Increase in resistance to sulfonylurea herbicides in *Alopecurus myosuroides* populations in north-eastern Poland

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Abstract

The number of herbicide-resistant populations of black-grass (*Alopecurus myosuroides* Huds.) in winter cereal crops in Europe is increasing, leading to serious restrictions in winter cereal production. The aim of this study was to evaluate the reaction of five *A. myosuroides* populations: four suspected as being herbicide-resistant and one susceptible to mesosulfuron + iodosulfuron-based herbicide (acetolactate synthase (ALS) inhibitor). Mutation events in domains A and B of the *als* gene, and the relationship between resistance to mesosulfuron + iodosulfuron and plant fitness in the early vegetative stage were also examined. The fitness was assessed on the basis of tillering, average leaf number, dry mass accumulation, chlorophyll content and photosystem II efficiency.

The whole-plant bioassay confirmed resistance to ALS inhibitors in four *A. myosuroides* populations, and the level of reduction of biomass accumulation after herbicide treatment corresponded with the number of *als* gene mutations conferring resistance in the population. Non-treated populations did not differ significantly in biomass accumulation, chlorophyll content or photosystem II efficiency. However, particular ALS-resistant populations revealed more intensive tillering than the susceptible population. In the examined *A. myosuroides* populations, resistance conferring mutations (Pro197Thr and Trp574Leu) of the *als* gene were identified. These mutations have not been found in Poland before. In two populations there were some survivors after mesosulfuron + iodosulfuron treatment, where no mutation in the *als* gene was identified. The survival rate in these populations after herbicide treatment suggests that these populations were composed of individuals with target-site resistance (TSR) and non-target-site resistance (NTSR) to mesosulfuron + iodosulfuron.

Key words: ALS inhibitors, black-grass, mesosulfuron + iodosulfuron, plant fitness, target-site resistance.

Introduction

Weed competition in crops is a serious problem in agriculture (Yu, Powles, 2014 a; Auškalnienė et al., 2018; Velička et al., 2018). In recent years, the eradication of weeds in crops has mostly been based on herbicides targeting particular metabolic pathways, e.g., acetolactate synthase (ALS, also referred to as acetohydroxyacid synthase, AHAS), acetyl-coenzyme A carboxylase (ACCase) and D1 protein within photosystem II, etc. (Powles, Yu, 2010). Due to high selectivity in major world crops, herbicides inhibiting ALS have recently attracted considerable interest. They stop the synthesis of branched-chain amino acids, leading to plant death. They are able to control many weed species, while exhibiting low mammalian toxicity (Yu, Powles, 2014 b).

Since selective herbicides have been applied, weed populations have developed several resistance mechanisms, grouped as target-site resistance (TSR) and non-target-site resistance (NTSR). TSR is caused by a mutation within a gene coding for an herbicide target-site enzyme (limiting herbicide binding) or by overproduction of the target enzyme. In the case of ALS resistant weeds with TSR, mechanism mutations have been identified in triplets coding for amino acids in eight positions on the ALS enzyme: Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654 (Heap, 2019). NTSR encompasses mechanisms that decrease the amount of active herbicide influencing the target site (Yu, Powles, 2014 a; b). In many cases, combined TSR and NTSR has been observed. This has also been found as a reaction to ALS-inhibiting herbicides based on mesosulfuron and iodosulfuron (Hull et al., 2014).

Fitness, or success in the field, can be defined as the ability to establish, survive and reproduce successfully in a given environment. Fitness alterations can be exploited to predict population dynamics and establish resistance management strategies (Darmency et al., 2014). Mutations conferring resistance to pesticides are generally expected to generate a fitness cost in the absence of this selection pressure (Vila-Aiub et al., 2009). However, increased fitness of herbicide-resistant populations has also been noted (Wang et al., 2010; Babineau et al., 2017).

Black-grass (*Alopecurus myosuroides* Huds.) is a weed that commonly occurs in winter cereals (Moss et al., 2011; Hull et al., 2014; Pacanoski, 2018). In Poland it is considered to be an archaeophyte that was introduced

in around the 15th century (Zając, Zając, 2014) and was described for the first time in 1919 by Raciborski and Szafer (Trzcińska-Tacik, Stachurska-Swakoń, 2011). Its occurrence was noted mostly in Silesia and north-eastern Poland.

In recent years *A. myosuroides* has become one of the most intrusive herbicide-resistant weeds in Europe (Domaradzki et al., 2010; Hull et al., 2014). The species has high seed productivity with around 500 viable seeds produced by one plant. Although about 20% of the seeds germinate in spring, most of the seeds germinate in autumn, therefore, winter cereals are threatened more (Maréchal et al., 2012).

The timing of germination and seedling growth of *A. myosuroides* from different populations varies; therefore, the species shows great plasticity and tolerance to changing habitat conditions. Abundant *A. myosuroides* populations in wheat have been identified in a number of European countries, including England, France and Germany (Delye et al., 2010; Rosenhauer et al., 2013; Hull et al., 2014). Recent papers have indicated its adaptation ability to develop resistance mechanisms to a range of selective herbicides. Since the first use of mesosulfuron + iodosulfuron herbicide in the UK, by 2013 the resistance of *A. myosuroides* to mesosulfuron + iodosulfuron had been confirmed on over 700 farms in 27 counties of England (Hull et al., 2014).

The same is true in Poland. While Domaradzki and Rola (2006) discuss successful *A. myosuroides* eradication with mesosulfuron + iodosulfuron herbicides in the district of Opole (south-western Poland) in 2006, there were reports in 2011 of a resistant population with TSR and a mutation in the A domain of ALS (*als*) gen (Krysiak et al., 2011). In 2016, resistant populations were not only detected in south-western Poland but also in the north of the country, i.e. in Kmiecin (54°11′18″ N, 19°08′36″ E), Sułowo (54°05′47″ N, 20°49′40″ E) and Łabędnik (54°11′04″ N, 20°58′08″ E) (Adamczewski et al., 2016).

Unfortunately, *A. myosuroides* is a very plastic species, and there are populations in many countries that have evolved resistance not only to ALS inhibitors but to ACCase, photosystem II, long-chain fatty acid and lipid synthesis inhibitors as well (Heap, 2019). This multidimensional resistance impedes and decreases winter crop production and may lead to the ultimate cessation of production of winter cereals, as has been the case in some counties in England (Moss et al., 2011; Hull et al., 2014).

The objective of the present study was: (i) to evaluate the reaction of examined populations to mesosulfuron + iodosulfuron, (ii) detect mutations in domains A and B of the *als* gene conferring resistance to acetolactate synthase (ALS) inhibitors, and (iii) identify the relationship between resistance to mesosulfuron + iodosulfuron and tillering, average leaf number, dry mass accumulation, chlorophyll content and photosystem II efficiency. We tested the following hypotheses: (i) ALS-inhibitor resistance of examined *Alopecurus myosuroides* populations is conditioned both by target-site resistance (TSR) and non-target-site resistance (NTSR), (ii) ALS-inhibitor resistance in black-grass is increasing in Poland, and (iii) there are differences in fitness among the analysed *A. myosuroides* populations.

Materials and methods

Plant material. Four populations of black-grass (*Alopecurus myosuroides* Huds.) were tested. Field samples of four populations suspected to be resistant to acetolactate synthase (ALS)-inhibiting herbicides were collected in 2016 from the districts of Bartoszyce (54°14′56″ N, 20°48′29″ E) (RI and RII populations) and Kętrzyn (54°04′37″ N, 21°22′31″ E) (RIII and RIV populations) in Warmian-Masurian Voivodeship, Poland. Samples were suspected to be resistant based on interviews with farmers and chemical advisors working in the crop protection supply chain. A susceptible (S) population was acquired from Błotnik in the district of Gdańsk in Pomeranian Voivodeship (54°16′33″ N, 18°52′30″ E), Poland. Each sample contained more than 100 ears from at least 30 plants. Individuals from winter wheat fields with very poor herbicide control of *A. myosuroides*, despite being sprayed with full label doses, were harvested and classified for further greenhouse and laboratory tests, which were conducted in 2017.

Whole-plant resistance bioassay. Seeds of *A. myosuroides* were stored in a refrigerator for seven days at a temperature of approximately −4°C in order to interrupt seed dormancy. The seeds were then sown into river sand and after germination moved to pots of a volume of 0.5 dm^3 filled with a mineral sandy-clayey soil. Five pots per sample, each containing five seedlings, were used for the experiment.

The seedlings at a stage of 2–3 leaves (two weeks after sowing) were sprayed with herbicide Atlantis 12 OD (a.i. mesosulfuron + iodosulfuron) following the manufacturer's recommendations, i.e. single (1LD = 2.4 g ha^{-1} of iodosulfuron + 12 g ha^{-1} of mesosulfuron) and double (2LD = 4.8 g ha^{-1} of iodosulfuron + 24 g ha^{-1} of mesosulfuron) doses. The herbicide was applied with a sprayer equipped with a single nozzle calibrated to deliver 300 L ha^{-1} at a spraying pressure of 200 kPa. After six weeks, shoot dry mass (DM) was determined and samples for molecular analyses collected.

Analysis of selected morphological and physiological parameters. Plants for the evaluation of morphological traits and photosynthetic apparatus efficiency were prepared in the same manner as for the whole-plant bioassay. Four weeks after germination, the number of leaves and number of tillers in each plant were determined. After a further four weeks, the relative chlorophyll content was assessed using a chlorophyll content meter CCM-200 (Opti-Science, USA), chlorophyll *a* fluorescence was measured using a fluorimeter Handy-PEA (Hansatech Instruments Ltd., UK), and the number of tillers was determined.

Molecular analysis. Molecular analysis was undertaken in order to check the presence of the target-site resistance (TSR) mechanism of resistance to ALS (mesosulfuron + iodosulfuron) inhibitors in analysed populations of *A. myosuroides*. The DNA was extracted from three plants from each of the 1LD and 2LD treatments using the CTAB (cetyltrimethylammonium bromide) method (Doyle, Doyle, 1987). Primer sequences for amplification of domains A and B of the *als* gene were as described by Krysiak et al. (2011). Genomed S.A. (Poland) was commissioned to undertake purification of the PCR product and sequencing. Chromatograms were analysed using software *FinchTV* (Geospiza, USA). The obtained sequences were compared using the online-accessed software *ClustalW* (GenomeNet, Japan).

Statistical analysis. To compare results, a one-way analysis of variance (ANOVA) was performed using software *Statgraphics Plus*, version 4.1 (Statpoint Technologies Inc., USA). Differences between means of samples were evaluated by HSD (honestly significant difference) Tukey test at α = 0.05. The presented data are means ± standard error (SE). The number of biological replications (single plant in a container) was five.

Results and discussion

Photosynthetic apparatus efficiency, assessed as the chlorophyll content index and chlorophyll *a* fluorescence of non-treated susceptible and resistant populations of *A. myosuroides*, did not differ significantly (Table 1).

Similar results were obtained by Kaiser et al. (2013). Six *A. myosuroides* populations of different herbicide susceptibility did not show significant differences in maximum quantum efficiency of photosystem II (F_v:F_m) during seven days of measurements. Shoot dry mass accumulation in all populations was not affected significantly (Fig. 1).

Table 1. Efficiency of the photosynthetic apparatus and morphological parameters of *Alopecurus myosuroides* plants tested in a greenhouse pot trial

Population	CCI	F_v:F_m	PI	L	Tillers
					4 / 8 weeks
S	1.39 a*	0.821 a	1.93 a	3.48 c	0.44 a / 4.16 a
RI	1.40 a	0.822 a	2.20 a	3.40 bc	0.52 a / 4.84 b
RII	1.51 a	0.821 a	2.14 a	3.16 ab	0.36 a / 5.24 b
RIII	1.35 a	0.815 a	1.87 a	3.24 abc	0.52 a / 4.88 b
RIV	1.45 a	0.811 a	1.85 a	3.08 a	0.76 a / 5.24 b

Note. S – susceptible; CCI – chlorophyll content index; F_v:F_m – maximum quantum efficiency of photosystem II; PI – performance index of photosystem II; L – number of leaves per plant (measured 4 weeks after transplantation); tillers – number of tillers per plant; 4 / 8 weeks – 4 and 8 weeks after transplantation; means, letters indicate homogenous groups; * – data followed by the same letter do not differ significantly.
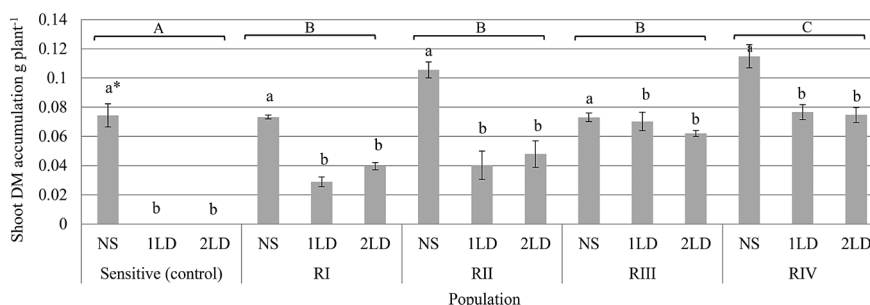
Note. Means ± SE, n = 5; NS – non-sprayed, 1LD – 100% of the label dose (2.4 g ha^{-1} of iodosulfuron + 12 g ha^{-1} of mesosulfuron), 2LD – 200% of the label dose (4.8 g ha^{-1} of iodosulfuron + 24 g ha^{-1} of mesosulfuron); * – data followed by the same letter do not differ significantly (capital letters – population, lowercase letters – treatment).
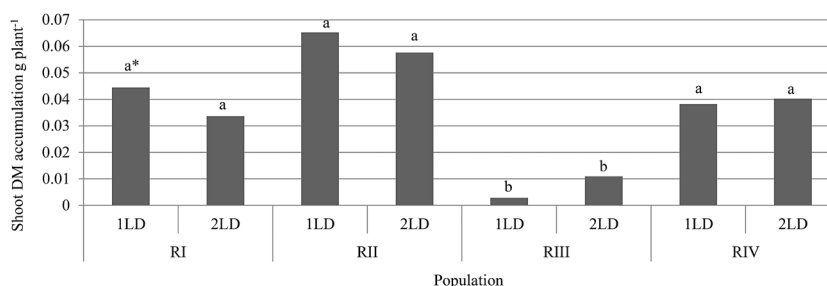
Figure 1. Shoot dry mass (DM) accumulation in *Alopecurus myosuroides* seedlings treated with iodosulfuron + mesosulfuron in comparison to the non-treated seedlings of each population

Four weeks after germination, the number of leaves per plant was highest in the susceptible (S) population; RII and RIV populations revealed significantly lower leaf numbers. The highest number of tillers was noted in RIV; however, the differences were not significant (Table 1). After 8 weeks, all resistant populations showed significantly higher tiller number than the susceptible (S) population. There were no significant differences between any of the resistant populations; however, RII and RIV populations exhibited more abundant tillering on average with 5.24 in both populations. As a consequence, their shoot dry mass accumulation was higher (Fig. 1). Shoot dry mass accumulation in non-treated plants did not differ between any of the populations. Moreover, it was significantly higher than in herbicide-treated plants in each biotype.

These findings indicate that despite the developed resistance to ALS inhibitors, plants of all biotypes were affected by the mesosulfuron + iodosulfuron mixture. However, the reaction of a particular biotype depended on its resistance level. The lowest shoot dry mass decrease was noted in RIII population at 0.0029 g (4%) and 0.011 g (15%) DM per plant in 1LD and 2LD treatments, respectively. The RII population showed the greatest shoot dry mass decrease (Fig. 2); however, the differences between 1LD and 2LD treatments were not significant.

Note. 1LD – 100% of the label dose (2.4 g ha^{-1} of iodosulfuron + 12 g ha^{-1} of mesosulfuron), 2LD – 200% of the label dose (4.8 g ha^{-1} of iodosulfuron + 24 g ha^{-1} of mesosulfuron); * – data followed by the same letter do not differ significantly.

Figure 2. The decrease in shoot dry mass (DM) accumulation of herbicide-treated *Alopecurus myosuroides* seedlings in comparison to the non-treated seedlings of each population

The registration procedure of herbicides determines the scale of weed susceptibility evaluation in percentage terms, taking into account population size, soil coverage, plant height and vigour, i.e. assessed weed mass (EPPO standard PP 1/181(4), 2012). Efficacy below 70% indicates intermediate susceptibility to a particular herbicide. None of the examined RI, RII, RIII and RIV populations showed shoot dry mass diminishing to such a level. Thus, irrespective of other physiological parameters, all these biotypes should be considered as being resistant to mesosulfuron + iodosulfuron. At the end of the experiment, all the examined plants were in a good condition, revealing typically green leaves and the potential to grow further without any disturbances.

In the examined *A. myosuroides* populations, mutations in one or two domains of the *als* gene were found (Table 2). In 2011, a mutation in domain A of the *als* gene was found in Poland (Krysiak et al., 2011). At position 197 of herbicide-resistant *A. myosuroides*, a sequence CAC instead of CCC was found, leading to the replacement of proline by histidine. In the present study, proline-encoding CCC was replaced by threonine-encoding ACC. Additionally, a mutation in domain B (Trp574Leu) was found. These types of mutations have been found already in *A. myosuroides* populations elsewhere in the world (Heap, 2019). However, to our knowledge, this is the first report of Pro197Thr and Trp574Leu in ALS-resistant *A. myosuroides* in Poland. This suggests the increasing resistance of *A. myosuroides* populations in Poland to ALS-inhibiting herbicides. A similar situation has been observed in numerous countries worldwide (Heap, 2019).

The resistance level expressed by decreased biomass accumulation after herbicide treatment was connected with a number of specimens showing a mutation in the *als* gene as well as with the number of mutations in a specimen. The lowest biomass accumulation decrease was noted in RIII population (Fig. 2). Simultaneously, the largest number of plants with two mutations in the *als* gene was found among survivors in RIII population (Table 2). A similar relationship has been identified by Deng et al. (2017) in *Descurainia sophia* biotypes resistant to ALS inhibitor-type herbicides. The RII population showed the greatest reduction of biomass accumulation in the herbicide-treated samples. Here 50% of the analysed seedlings that survived had no mutation in any domain of the *als* gene.

Mutations in the *als* gene confer TRS. Based on the survival rate in RI and RII populations after 2LD treatment, it may be inferred that these populations contained seedlings with TSR and NTSR to mesosulfuron + iodosulfuron. Compared with TSR, NTSR is understudied, because its mechanisms involve multiple gene activities (Petit et al., 2010), making research more complicated. Gardin et al. (2015) have studied NTSR in *A. myosuroides*, but the analysis of differences in transcriptomic herbicide response between resistant and susceptible plants did not allow for an identification of processes directly explaining NTSR. Nevertheless, the fitness of resistant specimens may be significantly affected by mutation type (Vila-Aiub et al., 2009; Delye et al., 2013). Therefore, the present study analysed possible links between morphological traits of *A. myosuroides* and a resistance trait to mesosulfuron + iodosulfuron.

The present study's results suggest that resistant biotypes are able to reach particular phenological stages, including maturity, faster. This enables seed dispersal before crop plant harvesting, avoiding hybridisation between resistant and susceptible biotypes and achieving

Table 2. Mutations observed in the *als* gene of *Alopecurus myosuroides* plants

Population	Dose of herbicide	Plant	Pro197	Trp574
S	–	1	CCC	TGG
		2	CCC	TGG
		3	CCC	TGG
RI	1LD	1	ACC	TTG/TGG
		2	CCC	TGG
		3	CCC	TGG
	2LD	1	ACC	TGG
		2	ACC	TTG/TGG
		3	ACC	TGG
RII	1LD	1	ACC/CCC	TGG
		2	CCC	TGG
		3	CCC	TTG/TGG
	2LD	1	CCC	TGG
		2	CCC	TTG/TGG
		3	CCC	TGG
RIII	1LD	1	ACC	TTG/TGG
		2	ACC	TTG/TGG
		3	ACC/CCC	TGG
	2LD	1	CCC	TTG
		2	ACC	TTG/TGG
		3	TCC	TTG/TGG
RIV	1LD	1	ACC/CCC	TGG
		2	GCC/CCC	TGG
		3	CCC	TTG
	2LD	1	CCC	TTG
		2	ACC/CCC	TTG/TGG
		3	ACC	TTG/TGG

S – susceptible; 1LD – 100% of label dose (2.4 g ha^{-1} of iodosulfuron + 12 g ha^{-1} of mesosulfuron); 2LD – 200% of label dose (4.8 g ha^{-1} of iodosulfuron + 24 g ha^{-1} of mesosulfuron)

a higher level of resistance since larger plants reveal lower herbicide vulnerability (Manalil et al., 2011). The more advanced the plant development, the greater the activity of the GST enzyme (Milner et al., 2001), influencing the effectiveness of herbicides applied in the spring. Moreover, larger weed plants with a higher number of tillers compete more effectively with crop plants, including winter wheat. Different tillering ability may result in a wide range of economic harm caused by *A. myosuroides*, leading to up to 5% yield loss with 12 or 25 individuals per m², as reported by Keller et al. (2014) and Domaradzki et al. (2010), respectively.

Previous results concerning differences in fitness between resistant and susceptible populations are not unequivocal. Some authors have reported that resistant biotypes show a lower biomass rate and/or lower seed production (Vila-Aiub et al., 2009), or herbicide resistance not coinciding with the above-mentioned morphological parameters in comparison to herbicide-sensitive populations (Vila-Aiub et al., 2009; Keshtkar et al., 2017). A negative impact of Pro197 and Trp574 mutations in the *als* gene on plant fitness in *A. myosuroides* has not yet been confirmed. In contrast, Trp574Leu mutation in *Raphanus sativus* has resulted in visible fitness reduction in the population with a mutated *als* gene, showing 22–38% and 21–47% lower seed numbers and yield per plant than the susceptible biotype (Vercellino et al., 2018). Up to now it has not been confirmed that ALS inhibitor-resistant biotypes of *A. myosuroides* show a faster biomass increment and phenological development than susceptible biotypes. However, Delye et al. (2013) have observed later germination and a decrease in fatal germination in *A. myosuroides* specimens with Leu1781 mutation conferring resistance to ACCase inhibitors.

It is expected that individuals with delayed germination have more chance to survive in the field than fast germinating ones thanks to a greater probability of escaping pre-sowing cultural practices. In contrast, in ALS-resistant *Apera spica-venti* showing NTSR, earlier germination and earlier flowering have been found (Babineau et al., 2017). More rapid growth kinetics has also been observed for ACCase inhibitor-resistant *Setaria viridis* (Wang et al., 2010).

Conclusions

1. In two of four resistant populations in all survivors after herbicide treatment resistance conferring mutations in the *als* gene were identified; however, in the remaining two populations there were survivors without such mutations. Therefore, acetolactate synthase (ALS)-inhibitor resistance of examined black-grass (*Alopecurus myosuroides* Huds.) populations is conditioned both by target-site resistence (TSR) and non-target-site resistance (NTSR).

2. Resistance conferring mutations not identified in Poland before were found in the *als* gene of the mesosulfuron + iodosulfuron resistant populations of *A. myosuroides*, which indicates the growing problem of *A. myosuroides* resistance to ALS inhibitors in Poland.

3. Aboveground biomass accumulation and photosynthetic apparatus efficiency did not differ between the *A. myosuroides* populations examined; however, some resistant populations revealed more intensive tillering in comparison to the sensitive population, which may result in earlier development and more successful competition with crop plants.

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Alopecurus myosuroides populiacijų padidėjęs atsparumas sulfonilurėjos grupės herbicidams Šiaurės Rytų Lenkijoje

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Santrauka

Europoje žieminių javų pasėliuose daugėja herbicidams atsparių pelinio pašiaušėlio (*Alopecurus myosuroides* Huds.) populiacijų, o tai kelia problemų žieminių javų augintojams. Tyrimo tikslas – įvertinti pelinio pašiaušėlio penkių populiacijų, iš kurių keturios gali būti atsparios herbicidams ir viena jautri mezosulfuro + jodosulfurono herbicidui (acetolaktato sintezės (ALS) inhibitoriui). Taip pat buvo tirtos *als* geno mutacijos A bei B srityse ir ryšys tarp atsparumo mezosulfuronui + jodosulfuronui ir augalų išsivystymo ankstyvuoju vegetacijos tarpsniu. Augalų išsivystymas buvo įvertintas pagal jų krūmijimąsi, vidutinį lapų skaičių, sausos masės kaupimą, chlorofilo kiekį ir II fotosintezės efektyvumą.

Viso augalo tyrimas patvirtino keturių *A. myosuroides* populiacijų atsparumą ALS inhibitoriams, o biomasės kaupimosi sumažėjimas po herbicido panaudojimo atitiko *als* atsparumą suteikiančių genų mutacijų skaičių. Herbicidais neapdorotos populiacijos reikšmingai nesiskyrė nei sukauptos biomasės ir chlorofilo kiekiais, nei II fotosintezės efektyvumu. Tačiau tam tikri ALS atsparūs biotipai krūmijosi intensyviau nei jautrūs biotipai. Tirtose *A. myosuroides* populiacijose buvo nustatytos atsparumą suteikiančio *als* geno mutacijos (Pro197Thr ir Trp574Leu), kurios Lenkijoje anksčiau nebuvo nustatytos. Dviejose populiacijose buvo keletas po apdorojimo mezosulfuronu + jodosulfuronu išgyvenusių augalų, kuriems nebuvo nustatyta *als* geno mutacija. Šių populiacijų išgyvenimo lygis po apdorojimo herbicidais rodo, kad jas sudarė individai, turintys tikslinį vietos ir netikslinį atsparumo mezosulfuronui + jodosulfuronui pobūdį.

Reikšminiai žodžiai: ALS inhibitoriai, augalų stiprumas, mezosulfuronas + jodosulfuronas, pelinis pašiaušėlis, tikslinis vietos atsparumas.