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The mechanism of resistance to ALS-inhibiting herbicides in biotypes of wind bent grass (*Apera spica-venti* L.) with cross and multiple resistance

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Abstract. The aim of the greenhouse experiments was to study the mechanism of cross- and multiple resistance of *A. spica-venti* to inhibitors of acetolactate synthesis (ALS). To determine the type of resistance to sulfonylurea herbicides, green mass of over ground parts of *A. spica-venti* plants was collected and examined. Of 3 tested weed biotypes characterized by cross resistance to sulfonylurea herbicides, 2 biotypes (R2 and R3) showed ALS gene point mutation at position Pro-197 conferring ALS target-site-resistance. Within the group of 3 biotypes showing multiple resistance to ALS and acetylCoA carboxylase (ACCase) inhibitors the biotypes R4 and R6 with specific gene point mutation at position Pro-197 were also detected. The sulfometuron showed to be a useful tool to study weed resistance to sulfonylurea herbicides.

key words: *Apera spica-venti*, herbicides, chlorsulfuron, fenoxaprop-P-ethyl, sulfometuron, mechanism of resistance

INTRODUCTION

Research into weed resistance to herbicides has been conducted at the Institute of Plant Protection for many decades now. The big number of studies concerned wind bent grass (Adamczewski and Kierzek, 2007; Adamczewski and Matysiak, 2009, 2010; Adamczewski et al., 2007, 2009; Adamczewski, 2009; Krzakowa and Adamczewski, 2007) has been done. Up to now resistance to sulfonylurea herbicides has been observed most commonly. A total of 393 herbicide-resistant weed species have been recorded worldwide, of which almost 1/3 (127 species) are resistant to ALS-inhibiting herbicides (www.weedscience.com). Such a high number of species resistant to herbicides with this mode of action probably results from the fact that sulfonylureas act in plants at the beginning of the metabolic pathway and ALS-inhibiting herbicides are commonly used in agricultural practice.

Research concerning the mechanism of weed resistance to herbicides is relatively expensive and requires well-equipped biochemical laboratories, which are generally lacking in research centres working on weed resistance. The mechanism of weed resistance to herbicides has been extensively described in literature and it can be divided into two groups, i.e. resistance resulting from a mutation in the DNA-sequence of the herbicide target enzym (target-site-resistance) and resistance not related directly to the herbicide target (non-target-site-resistance), which is most frequently connected with metabolic resistance (Moss et al., 2003; Massa et al., 2011).

The mechanism of target-site resistance to sulfonylureas in weeds is one of the better known and it is connected with the Pro-197 mutation. Proline found at position 197 is substituted by other amino acids such as: Ala, His, Liz, Izo, Leu, Met, Gln, Arg, Ser, Ttp, Tre. Experiments conducted by Yuan et al. (2007, 2010), Yu et al. (2010), Hull and Moss (2007) and Jander et al. (2003) indicate that with the use of sulfometuron on ALS-resistant weeds we may determine the mechanism of resistance – whether it is target-site or non-target-site-resistance. Sulfometuron (Oust 75 WG by DuPont) is a non-selective herbicide controlling all weeds, but it is not effective in the case of mutations in position Pro-197.

The aim of the investigations was to determine the mechanism of resistance to sulfonylurea herbicides in three biotypes of wind bent grass with cross resistance to ALS herbicides and three biotypes with multiple resistance to ALS and ACCase inhibitor herbicides.

MATERIAL AND METHODS

Collection of seed samples. Seed samples of wind bent grass were collected for analyses in the years 2007–2009

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from fields, in which farmers reported poor efficacy of herbicides. The manner of seed collection in wind bent grass was described in a study by Adamczewski and Kierzek (2007).

Greenhouse experiments. Collected plants were threshed and cleaned under laboratory conditions and thus prepared material was the object of greenhouse experiments. In the first stage of the investigations 4 herbicides were applied at 1 full recommended dose: chlorsulfuron (Glean 75 WG), isoproturon (Arelon Dyspersyjny 500 SC), mesosulfuron + iodosulfuron (Atlantis 04 WG) as well as fenoxaprop-P-ethyl (Puma Universal 069 EW). Results of that analysis made it possible to select biotypes exhibiting cross resistance to sulfonylurea herbicides and biotypes with multiple resistance to inhibitors of acetolactate synthase (ALS) and inhibitors of acetylCoA carboxylase (ACCase). Three biotypes with cross resistance to ALS herbicides (R1, R2, R3) and 3 biotypes with multiple resistance to ALS and ACCase herbicides (R4, R5, R6) were selected for further analyses. One biotype (R3) with cross resistance in the analyses conducted at the Warsaw University of Life Sciences had a mutation of Pro-197-Ser (Krysiak et al., 2011). In order to determine the dose-response curve calculation of the control and resistance factor as well as verify the resistance mechanism of 6 selected biotypes (R1, R2, R3, R4, R5 and R6) chlorsulfuron (Glean 75 WG), fenoxaprop-P-ethyl (Puma Universal 069 EW) and sulfometuron (Oust 75 WG) were used in 6 dosages (shown in figures) in the experiment. A sensitive biotype (S) coming from Winna Gora, from an area which is not agriculturally utilized, was used as a standard. Greenhouse experiments were performed in four replications in plastic pots of 0.5 l and 9 cm in diameter. Horticulture soil mixed with sand at a 3 : 1 ratio was used in the experiments. The soil contained 1.89% organic matter and pH was 6.2. Approximately 18–20 seeds were sown to each pot, after emergence plants were thinned leaving 12 plants per pot. Temperature in the greenhouse was 20–25°C and the length of day and night was set at 16/8 h. Herbicides were sprayed with a greenhouse sprayer at the phase of 4–5 leaves of wind bent grass. Efficacy of applied herbicides was evaluated 4 weeks after their application, determining the fresh weight of shoot biomass of 12 plants. The percentage of fresh weight loss was determined in relation to the control. Results were evaluated statistically using the analysis of variance, for each biotype the regression curve was calculated at the confidence level of 0.05%. Statistical analyses were performed with the use of the Polo Plus computer programmer and logit analysis was used (Robertson et al., 2002). This programmer automatically plots the curve and performs calculations of the effective dose (ED50), causing a 50% reduction of fresh weight. On this basis the resistance factor (RF) was determined, which is a ratio of the dose causing a 50% reduction of fresh weight of plants in the resistant biotype to the dose causing a similar effect in plants of the sensitive biotype.

RESULTS

Populations with cross resistance to acetolactate synthase (ALS). The experiments were conducted using three biotypes resistant to sulfonylurea herbicides, i.e. R1, R2 and R3. In molecular analyses with biotypes of wind bent grass collected at the Institute of Plant Protection, performed by Krysiak et al. (2007, 2011), it was shown that the mechanism of resistance in biotype R3 is of the mutation (target-site) type and results from a mutation of Pro-197 to Pro-197-Ser. In contrast, the mechanism of resistance at populations R1 and R2 was not known. After the application of several doses of chlorsulfuron the 3 tested biotypes showed a high degree of resistance to this herbicide (Fig. 1). Only after the application of high doses of this herbicide (150–225 g ha^{-1}) effective control was observed in these biotypes. In turn, to control the reference standard, the sensitive biotype (S), the application of a dose of 18.75 g chlorsulfuron (25 g Glean 75 WG) was sufficient. The resistance factor in relation to chlorsulfuron at resistant biotypes ranged from 18.9 to 21.0 (Table 1). After the application of sulfometuron the response of biotypes resistant to ALS-inhibiting herbicides (R1, R2, R3) was different (Fig. 2). It appeared that only 2 biotypes, i.e. R2 and R3, were poorly controlled by the applied sulfometuron. The mechanism of resistance in biotype R2, similarly as in biotype R3, results from a mutation at position Pro-197. In contrast, population R1 turned out to be sensitive to sulfometuron, which indicates that the mechanism of resistance is not of the target-site type, probably resistance to sulfonylureas is the effect of metabolism. The Figure 3 shows the activity of two herbicides, sulfometuron and chlorsulfuron on plants of three *A. spica-venti* biotypes, S susceptible biotype and the R1 and R2 biotypes resistant to ALS herbicides. Resistance factor for biotypes R2 and R3 ranged from 35.6 to 36.1, while for biotype R1 it was only 1.1 (Table 1).

Table 1. Detection parameters of resistant (R1, R2, R3) and sensitive biotypes (S) of *A. spica-venti* on ALS herbicides.

Biotype	Chlorsulfuron		Sulfometuron		Mechanism of resistance
	ED50 [g ha^{-1}]	R/F	ED50 [g ha^{-1}]	R/F	
R1	159.6	20.7	4.8	1.1	(Non-target-site)
R2	161.8	21.0	153.0	35.6	(Mutation – Pro 197)
R3	145.4	18.9	156.0	36.1	(Mutation – Pro 197)
S	7.7	–	4.3	–	–

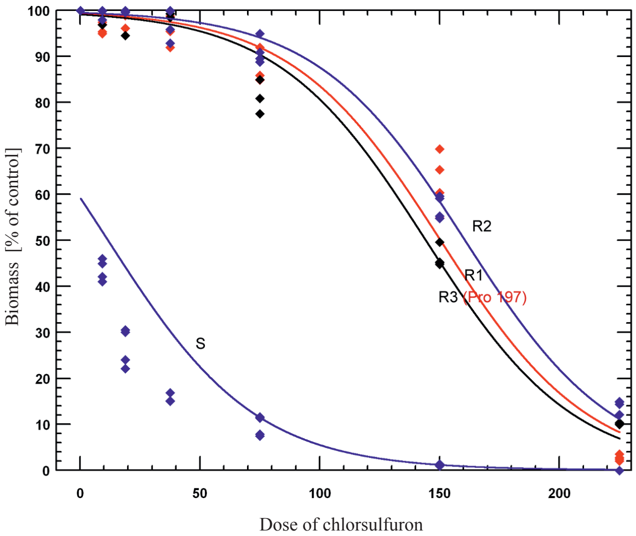

Fig. 1. The effect of chlorsulfuron on fresh-weight reduction of resistant (R1, R2, R3) and susceptible (S) biotypes of *A. spica-venti.*

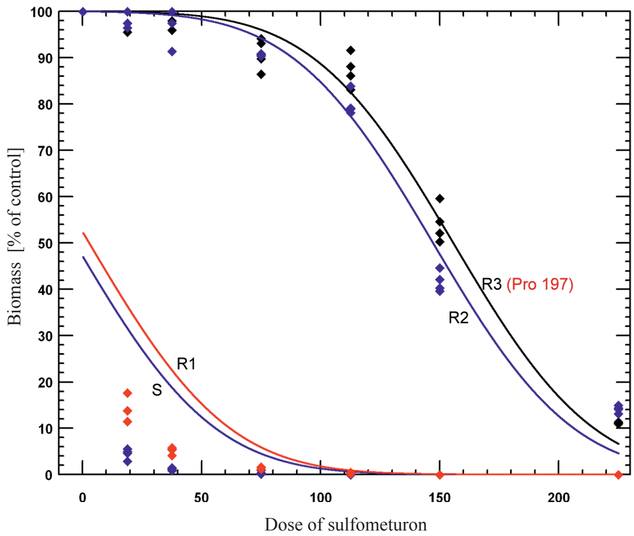

Fig. 2. The effect of sulfometuron on fresh-weight reduction of resistant (R1, R2, R3) and susceptible (S) biotypes of *A. spica-venti.*

Table 2. Detection parameters of resistant (R4, R5, R6) and susceptible (S) biotypes of *A. spica-venti* on ALS herbicides.

Biotype	Chlorsulfuron		Fenoxaprop-P-ethyl		Sulfometuron		Mechanism of resistance
	ED50 [g ha^{-1}]	RF	ED50 [g ha^{-1}]	RF	ED50 [g ha^{-1}]	RF	
R4	143.7	27.6	144.5	16.8	137.1	15.6	(Mut. Pro-197)
R5	116.4	22.4	95.2	10.9	9.9	1.1	(Non-target-site)
R6	102.5	19.7	128.8	15.0	94.3	10.7	(Mut. Pro-197)
S	5.2	–	8.6	–	8.8	–	–

S – sensitive biotype, R1, R2 – resistant biotypes on sulfonylurea herbicides
1 – untreated, 2 – chlorsulfuron, 3 – sulfometuron

Fig. 3. Influence of ALS inhibiting herbicides on susceptible and resistant *A. spica-venti* biotypes.

Biotypes with multiple resistance to inhibitors of acetolactate synthase (ALS) and of acetylCoA carboxylase (ACCase). In this series of experiments 3 herbicides were applied: chlorsulfuron, fenoxaprop-P-ethyl and sulfometuron on 3 biotypes of wind bent grass resistant to ALS- and ACCase-inhibiting herbicides, i.e. exhibiting multiple resistance.

The analyzed biotypes of wind bent grass, i.e. R4, R5 and R6, were characterized by a relatively highly varied degree of resistance to tested herbicides. The highest degree of resistance was found for biotype R4 (Figs 4, 5, 6). The resistance factor for this biotype was 27.6 for chlorsulfuron, 16.8 for fenoxaprop-P-ethyl and 15.6 for sulfometuron (Table 2). The biotype which was less resistant to chlorsulfuron was R6, while biotype R5 was less resistant to fenoxaprop-P-ethyl. In order to obtain a 50% reduction of fresh weight in resistant biotypes of wind bent grass the required dose ranges from 102.5 g to 143.7 g chlorsulfuron and from 95.2 to 144.5 g fenoxaprop-P-ethyl. After using the sulfometuron herbicide the biotype R5 was effec-

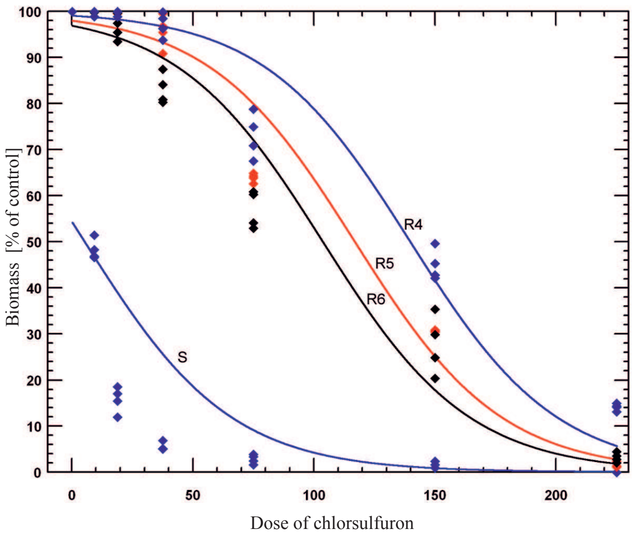

Fig. 4. The effect of chlorsulfuron on fresh-weight reduction of resistant (R4, R5, R6) and susceptible (S) biotypes of *A. spica-venti*

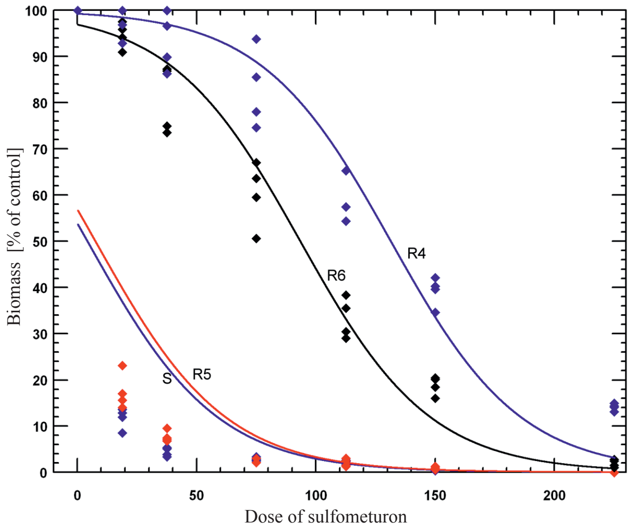

Fig. 6. The effect of sulfometuron on fresh-weight reduction of resistant (R4, R5, R6) and susceptible (S) biotypes of *A. spica-venti*

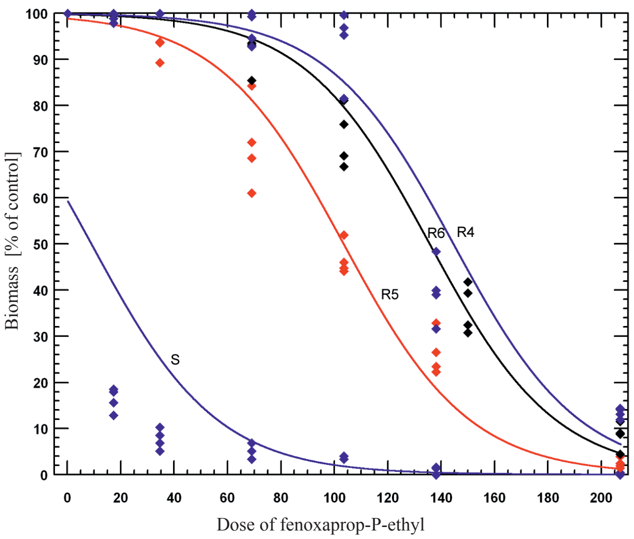

Fig. 5. The effect of fenoxaprop-P-ethyl on fresh-weight reduction of resistant (R4, R5, R6) and susceptible (S) biotypes of *A. spica-venti*

tively controlled, and its resistance factor was 1.1. These results indicate a different mechanism of resistance to ALS herbicides from the resistance mutation (target-site). Probably the resistance of this biotype is sulfonylurea herbicide metabolic type. The use of herbicide sulfometuron allowed to recognize the mechanism of resistance to sulfonylurea herbicides in the case of 3 tested biotypes of wind bent grass (Fig 6, Table 2). The biotypes R4 and R6 showed the type of ALS target-site-resistance. These biotypes were characterized by ALS gene point mutation at position 197, it means that amino acid proline was replaced by another amino acid. The action of sulfonylurea herbicides is conditioned by the presence of proline amino acid at position 197 in the amino acid sequence of weed plants. Due to a point mutation the amino acid proline may be replaced with another amino acid. Therefore, the applied sulfonylurea herbicides do not interfere the metabolism of wind bent grass plants. Resistance factor to sulfometuron of biotype R4 was 15.6, and 10.7 of biotype R6. The results of herbicides applied at the three different biotypes of wind bent grass are shown in Table 2.

The experiments made with three biotypes R4, R5 and R6 of wind bent grass representing different types of multiple resistance at inhibitors of acetolactate synthase (ALS) and inhibitors of acetylCoA carboxylase (ACCase), with the use of three herbicides (chlorsulfuron, fenoxaprop-P-ethyl and sulfosulfuron) allowed the mechanism of resistance to sulfonylurea herbicides to be defined. However, the study gives no indication what might be the mechanism of acetyl CoA carboxylase inhibitors resistance (ACCase). To obtain this information further studies like molecular tests are needed.

DISCUSSION

The effective control of grass weeds in winter cereal crops currently relies on post-emergence ALS and ACCase herbicides. In spite of resistance evolution worldwide, to date the majority of grass biotypes infesting cereal fields are sensitive to those two herbicide modes of action. However, once selected, resistance can spread very quickly, especially when dominant target site resistance is involved

(Moss et al., 2003). In order to contain the proliferation of resistance to these two important herbicide modes of action, it is imperative to know what kind of mechanism of resistance in *A. spica-venti* is present. Results from the whole-plant bioassays showed that the efficacy of treatment with ALS inhibitor chlorsulfuron confirmed resistance in all *A. spica-venti* biotypes (R1-R6). Biotypes R1, R2 and R3 were cross resistant to other sulfonylureas (ALS-inhibiting herbicides). But biotypes R4, R5 and R6 also showed resistance to ACCase inhibitors. According to the earlier examination (Krysiak et al., 2011) the resistance mechanism of biotype R3 was found to involve target site resistance with mutation at Pro-197-Ser. In the case of remaining wind bent grass biotypes the mechanism of resistance to herbicides has not been recognized yet. The phenomenon of cross resistance of *Centaurea cyanus* to sulfonylurea herbicides has been studied earlier (Adamczewski and Kierzek, 2011). Work carried out by Hull and Moss (2007) with *Alopecurus myosuroides*, Yu et al. (2010) with *Lolium rigidum* indicated that sulfometuron, a non selective herbicide, used at recommended dose does not control resistant grass weeds with mutation Pro-197. In accordance with above mentioned authors, sulfometuron controls grass weeds with non target site resistance (metabolic resistance). The mutation Pro-197 gives high level of resistance to sulfonylureas. Whole plant bioassays indicated that not only biotype R3 but also biotypes R2, R4 and R6 are target site resistant for ALS herbicides with mutation Pro-197, but the remaining biotypes R1 and R5 are non target site resistant. These two biotypes probably show the metabolic-type resistance. Hamouzova et al. (2011) reported that sulfometuron used for resistance work with *A. spica-venti* does not control all biotypes which were resistant to sulfonylurea herbicides. Massa et al. (2011) found the two new target sites resistances in *A. spica-venti*: biotype Pro-197-Asn and Arg-377-His. The results indicated new evolutionary resistance patterns in weed biotype. Comparable results of the studies on resistance of the weed species *Alopecurus myosuroides* and *Lolium rigidum* to sulfonylurea herbicides were reported by Hull and Moss (2007) and Yu et al. (2010). These authors also used sulfometuron for resistance work and they showed that the amino acid proline in position 197 was replaced by amino acids arginine, alanine, glutamine and serine.

CONCLUSION

1. The herbicide sulfometuron can be regarded as a useful tool to study the mechanism of weed resistance to sulfonylurea herbicides.

2. Within the group of 3 *A. spica-venti* biotypes showing cross resistance to ALS herbicides two biotypes R2 and R3 revealed a specific point mutation at position Pro-197.

3. In biotypes R4 and R6 of *A. spica-venti* characterized by multiple resistance to ALS and ACCase inhibitors a single point mutation at position Pro-197 was also found.

4. The use of sulfometuron herbicide to study the mechanism of resistance to herbicides did not allow the identification of an amino acid which replaced proline at position Pro-197.

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