ORIGINAL ARTICLE

A strategy of chemical control of *Apera spica-venti* L. resistant to sulfonylureas traced on the molecular level

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Abstract

Three populations of silky bent grass (*Apera spica-venti* L.) were tested – one that is susceptible and two that are resistant to sulfonylureas. This study assessed the efficacy of control by different herbicides in a pot experiment and estimated the molecular status of resistance to sulfonylureas in analysed populations and its effect on the efficacy of different chemical treatments. The three most effective herbicide rotation schemes were: 1) chlorsulfuron + isoproturon, ethametsulfuron + metazachlor + quinmerac, chlorsulfuron + isoproturon; 2) prosulfocarb + diflufenican, ethametsulfuron + quizalofop-p-ethyl, prosulfocarb + diflufenican; 3) diflufenican + flufenacet, quizalofop-p-ethyl, diflufenican + flufenacet. In most cases it was more difficult to destroy 100% of the resistant population from Modgarby where the majority of plants had no mutation in the *als* gene. In the resistant population from Babin there were significantly more individuals with mutation in the *als* gene, therefore exhibiting target-site resistance.

Key words: ALS inhibitors, non-target-site resistance, silky bent grass, target-site resistance, weed control

Introduction

Silky bent grass (*Apera spica-venti* L.) is an annual overwintering grass that is considered one of the most important and widespread weeds in winter cereals in central and northern Europe.

Chemical control of this species has often been carried out without sufficient rotation of active substances, which has resulted in the selection of herbicide-resistant biotypes. The first case of silky bent grass resistance to acetolactate synthase (ALS) inhibitors was reported in 2002 by Rola and Marczewska. Currently ALS-resistant biotypes of this species have been found in Austria, Czech Republic, Germany, France, Sweden, Demark and Lithuania (Heap 2017). Unfortunately populations of *A. spica-venti* resistant to more than one mechanism of action have already been identified. In Poland silky bent grass resistant to ALS and acetyl-coenzyme A carboxylase (ACCase) inhibitors has been

observed and in Germany a biotype resistant to three sites of action – ALS, ACCase and photosystem-II (PSII) inhibitors – has been identified (Heap 2017). Resistance in *A. spica-venti* biotypes can also be found against pre-emergence herbicides such as diflufenican and pendimetalin, as well as in populations that are already resistant to ACCase and ALS inhibitors (Petersen *et al.* 2012).

Resistance to herbicides can be divided into two major types: target-site or non-target-site resistance. So far seven mutation sites in the *als* gene endowing target-site resistance to ALS inhibitors in different plant species have been reported: Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654 (Powles and Yu 2010; Massa *et al.* 2011). Metabolic resistance to ALS inhibitors can be due to increased activity of endogenous cytochrome P450 monooxygenases

(P450s), glucosyl transferases (GTs) or glutathione S-transferases (GSTs), which can metabolise herbicides (Yu and Powles 2014; Mahmood *et al*. 2016; Duhoux *et al*. 2017). This mechanism, potentially conferring resistance to many herbicides, is a particular threat to herbicide sustainability and thus global crop production.

The aim of this study was to determine if herbicide rotations involving active substances other than ALS inhibitors would be more effective on a population of silky bent grass with a predominance of target-site resistance to sulfonureas than on a population with a predominance of non-target-site resistance.

Materials and Methods

Plant material

Three populations of *A. spica-venti* were used in the study: a population susceptible to sulfonylureas (S), a resistant population from Babin, Poland (RB) and a resistant population from Modgarby, Poland (RM). Seeds of the susceptible populations originated from Herbiseed. Seeds from resistant populations of silky bent grass came from commercial fields in Poland where cereals had been grown for several years with chemical weed management (including ALS inhibitors) and where unsatisfactory herbicide performance was reported. All seeds were provided by DuPont.

Whole-plant bioassays

Seeds were sown in pots with compost soil and were well-watered. At the 1-leaf stage seedlings were planted in pots (100 × 20 × 20 cm) with sandy loam soil.

Ten plants from each population were placed in individual pots. The experiment was set up in three replications. From September to November plants were grown in a glasshouse at 15°C/10°C day/night under natural light conditions. From December to the stage of seed collection pots were kept outdoors. Plants were bottom watered if the soil surface was dry except during spring and summer when plants were watered daily to field capacity by drip irrigation. Plants were sprayed at the 2 to 3-leaf stage. Herbicides were applied with a sprayer equipped with a single nozzle calibrated to deliver 300 l · ha⁻¹ at a spraying pressure of 200 kPa. The herbicide treatments applied are presented in Table 1. Treatments for the three consecutive years of the study were chosen according to the crop rotation frequently practiced by farmers, i.e. winter wheat, winter rape, winter wheat.

Herbicide efficacy was assessed 15 and 30 days after treatment and compared with the untreated control. Plants were counted before and after treatment and the *A. spica-venti* control efficacy is expressed as a percentage of plants that were destroyed by the treatment. Plants that survived were grown to maturity. Pots from the same treatment and population were covered with white polypropylene tissue before flowering to avoid cross-pollination with other treatments and populations. At maturity seeds from each treatment and population were collected and sown back into the same pots from where they had been harvested. In some treatments no seeds were harvested because all the plants had been destroyed by the herbicides. In these cases germination of seeds from the soil bank was simulated: seeds from original populations were sown in small pots with sandy loam soil, kept well-watered and at the 1-leaf stage seedlings were planted in the pots where all the plants had been destroyed by the herbicides.

Table 1. Herbicide treatments applied

Years 1 and 3			Year 2		
Active ingredient [a.i.]	HRAC group	Applied doses [g a.i. · ha⁻¹]	Active ingredient [a.i.]	HRAC group	Applied doses [g a.i. · ha⁻¹]
chlorsulfuron	B	18.75	ethametsulfuron	B	18.75
chlorsulfuron + diflufenican	B+F1	15+50	ethametsulfuron + aminopyralid + clopyralid + picloram	B+O+O+O	18.75+12+72+24
chlorsulfuron + prosulfocarb	B+N	15+1,600	ethametsulfuron + metazachlor	B+K3	18.75+750
chlorsulfuron + isoproturon	B+C2	15+1,000	ethametsulfuron + metazachlor + quinmerac	B+K3+O/L	18.75+800+200
prosulfocarb + diflufenican	N+F1	1,600+50	ethametsulfuron + quizalofop-p-ethyl	B+A	18.75+35
diflufenican + flufenacet	F1+K3	112+112	quizalofop-p-ethyl	A	35

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Molecular analysis of the *als* gene

The DNA of three plants from each original population and from each progeny was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987) with minor modifications. Primer sequences for amplification of domains A and B and the area between these domains of the *als* gene were as described by Krysiak *et al.* (2011). Genomed S.A. (Warsaw, Poland) was commissioned to undertake purification of the PCR product and sequencing. Chromatograms were analysed using the FinchTV program. The obtained sequences were compared in the ClustalW online-accessed program.

Results

Whole-plant bioassays

The efficacy of silky bent grass control in the first year is presented in Figure 1. The most effective treatment was chlorsulfuron + isoproturon. Prosulfocarb + diflufenican and diflufenican + flufenacet were also satisfactorily effective in controlling *A. spica-venti*. Chlorsulfuron, both alone and in a mixture with diflufenican controlled only the S population.

Apera spica-venti control efficacy by herbicides applied in the second year is presented in Figure 2. The most effective mixtures were: ethametsulfuron + metazachlor,
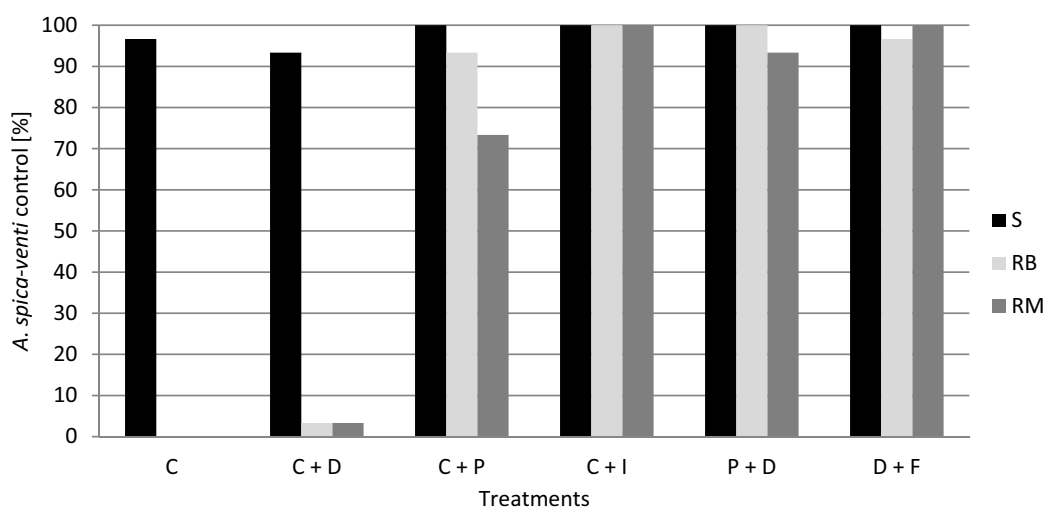

Fig. 1. Efficacy of *Apera spica-venti* control in the first year assessed 30 days after treatment. S – population sensitive to sulfonylureas, RB – population resistant to sulfonylureas from Babin, RM – population resistant to sulfonylureas from Modgarby, C – chlorsulfuron, D – diflufenican, P – prosulfocarb, I – isoproturon, F – flufenacet
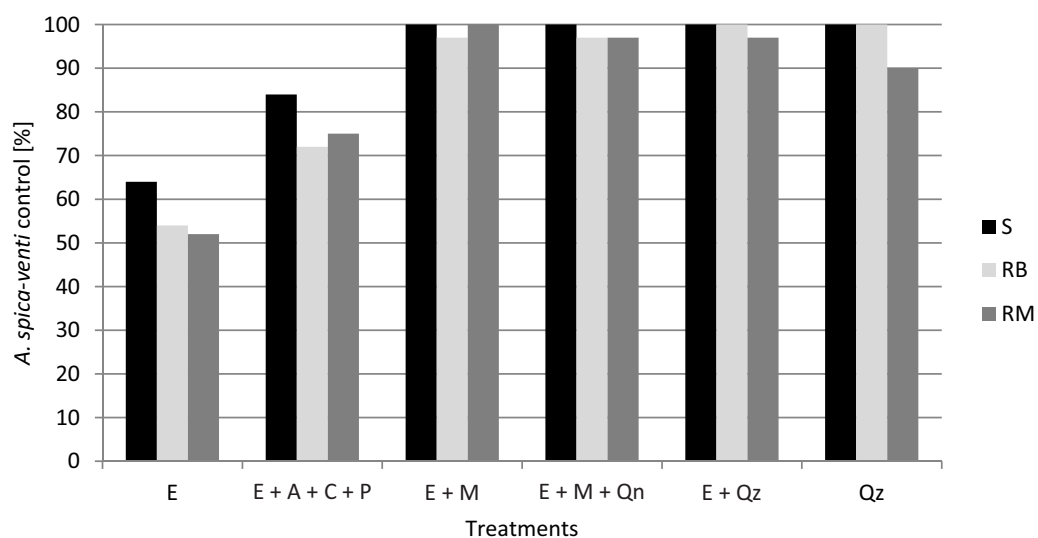

Fig. 2. Efficacy of *Apera spica-venti* control in the second year assessed 30 days after treatment. S – population sensitive to sulfonylureas, RB – population resistant to sulfonylureas from Babin, RM – population resistant to sulfonylureas from Modgarby, E – ethametsulfuron, A – aminopyralid, C – clopyralid, P – picloram, M – metazachlor, Qn – quinmerac, Qz – quizalofop
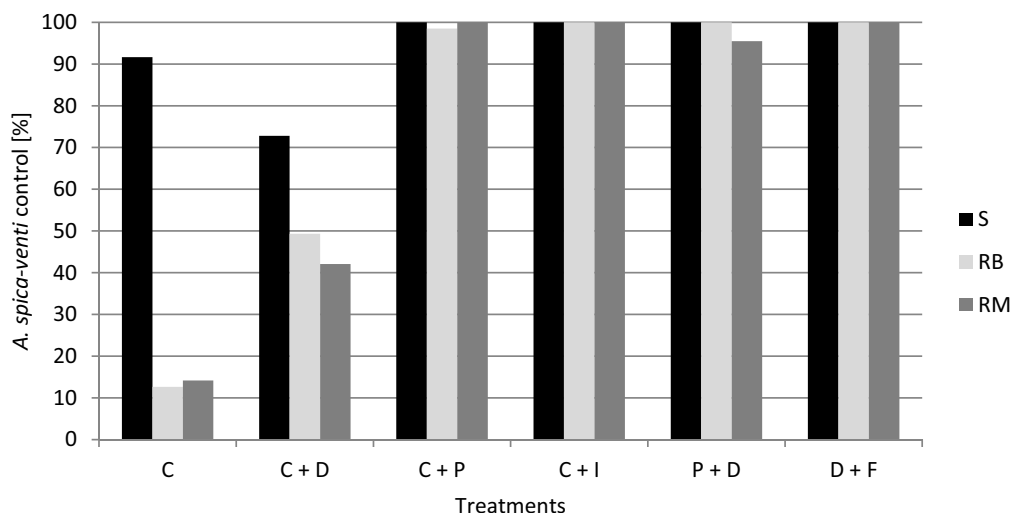
Fig. 3. Efficacy of *Apera spica-venti* control in the third year assessed 30 days after treatment. S – population sensitive to sulfonylureas, RB – population resistant to sulfonylureas from Babin, RM – population resistant to sulfonylureas from Modgarby, C – chlorsulfuron, D – diflufenican, P – prosulfocarb, I – isoproturon, F – flufenacet

ethametsulfuron + metazachlor + quinmerac and ethametsulfuron + quizalofop-P-ethyl. Quizalofop-P--ethyl alone was also effective in *A. spica-venti* control, however 100% control was obtained for only the S and RB populations. For the RM population, efficacy of control was lower and the surviving plants were large and showed no symptoms of injury or developmental delay. In two of the treatments: ethametsulfuron alone and ethametsulfuron + aminopyralid + clopyralid + picloram – control of the *Apera* plants was not satisfactory.

Efficacy of silky bent grass control in the third year is presented in Figure 3. The most effective mixtures were: chlorsulfuron + isoproturon and diflufenican + flufenacet where 100% control was noted. Chlorsulfuron + prosulfocarb and prosulfocarb + diflufenican were also satisfactorily effective in *A. spica-venti* control. In the treatments of chlorsulfuron alone and chlorsulfuron + diflufenican, the majority of the resistant plants survived.

Molecular analysis of the *als* gene

For domains A and B of the *als* gene, the PCR products were approximately 380 and 520 bp, respectively. The PCR product of the area between domains A and B was approximately 1,200 bp. Codons 122-205, 367-377 and 574-653 were clearly legible.

Analysis of the *als* sequence in three plants from each original population did not reveal any mutation endowing resistance in the S population. In the RB population there were two plants that showed Pro to Thr substitution at position 197 (heterozygotes) and one plant without mutation in the *als* gene (Table 2). In the RM population after the first year of the study

no mutations endowing resistance in domain A were detected. After the second and third years in the RM population one out of three plants analysed had Pro to Thr substitution at position 197 (Table 2). In domain B and in the area between domains A and B no substitutions at Asp376, Arg377, Trp574, Ser653 or Gly654 codons were detected. In progenies treated with herbicides, in the RB population the majority of plants showed mutations in the codon 197 of domain A. In these plants proline was substituted by threonine. In progenies of the RM population, the situation was different in that the majority of the plants had no resistance-endowing mutations in domains A and B or in the intradomain area. Only in progenies treated with chlorsulfuron + diflufenican/ethametsulfuron + aminopyralid + clopyralid + picloram/chlorsulfuron + diflufenican were plants with mutations in domain A identified. In these individuals proline was substituted by threonine, alanine or serine (Table 2). All substitutions concerned the first position of the 197 codon. The majority of plants with this mutation were heterozygotes at this codon, however some homozygotes were also identified (Table 2).

Discussion

Active ingredients were chosen in order to evaluate the level of their control of *A. spica-venti* resistant and susceptible populations in frequent rotations of winter wheat/winter rape/winter wheat. On the basis of the results it can be stated that the most effective herbicide rotation schemes were: 1) chlorsulfuron + isoproturon, ethametsulfuron + metazachlor

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Table 2. Mutations observed in the *als* gene at position 197 in progenies of silky bent grass obtained after each year of treatment. Numbers indicate the number of plants possessing mutations. In each progeny and treatment the *als* gene of 3 plants was sequenced, if available. Some combinations produced no progenies because all the plants had been destroyed by herbicides, therefore these plants were not examined (n.e.)

Progenies after year 1

Treatment	S	RB	RM
control	0	2 Pro/Thr	0
chlorsulfuron	0	3 Pro/Thr	0
chlorsulfuron + diflufenican	1 Pro/Thr	2 Pro/Thr	1 Pro/Thr 1 Pro/Ala
chlorsulfuron + prosulfocarb	n.e.	3 Pro/Thr	0
chlorsulfuron + isoproturon	n.e.	n.e.	n.e.
prosulfocarb + diflufenican	n.e.	n.e.	0
diflufenican + flufenacet	n.e.	2 Pro/Thr	n.e.

Progenies after year 2

Treatment	S	RB	RM
control	0	2 Pro/Thr	1 Pro/Thr
ethametsulfuron	0	2 Pro/Thr	0
ethametsulfuron + aminopyralid + clopyralid + picloram	0	3 Pro/Thr	1 Pro/Ser
ethametsulfuron + metazachlor	n.e.	2 Pro/Thr	0
ethametsulfuron + metazachlor + quinmerac	n.e.	n.e.	0
ethametsulfuron + quizalofop	n.e.	n.e.	0
quizalofop	n.e.	1 Thr/Thr 1 Pro/Thr	0

Progenies after year 3

Treatment	S	RB	RM
control	0	2 Pro/Thr	1 Pro/Thr
chlorsulfuron	0	2 Pro/Thr	0
chlorsulfuron + diflufenican	0	3 Pro/Thr	1 Pro/Ala
chlorsulfuron + prosulfocarb	n.e.	2 Pro/Thr	0
chlorsulfuron + isoproturon	n.e.	n.e.	0
prosulfocarb + diflufenican	n.e.	n.e.	0
diflufenican + flufenacet	n.e.	n.e.	n.e.

S – population sensitive to sulfonylureas, RB – population resistant to sulfonylureas from Babin, RM – population resistant to sulfonylureas from Modgarby

+ quinmerac, chlorsulfuron + isoproturon; 2) prosulfocarb + diflufenican, ethametsulfuron + quizalofop-p-ethyl, prosulfocarb + diflufenican; 3) diflufenican + flufenacet, quizalofop-p-ethyl, diflufenican + flufenacet; 4) chlorsulfuron + prosulfocarb, ethametsulfuron + metazachlor, chlorsulfuron + prosulfocarb.

In the second year no herbicide treatment showed 100% efficacy in *Apera* control in any of the populations tested. Ethametsulfuron + metazachlor and ethametsulfuron + quizalofop-p-ethyl proved to be the most efficient – 100% control of two populations and 97% control of the third population. The level of control for ethametsulfuron + metazachlor + quinmerac was also high – 100% for the S population and 97% for the RB and RM populations. Moreover the plant that survived treatment with ethametsulfuron + metazachlor + quinmerac in the RB population was delayed in its development and produced no seeds. Herbicides with different modes of action are widely recommended as a way of tackling the ALS-resistant phenomenon in weeds. Chemicals from the HRAC group K3 (inhibitors of very long chain fatty acids synthesis) can be an alternative to ALS inhibitors in weed control (Beckie and Tardiff 2012).

In all the plants where mutations were detected, the substitutions concerned codon Pro197. This mutation, which occurs the most frequently, is known to confer resistance to sulfonylureas, with 11 documented amino acid substitutions endowing resistance to ALS-inhibiting herbicides (Powles and Yu 2010; Krysiak *et al.* 2011; Massa *et al.* 2011).

The mechanism of ALS resistance in the RB and RM populations is probably mixed. In the RB population there were significantly more individuals with target-site resistance, however it cannot be excluded that in this population there was a certain level of non-target-site resistance because some individuals without resistance-endowing mutation in the *als* gene were noted among the survivors. However the RM population seemed to be more diversified. The majority of plants from this population had no mutation in the *als* gene, but in plants possessing mutation at position 197, three types of substitution were identified (Pro/Thr, Pro/Ala and Pro/Ser) while in the RB population only the Pro/Thr mutation was detected.

In the RM and RB populations, not all of the tested individuals showed mutations in domains A or B or in the intradomain area, although they had survived the herbicide treatment (Stankiewicz-Kosyl *et al.* 2013). Similar results were obtained by Krysiak *et al.* (2011) and Hamouzova *et al.* (2014). The probable cause of this might be the existence of another mechanism of resistance, for example enhanced metabolism. In rigid ryegrass (*Lolium rigidum* Gaudin) populations in Australia, 70% of the populations exhibited target-site and non-target-site resistance to ACCase herbicides (Yu *et al.* 2014). Even in one individual different mechanisms of resistance can coexist (Han *et al.* 2016). In the same population one to several different mutations can be noted (Yu *et al.* 2008).

One plant with substitution Pro197 to Thr was detected in the first year in the susceptible population among plants that survived the combination of chlorsulfuron + diflufenican. It is known that herbicides do not generate mutations in plants, but select plants with mutations already existing in a population. Research conducted on *L. rigidum* populations never previously exposed to ALS inhibitors revealed a high initial frequency of individuals resistant to these herbicides (Preston and Powles 2002). In the herbarium collection of black-grass (*Alopecurus myosuroides* Huds.), one plant with a mutation conferring ACCase resistance was identified. This specimen was collected in 1888, thus before any herbicides were on the market (Delye *et al.* 2013b).

When control efficacy between treatments was compared, it was observed that it was more difficult to destroy 100% of the RM population. In this population more plants without target-site resistance were identified and it is known from the literature that populations with non-target-site resistance are more difficult to control with the use of different herbicides (Beckie and Tardif 2012; Delye 2013). This suggests that the efficacy of the herbicide treatment depends on the resistance pattern of silky bent grass in the field. Similar results were obtained for black-grass (Rosenhauer *et al.* 2014).

Few new active ingredients are appearing on the market and therefore mixtures of existing ones can help in prevention and control of weed resistance. However this presents a significant challenge to chemical companies and farmers. Including flufenacet, metazachlor, isoproturon and prosulfocarb in the herbicide rotation schemes can be useful for control of silky bent grass populations resistant to ALS inhibitors. In the same population of silky bent grass individuals with target-site and non-target-site resistance to ALS inhibitors can coexist. Moreover the population with a prevalence of individuals without resistance endowing mutations in the *als* gene is more difficult to control than the population where most individuals exhibit target-site resistance to ALS inhibitors. Therefore the molecular status of resistant plants can be helpful in identifying the appropriate herbicide treatment.

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