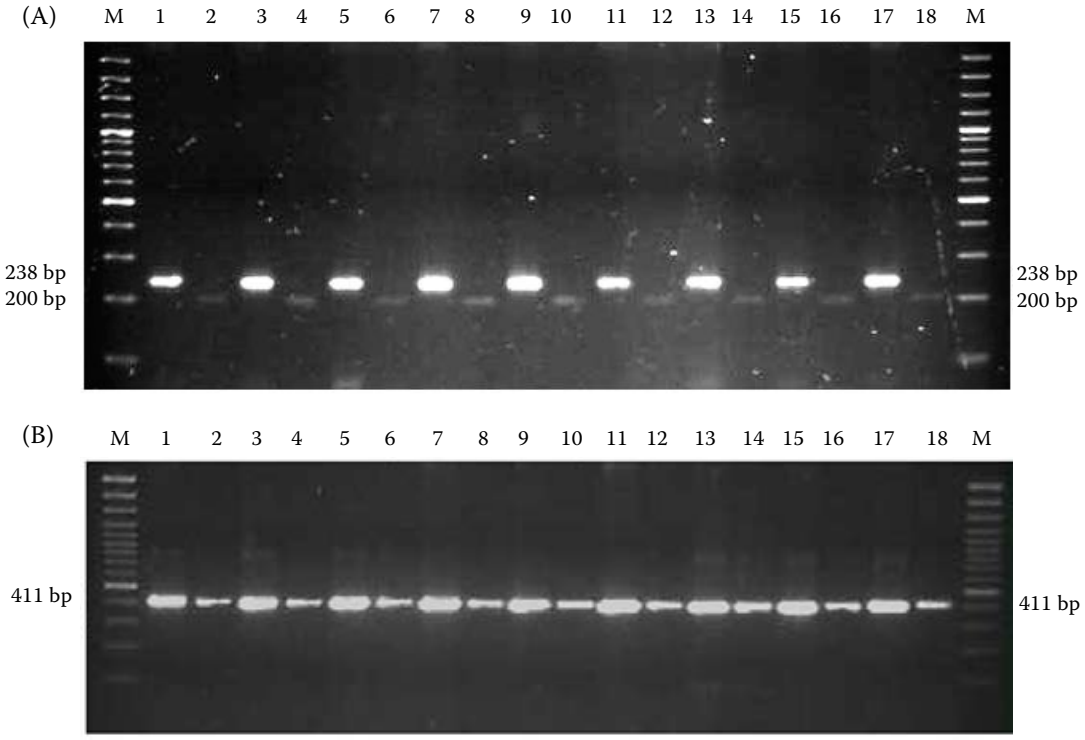


Figure 3. Foliage weight from each collection: 1 – 21 days; 2 – 30 days; 3 – 40 days after treatment; 4 – non-treated control

furon-based herbicide has been used in the Czech Republic since 1982. Several populations of blackgrass showed a resistant phenotype to chlorsulfuron. As a result of this phenomenon the farmers of many localities in Southern Bohemia stopped using chlorsulfuron for the blackgrass control. Three of such resistant populations were evaluated here by biological and molecular methods. The suggested dose (according to a herbicide register) of chlorsulfuron is 20.0 g/ha and 7.0 g/ha for



Lane M – DNA size marker 100 bp
Odd lanes – PCR products for each A. *myosuroides* population (O9, O11, Z) and doses – 12 g/ha (1, 3, 5); 22.5 g/ha (7, 9, 11); 37.5 g/ha (13, 15, 17)
Even lanes – dCAPS patterns cleavage by BamHI of codon P197 (A) and non-cleavage by BstXI of codon W574 (B)

Figure 4. dCAPS patterns of codon P197 (A) and W574 (B) of *Alopecurus myosuroides*

post-emergence and pre-emergence application, respectively. The treatment dose in our experiment was 2.5 times higher than the usual post-emergence dose. Our bioassay clearly showed that the tested populations of blackgrass survived at 37.5 g/ha chlorsulfuron. Plants resistant to chlorsulfuron were reported in previous studies. Tan *et al.* (2007) tested some populations of *Lolium rigidum* and found 84–100 % of surviving plants at the dose of 15.0 g/ha. Adkins *et al.* (1997) reported the *Fallopia convolvulus* (F) resistance to chlorsulfuron. Eighty-five per cent of plants survived and their dry weight reached 141% when evaluated 30 days after chlorsulfuron treatment at 15.0 g/ha. A small proportion of blackgrass individuals of a population from England survived a sulfometuron treatment at 50.0 g/ha and a mixed treatment of mesosulfuron at 12.0 g/ha and iodosulfuron at 2.4 g/ha. Some of these plants had their foliage weight reduced. However, the plants showed a resistant phenotype (Marshall & Moss 2008). dCAPS pattern of P197 codon of our tested populations indicates that the resistance is not confirmed at this site. By contrast, the pattern of W574 indicated that the resistance may be confirmed by the mutation of W574 codon. Our results suggest that the resistance may be regulated by the mutation of tryptophan at the W574. Similar results were described previously by Delye and Boucansaud (2008) and by Marshall and Moss (2008). However, Marshall and Moss (2008) reported a single blackgrass population from England (Wilts04) showing a resistant phenotype to sulfometuron in screening tests without any evidence of dCAPS polymorphism. This mechanism of resistance may therefore be different from the mutation of the above-mentioned codon and most probably a metabolism-based one.

All tested blackgrass populations showed a resistant phenotype to chlorsulfuron in biological tests. In the molecular assay, dCAPS was conducted targeting the ALS gene to confirm this status. No polymorphism in codon P197 was found out. The codon W574 was found to be polymorphic, and the mutation of tryptophan in this codon most probably confers the resistance to our tested blackgrass populations.

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