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Occurrence and Mechanism of *Papaver rhoeas* ALS Inhibitors Resistance in Poland

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Abstract: Herbicide resistance in weeds, including corn poppy (*Papaver rhoeas* L.), is an increasing problem compromising global crop production. The aims of this study were to evaluate the susceptibility of *P. rhoeas* populations in Poland to acetolactate synthase (ALS) inhibitors and elucidate their mechanisms of resistance. Between 2017 and 2020, 157 seed samples were collected nationwide and a dose-response study with various ALS-inhibiting herbicides was performed in glasshouses. This revealed 14 resistant populations with R/S ranges of 2.3–1450.2, 9.5–398.5 and 2–2.5 for tribenuron, iodosulfuron and florasulam, respectively. Eight of them were cross-resistant to both tribenuron and iodosulfuron, three and one populations were singly resistant to tribenuron and iodosulfuron, respectively, and one population had reduced susceptibility to florasulam only. In one population, cross-resistance to tribenuron, iodosulfuron and florasulam was identified. The ED_{50} of many populations susceptible to ALS inhibitors was close to half the recommended dose of the herbicides tested. In seven out of eight resistant *P. rhoeas* populations analysed, target-site resistance was identified. Six amino acid replacements were found (Ala197, Arg197, His197, Leu197, Ser197 and Thr197). In one population resistant to ALS inhibitors, no mutations in the *ALS* gene were detected. An efficient anti-resistance strategy is needed to reduce the development of herbicide resistance in *P. rhoeas* in Poland.

Keywords: corn poppy; tribenuron-methyl; iodosulfuron-methyl; florasulam; target-site resistance; non-target-site resistance

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1. Introduction

The problem of weed resistance to herbicides is becoming increasingly common. Worldwide, resistant biotypes have been identified in over 266 weed species in 71 countries [1]. In winter crops in Europe, this problem mainly concerns species such as silky bentgrass, blackgrass and corn poppy. This phenomenon is based on their ability to survive and keep growing despite the application of a dose of herbicide that is considered lethal [2], and is mainly caused by simplified crop rotations and excessive use of the same active substances or herbicides with the same mechanism of action. As a result of selection pressure, the number of resistant individuals is growing, which in turn leads to higher crop production costs [1,3–5].

Corn poppy (*Papaver rhoeas* L.) is one of the most common winter crop weeds in Europe [6,7]. *P. rhoeas* dominates in winter wheat where it causes the greatest yield losses. In some countries, it is also a serious weed in winter barley, rye and winter oilseed rape [8–11]. It is a species that has adapted very well to commonly used, simplified agricultural systems, including direct sowing [7,12,13]. Seeds remain viable for a long time. According to Cirujeda et al. [14,15], 53% of seeds germinated after remaining at a depth of 20 cm for more than six years. *P. rhoeas* is a very fertile species, and if there is a lack of competition it can produce up to 800 thousand seeds per plant [16], while in the crop canopy one plant can produce 10,000–20,000 seeds. The ability of *P. rhoeas* to spread and persist in farmland can be attributed to the formation of a seed bank that is viable for many years, an extended germination period, and high seed production [16]. In countries with a temperate climate, both spring and autumn-emerging plants are able to produce seeds, increasing the harmfulness of this weed species [6], but plants emerging in autumn produce more seeds and are more vigorous and competitive [9].

For over 60 years, the synthetic auxin herbicide 2,4-D has been the most frequently used active ingredient to control *P. rhoeas*. Acetolactate synthase (ALS) inhibitors are another group commonly used in the control of this species. The first reports concerning resistance to these compounds appeared in Spain in the early 1990s, before being confirmed in Greece, Italy, France and the UK [17–19]. So far, *P. rhoeas* biotypes resistant to synthetic auxin herbicides, ALS inhibitors, or both, have been identified in ten European countries [1]. Resistance to these herbicides can be due to target-site resistance (TSR) or non-target-site resistance (NTSR) mechanisms. The TSR mechanism largely involves mutation(s) in a herbicide's target site of action, resulting in the protein's complete or partial insensitivity to the active ingredient, while the NTSR mechanisms usually result from enhanced metabolism of the herbicide [20,21]. In the case of 2,4 D resistance in *P. rhoeas*, the NTSR mechanism is proposed [18,22,23]. In one of populations of this species two independent NTSR mechanisms are reduced transport and enhanced metabolism [24]. The situation is more complex in *P. rhoeas* biotypes resistant to ALS inhibitors. In such populations, TSR only [19,25–27], NTSR only [28] or both mechanisms of resistance, can be observed and, in some cases, even in the same plant [29,30]. In populations from Greece, France, Italy, Spain and the UK, an *ALS* gene mutation in codon Pro197 (resulting in substitutions Leu, His, Arg, Ser, Thr) and Trp574 (substitution by Leu) has been observed to confer resistance to tribenuron, but no or moderate resistance to florasulam [25–27,29]. Determining whether the TSR or the NTSR mechanism is involved is essential for the correct selection of weed control method. In recent years in Europe, five active substances (cyanazine, simazine, prometryne, oxadiargyl, isoproturon) used in the control of *P. rhoeas* have been withdrawn; currently, only herbicides from groups 2 (ALS inhibitors), 5 (photosystem II inhibitors) and 4 (synthetic auxins) [2] are frequently used, with weeds developing resistance to the first two groups relatively easily [31]. The use of herbicide mixtures is an easy element of the anti-resistance strategy to implement [32,33]. Nevertheless, herbicide rotation or mixing might not be effective if applied at insufficient time intervals [4]. Moreover, it is recommended that residual herbicides are used more often, along with integrated weed management (IWM). More effective weed control can also be achieved by improving the formulation or breeding of more competitive crop varieties [34,35].

The objective of this study was to evaluate the susceptibility of *P. rhoeas* populations in Poland to ALS inhibitors, and to identify mutations in the *ALS* gene of analysed populations with the aim of elucidating their mechanism of resistance.

2. Materials and Methods

2.1. Collection of P. rhoeas Seeds

P. rhoeas seeds for the study, potentially resistant to herbicides, were collected between 2017 and 2020 from 157 farms in regions in Poland where there is frequent infestation by this species. Seeds were collected on farms where farmers had reported difficulty controlling *P. rhoeas*. Where the weed was distributed uniformly across the whole field, a 100 × 50 m plot was designated. Ripe *P. rhoeas* poppies were harvested from a few spots and combined into one sample in a paper bag [36,37]. At least 100 fully developed infructescences were harvested in one field. On each occasion, the geographic coordinates of the collection place were noted. The collected samples were stored in a ventilated, dry room until they were air-dried. The samples were then cleaned and stored in paper bags until use. In order to break dormancy, the seeds were kept in a cool room at 4 °C for 7 days [37].

2.2. Biological Tests in Glasshouses

Biological tests were carried out in the period 2018–2020 in ventilated glasshouses with a 14/10 h photoperiod during early spring and autumn, and in natural sunlight from June to July. The temperature in the glasshouses ranged from 16 to 25 °C. Multipots, with individual pots 5.5 cm in diameter, were filled with a mixture of commercial vegetable substrate Kronen® (Lasland sp. z o.o. Grądy, Poland) mixed with sand at a proportion of 2:1. A few *P. rhoeas* seeds were sown in a single pot, and after emergence the number of plants was regulated to three per pot. The experimental layout was a completely randomised design with three replicates per dose. The resistance/susceptibility of *P. rhoeas* was tested against three post-emergent herbicides (Table 1).

Table 1. Description of herbicides used in the biological tests.

Active Substance	Commercial Product	Producer	Content of Active Substancein Commercial Products	Recommended Dose of Commercial Product	Field Dose (1N) of Active Substance
Tribenuron methyl	Lumer 50 WG	ADAMA, PL	500 g kg^{-1} (50%)	30 g ha^{-1}	15 g ha^{-1}
Florasulam	Saracen 050 SC	Cheminova, PL	50 g L^{-1} (4.81%)	0.1 L ha^{-1}	5 g ha^{-1}
Iodosulfuron	Autumn 10 WG	Bayer, PL	100 g kg^{-1} (10%)	100 g ha^{-1}	10 g ha^{-1}

Distilled water solutions of the herbicides (100 mL) were prepared using the dilution method, where the highest dose of herbicide (32 times the field dose) was a stock solution. The herbicides were applied using two precision bench sprayers (FHU KAMA, Skarżysko-Kamienna, Poland and APORO Sp. z o.o., Poznań, Poland), each with a boom equipped with one flat-fan hydraulic Teejet XR 11002 VP nozzle, and calibrated to the same parameters, i.e., delivering 200 L ha^{-1} of spray at a pressure of 200 bars.

2.2.1. Discriminate Dose Experiments

To verify whether the collected *P. rhoeas* populations were herbicide-resistant, plants in the 2-leaf stage, BBCH 12 [37], were sprayed with a field dose (1N) of each herbicide (Table 1) and with distilled water (control treatment).

The reduction in visually estimated biomass (VEB) on a scale of 0–100% was assessed three weeks after spraying [37]. Plants were assessed as susceptible at VEB = 51–100%. Plants at VEB $\leq$ 50% were classified as potentially resistant and qualified for whole-plant dose-response bioassays.

2.2.2. Whole-Plant Dose-Response Bioassays

At the 2-leaf growth stage BBCH 12 [37], potentially resistant *P. rhoeas* plants were sprayed with seven doses of the herbicide: 1/2N, 1N, 2N, 4N, 8N, 16N or 32N (with N referring to the field dose). On each occasion, a susceptible population representative of the Polish region was taken for comparison and also sprayed with the following herbicide doses: 1/16N, 1/8N, 1/4N, 1/2N, 1N, 2N and 4N. Control plants were sprayed with distilled water only (0N).

Three weeks after herbicide application, the aboveground plant biomass was cut and immediately weighed (fresh biomass) using a laboratory balance (Radwag, Radom, Poland) with an accuracy of three decimal places.

The aim of the whole-plant dose-response bioassay was to establish the ED_{50} dose, i.e., the dose causing a 50% reduction in the biomass of a herbicide-treated plant. ED_{50} was calculated using the 'drc' package [38] in R ver. 4.0.1 [39]. The resistance index (RI) classification was based on the modified scale of Beckie and Tardif [40] for ALS inhibitors. The RI [41], defined as the ratio of ED_{50} values of the R and S populations, was S—susceptibility (<2); r—reduced susceptibility (2–2.9); R—low resistance (3–4.9); RR—moderate resistance (5.0–9.9); RRR—high resistance (10.0–68.1); RRRR—very high resistance (>68.2). ED_{50} for the susceptible biotype (9547) was 7.04 g ha^{-1} for tribenuron, 2.34 g ha^{-1} for florasulam and 4.67 for iodosulfuron. Dose-response curves were plotted using a logarithmic scale for herbicide dose (*X*-axis).

2.3. Molecular Analysis of the ALS Gene

Molecular analysis was undertaken to check the presence of the TSR mechanism of resistance to ALS inhibitors in the populations of *P. rhoeas* analysed. The second aim was to assess the variability of *ALS* gene sequences in the populations studied. In total, eight populations resistant to ALS inhibitors, and seven susceptible to them, were analysed. DNA was extracted from three plants originating from the RI test with tribenuron-methyl. For analysis of populations classified as susceptible, leaves were taken from plants not treated with herbicide (0N). For analysis of populations classified as being resistant to tribenuron-methyl, leaves were taken from survivors of dose 4N. If fewer than three plants survived dose 4N, plants from 2N or 1N were also taken (which was the case for population 8727). Three populations resistant to ALS inhibitors (10,303, 8727, 8961) and one susceptible population (9329) were studied in more detail. Population 10,303 with a mutation in codon 197 of the *ALS* gene was analysed after treatment with tribenuron-methyl and iodosulfuron (additional 12 survivors). In population 8727, only one individual (out of three) with a mutation in the *ALS* gene was identified; therefore, an additional five survivors after tribenuron-methyl and iodosulfuron were analysed. In population 8961, different genotypes in codon 197 of the *ALS* gene were identified; therefore, a further three survivors were studied.

DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method [42]. The concentration of each DNA sample was measured, diluted to a final concentration of 100 ng μL^{-1}, and used immediately for the polymerase chain reaction (PCR) test or stored at −20 °C until use. Primer sequences for amplification of domains A and B of the *ALS* gene were as described by Marshall et al. [27]. Primers used for the amplification of the intradomain area were designed in the present study based on the *P. rhoeas* GeneBank sequence (AJ577316) using Primer3web ver. 4.1.0 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) software accessed online. The sequences were as follows: M376F 5′-agtgaagaattgagacggtt-3′ and M376R 5′-cccttctgttaattcgtcca-3′.

Amplifications were done in 30 µL with 100 ng of genomic DNA template, 0.5 µM of each primer, 0.2 mM of each dNTP, 1× Phusion HF Buffer and 0.02 u μL^{-1} of Phusion HF DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions for primers designed by Marshall et al. [27] (2010) were modified, and a two-step protocol was applied as follows: 98 °C 30 s followed by 35 cycles of 98 °C 10 s, 72 °C 30 s and a final extension of 10 min at 72 °C. Cycling conditions for the amplifications of the intradomain

area were: 98 °C 30 s followed by 35 cycles of 98 °C 10 s, 58 °C 30 s, 72 °C 30 s and a final extension of 10 min at 72 °C. Cycling reactions were performed using SimplyAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, USA).

Genomed S.A. (Warsaw, Poland) was commissioned to undertake purification of the PCR product and sequencing. Chromatograms were analysed using FinchTV software (Geospiza, Washington, DC, USA). The obtained sequences were compared using ClustalW software (GenomeNet, Kyoto, Japan) accessed online. The amino acid numbering refers to the *Arabidopsis thaliana ALS* sequence (GenBank: X51514).

3. Results

3.1. Reaction of P. rhoeas Populations to ALS Inhibitors

The reaction of *P. rhoeas* populations in the whole-plant dose-response bioassays to ALS inhibitors varied (Table 2). A total of 14 out of 157 populations showed resistance, or at least reduced susceptibility, to the active ingredients tested. Populations were most often (12 out of 14) resistant to tribenuron-methyl. The R/S ratio ranged from 2.3 (10,374) to 1450.2 (10,186). As many as eight out of 12 populations demonstrated a high (RRR) and very high (RRRR) level of resistance to this active ingredient. These populations originated from the provinces of Warmia-Mazuria, Lower Silesia, West Pomerania and Lublin (Figure 1). A total of 10 out of 14 populations were found to be resistant to iodosulfuron-methyl, with ED_{50} values ranging from 9.5 to 398.5. Two of these populations (10,410, 10,612) showed reduced susceptibility to this active ingredient (R/S < 2.9) and four of them were found to be resistant to iodosulfuron at a high or very high level (R/S > 10.1). During the whole-plant dose-response bioassays to florasulam, only two populations (8833, 10,416) with reduced susceptibility to this active ingredient were found, with ED_{50} values of 4.7 and 6.0, respectively. These populations were found in the provinces of Świętokrzyskie and Lublin (Figure 1). Population 10,416 from the province of Lublin was diagnosed as being cross-resistant to tribenuron-methyl, iodosulfuron-methyl and florasulam. Its level of resistance was different for each of the active ingredients tested: high for tribenuron-methyl, moderate for iodosulfuron-methyl and reduced susceptibility to florasulam. Eight populations were found to be resistant to tribenuron-methyl and iodosulfuron-methyl, with a high or very high level of resistance to both active ingredients in four of these populations. Three populations were resistant to tribenuron-methyl only, while one population was diagnosed as resistant to iodosulfuron-methyl only, and one to florasulam only.

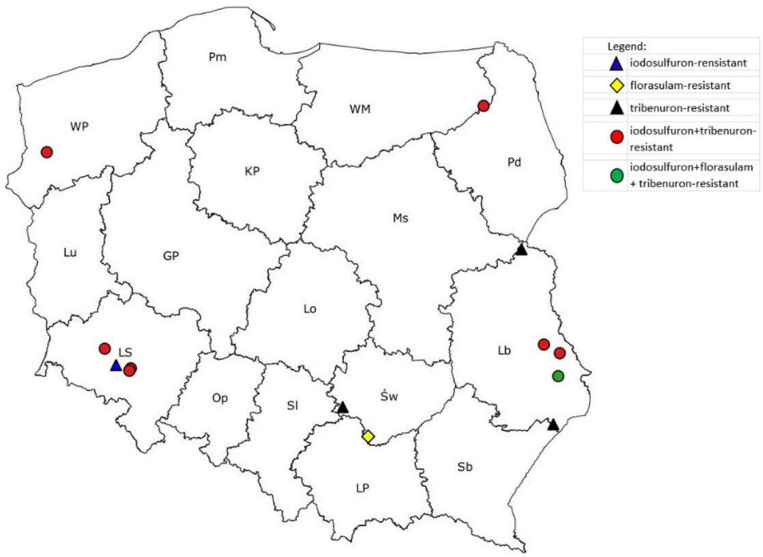

Figure 1. Locations in Poland where herbicide-resistant populations of *P. rhoeas* L. were found. Abbreviations: GP—Greater Poland, KP—Kuyavia-Pomerania, LP—Lesser Poland, LS—Lower Silesia, Lb—Lublin, Ms—Masovia, Pd—Podlasie, Pm—Pomerania, Sb—Subcarpathia, Św—Świętokrzyskie, WM—Warmia-Masuria, WP—West Pomerania, Op—Opole, Sl—Silesian, Lo—Łódź, Lu—Lubusz.

Table 2. Populations of *P. rhoeas* resistant to acetolactate synthase inhibitors characterised by ED_{50} (effective dose causing 50% shoot fresh weight reduction in the treated plants) and resistance index (RI).

Population	Province	Tribenuron-Methyl			Iodosulfuron-Methyl-Na			Florasulam		
		RI	ED_{50} (g ha^{-1})	R/S	RI	ED_{50} (g ha^{-1})	R/S	RI	ED_{50} (g ha^{-1})	R/S
8491 *	Lb	RR	47.9 ± 14.4 **	6.8	S	X	X	S	X	X
8727 *	Lb	RR	50.6 ± 10.2	7.2	S	X	X	S	X	X
8833	Św	S	X	X	S	X	X	r	4.7 ± 1.6	2.0
8935	LS	S	X	X	R	16.7 ± 1.07	3.6	S	X	X
8961 *	WM	RRRR	545.6 ± 109.1	77.5	RR	23.2 ± 5.8	5.0	S	X	X
9068 *	LS	RRR	201.1 ± 20.1	28.6	RRRR	398.5 ± 14.2	85.4	S	X	X
9069 *	LS	RRRR	4089 ± 531.6	580.7	RRR	283.7 ± 37.4	60.8	S	X	X
9440	Św	RR	35.7 ± 5.3	5.1	S	X	X	S	X	X
10186	LS	RRRR	10,212 ± 2246.6	1450.2	RRR	101.0 ± 16.2	21.6	S	X	X
10303 *	WP	RRR	63.9 ± 11.5	3.2	RRR	136.3 ± 30.0	29.2	S	X	X
10374	Lb	R	16.3 ± 2.3	2.3	R	23.0 ± 4.4	4.9	S	X	X
10410 *	Lb	RRR	164.1 ± 34.5	23.3	r	10.7 ± 2.0	2.3	S	X	X
10416 *	Lb	RRR	180.6 ± 11.7	25.7	RR	24.3 ± 4.6	5.2	r	6.0 ± 1.8	2.5
10612	LS	RRRR	>480	>68.2	r	9.5 ± 1.22	2.0	S	X	X

RI (resistance index) on the basis of R/S value: S—susceptibility (<2); r—reduced susceptibility (2–2.9); R—low resistance (3–4.9); RR—moderate resistance (5.0–9.9); RRR—high resistance (10.0–68.1); RRRR—very high resistance (>68.2) (the modified scale of Beckie and Tardif [40]); X—not calculated; * populations subjected to molecular analysis; ** standard error.

The field dose of tribenuron-methyl reduced the fresh biomass of resistant populations to a lesser extent than that of the susceptible populations. The reduction ranged from 40.7% in cases of very high resistance (RRRR) to 67.7% in cases of low resistance (R) compared with the susceptible population (Figure 2). The highest tested dose of tribenuron-methyl (32N) caused a reduction of just 33.5% in the population with a very high level of resistance (10,186). Populations diagnosed as R and RR reacted similarly to the tribenuron-methyl dose at the level of double field dose or higher.

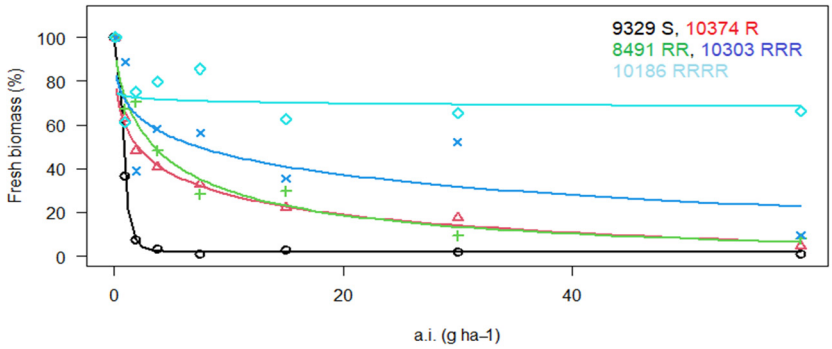

Figure 2. Dose-response curves of *P. rhoeas* populations resistant (R, RR, RRR, RRRR) and susceptible (S) to tribenuron-methyl.

Iodosulfuron-resistant populations responded to the recommended dose with a smaller reduction in fresh weight of 70% or more compared with the susceptible population (Figure 3). Populations with a high or very high level of iodosulfuron-methyl resistance responded similarly to the application of this active ingredient. The highest tested dose of iodosulfuron-methyl (32N) caused fresh weight reductions of 63.8% and 45.9%, respectively.

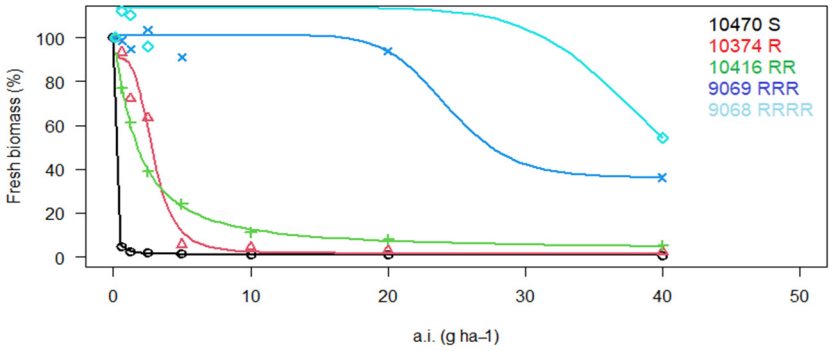

Figure 3. Dose-response curves of *P. rhoeas* populations resistant (R, RR, RRR, RRRR) and susceptible (S) to iodosulfuron-methyl-Na.

The ED_{50} values of *P. rhoeas* populations susceptible to tribenuron-methyl ranged from 0.01 to 8.07 (Figure 4a). It should be noted that there was a large group of populations with an ED_{50} close to or above 0.5N (7.5 g ha^{-1}). The ED_{50} values of two tribenuron-methyl susceptible populations (9486 and 10,607) were more than half of the recommended field dose of this active ingredient. ED_{50} values of 11 populations were slightly below the level of 0.5 N and ranged from 6.59 to 7.4. The ED_{50} values of *P. rhoeas* populations susceptible to iodosulfuron-methyl ranged from 0.01 to 7.13 (Figure 4b). The ED_{50} values of two iodosulfuron-methyl susceptible populations were more than half the recommended field dose of this active ingredient (5 g ha^{-1}), while the value for population 10,407 was as high as 7.13. The ED_{50} values of 18 populations were slightly lower than 0.5 N and ranged from 4.51 to 4.86. The ED_{50} values of populations susceptible to florasulam ranged from 0.01 to 3.70 (Figure 4c). Three populations were characterised by ED_{50} values above 0.5 N

(2.56–3.70). The ED_{50} values of 13 populations were slightly less than half the recommended dose of florasulam (2.5 g ha^{-1}) and ranged from 2.22 to 2.41.

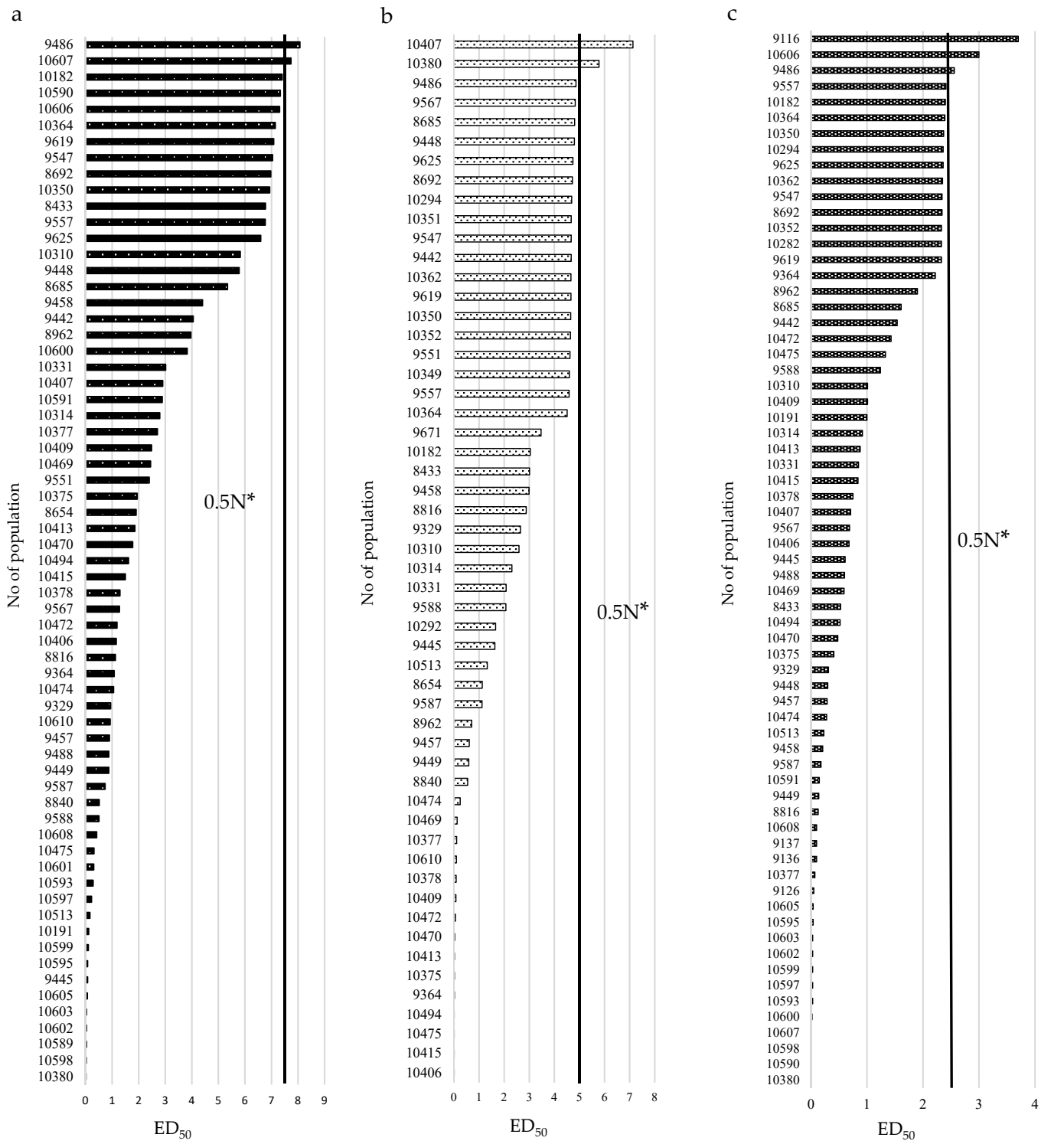

Figure 4. Populations of *P. rhoeas* susceptible to tribenuron-methyl (**a**), iodosulfuron-methyl-Na (**b**) and florasulam (**c**) characterised by ED_{50} (effective dose causing 50% shoot fresh weight reduction in the treated plants). * Half of the recommended field dose of tribenuron-methyl, iodosulfuron-methyl-Na and florasulam.

3.2. Molecular Analysis of the ALS Gene

DNA was isolated from 69 individuals belonging to 15 populations: eight resistant to ALS inhibitors, and seven susceptible to them (Table 3). Populations were qualified as resistant or susceptible according to the RI test. No substitution at codon Pro197 was found in plants from susceptible populations. However, six amino-acid replacements were identified at this position (Ala, Arg, His, Leu, Ser, Thr) in populations resistant to ALS inhibitors. In populations 8961, 9068, 9069, 10,303, 10,410 and 10,612, all analysed individuals had a substitution in codon Pro197. In the majority of populations with a mutation in codon 197, more heterozygous mutants were identified than homozygous mutants. Population 9069 was the only exception, with all three survivors analysed being homozygous mutants. Population 8961 was the most diversified in codon Pro197, where three different substitutions (Ala197, His197, Ser197) were found. In the remaining resistant populations, all plants within the population had the same type of substitution. In resistant population 8727, the substitution Leu197 was identified in one plant only, and in population 8491 no substitutions were present in codon Pro197. In the majority of individuals without mutations in codon 197 (20 out of 34), the sequence of the *ALS* gene was identical to the wild type of the gene in *P. rhoeas* (GenBank: AJ577316) (Table 3).

In three populations resistant to ALS inhibitors, more than three individuals were analysed: 15, 8 and 6 individuals from populations 10303, 8727 and 8961, respectively. Despite analysing 15 individuals from population 10303, only one type of substitution was found: nine heterozygotes (CST) and six homozygotes (CGT) resulting in the substitution Arg197. Population 8961 was diversified in codon 197. Three individuals had one substitution—KCT and SCT in one and two individuals, respectively—however in three plants a double substitution was identified (SMT), resulting in the replacement Ala/His197. In population 8727, in one plant out of eight, the substitution Leu197 was identified. This was a heterozygous mutant. The remaining survivors in this population had no mutation in codon 197 (Table 3).

Of all the individuals analysed, other than in Pro197, no non-synonymous substitutions in other codons of the *ALS* gene were identified as being responsible for ALS inhibitor resistance in *P. rhoeas* or other weed species (Ala122, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654). In 43 out of 69 individuals, at least one substitution was identified apart from codon 197; however, these substitutions were present in populations resistant and susceptible to ALS inhibitors. The mutations concerned codons Ile136, Glu144, Ile211, Ile411, Gly417, Gly438, Met445 and Leu453. Replacement Met445Lys was the most frequent—identified in 10 out of 69 individuals (five individuals with a mutation in codon 197 and five individuals without a change in this codon). Non-synonymous mutations were identified in five codons: Glu144Lys, Ile411Val, Gly417Met, Gly438Ala and Met445Lys. These substitutions were present in individuals from populations resistant to ALS inhibitors, but also in at least one individual from a population rated as susceptible on the basis of the biological test.

In two individuals from population 8491 rated as resistant to tribenuron methyl, non-synonymous substitutions were identified. In this population, no mutation in codon 197 was found. However, these substitutions (Ile411Val, Gly417Met, Gly438Ala and Met445Lys) were not responsible for resistance to ALS inhibitors because they were also identified in susceptible populations.

Table 3. Genetic variability in the acetolactate synthase of *P. rhoeas* populations. Only positions related to *ALS* resistance (Ala122, Pro197, Ala205, Asp376, Trp574, Ala653 and Gly654) and others where mutations were found are represented. All sequences were compared with the wild type *P. rhoeas ALS* gene (GenBank: AJ577316). IUPAC code was used for the nucleotide nomenclature. Dots indicate codons identical to those of the wild type *ALS* gene. ALS-resistant populations are underlined.

Ala 122 GCA	**Ile 136 ATT**	**Glu 144 GAA**	**Pro 197 CCT**	**Ala 205 GCA**	**Ile 211 ATT**	**Asp 376 GAT**	**Arg 377 CGT**	**Ile 411 ATC**	**Gly 417 GTG**	**Glu 427 GAA**	**Gly 438 GTG**	**Met 445 ATG**	**Leu 453 TTG**	**Trp 574 TGG**	**Leu 648 TTG**	**Ala 653 GCT**	**Gly 654 GGT**	**No. of occ.***	**Population** **Wild Type *ALS***
GCA	ATT	GAA	CCT	GCA	ATT	GAT	CGT	ATC	GTG	GAA	GTG	ATG	TTG	TGG	TTG	GCT	GGT	20	8816 8727 8491 8483 9329 10,188 10,310 10,314 10,331
. . .	. . .	. . .	CST **	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	6	10,303
. . .	. . .	. . .	CST	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	YTG	. . .	. . .	. . .	. . .	1	10,303
. . .	. . .	. . .	CGT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	CTG	. . .	. . .	. . .	. . .	4	10,303
. . .	. . .	. . .	CGT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	AWG	CTG	. . .	. . .	. . .	. . .	1	10,303
. . .	. . .	. . .	CGT	. . .	. . .	. . .	. . .	. . .	RTG	. . .	. . .	AWG	YTG	. . .	. . .	. . .	. . .	1	10,303
. . .	. . .	. . .	CST	. . .	. . .	. . .	. . .	RTC	. . .	. . .	GYG	AWG	. . .	. . .	. . .	. . .	. . .	1	10,303
. . .	. . .	. . .	CST	. . .	. . .	. . .	. . .	. . .	RTG	. . .	. . .	AWG	. . .	. . .	. . .	. . .	. . .	1	10,303
. . .	. . .	. . .	CYT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	1	8727
. . .	. . .	. . .	TCT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	1	10,410
. . .	. . .	. . .	YCT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	4	10,410 10,416
. . .	. . .	. . .	ACT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	4	9068 9069
. . .	. . .	. . .	MCT	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	2	9068
. . .	. . .	. . .	KCT	. . .	ATY	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	1	8961
. . .	. . .	. . .	SCT	. . .	ATY	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	2	8961
. . .	. . .	. . .	SMT	. . .	ATY	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	3	8961
. . .	. . .	. . .	YCT	. . .	. . .	. . .	. . .	RTC	RTG	. . .	. . .	AWG	. . .	. . .	. . .	. . .	. . .	1	10,416
. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	RTC	RTG	. . .	GYG	AWG	. . .	. . .	. . .	. . .	. . .	3	8491 8483
. . .	ATW	. . .	. . .	. . .	. . .	. . .	. . .	RTC	RTG	. . .	GYG	AWG	. . .	. . .	. . .	. . .	. . .	1	10,314
. . .	. . .	. . .	. . .	. . .	ATY	. . .	. . .	. . .	. . .	. . .	. . .	AWG	. . .	. . .	. . .	. . .	. . .	1	10,310
. . .	. . .	. . .	. . .	. . .	ATY	. . .	. . .	RTC	RTG	. . .	GYG	AWG	. . .	. . .	. . .	. . .	. . .	1	8816
. . .	. . .	. . .	. . .	. . .	ATY	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	3	8816 8727 10,314
. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	RTC	. . .	. . .	. . .	AWG	. . .	. . .	. . .	. . .	. . .	2	10,188 10,331
. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	YTG	. . .	. . .	. . .	. . .	1	9329
. . .	ATW	RAA	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	1	9329
. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	CTG	. . .	. . .	. . .	. . .	1	9329

* Number of occurrences, ** S = C/G, Y = C/T, W = A/T, R = A/G, M = A/C, K = G/T.

4. Discussion

P. rhoeas is widespread throughout Europe and mainly infests winter cereals [11]. In Poland, a rising number of cases have also been noted in winter rape [43]. The genetic diversity of *P. rhoeas* indicates its high adaptation potential to changing environmental conditions and its ability to colonise new habitats [44,45]. Studies from Poland during the second half of the last century indicate that this species is most abundant in fertile soils that are rich in humus and calcium carbonate [46–49]. However, studies conducted in recent years indicate that *P. rhoeas* is increasingly colonising soils that are rich in nutrients, but that are also slightly acidic or neutral [43]. In regions where resistant *P. rhoeas* populations were identified in this study, the soils are rich in calcium carbonate, and this species has been infesting crops there for a long time [46,50–52]. For several years, farmers have had problems with effective control of this species.

Since the end of the last century Europe has been struggling with the problem of *P. rhoeas* resistance to chemical control. This problem is particularly serious in cereal crops in the Mediterranean region [11]. In Poland, this species' resistance to ALS inhibitors was first reported by Adamczewski et al. [53] in a study concerning *P. rhoeas* resistant to tribenuron-methyl. The research described in the present paper shows that the issue of resistance of *P. rhoeas* to herbicides is worsening in Poland. Out of 157 seed samples tested, resistance was found in 14 cases, and was often cross-resistance to different chemistries of ALS inhibitors. This phenomenon is in line with the general trend of the decreasing effectiveness of post-emergence herbicide treatments in the control of common cereal weeds. Describing the development of resistance of *Alopecurus myosuroides* and *Apera spica-venti* in Germany over a period of 15 years, Petersen and Raffel [54] also found a significant rise in the percentage of resistant populations, including multiple-resistant populations. The problem worsened after 2017, which the authors attribute to the selective pressure of ALS inhibitors. Among the *P. rhoeas* samples analysed in the present study, a significant percentage of populations required about half of the field herbicide dose for effective elimination. These populations can be described as being "in the waiting room" for acquisition of resistance to ALS inhibitors, and the fact that they have been selected for research indicates that farmers have already identified issues with the control of this species in their fields.

In seven out of eight resistant *P. rhoeas* populations analysed, TSR due to mutations was identified. This was due to mutations in codon 197 of the *ALS* gene. Six amino acid replacements were found (Ala197, Arg197, His197, Leu197, Ser197 and Thr197). This is the first molecular analysis of Polish *P. rhoeas* populations resistant to ALS inhibitors. These genotyping results are consistent with earlier studies of other European populations, with the same mutations identified in codon 197 of the *ALS* gene [1]. Délye et al. [29] and Kati et al. [19] also describe mutations in codon 574 of the *ALS* gene in *P. rhoeas*. In the present study, no replacement was found in this codon. As the research conducted in Europe shows, the mutation in codon 574 is much rarer. This mutation has previously been observed in a single plant from one population in Italy [29] and in two populations in Greece [19], while in Spain, after sequencing more than 1000 plants, it was never found [13,30]. In the present study, no mutation was found in the other codons known to be responsible for ALS inhibitor resistance: 122, 205, 376, 377, 653 and 654 [1]. Similarly, these six codon replacements were not found either by other teams studying the molecular basis of *P. rhoeas* resistance to ALS inhibitors.

Only one of seven populations with TSR analysed at the molecular level in this study showed high diversity in codon 197 of the *ALS* gene. In population 8961, four out of six individuals were found to be trans-heterozygotes (i.e., they contained two different types of mutant *ALS* alleles). Similar variability has been observed for some Greek, Italian and Spanish *P. rhoeas* populations resistant to ALS inhibitors [26,29,30].

The presence of the mutated codon 197 in all analysed survivors within the population provided high resistance to tribenuron-methyl. The Pro197 amino-acid residue is directly involved in anchoring the aromatic ring of sulfonylureas. Any substitution in this position

will affect sulfonylureas binding, resulting in a high level of resistance to this active ingredient [55]. In contrast, the presence of the mutated *ALS* allele in codon 197 did not confer resistance to florasulam. Reduced susceptibility was only observed in population 10,416. This population's low level of susceptibility to florasulam could be due to the presence of individuals with Ser197 replacement. As proposed by Délye et al. [29], Ser197 does not confer resistance of *P. rhoeas* to this herbicide, although it can reduce the sensitivity of the plants carrying it. An NTSR component of population 10,416's resistance to ALS inhibitors cannot be excluded.

The populations of *P. rhoeas* analysed in this study were found to be variable not just in codon 197 of the *ALS* gene. Analysis of this gene sequence of survivors revealed five codons with non-synonymous mutations (Glu144Lys, Ile411Val, Gly417Met, Gly438Ala and Met445Lys), other than those known from the literature, to contribute to ALS resistance. As these substitutions were not just identified in ALS resistant populations but in susceptible populations too, and it was assumed that these new mutations were not involved in the resistance response. Variation unconnected to resistance in other codons of this gene (Gly281, Gly427 and Leu648) was detected in earlier studies of *P. rhoeas* [25,30]; however, in the Polish populations these codons were identical to the wild type (GenBank: AJ577316).

Populations 9068 and 9069 originated from the same village, but from different fields; therefore, it is possible that these two cases of resistance are not independent, but rather are due to the spread of seeds or pollen of the resistant biotype. The sequence of the *ALS* gene in individuals from these two populations is identical. The only difference is the sequence of codon 197. Homozygote mutants (ACT) or heterozygotes (MCT) were found in survivors after the RI test; however, these codons resulted in the same substitution Thr197. Other cases of resistance seem to be independent appearances, even in populations 10,410 and 10,416. Individuals from these populations had the same replacement Ser197; however, they originated from villages around 90 km apart and the sequence of the *ALS* gene of these plants was not identical due to some non-synonymous mutations unconnected to resistance. Populations 10,410 and 10,416 also differed in their susceptibility to florasulam. The results of this study confirm the thesis formulated by Délye et al. [29] for Italian populations of *P. rhoeas* resistant to ALS inhibitors, that mutant *ALS* alleles evolve by multiple, independent appearances.

In population 8491, none of the plants that survived the 4N dose of tribenuron had mutations in codons of the *ALS* gene known to be responsible for ALS resistance. In the case of population 8727, seven out of eight survivors analysed had no such mutation either. Therefore, ALS resistance in population 8491 is probably due to NTSR, with TSR and NTSR coexisting in population 8727. Other TSR mechanisms of ALS resistance have been already diagnosed in European populations of *P. rhoeas* [24,28–30]. In one of the populations analysed by Rey-Caballero et al. [30], only one of 51 plants presented a substitution in codon 197. The results obtained for the Polish population 8727 are similar to the situation described for Mediterranean populations, where both target site and non-target site mechanisms conferring resistance to ALS inhibitors can coexist in the same weed population in *P. rhoeas*.

Compared with western Europe, the situation with *P. rhoeas* in Poland is in its infancy. However, the increasing area of winter oilseed rape each year, and its consistently very high share in the structure of winter cereals [56], mean that *P. rhoeas* is proliferating in arable fields [52], significantly increasing the likelihood of exacerbating herbicide resistance in this species. To prevent this, the desired action would be to implement methods of integrated protection and resistance management, the effectiveness of which has been reported in numerous studies [7,54,57,58]. As described by [17], ALS and 2,4-D resistance in some populations of this species coexist. According to [30], selection pressure with ALS non-SU inhibitors carries the risk of promoting the evolution of NTSR mechanisms in *P. rhoeas*. It is necessary to change weed harmfulness thresholds and strive to reduce the seed bank as much as possible. The reality for most growers today is that weed seed banks in their fields are probably resistant to one or more herbicide site(s) of action. A zero-tolerance policy ('take no prisoners') is now being advocated [59,60].

5. Conclusions

In the evaluation of the reaction of *P. rhoeas* to ALS inhibitors, resistance was found in 14 populations from the provinces of Lublin, Świętokrzyskie, Warmia-Mazuria, Lower Silesia and West Pomerania. Most of them showed resistance to tribenuron, 10 were resistant to iodosulfuron and two to florasulam. Worryingly, eight populations had cross-resistance to tribenuron and iodosulfuron, and half of them showed high (RRR) or very high (RRRR) resistance to both active substances. In one population, cross-resistance to tribenuron, iodosulfuron and florasulam was identified. ED_{50} values for tribenuron, iodosulfuron and florasulam of many susceptible *P. rhoeas* populations fluctuated at around half of the recommended dose (0.5 N), which could be a concern, indicating that resistance to these active substances is developing.

In six out of eight analysed resistant *P. rhoeas* populations, TSR was identified in all plants from the population. This was due to mutations in codon 197 of the *ALS* gene. In one out of eight populations resistant to ALS inhibitors, the presence of the NTSR mechanism is proposed as the main mechanism of resistance due to the lack of mutations in the *ALS* gene of studied survivors from the biological test. In one *P. rhoeas* population, TSR and NTSR to ALS inhibitors probably coexist. In some codons of the *ALS* gene, diversity was detected, but it was not related to herbicide resistance to ALS inhibitors as these mutations were observed in populations rated as being resistant or susceptible to ALS inhibitors. Populations with the NTSR mechanism are very prone to developing resistance to different mechanisms of action. Therefore, an intensive *P. rhoeas* anti-resistance strategy should be implemented in agricultural practice, aimed at preventing the build-up of resistance to ALS inhibitors and multiple resistance.

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