agronomy

MDPI

Article

Impact of ALS Herbicide-Resistant Perennial Ryegrass (*Lolium perenne*) Population on Growth Rate and Competitive Ability against Wheat

Aristeidis P. Papapanagiotou [1,*], Dimitrios Loukovitis [2], Eleni Anthimidou [3] and Ilias G. Eleftherohorinos [3]

[1] Laboratory of Agronomy, Department of Agriculture, University of Western Macedonia, Terma Kontopoulou Str., 53100 Florina, Greece
[2] Department of Fisheries and Aquaculture, School of Agricultural Sciences, University of Patras, New Buildings, 30200 Messolonghi, Greece; dloukovi@upatras.gr
[3] Laboratory of Agronomy, Department of Field Crops and Ecology, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; anthimidou.lena@gmail.com (E.A.); eleftero@agro.auth.gr (I.G.E.)
* Correspondence: apapanagiotou@uowm.gr

Abstract: Three perennial ryegrass (*Lolium perenne*) populations (R1, R2, and R3) with suspected resistance (R) to acetolactate synthase (ALS) or acetyl-CoA carboxylase (ACCase) herbicides were collected from wheat (*Triticum aestivum*) fields in northwestern Greece to study the underlying mechanisms of resistance and their impact on growth rate and competitive ability against wheat. Preemergence and postemergence plant dose–response assays showed that the R1 population was cross-resistant to the ALS inhibitors chlorsulfuron, mesosulfuron + iodosulfuron, and pyroxsulam, but susceptible (S) to imazamox. However, all populations were susceptible to the ACCase inhibitors clodinafop-propargyl, clethodim, diclofop-methyl, and pinoxaden. The analysis of the *ALS* gene sequence revealed a substitution of Pro197 by His or Leu in the ALS enzyme in *L. perenne*, which is reported for the first time in this weed and indicates a potential mechanism of target site-mediated resistance. The R1 population grown in the absence or presence of wheat competition displayed similar aboveground biomass and tiller number trends, and therefore similar estimated growth rates. In addition, the aboveground biomass of wheat was similarly reduced by both the R1 and S populations, supporting the evidence of their similar competitive ability against wheat. In general, these findings indicate that there is no clear evidence for the fitness advantage of R1 over the S population.

Keywords: ALS inhibitors; fitness trait; resistance evolution; weed–crop competition

Citation: Papapanagiotou, A.P.; Loukovitis, D.; Anthimidou, E.; Eleftherohorinos, I.G. Impact of ALS Herbicide-Resistant Perennial Ryegrass (*Lolium perenne*) Population on Growth Rate and Competitive Ability against Wheat. *Agronomy* **2023**, *13*, 1641. https://doi.org/10.3390/agronomy13061641

Academic Editors: Thomas K. Gitsopoulos and Nicholas Emmanuel Korres

Received: 28 March 2023
Revised: 15 June 2023
Accepted: 16 June 2023
Published: 19 June 2023

1. Introduction

The ability of perennial ryegrass (*Lolium perenne* (L.) ssp. *perenne*), Italian ryegrass (*L. multiflorum* Lam. or *L. perenne* (L.) ssp. *multiflorum* (Lam.) Husnot), and rigid ryegrass (*L. rigidum* L.) to rapidly adapt in agricultural and non-agricultural areas has gained particular interest from weed scientists [1]. These species are consistently included among the most serious weeds of winter cereal and perennial crops, such as orchards, olive groves, vineyards, and alfalfa (*Medicago sativa* L.) [2–4]. According to their distribution, they are native to Europe, temperate Asia, and North Africa, but their presence has also spread over the past two centuries to southern parts of Africa, Australia, South America, New Zealand, and North America [5–7].

Ryegrass species have a diploid ($2n = 2x = 14$) chromosome number and are characterized by self-incompatibility, rendering them obligate out-crossers. Due to the free cross-pollination occurring between *L. perenne* and *L. multiflorum*, numerous hybrids with intermediate characteristics have been naturally developed, which are characterized by

high genetic variability, adaptability in agricultural landscapes, high phenotypic plasticity, and prolific seed production [7–9].

The control of various ryegrass species in winter cereal crops relies mainly on herbicides inhibiting the acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) enzyme activity, applied either alone or in mixtures. Unfortunately, these two herbicide groups are associated with the highest risk of the rapid evolution of target-site resistance, which is most commonly due to a single point mutation in the *ALS* or *ACCase* gene that results in the substitution of an amino acid in the ALS or ACCase target enzyme [10–12]. The herbicide resistance genes of ryegrass species spread easily via wind-mediated pollen movement due to their self-incompatible and obligate out-crossing traits [13,14]. In addition, mutant alleles conferring resistance to ryegrass are generally not linked and, therefore, allow different combinations of resistance mechanisms to accumulate independently [11]. Consequently, multiple herbicide-resistant ryegrass plants and populations evolve very rapidly, exhibiting complex herbicide resistance patterns, due to both target site-based resistance (TSR) and non-target site resistance (NTSR) mechanisms [15–17]. The mechanisms conferring TSR include an alteration of the herbicide target enzyme caused by point mutations within the coding gene or an overproduction of the target enzyme [18–20]. On the other hand, NTSR refers to all other mechanisms evolved within a weed that result in reduced herbicide activity, such as reduced uptake or translocation, increased sequestration, or enhanced metabolism [18,21,22]. Ultimately, the evolution of weeds with multiple herbicide resistance due to the accumulation of various resistance mechanisms reduces the options for herbicide rotation with different modes of action to manage these weeds [11].

Target site-mediated resistance in *L. perenne* species has already evolved to ALS inhibitors in France [23] and in California [24], whereas populations from Chile were found to have TSR to ACCase and EPSPS, and NTSR to the ALS inhibitor iodosulfuron [11]. In addition, an *L. perenne* population in Argentina evolved cross-resistance to pinoxaden, clethodim, and quizalofop due to the Asp-2078-Gly mutation [25], whereas *Lolium* ssp. (*L. perenne*, *L. multiflorum*, and their hybrids) populations from this country were found to have multiple resistance to pinoxaden and iodosulfuron+mesosulfuron, which was mainly due to cytochrome P450-mediated herbicide metabolism [26]. Also, three *L. perenne* populations in Texas were found to be multiple resistant to diclofop-methyl and mesosulfuron [27], whereas an *L. perenne* population from New Zealand was found to have cross-resistance to pinoxaden and quizalofop-p-ethyl due to both the target-site mechanism (isoleucine to valine replacement at position 2041 of the *ACCase* gene) and cytochrome P450-based metabolism [28]. Moreover, many *L. perenne* populations in southwestern North Carolina evolved cross-resistance to diclofop and pinoxaden, five of which were multiple-resistant to diclofop, pinoxaden, and mesosulfuron, and two were cross-resistant to imazamox, mesosulfuron, and pyroxsulam [29]. Finally, *L. perenne* populations with multiple resistance to both ACCase and ALS inhibitors were reported in cereal crops grown in Germany and Denmark, whereas populations of this weed in Portugal, New Zealand, and Argentina were found to be resistant to the EPSPS inhibitor glyphosate [10,30].

Plant traits (e.g., seed germination, seed production, early vigor, tillering, growth rate, biomass production, and competitive ability) linked to herbicide-resistant alleles are key players in the evolution of adaptive alleles fixation, but the rate of this process depends on their opposing cost and benefit effects [31]. More specifically, the benefits of the resistance alleles are clear and usually appear very fast, as they allow plants to survive and reproduce under herbicide selection, while the cost effects of the resistance alleles on the surviving resistant weed plants are unclear and usually appear slowly because of their reduced adaptive and establishing potential compared to crops and other weed plants. Regarding fitness/adaptation penalty associated with herbicide resistance alleles, Vila-Aiub et al. [32] reported that these are evident among plant species, but their expression depends on particular herbicide resistance gene and allele, the genetic background, the dominance of the fitness cost, and the abiotic and biotic environmental conditions.

Lolium perenne is less abundant than *L. rigidum* in winter cereal crops in Greece. However, some cereal farmers in northwestern Macedonia, Greece, observed poor control of this weed after spraying their crops with ALS-inhibiting herbicides during the 2020 growing season. This information prompted the current study, with objectives to (1) investigate whether three *L. perenne* populations from the study area have evolved resistance to ALS-inhibiting herbicides, (2) evaluate the potential preemergence and postemergence herbicides as alternatives for the management of these populations, (3) elucidate the possible target site-mediated resistance mechanism, (4) assess the growth rate (aboveground biomass and tiller number) of an R and a reference S *L. perenne* population without competition, and (5) compare their competitive ability against wheat.

2. Materials and Methods

2.1. Plant Material

Lolium perenne seeds were collected from three cereal monoculture fields located in northwestern Greece (Table 1), where the preemergence chlorsulfuron or the postemergence mesosulfuron-methyl + iodosulfuron-methyl-sodium or pyroxsulam resulted in an insufficient control. More specifically, mature weed seeds were collected by hand from 70 to 80 plants grown in different patches within each field, prior to the harvest of winter wheat. In addition, an *L. perenne* population from an adjacent non-cultivated area, which was never exposed to herbicides, was also included and served as a reference S population. The collected seeds from each field were pooled together to form the field population, air-dried and threshed at the laboratory, and subsequently placed in paper bags and stored in a cold room at 5–7 °C until needed.

Table 1. Location, code, and geographical position of *Lolium perenne* populations evaluated in the present study.

Location	Code of Population	Position
Florina	R1	40°83351 N, 21°46629 E
Florina	R2	40°88353 N, 21°51354 E
Florina	R3	40°80726 N, 21°48755 E
Florina	S	40°87470 N, 21°51652 E

2.2. Plant Dose–Response Bioassays to Preemergence Herbicides

A pot experiment was carried out at the farm of the University of West Macedonia, Florina, during the middle of autumn (10 October to 21 November) 2020 to access three *L. perenne* suspected R populations (R1, R2, R3) for possible ALS-resistance evolution to the sulfonylurea herbicide chlorsulfuron, which has been used for many years as a preemergence standard practice for weed control in winter cereals grown in northwestern Greece. The experiment was carried out in plastic pots (0.9 L vol.) filled with a mixture of clay loam soil (31.6% clay, 48.0% silt, 20.4% sand, 1.3% organic matter, and 7.8 pH): peat: sand mixture (3:1:1, *v*/*v*). In each pot, approximately 30 *L. perenne* seeds were sown and covered carefully with about 1 cm of the soil mixture. The three putative R populations were treated immediately after pot seeding with the recommended (1X), 2X, and 4X field label rates of the preemergence application of chlorsulfuron (Glean 75 WG, DuPont Hellas, Athens, Greece), photosystem II (PS II) inhibitor chlorotoluron + phytoene desaturase (PDS), inhibitor diflufenican (Constel F® SC, Alpha Agricultural Supplies S.A., Athens, Greece), and very long chain fatty acids (VLCFAs) inhibitor prosulfocarb (Boxer 80 EC, Syngenta Hellas, Oinofyta, Greece), whereas the S population was treated with 1/4X, 1/2X and 1X (recommended) rates of the herbicides (Table 2). The untreated control was also included for each population. The tested herbicides were applied with a portable field plot sprayer (AZO-Sprayers, Ede, The Netherlands), with a 2.4 m wide boom, equipped with six 8002 flat-fan nozzles (Teejet Spray System Co., Wheaton, IL, USA), operating with pressurized propane. The sprayer was calibrated to deliver 300 L ha^{-1} at a pressure of 280 kPa. After

treatment, the herbicides were incorporated into the soil mixture with uniform irrigation. Pots were placed in a random pattern in a net-protected area outdoors and were watered as needed. The mean monthly temperature and total monthly rainfall data recorded near the experimental area are given in Figure 1. To ensure similar growing conditions for all plants, the pots were re-randomized every week.

Table 2. Fresh weight reduction (% of untreated control) of the putative R1, R2, and R3 *L. perenne* populations due to the herbicides chlorsulfuron, chlorotoluron + diflufenican, and prosulfocarb applied preemergence at the recommended 2-fold and 4-fold rates. The S population was treated with one-fourth, half, and recommended rates of the same herbicides. The values of each herbicide rate are means of six replicates.

Herbicide	Rate	Populations			
		R1	R2	R3	S
	g ai ha^{-1}	----------% of control----------			
Chlorsulfuron	3.75				86
	7.5				93
	15 [a]	0	71	86	100
	30	0	77	96	
	60	4	84	98	
Chlorotoluron + diflufenican	400 + 25				97
	800 + 50				100
	1600 + 100	100	100	100	100
	3200 + 200	100	100	100	
	6400 + 400	100	100	100	
Prosulfocarb	1000				96
	2000				98
	4000	100	100	100	100
	8000	100	100	100	
	16,000	100	100	100	
$LSD_{0.05}$			2		1

[a] The rates in boldface are the recommended field label rates of the herbicides.

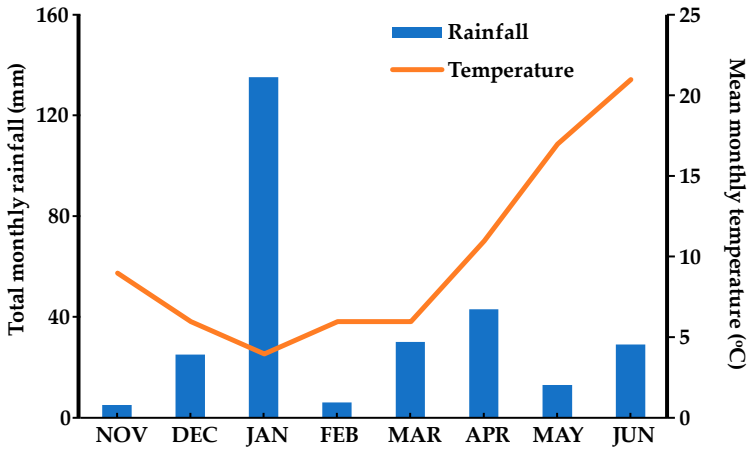

Figure 1. Mean monthly temperature and total monthly rainfall data recorded during the 2020–2021 growing season near the experimental area.

The experiment was performed twice following a randomized complete block design (RCBD) with three replicated pots per herbicide treatment and untreated control. Six weeks after the herbicide treatments, the *L. perenne* control was assessed by measuring the above-ground biomass (fresh weight) of all emerged plants in each pot and expressing the data as a percentage of the fresh weight of the untreated control. A combined ANOVA was performed over the two experiments to evaluate the R populations, using a 3 (populations) × 3 (herbicides) × 3 (herbicide rates) split plot approach, where the populations were the main plots and the three herbicides were the three rates of the sub-plots. Also, a combined ANOVA was performed over the two experiments to evaluate the S *L. perenne* population using a 3 (herbicides) × 3 (herbicide rates) factorial approach. The homogeneity of variances was tested using Barlett's test [33], which indicated that the departure of normality was not significant, thus allowing the data to be pooled and analyzed in the two experiments. Fisher's protected LSD (least significant difference) test was used to compare the differences between means ($p < 0.05$). Based on this comparison, the putative R1 population was characterized as resistant to chlorsulfuron, whereas R2 and R3 were moderately susceptible. Regarding the S population, the obtained results confirmed its susceptibility.

2.3. Plant Dose–response Biossays to Postemergence ALS- and ACCase-Inhibiting Herbicides

The R1 and S *L. perenne* populations (as characterized previously) were further studied in pot experiments from late winter to spring 2021 at the same location as before. The methodology for the pot establishment (pot size, soil type, sowing, and growing conditions) were as described for the preemergence herbicide experiments. When *L. perenne* plants reached the two-leaf stage, they were thinned down to six plants per pot. The R1 population was treated with the recommended (1X), 2X, and 4X field label rates of the postemergence application of ALS-inhibiting herbicides mesosulfuron-methyl + iodosulfuron methyl-sodium (Atlantis® WG, Bayer Crop Science Hellas, Athens, Greece), pyroxsulam (Senior 75 WG, Dow Elanco Hellas, Acharnes, Greece), and imazamox (Pulsar SL, BASF Hellas, Marousi, Greece), whereas the S population was treated with the 1/4X, 1/2X, and X (recommended) rates of the same herbicides (Table 3). The untreated control for each population was also included.

Table 3. Fresh weight reduction (% of untreated control) of the R1 *L. perenne* populations as affected by the ALS-inhibiting herbicides mesosulfuron + iodosulfuron, pyroxsulam, propoxycarbazone + iodosulfuron, and imazamox applied postemergence at the recommended 2-fold and 4-fold rates. The S population was treated with one-fourth, half, and recommended rates of the same herbicides. The values of each herbicide rate are means of six replicates.

Herbicide	Rate	Populations	
		R1	S
	g ai ha^{-1}	----% of control----	
Mesosulfuron + iodosulfuron	3.75 + 0.75		95
	7.5 + 1.5		100
	15 + 3 [a]	38	100
	30 + 6	67	
	60 + 12	92	
Pyroxsulam	46.9		94
	93.8		100
	187.5	32	100
	375	48	
	750	58	

Table 3. *Cont.*

Herbicide	Rate	Populations	
		R1	S
Imazamox	12.5		78
	25		100
	50	99	100
	100	100	
	200	100	
$LSD_{0.05}$		2	1

[a] The rates in boldface are the recommended field label rates of the herbicides.

The R1 population was also treated with the recommended (1X), 2X, and 4X field label rates of the postemergence application of ACCase-inhibiting herbicides clodinafop-propargyl (Sword® 240 EC, K&N Efthymiadis S.A., Thessaloniki, Greece), diclofop-methyl (Keylofop® 36 EC, FARMA-CHEM S.A., Thessaloniki, Greece), pinoxaden (Axial® 60 EC, Syngenta Hellas, Oinofyta, Greece), and clethodim (Select® 12EC, Arysta LifeScience, Athens, Greece), whereas the S population was sprayed with 1/4X, 1/2X, and 1X (recommended) rates of the same herbicides (Table 4). The untreated control for each population was also included. The application of the postemergence herbicides was similar to that described in the above-mentioned screening experiments with the preemergence herbicides. The *L. perenne* plants were at the GS21-22 (3–4 leaves, 1–2 tillers) of the Zadoks growth scale [34] at the time of postemergence herbicide application.

Table 4. Fresh weight reduction (% of untreated control) of the R1 *L. perenne* populations due to ACCase-inhibiting herbicides clodinafop-propargyl, diclofop-methyl, pinoxaden, and clethodim applied postemergence at the recommended 2-fold and 4-fold rates. The S population was treated with one-fourth, half, and recommended rates of the same herbicides. The values of each herbicide rate are means of six replicates.

Herbicides	Rate	Populations	
		R1	S
	g ai ha^{-1}	---% of control---	
Clodinafop-propargyl	16		48
	32		100
	64 [a]	99	100
	128	100	
	256	100	
Diclofop-methyl	225		100
	450		100
	900	100	100
	1800	100	
	3600	100	
Pinoxaden	11.25		76
	22.5		100
	45	100	100
	90	100	
	180	100	

Table 4. *Cont.*

Herbicides	Rate	Populations	
		R1	S
Clethodim	60		100
	120		100
	240	100	100
	480	100	
	960	100	
$LSD_{0.05}$		1	1

[a] The rates in boldface are the recommended field label rates of the herbicides.

Each experiment was repeated twice following an RCBD, with three replicates per herbicide treatment. Every week, the pots were re-randomized to ensure similar growth conditions for all the *L. perenne* plants. The efficacy of all herbicides tested was assessed four weeks after treatment by cutting the surviving plants at the ground level and determining the aboveground biomass (fresh weight). The fresh weight data were expressed as a percentage reduction of the non-treated control and subjected to analysis of variance (ANOVA). A combined ANOVA over two experiments was performed to evaluate the R1 or S population using a factorial approach (3 herbicides × 3 rates for the ALS inhibitors, and 4 herbicides × 3 rates for the ACCase inhibitors). As the tested homogeneity of variances with Bartlett's test [33] indicated that the departure of normality was not significant, the data were pooled and analyzed over the two experiments. Differences between means were compared at $p < 0.05$ using Fisher's protected least significant difference (LSD) test.

2.4. Sequencing of the ALS Gene Fragment

The *ALS* gene fragment covering potential mutation sites in the suspected R1 and S *L. perenne* populations was amplified, sequenced, and compared. *ALS* gene amplification was performed on plant material harvested from six R1 plants grown individually in pots and treated with the recommended rate of the preemergence application of chlorsulfuron, and from four untreated plants of the S population. The chlorsulfuron treatment was intended to eliminate possible ALS-susceptible plants from the R1 population. The S population plants were not treated because the initial screening experiments indicated that the recommended rate of chlorsulfuron provided 100% control in that population. Genomic DNA was isolated from three S and seven R1 population plants, using 40–50 mg of young leaf tissue and according to 'NucleoSpin® Plant II' DNA extraction kit protocol (MACHEREY-NAGEL, Düren, Germany). Leaf tissues from surviving R1 plants and from the untreated S plants were harvested, immediately stored at −28 °C, and subsequently used for DNA extraction. The quality and quantity of the isolated DNA were checked using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The PCR amplification of the *ALS* gene fragment from the genomic DNA samples, containing the Pro197 codon (400 bp), was achieved using the forward 5′-GCCACCAACCTCG TCTCC-3′ and reverse 5′-CCACCGCCAACATARAGAAT-3′ primer pair [35]. The cycling conditions consisted of an initial denaturation step of 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR was performed in 10 µL volumes containing 5 µL (1X) of OneTaq® 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 0.5 µL of each forward and reverse primer (0.5 µM each), and 1 µL of template DNA (20 ng).

The purified PCR products were single-stranded sequenced with BigDye Terminator v3.1 (Life Technologies, Waltham, MA, USA) cycle sequencing methodology on an ABI3500 Genetic Analyzer (Applied Biosystems™, Waltham, MA, USA), using the same primers as for PCR (forward primer). The *L. perenne* sequences were manually checked, aligned, and compared to the *Arabidopsis thaliana* nucleotide sequence for the *ALS* gene (GenBank

Accession Number: X51514) using BioEdit v7.2.6 software [36] to detect possible point mutations at the Pro197 codon of the *L. perenne ALS* gene. As the *ALS* gene fragment sequences revealed a point mutation at the Pro-197 position in all R1 plants only, further analysis for other point mutations was not made because the detected mutation was considered adequate to confirm the cross-resistance of the R1 population, which was found in the plant dose–response bioassays to preemergence and postemergence ALS-inhibiting herbicides [37].

2.5. Lolium perenne Growth Rate Experiment

A pot experiment was established from early winter (December 2020) to spring 2021, at the same location as before, to assess the comparative growth rate of the R1 and S populations in the absence of crop competition. The mean monthly temperature and total monthly rainfall data recorded near the experimental area are shown in Figure 1. Plastic pots (20 × 20 × 25 cm) were filled with a mixture of soil, peat, and sand, as described previously. All pots were seeded with approximately 30 uniformly sized seeds from the R1 and S populations. The R1-seeded pots were immediately treated with the recommended field rate of chlorsulfuron (15 g ai ha^{-1}), as described above (this rate did not cause any visually detectable growth penalty on surviving seedlings), whereas the S-seeded pots were left untreated. The emerging plants were irrigated and fertilized as needed throughout the experiment to ensure vigorous plant growth. At the 2-leaf stage, the *L. perenne* plants were thinned down to four uniform seedlings per pot spaced at 13–15 cm. Any emerging broad-leaved or undesired grass weeds in the pots were carefully hand-removed to avoid competition with *L. perenne*. The plant growth of the R1 and S populations was evaluated at five consecutive destructive samplings, which were performed 10, 16, 18, 20, and 22 weeks after seeding. At each sampling date, the R1 and S plants were cut at the soil surface, and their aboveground biomass (fresh weight) and tiller number were determined. The last sampling coincided with the maturity of R1 and S populations. The experiment was repeated twice using an RCBD, with three replicates for each sampling time. A combined ANOVA over the two experiments was performed since Bartlett's test [33] verified the homogeneity of the variances. A factorial approach was employed (2 populations × 5 samplings), while Fisher's protected LSD test ($p = 0.05$) was used to compare differences between means. Moreover, the data of the two determined growth parameters (pooled over the two experiments) were regressed against the sampling time. In these regression analyses, the dependent variable (y) was the aboveground biomass or tiller number of the *L. perenne*, and the independent variable (x) was the sampling time.

2.6. Lolium perenne Competition with Wheat

The experiment was conducted from 15 December 2020 to 15 May 2021 at the same location and followed a similar procedure as for the growth rate experiments. The mean monthly temperature and total monthly rainfall data recorded near the experimental area are shown in Figure 1. This experiment was based on a 'target-neighborhood design' to evaluate the competitive ability of R1 and S populations against wheat [37,38]. For this purpose, a template (Figure 2) was used to achieve identical distances between neighboring plants. At first, each pot was sown with 16 wheat seeds in two rows spaced 15 cm apart, with each row having 4 hills of 2 seeds per hill and a 6 cm distance between the hills. When the wheat plants were at the one-leaf stage, they were carefully thinned down to leave one wheat plant per hill; thus, a total of 8 per pot (160 plants/m^2). At the same time, *L. perenne* seedlings at the one- and two-leaf stages were transplanted into each pot at densities of 0 (weed-free crop control), with 2, 4, 6, or 8 plants per pot (Figure 2). The transplanted R1 seedlings were taken from separate seeded pots, which were treated with the preemergence application of chlorsulfuron at the recommended field rate (15 g ai ha^{-1}). All transplanted pots were placed in the previously described outdoor net-protected area for 120 d (15 January (weed transplanting time) to 15 May 2021), and received irrigation and fertilizer (40 kg N/ha and 50 kg P/ha as ammonium phosphate before seeding, and

25 kg N/ha at the end of February plus 25 kg N/ha middle of April as nitrate ammonium), as needed to maintain vigorous growth. Hand-weeding of all undesired weeds emerging in the pots was carefully performed throughout the experiment to avoid any competition from these species. The experiments were performed twice, based on an RCBD with three replicates per weed density.

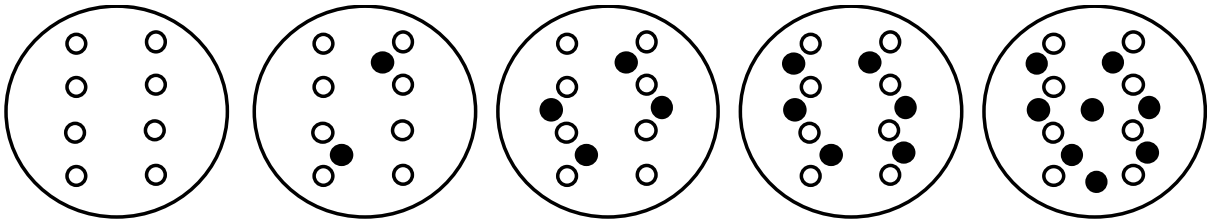

Figure 2. Schematic representation of the crop/weed density pattern (8:0, 8:2, 8:4, 8:6, 8:8) to assess plant responses of wheat grown in pure stands and in competition with the R1 or S *L. perenne* populations (wheat plants = open circles vs. R1 or S perennial ryegrass plants = black circles).

The evaluation of plant growth was based on the aboveground biomass of the wheat and *L. perenne* plants, which was determined by cutting them at the soil surface and measuring their fresh weight. Once again, the data obtained from the two experiments had equal variances as indicated by Bartlett's test [33] and were pooled together for ANOVA, following a factorial approach (2 populations × 4 weed densities for the *L. perenne* data, and 2 populations × 5 weed densities for the wheat data). Differences between means were compared using Fisher's protected LSD test ($p = 0.05$). In addition, the data for *L. perenne* or wheat aboveground biomass (pooled over the two experiments) were regressed against weed density. In this analysis, the fresh weight of *L. perenne* or wheat was the dependent variable (y), and the density of *L. perenne* was the independent variable (x).

3. Results

3.1. Plant Dose–Response to Preemergence Herbicides

The recommended two-fold and fout-fold rates of chlorsulfuron reduced the fresh weight of the R1, R2, and R3 populations by 0–4%, 71–84%, and 86–98%, respectively, whereas the respective rates of chlorotoluron + diflufenican or prosulfocarb provided 100% control of all putative R populations. In contrast, the rates 1/4X, 1/2X, or 1X (recommended rate) of the above-mentioned three herbicides resulted in 86–100%, 97–100%, and 96–100% fresh weight reduction of the S population, respectively (Table 2). Although GR_{50} values are more suitable to compare susceptibility between R and S populations, their estimation was not made in this experiment, as the highest (4X) rate of chlorsulfuron provided less than 50% growth reduction in the R1 population and its lower rate (1/4X) resulted in more than 50% growth reduction in the S population.

3.2. Plant Dose–Response to Postemergence ALS- and ACCase-Inhibiting Herbicides

The aboveground biomass of the R1 population was reduced by 38%, 67%, and 92% due to the recommended two-fold and four-fold rates of the ALS mesosulfuron + iodosulfuron, whereas the respective reductions by the same rates of pyroxsulam were 32%, 48%, and 58% (Table 2). As previously, GR_{50} values were not estimated in this experiment, but the results obtained clearly indicate that the two-fold rate of the recommended mesosulfuron + iodosulfuron reduced the growth by more than 50% of the R1 population, whereas the four-fold rate of the recommended pyroxsulam was needed for similar growth reduction. However, the respective imazamox rates reduced the aboveground biomass of the R1 population by 99%, 100%, and 100%. Regarding the S population, half or the recommended rate of the above-mentioned herbicides was able to reduce its fresh weight by 100% (Table 3).

The recommended (1X), 2X, and 4X rates of the ACCase clodinafop-propargyl, diclofop-methyl, pinoxaden, and clethodim reduced the fresh weight of the R1 population by 99–100%, whereas one-fourth, half, or the recommended rate of the same herbicides provided 90–100% control of the S population (Table 4).

3.3. Sequencing of the ALS Gene Fragment

The comparison of *ALS* gene fragment sequences in the seven R1 and three S *L. perenne* plants with the coding sequence of *Arabidopsis thaliana* (Accession number: X51514) revealed a point mutation in the R1 plants only (Figure 3). In particular, the substitution of the second cytosine by adenine at the Pro-197 (CCC) position was present in five individual ALS-resistant plants, which resulted in the replacement of Pro-197 by His (CAC). Four out of the five resistant plants were homozygous (RR, His-197), and one was heterozygous (RS, Pro-197-His). However, in the two remaining R1 resistant plants, the substitution of the second cytosine by thymine resulted in the replacement of Pro-197 by Leu (CTC) (one plant was homozygous (RR, Leu-197) and the other heterozygous (RS, Pro-197-Leu)). The analysis of the DNA sequence chromatographs for the three plants of the S population indicated homozygous plants for the wild-type allele at position Pro-197. As the detected point mutation in all R1 plants was considered adequate to confirm its cross-resistance to ALS inhibitors, which was found in plant dose–response bioassays, further analysis for other point mutations was not performed on the purified PCR products [39].

Pro197

S.1 TGGCCATCACGGGGCAGGT**CCC**GCGCCGCATGAT

S.2 TGGCCATCACGGGGCAGGT**CCC**GCGCCGCATGAT

S.3 TGGCCATCACGGGGCAGGT**CCC**GCGCCGCATGAT

R1.1 TGGCCATCACGGGGCAGGT**CAC**GCGCCGCATGAT

R1.2 TGGCCATCACGGGGCAGGT**CAC**GCGCCGCATGAT

R1.3 TGGCCATCACGGGGCAGGT**CAC**GCGCCGCATGAT

R1.4 TGGCCATCACGGGGCAGGT**CAC**GCGCCGCATGAT

R1.5 TGGCCATCACGGGGCAGGT**CMC**GCGCCGCATGAT

R1.6 TGGCCATCACGGGGCAGGT**CTC**GCGCCGCATGAT

R1.7 TGGCCATCACGGGGCAGGT**CYC**GCGCCGCATGAT

Figure 3. Alignment of perennial ryegrass *ALS* sequences using BioEdit v7.2.6 software. The first three DNA sequences correspond to S *L. perenne* plants, whereas the following seven samples are the R1 plants. The observed polymorphisms CAC, CTC, CMC, and CYC are marked in bold and correspond to the Pro-197 position of the *A. thaliana ALS* gene (X51514). More specifically, CAC = His, CTC = Leu, CMC (CAC = His or CCC = Pro), and CYC (CAC = His or CTC = Leu).

3.4. Lolium perenne Growth Rate Experiment

The aboveground biomass and tiller number of the R1 and S populations grown in the absence of crop competition increased with increasing sampling time, but the increase in both growth parameters was not proportional (Figure 4). In addition, the trends of the aboveground biomass and tiller of the R1 population were similar to those of the S population. This was confirmed by the similar estimated slopes from the linear equations, fitted to the aboveground biomass (R1 = 27.06 and S = 23.87) and tiller number (R1 = 8.07 and S = 7.33) of both populations against sampling time (Table 5). These results of the R1 and S plants could be attributed to the self-incompatibility [7–9] of this species and to the fact that its mutant alleles conferring resistance are generally not linked. Therefore, different

combinations of resistance mechanisms are accumulated in the weed populations [11], which result in high genetic variability, adaptability, and phenotypic plasticity.

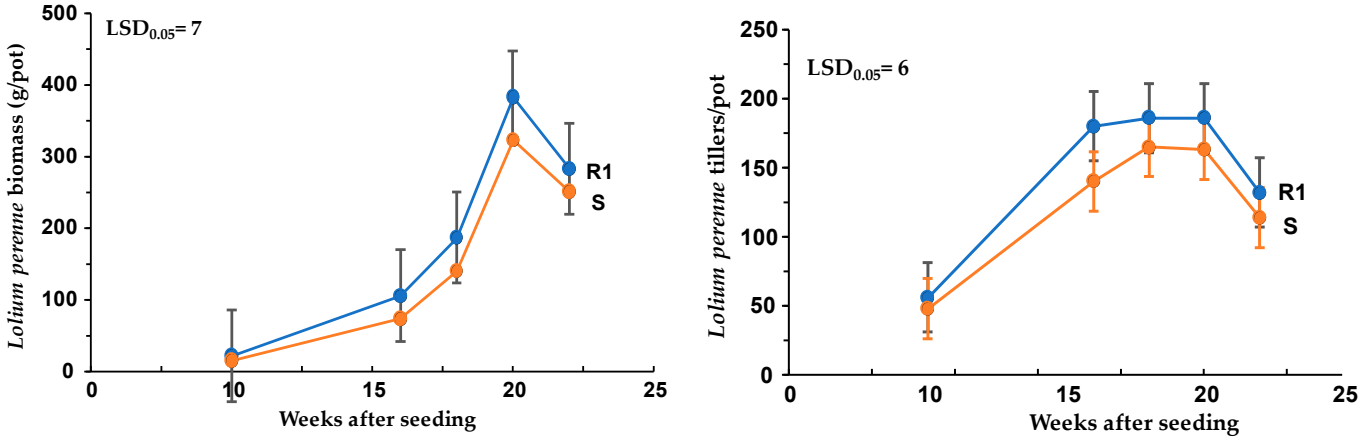

Figure 4. Aboveground biomass and tiller number produced by the R1 and S *L. perenne* populations grown in the absence of crop competition and monitored throughout the life cycle by five destructive samplings. Values are means of six replicates and bars indicate the standard errors.

Table 5. Intercepts, slopes, standard errors, confidence intervals, and coefficient of determination of the linear equations fitted on the regressed aboveground biomass or tiller number of R1 and S *L. perenne* populations (grown in the absence of competition) against sampling time.

L. perenne Populations	Intercept (a)	Slope (b)	±SE	CI (95%)	(R^2)
Aboveground biomass					
R1	−268.25	27.06	±8.62	−0.37, 54.49	0.77
S	−249.01	23.87	±7.77	−0.84, 48.59	0.76
Tiller number					
R1	9.26	8.07	±5.29	−8.76, 24.9	0.44
S	−0.16	7.33	±4.32	−6.42, 21.09	0.49

SE, standard error of slope b; CI, confidence interval of slope b; R^2, coefficient of determination.

3.5. Lolium perenne Competition with Wheat

The aboveground biomass of the R1 and S populations grown in competition with wheat increased with increasing weed density, but the R1 population produced more aboveground biomass than the S population (Figure 5). However, as their aboveground biomass followed a similar trend with increasing density, the estimated slopes from the linear equations fitted on the regressed *L. perenne* aboveground biomass data against density were similar (R1 = 18.85 and S = 19.35) (Table 6). As a result of these findings, the aboveground biomass of wheat grown in competition with R1 or S populations was similarly reduced with increasing weed density (Figure 5), which was strongly supported by the similarly estimated intercepts (165.8 and 162.4) and slopes (R1 = 16.1 and S = 15) from the linear equations fitted to the regressed wheat aboveground biomass data against weed density (Table 6).

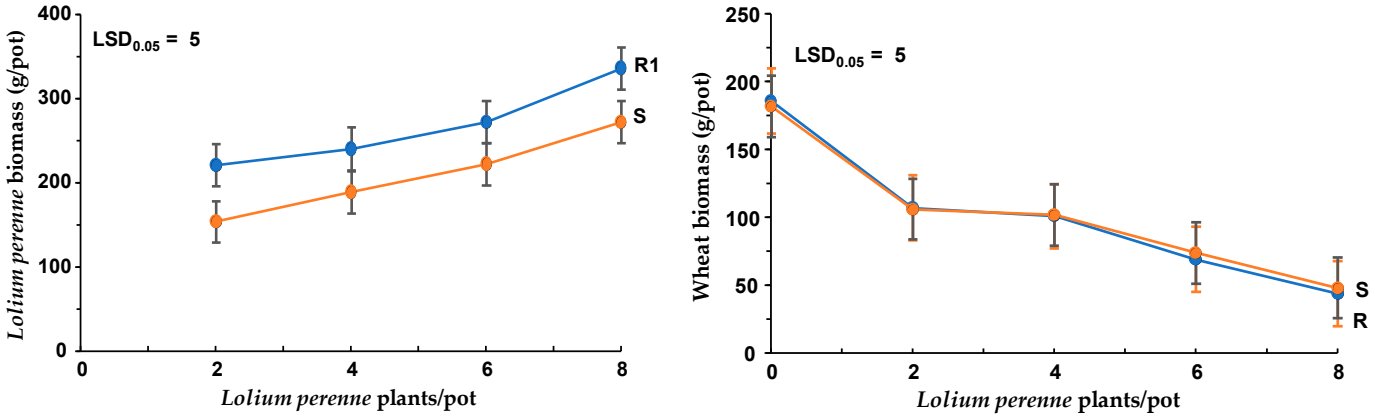

Figure 5. Aboveground biomass produced by the R1 and S *L. perenne* populations grown in competition with wheat, along with the respective wheat aboveground biomass. Values are means of six replicates and bars indicate the standard errors.

Table 6. Intercepts, slopes, standard errors, confidence intervals, and coefficient of determination of the linear equations fitted to the regressed aboveground biomass of the R1 and S *L. perenne* populations (grown in competition with wheat) against weed density, along with the respective wheat aboveground biomass regression parameters.

L. perenne Populations	Intercept (a)	Slope (b)	±SE	CI (95%)	R^2
L. perenne aboveground biomass					
R1-Wheat	173	18.85	± 3.62	3.27, 34.43	0.93
S-Wheat	112.5	19.35	± 1.36	13.49, 25.21	0.99
Wheat aboveground biomass					
Wheat-R1	165.8	−16.1	± 3.12	−26.02, −6.18	0.90
Wheat-S	162.4	−15	± 3.05	−24.7, −5.30	0.89

SE, standard error of slope b; CI, confidence interval of slope b; R^2, coefficient of determination.

4. Discussion

The fact that the recommended two-fold and four-fold rates of chlorsulfuron reduced fresh weight of the R1, R2, and R3 populations by 0–4%, 71–84%, and 86–98%, respectively, indicates that the R1 population has evolved resistance to chlorsulfuron, while R2 and R3 are moderately resistant and susceptible to this herbicide, respectively. However, the excellent control of these populations with chlorotoluron + diflufenican or prosulfocarb suggests their use as alternative chemical options to manage this weed [10]. Regarding the S population, the above herbicides showed good to excellent efficacy, even after the application of lower than their recommended field label rates. It is worth noting that, regardless of the high efficacy of the soil-applied chlorotoluron + diflufenican or prosulfocarb against this weed, their use in Greece is very limited because their efficacy against weeds and selectivity in winter cereals are dependent on weed species, crop cultivars, soil, and weather conditions.

The 38–67% and 32–48% fresh weight reduction in the R1 population by the recommended and two-fold rates of mesosulfuron + iodosulfuron and pyroxsulam supports the evidence that this population has evolved cross-resistance to these ALS-inhibiting herbicides, which makes their further use in some cases unsatisfactory for weed management. However, the fact that imazamox provided excellent control of this weed justifies its possible use in rotational Clearfield® crops (tolerant to imidazolinone herbicides). Also, the excellent control of the susceptible population with half of the recommended rate of the

above herbicides indicates that some susceptible populations of this weed can be controlled at lower than their recommended rates.

The R1 population fresh weight was reduced by 99–100% with the recommended field label rate of the ACCase-inhibiting herbicides clodinafop-propargyl, diclofop-methyl, pinoxaden, and clethodim, which indicates that this population with cross-resistance to ALS-inhibiting herbicides did not develop multiple resistance to ACCase inhibitors. In addition, the fact that one-fourth, half, or the recommended rate of the same herbicides provided 90–100% control of the S population shows that some S populations of this weed can be controlled at lower than their recommended rates.

The R1 *L. perenne* population evolution of cross-resistance to ALS-inhibiting herbicides could be attributed to overreliance on the preemergence application of chlorsulfuron or the postemergence application of mesosulfuron + iodosulfuron or pyroxsulam for many consecutive years. The high frequency of the ALS herbicide-resistant individuals occurring naturally in weed populations along with the rapid spread of resistance in *L. perenne* due to its obligate cross-pollination and self-incompatibility could account for the selection of cross-resistant populations by the repeated application of these herbicides [40,41]. Moreover, the fact that the control of this weed in Greece is mainly based on the ACCase and ALS inhibitors only, which are at high risk for herbicide-resistance evolution, the herbicide-resistant populations of this weed threaten the sustainability of cereal crop production, and this danger requires a well-thought approach to manage field-evolved resistance.

The lack of point mutations at codon Pro-197 of the sequenced *ALS* gene in the S population confirms its susceptibility to ALS inhibitors found in the whole-plant response experiments. However, the two identified point mutations in the *ALS* gene, causing amino acid substitutions at Pro-197 position in the ALS enzyme of the R1 plants, support the evidence of target site-based herbicide cross-resistance and confirm the plant resistance findings for this population. The Pro-197-His and Pro-197-Leu amino acid substitutions found in separate individuals of the R1 population, according to our knowledge, are reported for the first time in *L. perenne*, although their presence is very common in other weed species [10]. These two amino-acid substitutions conferred broad target-site cross-resistance to chlorsulfuron, mesosulfuron + iodosulfuron, pyroxsulam, and propoxycarbazone, which agree with results reported by Menegat et al. [23], who found that plants of a French *L. perenne* population with the ALS Asp-376-Glu genotype were cross-resistant against mesosulfuron + iodosulfuron, pyroxsulam and propoxycarbazone. However, Vázquez-García et al. [11] found that a *L. perenne* population from Chile was resistant to the ALS-iodosulfuron (due to non-target-site resistance mechanisms), and also multiple-resistant to the ACCase-diclofop-methyl (due to an Asp-2078-Gly point mutation) and the EPSPS-glyphosate (due to *EPSPS* gene amplification resulting in high enzyme activity). Finally, plant dose–response assays conducted by Singh et al. [27] indicated that three *L. perenne* populations in Texas were multiple-resistant to the ACCase-diclofop-methyl and the ALS-mesosulfuron-methyl.

The cross-resistance of the R1 population to sulfonylurea (chlorsulfuron, mesosulfuron + iodosulfuron) and triazolopyrimidine (pyroxsulam), but not to imidazolinone (imazamox) herbicides, is in contrast with the results reported by Saari et al. [22], who found that a *L. perenne* population from California was cross-resistant (due to less sensitive ALS target site enzyme) to both sulfonylurea (chlorsulfuron, sulfometuron, triasulfuron) and imidazolinone (imazapyr) herbicides.

The extremely high susceptibility of the R1 population to the ACCase inhibitors clodinafop-propargyl, diclofop-methyl, and pinoxaden, applied at even half of their registered field label rate, justifies their current use for the effective control of this weed in winter cereals. However, since the *L. perenne* population has evolved cross-resistance to ACCase inhibitors due to the Asp-2078-Gly substitution in the ACCase enzyme [11], measures should be taken for the rotational and appropriate use of these ACCase inhibitors.

The similar aboveground biomass and tiller number increasing trends of the R1 and S populations in the absence of crop competition strongly suggest that the R1 population has similar fitness compared that of the S population. In addition, the similar aboveground

biomass of the R1 and S populations grown in competition with wheat is in agreement with the results found in the growth rate experiment (with the absence of competition). The similar vegetative productivity trends of both R1 and S populations grown in the absence or presence of wheat competition could be attributed to their similar product biosynthesis, which may result from the similar catalytic activity of their ALS enzyme and/or similar substrate affinity, and/or similar feedback inhibition [32,41]. In general, the similarly calculated slopes for the R1 and S populations from the linear equations fitted on their aboveground biomass and tiller number grown in the absence or presence of wheat competition support the evidence for similar growth rates. In contrast to these results, Menegat et al. [23] found that a French *L. perenne* population, with cross-resistance to ALS inhibitors due to Asp-376-Glu substitution of the *ALS* gene, had no significant impact on shoot biomass and tiller number compared to the wild-type population, although its root biomass and ALS enzyme activity were 68 % and 48 % lower than those of the S population, respectively. In addition, Zangeneh et al. [42], studying one S and two R *L. rigidum* populations with target-site resistance to ACCase inhibitors, found that the R-2041-Asn population exhibited better fitness than the S and R-1781-Leu populations. Based on these results, it could be concluded that fitness cost endowed by field-selected *ALS* target-site resistance genes cannot be easily predicted, because it depends on weed species, the mutant allele allowing resistance, the genetic background, and the experimental and environmental conditions [43].

The lack of proportional aboveground biomass increases with increasing density of the R1 and S populations supports the evidence of both inter- and intra-species competition. In addition, their similar increasing aboveground biomass trends with increasing density were confirmed by their similar estimated growth rates. Finally, the similar wheat biomass growth in competition with either the R1 or S population suggests a similar competitive ability for both weed populations against wheat.

5. Conclusions

The results of this study indicate that the poor herbicide efficacy of several ALS-inhibiting herbicides tested against the R1 population was due to evolved ALS target-site cross-resistance at the Pro-197 position, suggesting that measures should be taken for reducing the further use of these herbicides. However, since the ACCase inhibitors were found to be very effective against the R1 population, they should be used carefully for the management of this weed due to their high risk of selecting herbicide resistance after repeated consecutive applications. Moreover, the similar vegetative productivity trend of both R1 and S populations grown in the absence or presence of wheat competition indicated a lack of fitness association with the two types of ALS mutation, which combined with similar growth rates and similar competitive ability.

Author Contributions: A.P.P.: conceptualization, principal investigation, data curation, writing—original draft; D.L.: amplification and sequencing of the ALS gene fragment; E.A.: formal analysis; I.G.E.: methodology, validation, formal analysis, reviewing, and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available in this article.

Acknowledgments: We thank the Assistant Professor of Weed Science Vaya Kati for her reviewing and editing. We also thank the Editor and the two anonymous reviewers for their constructive comments, which helped us to improve the quality of our manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Suzukawa, A.K.; Bobadilla, L.K.; Mallory-Smith, C.; Brunharo, C.A.C.G. Non-target-site resistance in *Lolium* spp. globally: A Review. *Front. Plant Sci.* **2021**, *11*, 609209. [CrossRef]
2. Collavo, A.; Sattin, M. Resistance to glyphosate in *Lolium rigidum* selected in Italian perennial crops: Bioevaluation, management and molecular bases of target-site resistance. *Weed Res.* **2012**, *52*, 16–24. [CrossRef]
3. Collavo, A.; Sattin, M. First glyphosate-resistant *Lolium* spp. biotypes found in a European annual arable cropping system also affected by ACCase and ALS resistance. *Weed Res.* **2014**, *54*, 325–334. [CrossRef]
4. Karn, E.; Jasieniuk, M. Nucleotide diversity at site 106 of *EPSPS* in *Lolium perenne* L. ssp. multiflorum from California indicates multiple evolutionary origins of herbicide resistance. *Front. Plant Sci.* **2017**, *8*, 777. [CrossRef] [PubMed]
5. Cool, M.; Hannaway, D.B.; Larson, C.; Myers, D. Perennial Ryegrass (*Lolium perenne* L.). Forage Fact Sheet. 2004, OR, USA: Oregon State University. Available online: https://forages.oregonstate.edu/php/fact_sheet (accessed on 16 January 2022).
6. Hannaway, D.B.; Evers, G.W.; Fales, S.L.; Hall, M.H.; Fransen, S.C.; Ball, D.M.; Johnson, S.W.; Jacob, L.H.; Chaney, M.; Lane, W.; et al. *Perennial Ryegrass for Forage in the USA*; Crop Science Society of America: Madison, WI, USA, 1997. [CrossRef]
7. Matzrafi, M.; Preston, C.; Brunharo, C.A. Review: Evolutionary drivers of agricultural adaptation in *Lolium* spp. *Pest Manag. Sci.* **2021**, *77*, 2209–2218. [CrossRef] [PubMed]
8. Gill, G.S.; Cousens, R.D.; Allan, M.R. Germination, growth, and development of herbicide resistant and susceptible populations of rigid ryegrass (*Lolium rigidum). Weed Sci.* **1996**, *44*, 252–256. [CrossRef]
9. Holt, J.S.; Weller, S.R.; Silvera, K.; Heap, I.M.; Heredia, S.M.; Martinez-Berdeja, A.; Palenscar, K.T.; Sweet, L.C.; Ellstrand, N.C. Taxonomic and life history bias in herbicide resistant weeds: Implications for deployment of resistant crops. *PLoS ONE* **2013**, *8*, e71916. [CrossRef]
10. Heap, I. The International Survey of Herbicide Resistant Weeds. Available online: http://www.weedscience.org (accessed on 30 April 2022).
11. Vázquez-García, J.G.; Alcántara-de la Cruz, R.; Palma-Bautista, C.; Rojano-Delgado, A.M.; Cruz-Hipólito, H.E.; Torra, J.; Barro, F.; De Prado, R. Accumulation of target gene mutations confers multiple resistance to ALS, ACCase, and EPSPS inhibitors in *Lolium* species in Chile. *Front. Plant Sci.* **2020**, *11*, 553948. [CrossRef]
12. Yu, Q.; Powles, S. Metabolism-based herbicide resistance and cross-resistance in crop weeds: A threat to herbicide sustainability and global crop production. *Plant Physiol.* **2014**, *166*, 1106–1118. [CrossRef]
13. Karn, E.; Jasieniuk, M. Genetic diversity and structure of *Lolium perenne* ssp. multiflorum in California vineyards and orchards indicate potential for spread of herbicide resistance via gene flow. *Evol. Appl.* **2017**, *10*, 616–629. [CrossRef]
14. Loureiro, I.; Escorial, M.-C.; Chueca, M.-C. Pollen-mediated movement of herbicide resistance genes in *Lolium rigidum*. *PLoS ONE* **2016**, *11*, e0157892. [CrossRef]
15. Powles, S.B.; Yu, Q. Evolution in action: Plants resistant to herbicides. *Annu. Rev. Plant Biol.* **2010**, *64*, 317–347. [CrossRef] [PubMed]
16. Li, W.; Wu, C.; Wang, M.; Jiang, M.; Zhang, J.; Liao, M.; Cao, H.; Zhao, N. Herbicide resistance status of Italian ryegrass (*Lolium multiflorum* Lam.) and alternative herbicide options for its effective control in the Huang-Huai-Hai Plain of China. *Agronomy* **2020**, *12*, 2394. [CrossRef]
17. Wu, C.; Song, M.; Zhang, T.; Zhou, C.; Liu, W.; Jin, T.; Zhao, N. Target-site mutation and cytochrome P450s confer resistance to multiple herbicides in Italian ryegrass (*Lolium multiflorum* Lam.) from China. *Crop Prot.* **2022**, *161*, 106068. [CrossRef]
18. Délye, C.; Jasieniuk, M.; Le Corre, V. Deciphering the evolution of herbicide resistance in weeds. *Trends Gen.* **2013**, *29*, 649–658. [CrossRef]
19. Torra, J.; Montuli, J.M.; Taberner, A.; Onkokesung, N.; Boonham, N.; Edwards, R. Target-site and non-target-site resistance mechanisms confer multiple and cross-resistance to ALS and ACCase inhibiting herbicides in *Lolium rigidum* from Spain. *Front. Plant Sci.* **2021**, *12*, 625138. [CrossRef]
20. Yu, Q.; Powles, S.B. Resistance to AHAS inhibitor herbicides: Current understanding. *Pest Manag. Sci.* **2014**, *70*, 1340–1350. [CrossRef]
21. Barroso, A.A.M.; Costa, M.G.S.; Neto, N.J.; Dos Santos, J.; Balbuena, T.S.; Carbonari, C.A.; Alves, P. Protein identification before and after glyphosate exposure in *Lolium multiflorum* genotypes. *Pest Manag. Sci.* **2018**, *74*, 1125–1133. [CrossRef]
22. Busi, R.; Porri, A.; Gaines, T.A.; Powles, S.B. Pyroxasulfone resistance in *Lolium rigidum* is metabolism-based. *Pest. Biochem. Physiol.* **2018**, *148*, 74–80. [CrossRef]
23. Menegat, A.; Baily, G.C.; Aponte, R.; Heinrich, G.M.T.; Sievernich, B.; Gerhards, R. Acetohydroxyacid synthase (AHAS) amino acid substitution Asp376Glu in *Lolium perenne*: Effect on herbicide efficacy and plant growth. *J. Plant Dis. Prot.* **2016**, *123*, 145–153. [CrossRef]
24. Saari, L.L.; Cot-Terman, J.C.; Smith, W.F.; Primiani, M.M. Sulfonylurea herbicide resistance in common chickweed, perennial ryegrass, and Russian thistle. *Pestic. Biochem. Physiol.* **1992**, *42*, 110–118. [CrossRef]
25. Yanniccari, M.; Gibón, R. Cross-resistance to acetyl-CoA carboxylase-inhibiting herbicides conferred by a target-site mutation in perennial ryegrass (*Lolium perenne*) from Argentina. *Weed Sci.* **2020**, *68*, 116–124. [CrossRef]
26. Yanniccari, M.; Gigón, R.; Larsen, A. Cytochrome P450 herbicide metabolism as the main mechanism of cross-resistance to ACCase- and ALS-inhibitors in *Lolium* spp. populations from Argentina: A molecular approach in characterization and detection. *Front. Plant Sci.* **2020**, *11*, 600301. [CrossRef] [PubMed]

27. Singh, V.; Maity, A.; Abugho, S.; Swart, J.; Drake, D.; Bagavathiannan, M. Multiple herbicide–resistant *Lolium* spp. is prevalent in wheat production in Texas lacklands. *Weed Technol.* **2020**, *34*, 652–660. [CrossRef]
28. Ghanizadeh, H.; Buddenhagen, C.E.; Harrington, K.C.; Griffiths, A.G.; Ngow, Z. Pinoxaden resistance in *Lolium perenne* L. is due to both target-site and non-target-site mechanisms. *Pestic. Biochem. Physiol.* **2022**, *184*, 105103. [CrossRef]
29. Chandi, A.; York, A.C.; Jordan, D.L.; Beam, J.B. Resistance to acetolactate synthase and acetyl Co-A carboxylase inhibitors in North Carolina Italian ryegrass (*Lolium perenne*). *Weed Technol.* **2011**, *25*, 659–666. [CrossRef]
30. Yanniccari, M.; Gomez-Lobato, M.E.; Istilart, C.; Natalucci, C.; Gimenez, D.O.; Castro, A.M. Mechanism of resistance to glyphosate in *Lolium perenne* from Argentina. *Front. Ecol. Evol.* **2017**, *5*, 123. [CrossRef]
31. Baucom, R.S.; Mauricio, R. Fitness costs and benefits of novel herbicide tolerance in a noxious weed. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13386–13390. [CrossRef]
32. Vila-Aiub, M.M.; Neve, P.; Powles, S.B. Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol.* **2009**, *184*, 751–767. [CrossRef]
33. Snedecor, G.W.; Cochran, W.G. *Statistical Methods*, 8th ed.; Ames IA:530 Iowa State University Press: Ames, IA, USA, 1989.
34. Zadoks, J.C.; Chang, T.T.; Konzak, C.F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]
35. Anthimidou, E.; Ntoanidou, S.; Madesis, P.; Eleftherohorinos, I. Mechanisms of *Lolium rigidum* multiple resistance to ALS- and ACCase-inhibiting herbicides and their impact on plant fitness. *Pestic. Biochem. Physiol.* **2020**, *164*, 65–72. [CrossRef]
36. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
37. Li, M.; Yu, Q.; Han, H.; Vila-Aiub, M.; Powles, S.B. ALS herbicide resistance mutations in *Raphanus raphanistrum*: Evaluation of pleiotropic effects on vegetative growth and ALS activity. *Pest Manag. Sci.* **2013**, *69*, 689–695. [CrossRef] [PubMed]
38. Papapanagiotou, A.P.; Spanos, T.; Zarrougui, N.E.; Livieratos, I.C.; Eleftherohorinos, I.G. Pro197 and Trp574 substitutions in the acetolactate synthase of corn marigold (*Glebionis segetum*) and their impact on competitive ability against barley. *Weed Technol.* **2023**, *37*, 165–173. [CrossRef]
39. Kaloumenos, N.S.; Tsioni, V.C.; Daliani, E.G.; Papavassileiou, S.E.; Vassileiou, A.G.; Laoutidou, P.N.; Eleftherohorinos, I.G. Multiple Pro-197 substitutions in the acetolactate synthase of rigid ryegrass (*Lolium rigidum*) and their impact on chlorsulfuron activity and plant growth. *Crop Prot.* **2012**, *38*, 35–43. [CrossRef]
40. Boutsalis, P.; Gill, G.S.; Preston, C. Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across Southeastern Australia. *Weed Technol.* **2012**, *26*, 391–398. [CrossRef]
41. Yu, Q.; Han, H.; Vila-Aiub, M.; Powles, S.B. AHAS herbicide resistance endowing mutations: AHAS functionality and plant growth. *Exp. Bot.* **2010**, *61*, 3925–3934. [CrossRef]
42. Zangeneh, H.S.; Chamanabad, H.R.M.; Zand, E.; Asghari, A.; Alamisaeid, K.; Travlos, I.S.; Alebrahim, M.T. Study of Fitness Cost in Three Rigid Ryegrass Populations Susceptible and Resistant to Acetyl-CoA Carboxylase Inhibiting Herbicides. *Front. Ecol. Evol.* **2016**, *4*, 142. [CrossRef]
43. Panozzo, S.; Scarabel, L.; Rosan, V.; Sattin, M. A new Ala-122-Asn amino acid change confers decreased fitness to ALS-resistant *Echinochloa crus-galli*. *Front. Plant Sci.* **2017**, *8*, 2042. [CrossRef]