Research Article

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Resistance to clethodim in Italian ryegrass (*Lolium perenne ssp. multiflorum*) from Mississippi and North Carolina

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Abstract

BACKGROUND: Clethodim, an acetyl-CoA carboxylase (ACCase)-inhibiting herbicide, is one of the few postemergence chemical control options available to growers of Mississippi to manage glyphosate and/or other herbicide resistant Italian ryegrass populations. Recently, clethodim failed to adequately control Italian ryegrass populations across Mississippi. A sethoxydim, also an ACCase inhibitor, -resistant Italian ryegrass population from North Carolina was cross-resistant to clethodim. This research characterized the magnitude and mechanisms of clethodim resistance in the Mississippi and North Carolina Italian ryegrass populations via whole-plant herbicide dose response, cross resistance, and metabolism studies, and molecular analysis.

RESULTS: Two clethodim-resistant biotypes from Mississippi, MS24 and MS37, were 10- and 4-fold resistant, respectively, relative to a susceptible (SUS1) biotype. A North Carolina biotype, NC21, was 40-fold resistant to clethodim compared to SUS1. Two additional biotypes from North Carolina, NC22 and NC 23, recorded shoot dry weight reduction of only 17–30% of nontreated at the highest clethodim dose of 2.17 kg ha^{-1}, (8×). The NC22 biotype was cross-resistant to sethoxydim, fluazifop, quizalofop, and pinoxaden. Metabolic inhibitors such as piperonyl butoxide and 4-chloro-7-nitrobenzofurazan did not affect resistance of MS37, MS51, and NC22 biotypes to fenoxaprop, clethodim, or pinoxaden. The MS37 biotype had three target site mutations, I2041N, C2088R, and G2096A. Another clethodim-resistant biotype from Mississippi, MS51, had only the C2088R substitution. The NC22 and NC23 biotypes had I1781L, I2041N, and D2078G replacements.

CONCLUSION: This study shows that the mechanism of resistance to clethodim in Italian ryegrass from Mississippi and North Carolina is due to target site modifications in the *ACCase* gene leading to broad cross-resistance to other ACCase-inhibiting herbicides.

Keywords: ACCase; acetyl CoA carboxylase; clethodim; herbicide; Italian ryegrass; *Lolium perenne ssp. multiflorum*; resistance

1 INTRODUCTION

Italian ryegrass [formerly *Lolium multiflorum*, now *L. perenne* L. ssp. *multiflorum* (Lam.) Husnot] also referred to as annual ryegrass, is native to temperate regions of Europe.[1] It is a herbaceous annual/biennial grass that is grown for silage, as a pasture crop, and as a cover crop along roadsides, rights-of-way, and industrial areas.[2–5] Perennial ryegrass (*L. perenne* L.), also native to Europe in addition to temperate Asia and North Africa,[6] is a forage and pasture crop like annual ryegrass, but differs in that it is used in grass and wildflower seed mixes,[7] and as a lawn grass.[8] Perennial ryegrass varieties have been particularly bred for preferred forage qualities such as higher total nonstructural carbohydrates that facilitated better digestion in the rumen of the grazing animals.[9]

Italian ryegrass readily naturalizes newly colonized areas, thereby, becoming a noxious weed in agricultural areas and an invasive species in native habitats.[10] It can germinate, emerge, and establish over a range of environmental conditions.[2,11] Italian ryegrass has become a management problem along roadsides due to its ability to hybridize with cultivated annual ryegrass species, escape cultivation, and by possession of a network of broad and shallow fibrous root system.[12–15]

Italian ryegrass has developed into an economically important weed affecting small grain and vegetable crops.[16–18] For example, winter wheat yield was decreased by 4700 kg·ha^{-1} when Italian ryegrass density increased from 0.7 to 3 plants·m^{-2}.[16] Competition from Italian ryegrass reduced winter wheat (*Triticum aestivum* L.) yield up to 92%.[17] Italian ryegrass densities of 600 to 1000 plants per meter row caused 100% yield loss in broccoli (*Brassica oleracea var. botrytis* L.).[18] Corn (*Zea mays* L.) density and yield were severely reduced due to competition from Italian ryegrass.[19]

Italian ryegrass control has traditionally been realized by chemical means.[2] Relentless selection pressure from herbicides

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has resulted in the evolution of resistance in Italian ryegrass to several herbicide mechanisms of action across varied cropping systems and several countries from Asia, Europe, North America, and South America.[20] Historically, glyphosate was frequently used for controlling Italian ryegrass and other weed flora in orchards and vineyards. In agronomic cropping areas across the US, including Mississippi, glyphosate was used as a preplant burndown and/or post-harvest treatment, prior to the commercialization of glyphosate resistant (GR) crops in the mid-1990s. With widespread adoption of the GR crops, multiple in-season postemergence (POST) applications of glyphosate have become common practice. This added selection pressure from glyphosate resulted in Italian ryegrass populations exhibiting increasingly less susceptibility. Evidence of evolved GR Italian ryegrass in row/agronomic crops was first reported from Washington County, Mississippi in 2005.[21] GR Italian ryegrass has since been documented in multiple states across the southeastern US.[20]

Glyphosate resistant Italian ryegrass populations have seriously jeopardized preplant burndown options in reduced-tillage row crop (corn, cotton (*Gossypium hirsutum* L.), rice (*Oryza sativa* L.), soybean [*Glycine max* Merr.)] production systems, thereby, delaying planting operations. Growers and land managers have increasingly relied on clethodim to combat GR Italian ryegrass invasions. Clethodim is an acetyl CoA carboxylase (ACCase, EC 6.4.1.2) inhibitor, belonging to the cyclohexanedione or 'dim' family (aryloxyphenoxypropionates or 'fop' and phenylpyrazolin or 'den' are two other ACCase-inhibiting herbicide families) used for POST control of volunteer corn and several annual and perennial weeds in multiple crops including soybean and cotton, in fall and spring.[22] In 2016 and 2017, several Italian ryegrass populations have not responded to commercial applications of clethodim in the Mississippi Delta (17-county region in northwestern Mississippi).[23]

In a different situation, Italian ryegrass was found to be resistant to sethoxydim, also an ACCase inhibitor, in an investigation of a single population from Iredell County alone, North Carolina.[24] This Italian ryegrass population originated from a commercial strawberry (*Fragaria* × *ananassa* Duchesne) operation, where it was used as a cover crop. Annually, prior to strawberry transplanting, the Italian ryegrass was controlled with sethoxydim. After 8 years of sethoxydim use, the Italian ryegrass was no longer adequately controlled. Preliminary greenhouse resistance screening studies indicated that this population is not controlled by clethodim, among other herbicides.

The objectives of this research were to measure the magnitude of resistance to clethodim and to determine the mechanism(s) of resistance to clethodim in Italian ryegrass populations from Mississippi and North Carolina.

2 MATERIALS AND METHODS

2.1 Plant materials

2.1.1 Mississippi

Italian ryegrass plants that survived spring burndown applications of clethodim were randomly collected from fields across the Mississippi Delta (Fig. 1) in March 2016. Plants were transplanted in to 10 ×10 ×10 cm plastic pots containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA). Plants were grown to maturity, seed harvested, air-dried in a greenhouse and stored at 2–8 °C until further use. There were 43 individual accessions generated from the spring collection. In a separate survey conducted in the summer of 2016, seed from Italian ryegrass plants was collected from randomly chosen sites (Fig. 1) across the Mississippi Delta. Seed was air-dried and stored as previously described until further use. Fifty-one accessions were gathered from the summer collection. Each summer accession represented a composite sample of seed from three to four plants growing within a 30-m radius.

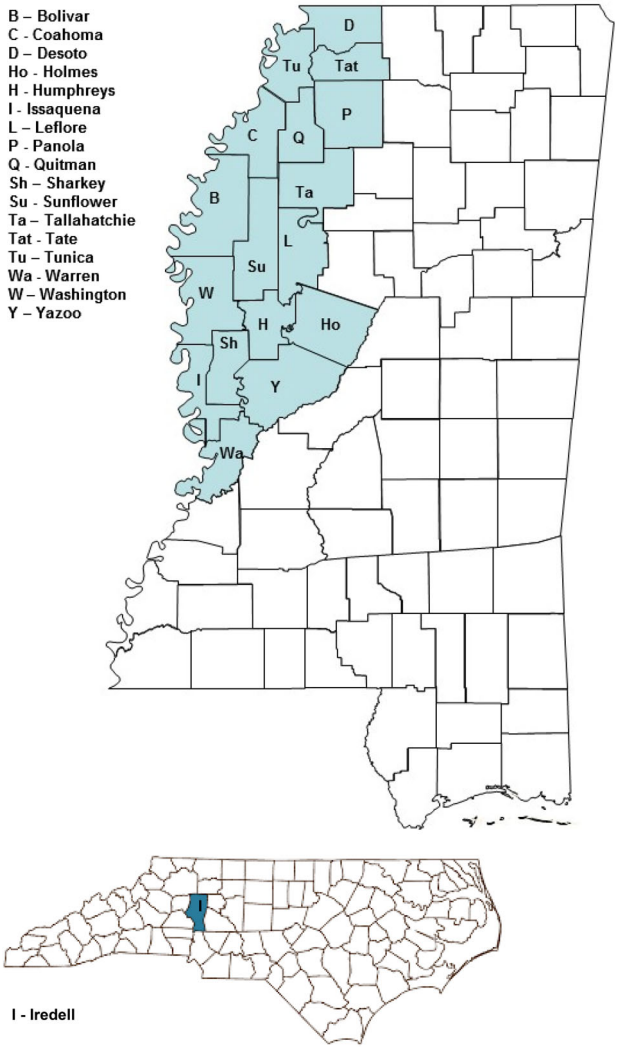

Figure 1. Map of counties in Mississippi and Iredell County, North Carolina where Italian ryegrass samples were collected.

In the Mississippi Delta, the traditional crop rotations are corn followed by cotton on lighter soils and soybean followed by rice on heavier soils. However, over the past 10 to 15 years, commodity prices, pest and weed problems, and weather conditions have driven crop rotations with no pattern; for example, corn could be followed by corn, cotton, or soybean. The spring and summer 2016 surveys were conducted exclusively in agronomic fields planted to corn, cotton, rice, or soybean the previous year or in the 2016 growing season.

2.1.2 North Carolina

Italian ryegrass seed from several plants that survived a POST application of sethoxydim applied on a strawberry field in Iredell County, North Carolina (Fig. 1) were bulked, air-dried, and stored as described above until further use.

2.2 Growing and herbicide treatment conditions

Italian ryegrass seeds were planted at 1-cm depth in 50-cm by 20-cm by 6-cm plastic trays containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA). Trays were watered, drained, and placed in a refrigerator at 2–8 °C for 48 h to break seed dormancy and induce germination.[21] No germination test was conducted for dormancy verification. Prior research experience with Italian ryegrass[21] necessitated treatment for dormancy as a routine procedure. Trays were then placed in a greenhouse maintained at 20/15 °C day/night temperatures with a 13-h photoperiod. Sodium halide lamps providing a photosynthetic photon flux density of 500 μmol m^{-2} s^{-1} were used for supplemental lighting. Italian ryegrass plants, 2 weeks after emergence, were transplanted into 6 cm × 6 cm ×6 cm pots containing the soil mix mentioned before. Plants were fertilized once with a nutrient solution (Miracle-Gro, The Scotts Company LLC, Marysville, OH, USA) containing 200 mg L^{-1} each of N, P_2O_5, and K_2O at one week after transplanting and sub-irrigated as needed thereafter. Italian ryegrass plants from all experiments were grown and maintained under the above conditions. Herbicide treatments were applied to Italian ryegrass plants at the 3- to 4-leaf growth stage with a moving-nozzle sprayer (DeVries Manufacturing Inc., Hollandale, MN, USA) equipped with 8002E nozzles (Spraying Systems Co., Wheaton, IL, USA) delivering 190 L ha^{-1} at 220 kPa. All herbicide treatments had a crop oil concentrate (Agridex, Helena Chemical Co., Collierville, TN, USA) at 1% vol. Treated plants were evaluated for survival 3 weeks after treatment (WAT) based on live/dead growing point and tillers in clethodim resistance screening experiments. Aboveground biomass of Italian ryegrass plants was collected 3 WAT, dried in an oven at 50 °C for 72 h, and weighed.

2.3 Clethodim resistance screening

Italian ryegrass plants, 18 per each spring- and summer-collected Mississippi accession, were screened with a 0.5× rate of clethodim (Select Max, Valent USA Corp., Walnut Creek, CA, USA), 0.068 kg ai ha^{-1}. The 0.5× rate was used to avoid losing plants with 'low' resistance. Since the level of resistance to an herbicide is largely due to the inherent resistance mechanism(s), plants with 'low' resistance may have a nontarget site-based resistance mechanism such as metabolism. Thereafter, another set of 18 plants per accession were treated with a 1.0× rate of clethodim, 0.136 kg ha^{-1}. Plants that survived 1.0× clethodim application were grown to maturity and subjected to a second series of screening experiments. Results revealed that all plants raised from seed of survivors of clethodim at 0.136 kg ha^{-1} were resistant at the 1.0× rate. Hereafter referred as biotypes MS24 (Washington County), MS37 (Leflore County, LC, USA), and MS51 (LC), plants raised from this second-generation seed were used in dose response and/or molecular studies. Three sets of plants of the North Carolina population, each set containing 18 plants, were screened with a 1.0× clethodim rate, and survivors that produced seed at maturity were screened again with the 1.0× rate. A 0.5× rate of clethodim was not used, assuming a higher level of resistance, with the plants having an 8-year exposure to sethoxydim. Three biotypes, NC21, NC22, and NC23, were developed from the second generation of survived plants and used in this research. A wild type Italian ryegrass biotype (from a roadside location in Stoneville, MS) confirmed to be susceptible to clethodim and other ACCase inhibitors (fenoxaprop, fluazifop, quizalofop, sethoxydim, and pinoxaden; data not shown), SUS1, was also included in all experiments for comparisons.

2.4 Clethodim dose response

Resistant and susceptible Italian ryegrass plants were treated with clethodim at 0, 0.14, 0.27, 0.54, 1.08, and 2.17 kg ai ha^{-1}. To ensure enough data points for the dose response curves, a lower rate of 0.07 kg ha^{-1} clethodim was included for SUS1. Herbicide doses higher than 1.0× were used on the SUS1 to ensure mortality and prevent sprouting of tillers, and doses lower than 0.5× were not included to avoid hormesis. There were five replications per treatment, with each replication represented by one plant per pot. The experiment was performed two times.

2.5 Cross-resistance to other ACCase inhibitors

Due to paucity of seed, only the NC22 among the clethodim-resistant biotypes was included along with the SUS1 biotype. Resistant and susceptible plants were treated with sethoxydim (Poast, BASF, Research Triangle Park, NC, USA) at 0.23 kg ai ha^{-1}, fluazifop (Fusilade DX, Syngenta Crop Protection, Greensboro, NC, USA) at 0.23 kg ai ha^{-1}, quizalofop (Targa, Gowan Co., Yuma, AZ, USA) at 0.083 kg ai ha^{-1}, and pinoxaden (Axial, Syngenta Crop Protection) at 0.064 kg ai ha^{-1}. All of the above rates represent labeled single highest application. There were eight replications per treatment, with each replication represented by one plant per pot. The experiment was conducted once.

2.6 Metabolism of ACCase inhibitors

Seed increase allowed inclusion of MS37 and MS51 biotypes in this study along with NC22 and SUS1 biotypes. Italian ryegrass plants of the above four biotypes were treated with fenoxaprop (Ricestar HT, Bayer CropScience, Research Triangle Park, NC, USA) at 0.12 kg ae ha^{-1}, clethodim at 0.136 kg ha^{-1}, and pinoxaden at 0.064 kg ha^{-1} alone and in separate combination with piperonyl butoxide (PBO-8, Zoecon/Wellmark International, Central Garden & Pet Co., Schaumburg, IL, USA, PBO) at 1400 g ha^{-1} prepared in methanol and applied 24 h prior to herbicide treatment, and 4-chloro-7-nitrobenzofurazan (Sigma-Aldrich, St. Louis, MO, USA, NBD-Cl) at 270 g ha^{-1} prepared in acetone and applied 48 h prior to herbicide treatment. There were five replications per treatment and the experiment was conducted two times.

2.7 *ACCase* sequencing

DNA was extracted using a modified CTAB extraction method[25] from 3- to 4-leaf Italian ryegrass plants of SUS1, MS37, MS51, NC 22, and NC23 biotypes, five plants per biotype. All plants of the resistant biotypes were from the second generation. Two primers, a forward (AW580: CAGTGGCAGACAGATTATTGT) and a reverse (AW581: CAATTCAGCAAACCGTATCGC) were used to amplify the *ACCase* gene. Two internal primers, AW582: GCATACAGCGTGAAGATCA and AW583: GAAGCCTCTCCAGTTAGCA, were also used because the carboxyltransferase domain was big enough to require internal primers for sequencing. The *ACCase-CT* was amplified using GoTaq Green PCR Master Mix (Promega, Madison, WI, USA). PCR cycle conditions were programmed for initial denaturation at 95 °C for 4 min; 30 cycles of 95 °C for 30s, 55 °C for 30s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were cleaned up using GeneJET PCR Purification Kit (Thermo Scientific, Vilnius, Lithuania). Purified DNA was sequenced (Roy J. Carver Biotechnology Center, DNA Services, Urbana, IL, USA) and data were analyzed and aligned in Geneious 11.0.3. Sequences of herbicide-resistant plants were submitted to NCBI GenBank (accession numbers MK000066-MK000069).

2.8 Statistical analysis

All data from dose response and cross resistance studies were subjected to ANOVA using PROC GLM in SAS 9.4 (SAS Institute, Cary, NC, USA). Data from the two runs of the dose response and metabolic inhibitors experiments were pooled because of nonsignificant influence of repeating the experiments. Nonlinear regression analysis was applied to define a three-parametric power equation of the form $y = y_0 + ax^b$ to relate the effect of herbicide dose (x) on shoot dry weight (y), where y_0 is an asymptote, a is a constant, and b is the slope of the curve. Equation parameters were computed using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). GR_{50} (dose required to reduce shoot dry weight by 50%) values were calculated by using values of individual parameters, derived from curve fitting in SigmaPlot, in the power equation at 50% reduction in shoot dry weight. Dose response curves were tested for parallelism using PROC GLIMMIX procedure in SAS. Treatment means in cross resistance and metabolic inhibitors experiments were separated using Fisher's protected LSD at $P = 0.05$.

3 RESULTS

3.1 Clethodim resistance screening

Preliminary screening experiments with a 0.5× rate of clethodim indicated that 42 out of 43 Mississippi accessions collected in spring 2016 had at least one surviving plant per accession with survival rate ranging from 11 to 100%. Additional screening of the 42 spring-collected accessions with a 1.0× rate of clethodim produced similar results as before in that majority of plants in all accessions survived (22–100%). A majority of the 43 tested (out of 51) accessions, randomly collected in the summer of 2016, were controlled with clethodim at a 1.0× rate with at least one plant surviving in only a third (14) of the treated groups. About 78–83% of Italian ryegrass plants from North Carolina survived the 1.0× rate of clethodim in the first screening experiment and all progeny of all first-generation survivors were likewise resistant to 1.0× clethodim. These results indicate the Italian ryegrass populations from Mississippi and North Carolina harbor genes endowing resistance to clethodim.

3.2 Clethodim dose response

Response of Italian ryegrass biotypes from Mississippi and North Carolina to clethodim is presented in Figures 2 and 3, respectively. Table 1 represents parameters of the nonlinear regression model used and corresponding confidence intervals. GR_{50} values for the MS24, MS37 and SUS1 biotypes were 0.4, 0.18, and 0.045 kg ha^{-1} of clethodim, respectively. The resistance index calculated from the above GR_{50} values indicated that the MS24 and MS37 biotypes were 10- and 4-fold resistant, respectively, relative to the SUS1 biotype. The NC21 biotype had a GR_{50} value of 1.81 kg ha^{-1} clethodim, pointing to a 40-fold resistance compared to SUS1. GR_{50} values for NC22 and NC 23 biotypes could not be calculated because even at the highest clethodim dose of 2.17 kg ha^{-1}, shoot dry weight reduction was only 30 and 17% of nontreated, respectively, making the chosen nonlinear regression model unsuitable for a meaningful curve fit to the response. Covariance analysis of predicted values of dose response data of biotypes MS24, MS37, and NC21 compared to the SUS1 biotype indicated a *P*-value of 0.8218, 0.5217, and 0.1479, respectively, implying a parallelism in dose response of the above three resistant biotypes with the SUS1 biotype. NC22 and NC23 biotypes were not included in this analysis. These results did not affect the magnitude of resistance.

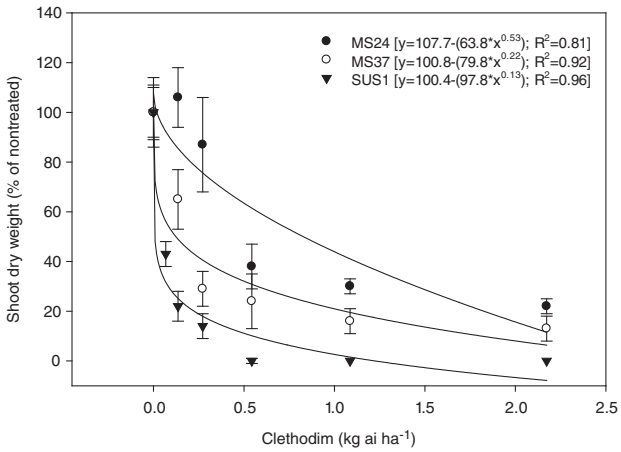

Figure 2. Clethodim dose response on shoot dry weight reduction of clethodim-resistant and -susceptible biotypes from Mississippi 3 weeks after treatment. GR_{50} (dose required to reduce shoot dry weight by 50%) values for MS24, MS37, and SUS1 biotypes were 0.4, 0.18, and 0.045 kg ai ha^{-1} of clethodim, respectively. Vertical bars represent standard error of mean.

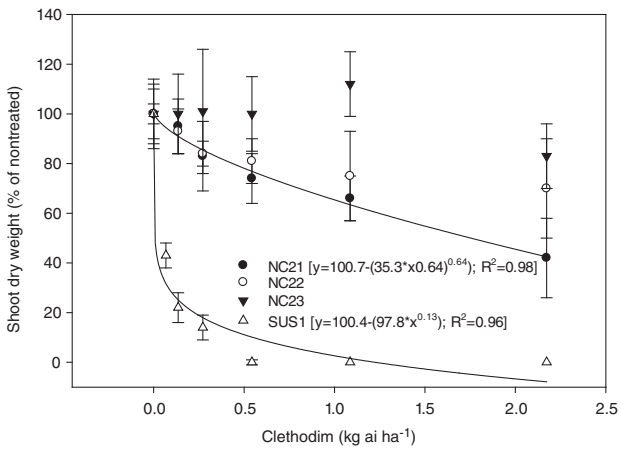

Figure 3. Clethodim dose response on shoot dry weight reduction of clethodim-resistant biotypes from North Carolina and a clethodim-susceptible biotype from Mississippi 3 weeks after treatment. GR_{50} (dose required to reduce shoot dry weight by 50%) values for NC21 and SUS1 biotypes were 1.8 and 0.045 kg ai ha^{-1} of clethodim, respectively. GR_{50} values for biotypes NC22 and NC23 could not be computed due to resistance above 50% of nontreated control at the highest clethodim rate evaluated. Vertical bars represent standard error of mean.

3.3 Cross-resistance

The NC22 biotype was cross-resistant to sethoxydim, fluazifop, quizalofop, and pinoxaden. All eight plants within each herbicide treatment survived the labeled rate with shoot dry weight being 121, 88, 123, and 118% of nontreated control with sethoxydim, fluazifop, quizalofop, and pinoxaden, respectively (data not shown). The SUS1 plants reached 100% mortality within one WAT (data not shown), irrespective of the herbicide. Shoot dry weight averaged 2–4% of nontreated control, across all herbicides (data not shown).

3.4 Metabolism of ACCase inhibitors

Response of the resistant and susceptible biotypes to selected herbicides in the absence and presence of PBO and NBD-Cl, two known metabolic inhibitors (MI) is presented in Table 2.

Table 1. Confidence intervals (95%) of parameters used in a power equation of the form $y = y_0 + ax^b$ to relate the effect of herbicide dose (x) on shoot dry weight (y)[a]

Biotype	y0	a	b
MS24	107.7 ± 66.7	−63.8 ± 82	0.53 ± 0.98
MS37	100.8 ± 40.8	−79.8 ± 46.2	0.09 ± 0.3
NC21	100.4 ± 28.5	−97.8 ± 32.1	0.13 ± 0.12
SUS1	100.7 ± 10.7	−35.3 ± 13.9	0.64 ± 0.34

[a] Confidence intervals were calculated by multiplying standard error of respective parameter with two-sided t value ($\alpha = 0.05$, degrees of freedom = # of observations-#of parameters).

At first glance, it is evident that the inhibitors did not affect how the herbicides inhibited the SUS1 plants. For example, the amount of shoot dry weight 3 WAT was similar across all treatments irrespective of herbicide or MI with reduction ranging from 83 to 92% of nontreated plants. More importantly, all SUS1 plants treated with herbicides alone or in combination with an MI were completely controlled recording 100% mortality.

All three resistant biotypes, in general, survived all herbicide treatments recording some level of inhibition of shoot growth, except MS37 and MS51 with fenoxaprop alone, and NC22 with clethodim alone and pinoxaden in combination with NBD-Cl which resulted in 12, 2, 1, and 27% increase in shoot dry weight compared to nontreated plants, respectively. Remarkably, all resistant plants across the three resistant biotypes survived the herbicide/MI treatments, with plants possessing an active growing point and/or tiller considered as surviving.

3.5 *ACCase* sequencing

ACCase from five plants per biotype of SUS1, Mississippi resistant biotypes MS37 and MS51, and North Carolina resistant biotypes NC22 and NC23 was sequenced and analyzed for amino acid changes at the following seven loci, with known mutations endowing resistance,[26] in the resistant biotypes compared to the SUS1 (wild type/susceptible) biotype: I1781L (isoleucine to leucine), W1999C (tryptophan to cysteine), W2027C (tryptophan to cysteine), I2041N (isoleucine to asparagine), D2078G (aspartate to glycine), C2088R (cysteine to arginine), and G2096A (glycine to alanine) (Table 3).

Two plants of the MS37 biotype did not exhibit any known mutations at any of the seven loci and there were no other amino acid changes in the sequenced *ACCase* region that only appeared in these resistant plants and not in the sensitive plants. Amongst the other three plants, one had the I2041N mutation, the second had the G2096A replacement, and the third had both C2088R and G2096Asubstitutions, mutations in all plants being heterozygous. All five plants of the Mississippi biotype MS51 possessed the C2088R mutation, with two in heterozygous and three in homozygous condition.

All plants of the North Carolina biotypes NC22 and NC23 had the D2078G replacement, with three plants of each biotype expressing heterozygously. In addition to the D2078G mutation, one plant each of NC22 and NC23 showed an additional mutation at the 1781 loci resulting in replacement of isoleucine with leucine. Further, one plant each of NC22 and NC23 displayed a third mutation, I2041N. All double and triple mutations were heterozygous in the NC22 and NC23 biotypes.

4 DISCUSSION

Level of resistance to clethodim in Italian ryegrass from Iredell County, North Carolina (40-fold, and perhaps, greater in NC22 and NC23 biotypes) was higher compared to the Mississippi biotypes (4- to 10-fold). The NC population was managed as a cover crop in a monoculture strawberry operation, where Italian ryegrass plants were exposed to full rates of sethoxydim, an ACCase inhibitor such as clethodim. The initial frequency of resistant individuals could have been less, but the proportion of resistant plants and level of resistance may have rapidly increased in a short period of time due to selection pressure from sethoxydim, small population, and lack of alternative control/suppression tactics. Conversely, the Mississippi biotypes evolved resistance in an agronomic production environment where stress from clethodim may have been delayed or masked by other management operations such as alternative herbicides, tillage, and crop factors, thereby, potential for increased resistance remaining dynamic rather than plateauing.

The NC22 biotype was cross-resistant to at least one ACCase inhibitor from each of the 'dims', 'fops', and 'den' families. Similar cases of Italian ryegrass cross-resistance to ACCase inhibitors were documented in Idaho and California.[27,28] In Idaho, 12% of populations tested had cross-resistance to several ACCase inhibitors including sethoxydim, clethodim, quizalofop, and pinoxaden.[27] Two Italian ryegrass populations from California that had multiple resistance sethoxydim, paraquat and glyphosate, were cross-resistant to fluazifop, fenoxaprop, and cyhalofop, with one population also resistant to clethodim displaying only a 14% reduction in above-ground biomass dry weight.[28] While cross-resistance studies were not conducted on any of the Mississippi resistant biotypes, expected results from treatment with other ACCase inhibitors may be assessed from the nature of mutations discovered from the sequencing studies described below.

Weed species can evolve resistance to herbicides due to metabolism of active ingredients to non-phytotoxic metabolites, catalyzed by enzyme systems such as cytochrome P450s (CYPs) and glutathione S-transferases, (GSTs), and to a lesser extent by glucosyl transferases (GTs) that impart tolerance to herbicides in many agronomic crops.[29,30] Three resistant biotypes, MS37, MS51, and NC22, and susceptible SUS1 biotype were treated with a representative herbicide from the three classes of ACCase inhibitors, fenoxaprop ('fop') clethodim ('dim'), and pinoxaden ('den') alone and in separate combination with PBO (a CYP inhibitor) and NBD-Cl (a GST inhibitor). Based on the results, it is evident that both PBO and NBD-Cl did not inhibit CYPs and GSTs, respectively, thereby, not reducing resistance (or making susceptible) to fenoxaprop, or clethodim, or pinoxaden in any of the three resistant biotypes tested. Therefore, our data indicates lack of involvement of herbicide metabolism in the resistance to ACCase inhibitors in the selected resistant biotypes from Mississippi and North Carolina. It is possible that other CYPs and/or GSTs could be involved that were not inhibited by PBO and NBD-Cl, given the common knowledge that CYPs and GSTs are large super families containing many types of enzymes with unique properties.

The Mississippi resistant biotypes had three amino acid substitutions, I2041N, C2088R, and G2096A, across them. Similarly, the North Carolina resistant biotypes had three mutations, I1781L, I2041N, and D2078G, between them. Some of the mutations discovered in the Mississippi and North Carolina biotypes have been reported previously in Italian ryegrass. Tehranchian et al. (2017) documented presence of an I1781L in two resistant biotypes from California.[28] In an ACCase inhibitor-resistant Italian ryegrass

Table 2. Treatment with metabolic inhibitors

Biotype	Herbicide	Metabolic inhibitor	Dry weight[a]	Mortality
			% of nontreated	%
SUS1	Fenoxaprop	None	15a	100
		PBO	11a	100
		NBD-Cl	16a	100
	Clethodim	None	8a	100
		PBO	12a	100
		NBD-Cl	9a	100
	Pinoxaden	None	10a	100
		PBO	17a	100
		NBD-Cl	12a	100
MS37	Fenoxaprop	None	112c	0
		PBO	64b	0
		NBD-Cl	61b	0
	Clethodim	None	61b	0
		PBO	40a	0
		NBD-Cl	49a	0
	Pinoxaden	None	68b	0
		PBO	51a	0
		NBD-Cl	58b	0
MS51	Fenoxaprop	None	102d	0
		PBO	88c	0
		NBD-Cl	79c	0
	Clethodim	None	46b	0
		PBO	40a	0
		NBD-Cl	35a	0
	Pinoxaden	None	76c	0
		PBO	40a	0
		NBD-Cl	71c	0
NC22	Fenoxaprop	None	83c	0
		PBO	63b	0
		NBD-Cl	63b	0
	Clethodim	None	101d	0
		PBO	44a	0
		NBD-Cl	82	0
	Pinoxaden	None	95d	0
		PBO	52a	0
		NBD-Cl	127e	0

[a] Treatment means within a biotype followed by same letter are not significantly different from each other according to Fisher's protected LSD at $P = 0.05$. Dry weight of nontreated plants is considered 100%.

population from Oregon, two mutations, I2041N and D2078G, were found.[31] A population from the UK, RG3, had a C2088R mutation.[32]

It is common knowledge that nature and location of one or more mutations in the gene encoding for an herbicidal target site determine the cross-resistance profiles of herbicides belonging to different chemical classes but with the same mode of action, as realized with ALS inhibitors,[33] ACCase inhibitors,[26,34,35] and other herbicide modes of action. The I1781L mutation conferred resistance to sethoxydim and cross-resistance to clethodim, fluazifop, fenoxaprop, and cyhalofop in Italian ryegrass from California.[28] Further, Italian ryegrass plants homozygous for mutant alleles at the 1781 loci were resistant to several 'fop' and 'dim' herbicides, and pinoxaden.[34] These findings are consistent with our results in that both the NC22 and NC23 biotypes had the 1781 mutation explaining resistance to clethodim, and in the case of NC22, cross-resistance to fluazifop, quizalofop, sethoxydim, and pinoxaden. NC23 can be expected to have a cross-resistance profile like NC22. The D2078G mutation imparted resistance to 'fop' and 'dim' herbicides in Australian rigid ryegrass populations.[34] The NC22 and NC23 plants, owing to the presence of the D2078G allele in either heterozygous or homozygous form, have been shown or expected, respectively, to have cross-resistance to multiple 'fop' and 'dim' herbicides, and pinoxaden.

The I2041N mutation found in MS37, also in NC22 and NC23 biotypes, was earlier documented in Italian ryegrass from Oregon that was resistant to clodinafop, sethoxydim, clethodim, and pinoxaden.[31] The C2088R mutation discovered in 20% of MS37 and 100% of MS 51 plants was earlier reported to provide resistance to several ACCase herbicides belonging to all three known families in Italian ryegrass from the UK[32] and in rigid ryegrass from Australia.[34] The G2096A mutation, found in MS37, was earlier reported to cause resistance to fops only.[36] While no mutations

Table 3. Amino acid substitutions due to mutations in the carboxyl transferase domain of acetyl-CoA carboxylase gene (*ACCase*) of selected clethodim-resistant Italian ryegrass biotypes from Mississippi and North Carolina

			Mutation loci[a]							
State of origin	Biotype	Plant #	I1781L	W1999C	W2027C	I2041N	I2041N	D2078G	C2088R	G2096A
Mississippi	SUS1	1	I	W	W	I	I	D	C	G
		2	I	W	W	I	I	D	C	G
		3	I	W	W	I	I	D	C	G
		4	I	W	W	I	I	D	C	G
		5	I	W	W	I	I	D	C	G
Mississippi	MS37	1	I	W	W	I	I	D	C	G
		2	I	W	W	I/N	I/N	D	C	G
		3	I	W	W	I	I	D	C	G/A
		4	I	W	W	I	I	D	C	G
		5	I	W	W	I	I	D	C/R	G/A
Mississippi	MS51	1	I	W	W	I	I	D	C/R	G
		2	I	W	W	I	I	D	R	G
		3	I	W	W	I	I	D	R	G
		4	I	W	W	I	I	D	C/R	G
		5	I	W	W	I	I	D	R	G
North Carolina	NC22	1	I	W	W	I	I	D/G	C	G
		2	I/L	W	W	I/N	I/N	D/G	C	G
		3	I	W	W	I	I	G	C	G
		4	I/L	W	W	I	I	D/G	C	G
		5	I	W	W	I	I	G	C	G
North Carolina	NC23	1	I	W	W	I	I	D/G	C	G
		2	I/L	W	W	I	I	D/G	C	G
		3	I/L	W	W	I/N	I/N	D/G	C	G
		4	I	W	W	I	I	G	C	G
		5	I	W	W	I	I	G	C	G

[a] I1781L (isoleucine to leucine), W1999C (tryptophan to cysteine), W2027C (tryptophan to cysteine), I2041N (isoleucine to asparagine), D2078G (aspartate to glycine), C2088R (cysteine to arginine), and G2096A (glycine to alanine).

were detected at the 1999 and 2027 loci in this research, a novel W1999S was reported in a Italian ryegrass population, UK21, from the UK.[37]

In summary, Italian ryegrass resistance to clethodim in populations from Mississippi and North Carolina has been documented in this research, with confirmed or potential for broad cross-resistance across several ACCase inhibitors. A more diversified integrated management program is warranted against the North Carolina Italian ryegrass population. The rest of this section discusses aspects and management of the ACCase inhibitor-resistant Italian ryegrass from Mississippi.

Glyphosate resistant Italian ryegrass populations in agronomic crop production systems were first reported in 2005 from Washington County.[21] Ever since, glyphosate resistance in Italian populations has spread to surrounding counties in Mississippi, adjoining states (Arkansas, Louisiana, Tennessee), and the southeastern US (North Carolina).[20] Several of these populations could be considered resistant to acetolactate synthase inhibitors (ALS) and/or ACCase inhibitors (diclofop) due to prior exposure to herbicides labeled for wheat (*Triticum aestivum* L.).

With glyphosate, ALS inhibitors, and now clethodim becoming ineffective, the only post emergence control option left for managing Italian ryegrass in Mississippi is paraquat. However, paraquat must be applied to Italian ryegrass very early in the spring (February–March) when plants are small and root systems are not very well established. Glufosinate is ineffective on Italian ryegrass due to prevailing low temperatures in the spring.

Italian ryegrass germinates and emerges in the fall and following spring in the Mississippi Delta. It is the fall-emerged plants that prove to be more combative than the spring flush. Therefore, fall-applied residual herbicides that include two or more unique herbicide modes of action are an effective tool despite added input costs. POST only applications in spring on fall-emerged populations that have overwintered is not a suitable option, especially, since the Mississippi Delta receives most of its annual rainfall, approximately 70%, in the winter months of November to February. A combination of conditions, such as absence of a fall-applied residual herbicide and prolonged adverse weather conditions preventing early spring application of paraquat, can be detrimental to growers due to proven competitiveness of Italian ryegrass against spring planted row crops such as corn.[19] Thus, it is prudent to implement management programs against Italian ryegrass in the fall under the premise that all populations are resistant to clethodim.

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