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Detection of herbicide resistance and a novel ALS-inhibitor mutation in Alabama Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) populations

Research Article

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Abstract

Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] is a significant weed in winter wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], and peanut (*Arachis hypogaea* L.) crops in Alabama. In response to reports of herbicide failure, field surveys were conducted in these cropping systems across Alabama in 2023. The objectives were to document the distribution of herbicide resistance in the collected *L. perenne* ssp. *multiflorum* populations. Populations were evaluated in a greenhouse for sensitivity to herbicides representing three modes of action: an acetolactate synthase (ALS) inhibitor (pyroxsulam), two acetyl-coenzyme A carboxylase (ACCase) inhibitors (fluazifop-butyl and clethodim), and a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor (glyphosate). Herbicide screenings were followed by dose–response assays of the most resistant *L. perenne* ssp. *multiflorum* population for each herbicide at eight rates (0.5, 1, 2, 4, 8, 16, 32, and 64×) compared with a susceptible population at six rates (0.0625, 0.125, 0.25, 0.5, 1, and 2×). Out of 44 populations evaluated, 21%, 11%, 25%, and 2% were found resistant to glyphosate, fluazifop-butyl, pyroxsulam, and clethodim, respectively. Resistance levels were confirmed to be 192-, 14-, 90-, and 738-fold for glyphosate, fluazifop-butyl, pyroxsulam, and clethodim, respectively. Mutation detection studies revealed specific mutations: Asp-2078-Gly in the *ACCase* gene, Pro-106-Ser in the *EPSPS* gene, and a novel Arg-421-Thr mutation in the *ALS* gene.

Introduction

The ryegrass or *Lolium* genus, native to the Mediterranean region of southern Europe, northwest Africa, and southwest Asia (Beddows 1973; Hubbard 1968), comprises eight species (Bararpour et al. 2017). Rigid ryegrass (*Lolium rigidum* Gaudin), perennial ryegrass (*Lolium perenne* L.), and Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] are considered highly valuable as forage, turf, and cover crops (Matzrafi et al. 2021). Factors contributing to the spread of weedy *Lolium* spp. across six continents (Bararpour et al. 2017) include assisted breeding programs targeting warmer regions (Matzrafi et al. 2021) and climate change. Climate change is likely to create suitable potential areas for *Lolium* species (Castellanos-Frias et al. 2016). Additionally, the presence of genetically diverse variants has accelerated acclimation (Matzrafi et al. 2021). Due to self-incompatibility, these three species naturally hybridize, leading to high genetic diversity and overlapping phenotypic traits (Pasquali et al. 2022). Extensive genetic diversity facilitates rapid evolution of adaptive traits that help *Lolium* spp. succeed in diverse agroclimatic and geographic regions, escape cultivation, and develop into feral and/or weedy biotypes (Jhala et al. 2021). The introduction of *L. perenne* ssp. *multiflorum* in the United States dates back to the early colonial days (Holt 1976), but its expansion as a pasture crop across the country likely occurred in the early 1930s (Evers 1995). Today, it is widely grown as a winter pasture crop due to its high palatability and nutritional value (Undersander and Casler 2014). However, feral biotypes of *L. perenne* ssp. *multiflorum* have become a problematic weed in many parts of the world including the United States.

In a survey conducted in 2023 by the Weed Science Society of America across the United States and Canada, *L. perenne* ssp. *multiflorum* has been reported as the 2nd most troublesome weed in winter cereal grains, 5th in spring cereal grains, and 10th among all grass crops, pasture, and turf weeds (Van Wychen 2023). Moreover, *L. perenne* ssp. *multiflorum* is one of the most competitive weed species in winter wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and vegetable crops (Appleby et al. 1976; Bell 1995; Nandula 2014). In a study conducted in California, *L. perenne* ssp. *multiflorum* at a density of 600 to 1,000 plants m^{-1} of crop row caused a 100% yield loss in broccoli (*Brassica oleracea* L. var. *botrytis* L.) (Bell 1995). Another study conducted in Oregon on wheat found that a density of 400 plants m^{-2} resulted in up to a 92% yield loss (Appleby et al. 1976). In Mississippi, *L. perenne* ssp. *multiflorum* has been reported to cause a 49% reduction in corn yield at a density of just 4 plants m^{-1} of crop row (Nandula 2014). However, managing herbicide-resistant *L. perenne* ssp. *multiflorum* significantly increases production costs for affected crops. For instance, successfully managing glyphosate-resistant (GR) *L. perenne* ssp. *multiflorum* in soybean [*Glycine max* (L.) Merr.] can cost more than US$100 ha^{-1} (Mississippi Soybean Promotion Board 2021).

Under southeast U.S. climatic conditions, *L. perenne* ssp. *multiflorum* behaves as a winter annual, typically germinating in the fall (October to November) when soil temperatures average between 10 and 18.3 C (Undersander and Casler 2014; Russell 2022). Additionally, a second flush of *L. perenne* ssp. *multiflorum* is often observed in early spring (January) in parts of the southern United States (Bagavathiannan et al. 2021), which underscores the importance of season-long weed management. *Lolium multiflorum* can also be considered a satellite weed for winter wheat, as it emerges around planting time, typically in the second week of October, and flowers from May to July, leaving behind a substantial soil seedbank (Hancock 2011). Cechin et al. (2021) reported that more than 95% of *L. perenne* ssp. *multiflorum* seeds, regardless of soil depth, become unavailable after approximately 1.5 yr, indicating its short seedbank persistence in soil. However, *L. perenne* ssp. *multiflorum* is a prolific seed producer, meaning that 5% of seeds are adequate for its persistent perpetuation. The standard management practice in the southeastern United States for controlling *L. perenne* ssp. *multiflorum* in winter wheat typically begins with a preplant burndown using thifensulfuron and tribenuron (WSSA Group 2) (FirstShot® SG), combined with nonselective herbicides such as glyphosate (WSSA Group 9) or paraquat (WSSA Group 22). For delayed preemergence to provide overlapping residual activity, a mix of flumioxazin (Group 14) and pyroxasulfone (Group 15) is recommended. To manage escaped *L. perenne* ssp. *multiflorum*, late-season postemergence (rescue) treatments using WSSA Group 1 herbicides, such as a premix of pinoxaden + fenoxaprop (Axial® Bold) combined with the Group 2 herbicide pyroxsulam, are advised (Russell 2022).

Given their effectiveness, economic benefits, rapid action, and flexibility, herbicides are the most common choice for farmers to manage weeds, including *L. perenne* ssp. *multiflorum* in wheat and other crops (Duke 2015; Russell 2022; Singh et al. 2020). However, the growing herbicide resistance issue in *L. perenne* ssp. *multiflorum* has complicated management by increasing complexity and expense. Worldwide, 75 cases of HR have been reported for *L. perenne* ssp. *multiflorum* across eight modes of action (MOAs) seven cropping systems (Heap 2024). In the United States, *L. perenne* ssp. *multiflorum* has been reported to be herbicide resistant (HR) to six MOAs, including acetyl-coenzyme A carboxylase (ACCase) inhibitors (WSSA Group 1), acetolactate synthase (ALS) (WSSA Group 2) inhibitors, enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors (WSSA Group 9), glutamine synthetase inhibitors (WSSA Group 10), very-long-chain fatty-acid (VLCFA) inhibitors (WSSA Group 15), and photosystem I (PSI) inhibitors (WSSA Group 22) (Heap 2024). Glyphosate had been an effective preplant treatment for growers for control of *L. perenne* ssp. *multiflorum* in the southern United States. However, a GR biotype with 3-fold resistance was reported in Mississippi in 2005, while another resistant biotype was reported in Tennessee in 2012 (Heap 2024). Resistance to fluazifop-butyl, a member of the aryloxyphenoxypropionate (FOP) family, in *L. perenne* ssp. *multiflorum* was reported in California in 2019 (Tehranchian et al. 2019) and resistance to clethodim, a member of the cyclohexanedione (DIM) family, has been confirmed in southeastern states, including Mississippi and North Carolina (Nandula et al. 2020).

After the development of GR and ACCase-resistant biotypes of *L. perenne* ssp. *multiflorum*, growers increasingly relied on ALS-inhibiting herbicides, particularly in wheat. However, resistance to chlorsulfuron was reported in Arkansas in 1995 (Heap 2024) and resistance to pyroxsulam was confirmed in North Carolina in 2007 (Chandi et al. 2011). ALS resistance is challenging due to the stronger selection pressure exerted by ALS herbicides than other modes, which facilitates the rapid selection and spread of resistance in weeds, including *L. perenne* ssp. *multiflorum* populations (Jones et al. 2021; Tranel and Wright 2002). Despite the continued reliance on pyroxsulam, glyphosate, clethodim, and fluazifop-butyl in wheat, soybean, corn, and preplant herbicide programs across Alabama, recent reports from neighboring states have documented cases of multiple and cross-resistance in *L. perenne* ssp. *multiflorum*. These findings signal an urgent need for proactive resistance management strategies in Alabama (Figure 1).

Although there are several reports of herbicide failures from growers in Alabama, no herbicide resistance survey has been conducted in Alabama to date. The wide range of genetic and phenological diversity exhibited by *L. perenne* ssp. *multiflorum* not only enables *L. perenne* ssp. *multiflorum* to adapt to any harsh field conditions but also helps select HR biotypes when exposed to similar MOAs repeatedly (Mortimer 1997). Therefore, the objectives of our study were to survey, screen, and confirm the distribution of HR *L. perenne* ssp. *multiflorum* in Alabama.

Materials and Methods

Collection and Storage of Plant Materials

Lolium multiflorum populations were collected from late spring to early summer of 2023 through a semi-stratified survey (Garetson 2017) designed to target wheat, soybean, peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.), and cornfields, as well as field borders, across Alabama (Figure 2). Some of the plants were survivors of in-season herbicide applications, identified based on the herbicide failure reports from growers. Others were assumed to be either potential survivors of season-long herbicide applications or late-emerged plants. A total of 20 to 25 seed heads from plants growing within an area of 10 to 15 m^2 were collected and combined to form each population. A distance of 3.2 to 4.8 km was maintained based on the previous weed survey between collection sites to ensure diversity among the populations (Bagavathiannan and Norsworthy 2016).

A total of 65 *L. perenne* ssp. *multiflorum* populations were collected late in the summer, by which point most populations had already completed their life cycle. As *L. perenne* is also common in the southeastern United States, all the *Lolium* populations collected were examined and confirmed to be *L. perenne* ssp. *multiflorum* following the methods of Maity et al. (2021) and Bararpour et al. (2017), largely based on the plant height, length of awn, leaf blade

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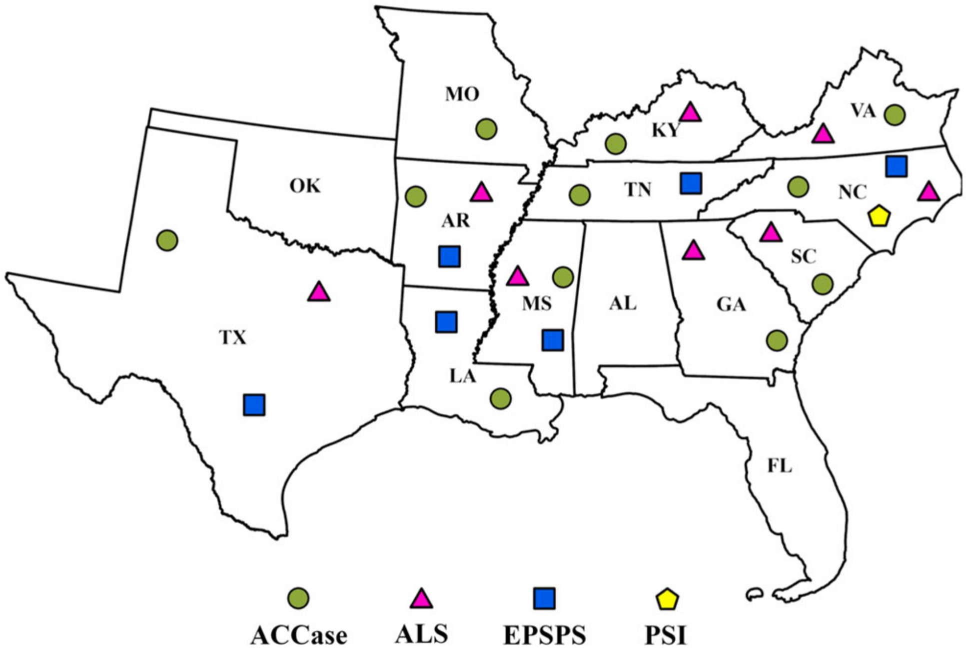

Figure 1. Geographic distribution of herbicide-resistant *Lolium perenne* ssp. *multiflorum* in the southern United States for acetolactate synthase (ALS), acetyl-CoA carboxylase (ACCase), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and photosystem I electron divertor (PSI) inhibitors. Colored shapes indicate resistance to a given mode of action reported within a specific state, but they do not conform to exact geographic coordinates. Information from Chandi et al. (2011), Heap (2024), Kuk and Burgos (2007), Nandula et al. (2007), Salas et al. (2013), and Taylor (2015).

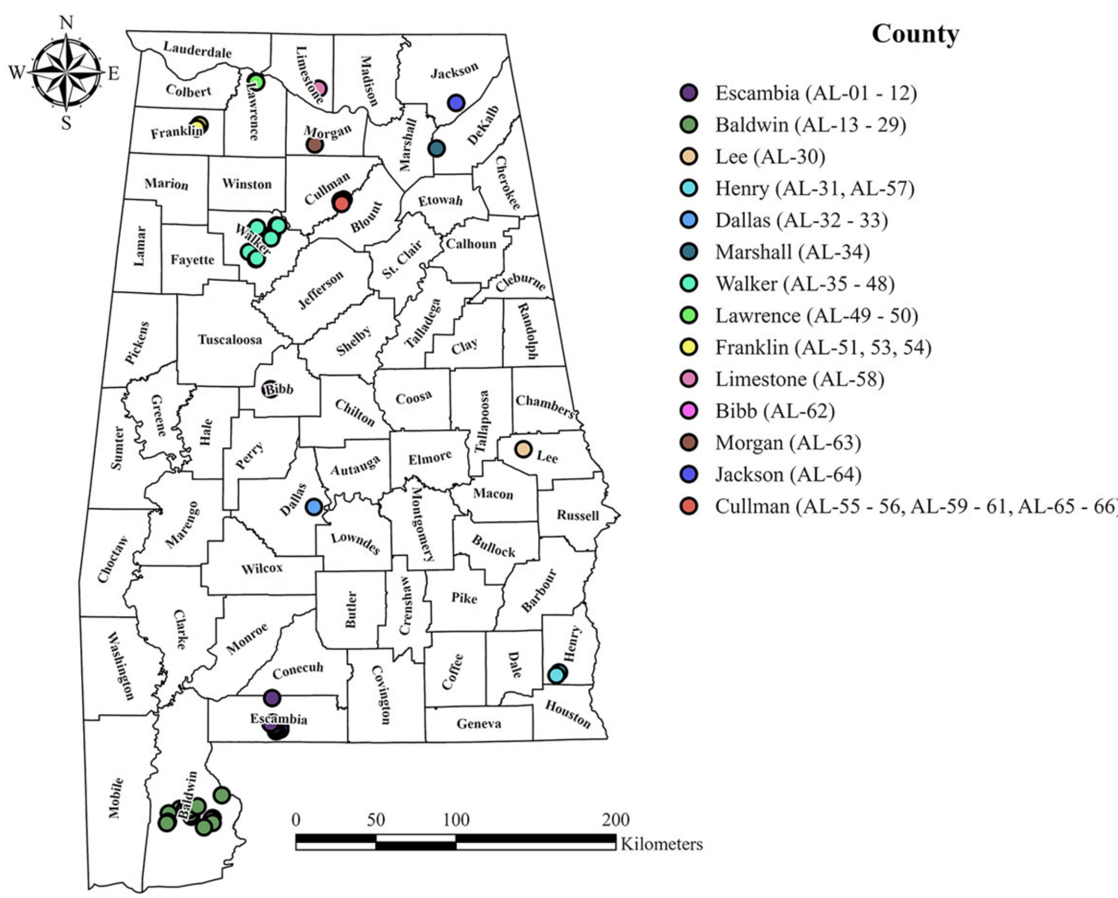

Figure 2. Geocoordinates of 65 *Lolium perenne* ssp. *multiflorum* populations collected from crop and non-cropped areas in Alabama in 2023.

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width, and plant growth habit. The seed heads were hand threshed immediately after collection, and the seeds were stored at room temperature (20 to 22 C) until they were needed for further experiments. Due to limited seed quantity and high seed dormancy, only 44 populations from Escambia, Baldwin, Lee, Henry, Dallas, Walker, Franklin, Cullman, Limestone Bibb, and Jackson counties were included in the herbicide screening study.

Herbicide Efficacy

Herbicide efficacy trials were conducted in the spring of 2024 at the Plant Science Research Center Greenhouse Complex (32.58°N, 85.48°W) located at Auburn University, Auburn, AL. Out of 44 populations, one population (AL-64) from northern Alabama without any known history of herbicide exposure was tested at field rate for all herbicides used in the study and, after results were analyzed, it was declared to be the susceptible standard. Each efficacy trial was conducted with two replications per population, each consisting of 24 plants. Populations were grown in 48-cell inserts placed inside trays (53.34 cm by 27.94 cm). Each tray was divided lengthwise into four sections, with each section containing 12 cells, for a total of 48 cells per tray. One section of 12 cells was allotted to each population, with two plants maintained per cell, allowing four populations to be grown adjacent to each other in a tray. Potting mix (Miracle-Gro® Potting Mix, Scotts Miracle-Gro, Marysville, OH) was used as growing medium, which was watered regularly as needed and fertilized with a soluble fertilizer (Osmocote® Pro 19-5-8, ICL Specialty Fertilizers, Charleston, SC) at the recommended rate at 2 wk after planting (WAP). Plants were maintained at a temperature range of 22 to 28 C. Photoperiod of 12 h was maintained, to compensate on cloudy days, supplemental light was provided by 1,000-W metal-halide bulbs delivering approximately 400 μmol m^{-2} s^{-1}.

Herbicide treatments included (1) ACCase inhibitors: fluazifop-butyl (Fusilade® DX, Syngenta, Greensboro, NC) and clethodim (Section® Three, Winfield Solutions, St Paul, MN); (2) the ALS inhibitor pyroxsulam (PowerFlex® HL Herbicide, Corteva Agriscience, Indianapolis, IN); and (3) the EPSPS inhibitor glyphosate (Roundup PowerMAX® 3 Herbicide, Bayer CropScience, St Louis, MO). All herbicides were applied at their recommended label rates: clethodim (283 g ai ha^{-1}), fluazifop-butyl (213 g ai ha^{-1}), pyroxsulam (233 g ai ha^{-1}), and glyphosate (1,133 g ae ha^{-1}). Herbicides were applied when the plants were at the 3- to 4-leaf seedling stage (~3- to 4-wk old), using a two-nozzle sprayer powered by a CO_2 cylinder and equipped with flat-fan nozzles (TeeJet® XR110015, TeeJet Technologies, Wheaton, IL) calibrated to deliver a spray volume of 140 L ha^{-1} at 289 kPa at a speed of 4.8 km h^{-1}. Injury ratings (based on a scale of 0% to 100%, where 0% means no injury, and 100% means completely dead) and plant mortality were recorded at 3 wk after treatment (WAT) to estimate the level of resistance, as described in Singh et al. (2020). Non-treated controls were included for initial screening. Survival rates of *L. perenne* ssp. *multiflorum* populations to respective herbicides were visualized using a modified box plot (violin plot) with the GGSTATSPLOT package in R v. 4.3.0 (Patil 2021; R Core Team 2023).

Dose–Response Assay

Following the initial assessment, populations with the highest survival rate and lowest injury from all four herbicides were selected for further dose–response assays to confirm the resistance in comparison to a susceptible biotype. The selected populations were AL-61 for glyphosate, AL-62 for pyroxsulam, and AL-65 for both clethodim and fluazifop-butyl-*P*-butyl. The susceptible population (AL-64) selected in the initial screening was used as a common standard for all herbicides. Plants for the dose–response studies were grown in 10.16-cm round pots using Miracle-Gro® Potting Mix (Scotts Miracle-Gro). Emergence was excellent, and thinning was carried out 1 wk after emergence to maintain a population of five plants per pot. The dose–response screening was then conducted using a spray chamber (Generation 4 Research Track Sprayer, DeVries Manufacturing, Hollandale, MN) equipped with a flat-fan nozzle (TeeJet® XR110015, TeeJet Technologies) calibrated to deliver a spray volume of 140 L ha^{-1} at 276 kPa at a speed of 4.8 km h^{-1}. The experiment was a completely randomized design with four replications, conducted across two independent trials. Eight rates were selected for resistant populations (0.5×, 1×, 2×, 4×, 8×, 16×, 32×, and 64×) and six (0.0625×, 0.125×, 0.25×, 0.5×, 1×, and 2×) for susceptible populations. At 4 WAT, plants were evaluated for injury percentages, followed by the shoot biomass collection. Shoot biomass was dried at 65 C for 3 d to record dry biomass.

Data for dose–response trials were analyzed in RStudio. Levene's test was used to check for the homogeneity of two independent trials. No significant difference was found, so data were pooled across the two runs for further analysis. The visual injury was regressed against herbicide dose using a three-parameter logistic regression equation (Equation 1) with the DRC package (Knezevic et al. 2007; Ritz et al. 2015) described later:

$$Y = d + \exp\{b[\log(x) - e]\} \quad [1]$$

In the equation, Y is the response variable (i.e., relative dry weight); x is the applied dose of respective herbicide; d is the upper limit; b is the relative slope around e; and e is GR_{50}, which is the dose of herbicide required for 50% growth reduction. The herbicide dose required to reduce growth by 50% (GR_{50}) was estimated, and the resistance index (R/S) was calculated as the ratio of GR_{50} values of resistant populations to that of the susceptible standard.

Target Site–Based Resistance Detection Studies

To investigate potential target-site resistance mechanisms associated with ACCase-, ALS-, and EPSPS-inhibiting herbicides, RNA was isolated from young leaf samples (approximately 0.1 g) of four *L. perenne* ssp. *multiflorum* populations: AL-61, AL-62, AL-65, and AL-64 (S). For resistant populations, plants surviving from the highest dose in the dose–response assay were used. The RNA extraction was performed using the Zymo-Spin™ II RNA Kit (Zymo Research, Irvine, CA), following the manufacturer's guidelines. The RNA quality and quantity were evaluated through gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). High-quality RNA was converted into complementary DNA (cDNA) using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA) via reverse transcriptase-polymerase chain reaction (RT-PCR). Sections covering known resistance-conferring mutations of the *ACCase*, *EPSPS*, and *ALS* genes were amplified using specific forward and reverse primers (Brunharo and Hanson 2018; Nandula et al. 2020; Tehranchian et al. 2019; Table 1).

PCR amplification was conducted in a 25-μl reaction volume using plant cDNA. The PCR reaction mixture consisted of 1× standard reaction buffer, 200 μM dNTPs, 0.5 μM of both forward

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Table 1. The six primer pairs used to detect single-nucleotide polymorphisms in *Lolium perenne* ssp. *multiflorum* populations.

Primer name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Population
ACCase (Nandula et al. 2020)	CAGTGGCAGACAGATTATTGT	CAATTCAGCAAACCGTATCGC	AL-65, AL-64
EPSPS (Brunharo and Hanson 2018)	AACAGTGAGGAYGTYCACTACATGCT	CGAACAGGTGGGCAMTCAGTGCCAAG	AL-61, AL-64
ALS1 (Tehranchian et al. 2019)	CTCCATCACCAAGCACAACTA	CCCAGTTAGCAGAGCATTCA	AL-62, AL-64
ALS2 (Tehranchian et al. 2019)	GGCACTGTCTACGCAAACTA	CCAGGAGTCTCAAGCATCTTC	AL-62, AL-64
ALS3 (Tehranchian et al. 2019)	CTGTCTTCGGCTGGTCTGG	CTCCTGGTGAGGGACGATGA	AL-62, AL-64
ALS4 (Tehranchian et al. 2019)	GGATGGTAGCTTCTTCATGAA	TCCTGCCATCACCTTCCATG	AL-62, AL-64

and reverse primers, 250 ng of cDNA, and 0.125 U Taq DNA polymerase (New England Biolabs). The thermal-cycling program included an initial activation step at 95 C for 30 s, followed by 35 cycles of 20 s at 95 C, 1 min of annealing at 58 to 62 C (depending on the primer), and 1 min at 68 C, with a final extension step of 5 min at 68 C. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide and run in Tris-acetate-EDTA buffer. The PCR product was sent to Eurofins Genomics (eurofinsgenomics.com) for PCR cleanup and Sanger sequencing. BioEdit (Hall 1999) software was used to align the sequences and identify mutations.

Table 2. Resistance levels of *Lolium perenne* ssp. *multiflorum* populations collected from Alabama[a].

Herbicide	Putatively resistant[b]	Potentially resistant[c]	Susceptible[d]
	% of the population		
Glyphosate	7	14	79
Clethodim	2	0	98
Fluazifop-butyl	4	7	89
Pyroxsulam	11	14	75

[a]A total of 44 populations of *L. perenne* ssp. *multiflorum* were subjected to 1× screening.
[b]Putatively resistant defined as populations with <20% injury.
[c]Potentially resistant defined as populations with 21–79% injury.
[d]Susceptible defined as populations with 80–100% injury.

Results and Discussion

Herbicide Resistance

Glyphosate

Out of the 44 populations subjected to the field rate of glyphosate, 21% survived with varying levels of injury (Table 2), among which three populations were from southern Alabama (Baldwin County) and six populations were from northern Alabama (Cullman and Limestone counties). Among the populations, 34% were classified as putatively resistant, and 66% as potentially resistant. Injury percentages among survivors in putative resistant populations ranged from 15% to 75% with a mean of 46%, while survival rates ranged from 45% to 83% with an average of 67%, which is higher compared with survival rates across all populations (17%) (Figure 3). All the populations from southern Alabama were classified as potentially resistant and were from peanut- and corn-cropping situations. Populations from northern Alabama soybean and cotton rotations varied from potentially to putatively resistant. Glyphosate has been extensively used in Alabama as a burndown herbicide before wheat, soybean, corn, and cotton planting, as well as for GR crops, potentially applying significant selection pressure for glyphosate resistance in *L. perenne* ssp. *multiflorum* populations. To our knowledge, this is the first report of glyphosate-resistant *L. perenne* ssp. *multiflorum* from Alabama.

The AL-61 population from Cullman County (GR soybean crop) was selected for dose–response study based on its high survival rate (76%) and low injury (18%) when treated with glyphosate at 1,133 g ae ha^{-1}. Dose–response assays revealed that the GR_{50} (herbicide dose causing 50% growth reduction) for the susceptible population was 16 g ae ha^{-1}, while it was 3,075 g ae ha^{-1} for the resistant population, indicating a 192-fold increase in resistance compared with the susceptible population (Table 3; Figures 4 and 5).

Table 3. GR_{50} values and herbicide resistance levels in *Lolium perenne* ssp. *multiflorum* populations collected from Alabama[a].

Herbicide	Population	GR_{50}[b]	SEM	RMSE	RI
		g ai/ae ha^{-1}			
Glyphosate	R	3,075[a]	184	10.6	192
	S	16[b]	12	1.5	
Fluazifop-butyl	R	119[a]	6	5.2	14
	S	8[b]	1	5.9	
Clethodim	R	705[a]	36	8.2	738
	S	1[b]	3	1.1	
Pyroxsulam	R	269[a]	9	7.1	90
	S	3[b]	2	1.9	

[a]GR_{50}, the dose of herbicide required for 50% growth reduction; R, resistant population for respective herbicide; RMSE, root square mean error; RI, resistance index, which is ratio of GR_{50} for the resistant and the susceptible populations; S, susceptible population for respective herbicide; SEM, standard error of the mean.
[b]Means compared within herbicide. Letters represent significant differences identified by separation of means using Tukey's honest significant difference (HSD) test (α = 0.05).

Globally, there have been 29 reports of GR *L. perenne* ssp. *multiflorum* (Heap 2024). In the United States, the first report of resistance came from Oregon in 2004, with a 5-fold resistance observed in orchard settings (Perez-Jones et al. 2005). In the southeastern United States, the first case was reported in Mississippi (2005), followed by Arkansas (2008), North Carolina (2009), Tennessee (2012), and Louisiana (2014) (Heap 2024). In California, resistance in *L. perenne* ssp. *multiflorum* has been documented ranging from 2- to 15-fold compared with susceptible populations (Jasieniuk et al. 2008). Similarly, Salas et al. (2012) reported 7- to 13-fold glyphosate resistance in Arkansas based on visual injury comparisons with susceptible populations. The

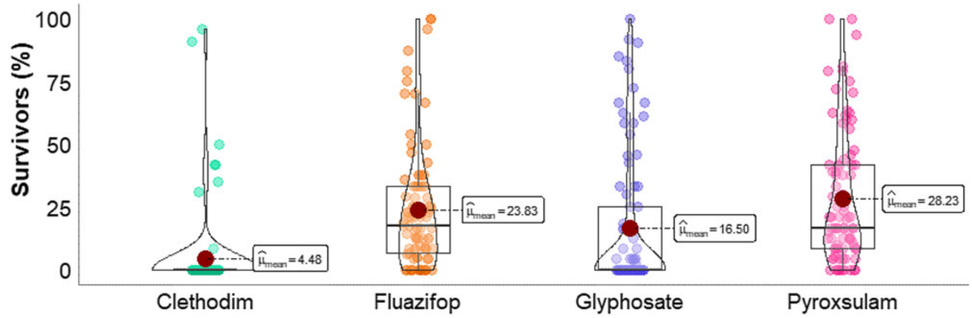

Figure 3. Percent survival of *Lolium perenne* ssp. *multiflorum* populations in response to different herbicides. The *y* axis represents the number of survivors in 44 different populations. This violin plot is a modified box plot in which the box represents the upper and lower quartiles, and the central line represents the median. The violin area shows the proportion of populations with survivors for each herbicide.

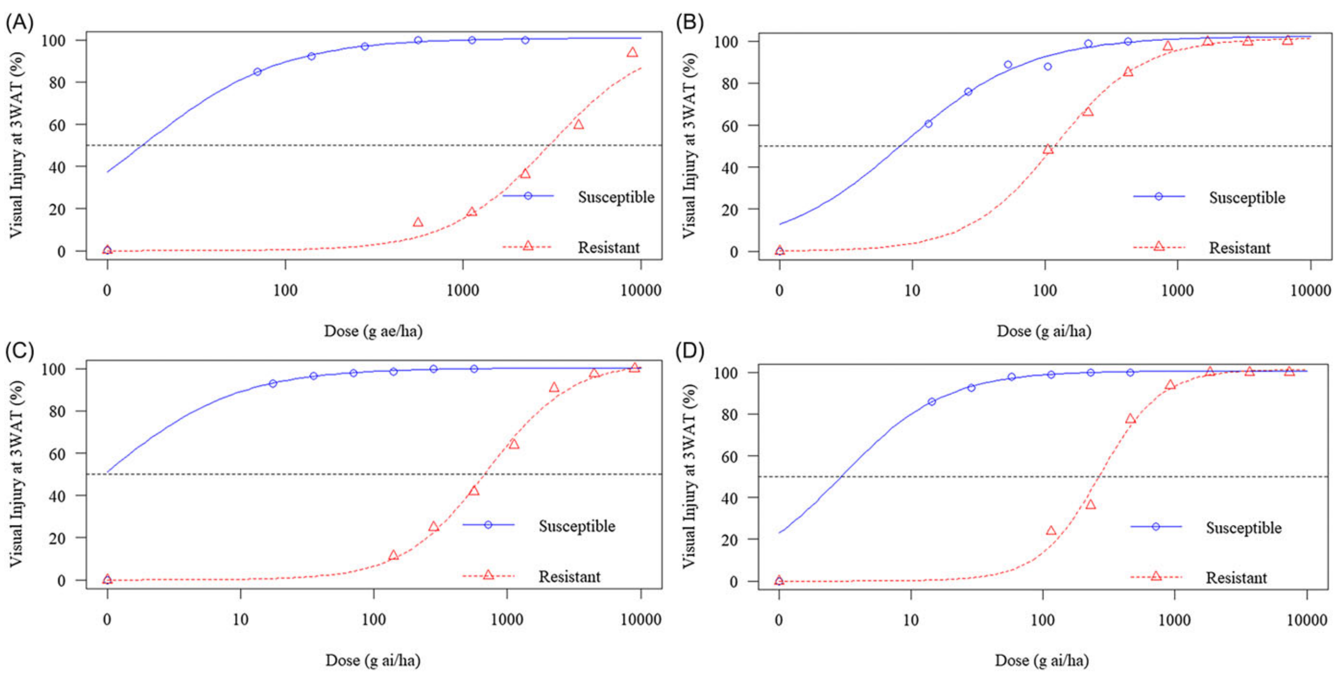

Figure 4. Dose–response of the resistant and susceptible *Lolium perenne* ssp. *multiflorum* populations to (A) glyphosate (population AL-61), (B) fluazifop-butyl (population AL-65), (C) clethodim (population AL-65), and (D) pyroxsulam (population AL-62). Injury (%) was based on visual assessment of respective treated populations compared with non-treated *L. perenne* ssp. *multiflorum*. Population AL-64 that was confirmed susceptible in the initial screening was used as the standard susceptible population for all herbicides.

extreme sensitivity of susceptible populations to glyphosate (GR_{50} = 16 g ae ha^{-1}) is an obvious factor contributing to the high level of resistance. The GR_{50} for susceptible *L. perenne* ssp. *multiflorum* populations was as high as 171 g ae ha^{-1} in Arkansas (Dickson et al. 2011) and 230 g ae ha^{-1} in California (Karn et al. 2018). In this study, 11× of 1,133 g ae ha^{-1} would be required to reduce the biomass of the resistant population 90%. Additionally, the significantly shorter awn length in putative GR populations may indicate a potential fitness cost (Yanniccari et al. 2016).

Pyroxsulam

Out of the 44 total populations, 11 survived the field rate of pyroxsulam (233 g ai ha^{-1}; Table 2). Five of the 11 populations were from southern Alabama (Escambia and Baldwin counties), 2 from central Alabama (Bibb and Dallas counties), and 4 from northern Alabama (Walker, Limestone, and Cullman counties). Populations from southern and central Alabama were from field borders as well as in fields of peanut, corn, and wheat; however, all putatively resistant populations from northern Alabama were from fields of cotton and soybean. Among these surviving populations, 45% were classified as putatively resistant and 65% as potentially resistant. The average survival rate across potentially and putatively resistant populations was 56% and across all populations it was 28% (Figure 3). Injury observed in potentially and putatively resistant populations ranged from 10% to 73%. The putatively resistant population (AL-62) for dose–response was selected based on a high survival (90%) and low injury (10%) rate from Bibb County collected from a field border. The resistance index, calculated from GR_{50} values, indicated a 90-fold resistance in the resistant population compared with the susceptible (Table 3; Figure 4).

Globally, 31 cases of resistance to ALS-inhibiting herbicides have been reported in *L. perenne* ssp. *multiflorum*, with 22% of these specifically resistant to pyroxsulam (Heap 2024). In the Texas Blacklands region, Singh et al. (2020) reported that 93% of the populations ($n = 64$) was resistant to the ALS herbicide

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mesosulfuron-methyl, with resistance levels as high as 37-fold. The 90-fold resistance observed in this study could be attributed to the frequent use of ALS-inhibiting herbicides in Alabama wheat production and the high sensitivity of the susceptible standard used. However, with 14% of the populations classified as potentially resistant and 75% still susceptible, resistance evolution appears to be slow in progress or the current *L. perenne* ssp. *multiflorum* management strategies in the region are still effective. This presents an opportunity for timely intervention and precautionary measures to prevent further resistance development.

Clethodim

Only 2% ($n = 1$ out of 44) of the populations collected from northern Alabama (Cullman County) were found to be putatively resistant to clethodim (Table 2). The mean survival rate across all populations was 4.48% (Figure 3). Dose–response studies revealed GR_{50} values of 705.24 g ai ha^{-1} for the resistant population and 0.95 g ai ha^{-1} for the susceptible population, indicating a 738-fold resistance in the resistant population (Table 3), which requires 12× the field rate to reduce 90% biomass of the resistant population.

Clethodim is a postemergence herbicide frequently used to manage GR *L. perenne* ssp. *multiflorum*. The soybean field in which the resistant population was collected had a history of repetitive use of clethodim to control annual grass weeds (personal communication with the wheat grower). Previously, resistance to clethodim was documented in the U.S. Pacific Northwest, where 5% of populations were resistant, with another 8% developing resistance (Rauch et al. 2010). In the southeastern United States, Nandula et al. (2020) observed up to 10-fold resistance in populations from Mississippi and up to 40-fold resistance in populations from North Carolina. In two additional populations, resistance levels could not be calculated because biomass reduction was only 17% to 30% at the highest tested dose (2,170 g ha^{-1}).

The extremely high resistance level (738-fold) observed in this study could be attributed to the high sensitivity of the susceptible standard, which showed a 93% biomass reduction at the lowest dose tested (17 g ha^{-1}). Furthermore, as glyphosate has become less effective in controlling *L. perenne* ssp. *multiflorum*, the use of clethodim has increased in Alabama as a burndown in soybean and cotton. Despite this, clethodim resistance was detected in only one population from northern Alabama, and the mean survival rate across all populations was low (4.48%). This suggests that while resistance is present, it remains rare, indicating that the systems may be integrated sufficiently to select against rapid resistance evolution to clethodim. Strong preventive measures are essential to prevent the spread of clethodim resistance, and the integration of other MOAs will be necessary to reduce the selection pressure exerted by clethodim.

Fluazifop-Butyl

Eleven percent of the total population survived fluazifop-butyl field rate, among which one population collected from a corn field in Baldwin County in southern Alabama was potentially resistant; the remaining four populations were from northern Alabama (Cullman County) from soybean fields (Table 2). Of the surviving populations, 45% were classified as putatively resistant and 55% as potentially resistant. Survival rates in putatively and potentially resistant populations ranged from 46% to 100% with a mean of 79%. A mean survival rate of 24% was observed across the populations (Figure 3). Population (AL-65) was selected for dose–response studies based on its high survival rate (98%) and low injury (13%); the same population was also used for dose–response studies for clethodim. The dose–response study indicated a 14-fold resistance in AL-65 compared with the susceptible (Table 3). No significant differences in seed traits were observed between fluazifop-butyl-resistant and fluazifop-butyl-susceptible populations.

Table 4. Cross- and multiple resistance in 44 *Lolium perenne* ssp. *multiflorum* populations collected from Alabama.

Mode of action[a]	Herbicide combination	Population with cross- or multiple resistance[b]	
		No.	%
EPSPS × ALS	Glyphosate × pyroxsulam	2	5
EPSPS × ACCase (fop)	Glyphosate × fluazifop-butyl	4	9
EPSPS × ALS × ACCase (dim) × ACCase (fop)	Glyphosate × pyroxsulam × clethodim × fluazifop-butyl	1	2

[a]ACCase, acetyl-coenzyme A carboxylase; ALS, acetolactate synthase; dim, cyclohexanedione family; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; fop, aryloxyphenoxypropionate family.
[b]Cross-resistance refers to resistance to different herbicide families with the same mode of action (MOA); multiple resistance refers to resistance to two or more unique herbicide MOAs. Resistance was determined based on plant survival (1–79% injury) at the recommended label rate for the given herbicide.

Fluazifop-butyl is recommended in Alabama for controlling annual and perennial grasses in cotton, soybean, and peanut (Alabama Cooperative Extension System, 2023a, 2023b, 2023c). Resistance to diclofop-methyl, a herbicide from the same aryloxyphenoxypropionate family as fluazifop-butyl, has become widespread (Heap 2024; Singh et al. 2020), largely due to its frequent use in wheat for grass weed control. This overreliance on diclofop has exerted extreme selection pressure on weeds, including *L. perenne* ssp. *multiflorum*. Previous studies have reported that both target-site and non–target site resistance mechanisms confer resistance to diclofop-methyl, which also extends resistance to fluazifop-butyl and other ACCase-inhibiting herbicides in *L. perenne* ssp. *multiflorum* (Kaundun et al., 2012, 2013).

Fluazifop-butyl resistance has been reported in California, where the labeled field rate reduced *L. perenne* ssp. *multiflorum* biomass by only 14% (Tehranchian et al. 2019). In the current study, the population (AL-65) that has the highest level of resistance to fluazifop-butyl also exhibited cross-resistance to clethodim, a common phenomenon among ACCase-inhibiting herbicides (Takano et al. 2020). Although resistance to fluazifop-butyl in Alabama is not as widespread as the resistance to glyphosate or pyroxsulam, it remains a viable option for controlling *L. perenne* ssp. *multiflorum*. However, proactive measures are essential to avoid overreliance on fluazifop-butyl and to ensure its continued efficacy through judicious use.

Cross-Resistance and Multiple Resistance

Fourteen percent of the populations ($n = 6$) exhibited putative multiple resistance to either the EPSPS inhibitor (glyphosate) and ALS inhibitor (pyroxsulam) or EPSPS inhibitor (glyphosate) and ACCase inhibitor (fluazifop-butyl) (Table 4). Two populations (AL-21 and AL-58) exhibited putative multiple resistance to ALS and EPSPS inhibitors from the Baldwin–peanut fields and Limestone–cotton fields, respectively. AL-21 was resistant to (injury = 13%) against pyroxsulam; however, it exhibited potential resistance (injury = 45%) against glyphosate, whereas AL-58 was putatively resistant (injury = 18%) to glyphosate and showed a moderate level of resistance (injury = 75%) to pyroxsulam. Four

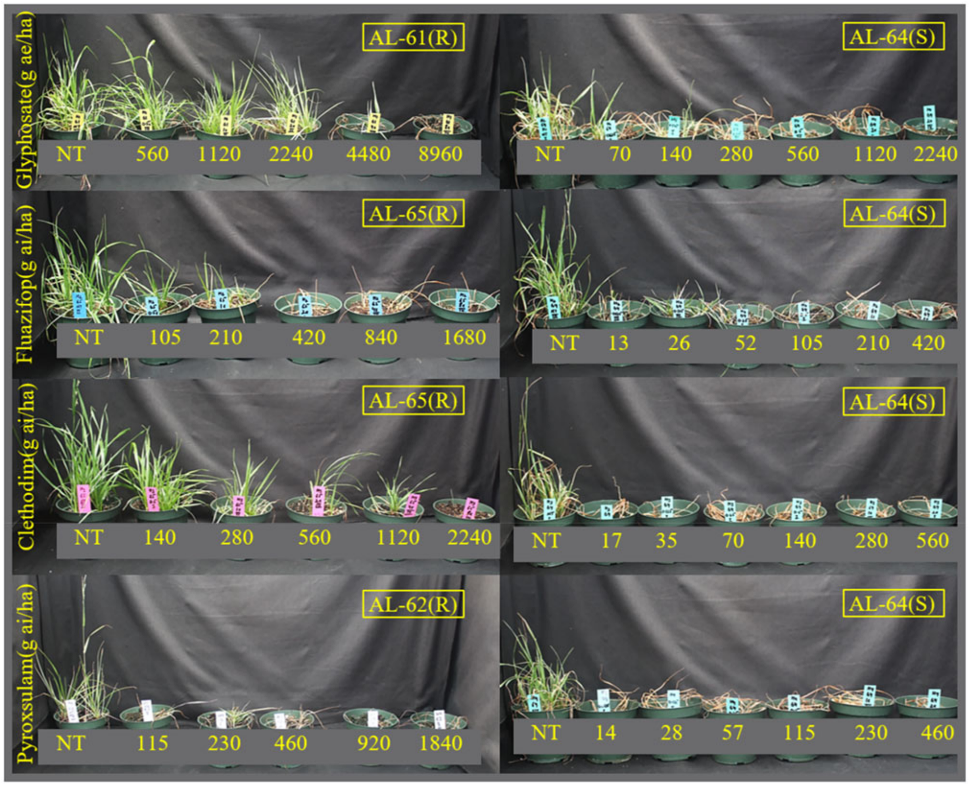

Figure 5. Dose–response of resistant *Lolium perenne* ssp. *multiflorum* populations, encompassing both resistant (R) and susceptible (S) populations, to varying rates of four herbicides: glyphosate, fluazifop-butyl, clethodim, and pyroxsulam. Herbicide application rates range from 1× to 8× the recommended label rate for resistant populations, and from 0.065× to 2× the recommended rate for the susceptible population. The populations tested include AL-61, AL-62, and AL-65, which are resistant (R), and AL-64, which is known susceptible (S). NT, non-treated control plants.

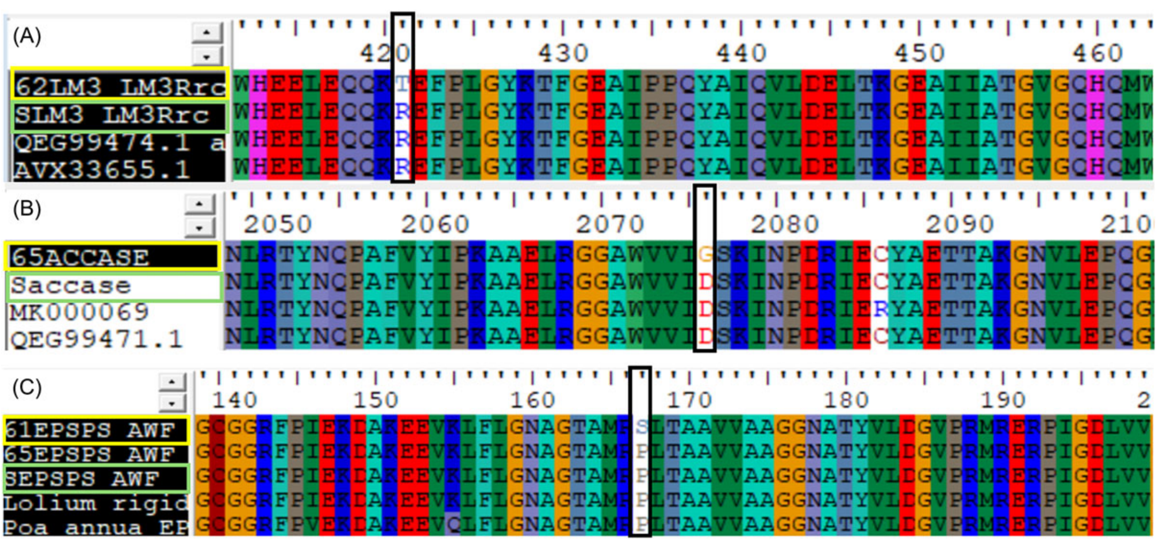

Figure 6. Multiple sequence alignments in the *Lolium perenne* ssp. *multiflorum* populations from BioEdit: (A) *ALS* gene of AL-62, (B) *ACCase* gene of AL-65, and (C) *EPSPS* gene of AL-61. Sequences from resistant populations are highlighted with yellow rectangles, while susceptible population (AL-64) respective gene sequences are highlighted with green rectangles. Other sequences from NCBI are included for cross-validation.

populations (AL-20, AL-59, AL-61, and AL-66) exhibited putative multiple resistance to ACCase and EPSPS inhibitors. Out of the four populations, one was from southern Alabama (Baldwin County), and three from Northern Alabama (Cullman County). The population from southern Alabama was from a cornfield, while all populations from northern Alabama were from soybean fields. All the populations were potentially resistant to both MOAs, except AL-61, which was putatively resistant to both MOAs.

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One population (AL-65) expressed cross-resistance to both aryloxyphenoxypropionate (fluazifop-butyl) and cyclohexanedione (clethodim) families of ACCase inhibitors, as well as multiple resistance to ACCase (clethodim and fluazifop-butyl), ALS (pyroxsulam), and EPSPS inhibitors (glyphosate). The resistant population was collected from a soybean field in Cullman County. It exhibited the highest survival rate in response to clethodim and fluazifop-butyl and had a target-site mutation (Asp-2078-Gly) in the *ACCase* gene. However, non–target site resistance may also be involved in conferring herbicide resistance in *L. perenne* ssp. *multiflorum*, as reported by previous literature (Kaundun et al., 2012, 2013). According to the Heap (2024) database, 28% of total HR cases ($n = 36$) reported against *L. perenne* ssp. *multiflorum* in the United States involve multiple resistance. In North Carolina, *L. perenne* ssp. *multiflorum* has been reported resistant to ALS, ACCase, EPSPS, and PSI inhibitors (Heap 2024). Similarly, in California, resistance to EPSPS, ACCase, and PSI inhibitors has been documented (Tehranchian et al. 2019). *Lolium perenne* ssp. *multiflorum* populations in California have also shown cross-resistance to ACCase-inhibiting herbicides, including fluazifop-butyl and clethodim (Tehranchian et al. 2019), which is consistent with the findings of this study.

Target-Site Mutation Detection

Sequencing of the ACCase region in the AL-65 population revealed a Asp-2078-Gly mutation (Figure 6B), which has previously been documented in *L. perenne* ssp. *multiflorum* populations from Leicestershire, UK (Kaundun 2010) and is associated with resistance to ACCase-inhibiting herbicides. In the ALS region of the AL-62 population, a novel Arg-421-Thr mutation was identified (Figure 6A), which has not been reported in previous studies. It was a transversion, which refers to a point mutation in DNA in which a single (two-ring) purine (A or G) is changed for a (one-ring) pyrimidine (T or C), or vice versa. Additionally, the *EPSPS* gene region of the AL-61 population exhibited a Pro-106-Ser mutation (Figure 6C), a mutation also reported by Brunharo and Hanson (2018) in GR *L. perenne* ssp. *multiflorum* populations.

The herbicide resistance in *L. perenne* ssp. *multiflorum* populations from Alabama was confirmed across all four herbicides evaluated, with molecular data corroborating these findings. However, resistance levels and distribution varied considerably among the tested herbicides. Fluazifop-butyl and pyroxsulam resistance was more widespread than glyphosate and clethodim resistance. Despite these differences, significant resistance was evident for all herbicides, likely influenced by the high sensitivity of the susceptible control population. To mitigate further spread of herbicide resistance in Alabama, it is critical to implement an integrated weed management strategy. Proactive measures such as using overlapping residual herbicides, establishing cover crops, timely postemergence herbicide applications, and integrating emerging technologies like harvest weed seed control are urgently required in the region.

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