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Physiological and Molecular Basis of Acetolactate Synthase-Inhibiting Herbicide Resistance in Barnyardgrass (*Echinochloa crus-galli*)

Dilpreet S. Riar,*[,†] Jason K. Norsworthy,[†] Vibha Srivastava,[†] Vijay Nandula,[§] Jason A. Bond,[#] and Robert C. Scott[⊥]

[†]Department of Crop, Soil, and Environmental Sciences, University of Arkansas, 1366 West Altheimer Drive, Fayetteville, Arkansas 72704, United States

[§]Crop Production Systems Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 141 Experiment Station Road, P.O. Box 350, Stoneville, Mississippi 38776, United States

[#]Delta Research and Extension Center, Mississippi State University, 82 Stoneville Road, Stoneville, Mississippi 38776, United States

[⊥]Department of Crop, Soil, and Environmental Sciences—Extension, University of Arkansas, P.O. Box 357, Lonoke, Arkansas 72086, United States

ABSTRACT: Barnyardgrass biotypes from Arkansas (AR1 and AR2) and Mississippi (MS1) have evolved cross-resistance to imazamox, imazethapyr, and penoxsulam. Additionally, AR1 and MS1 have evolved cross-resistance to bispyribac-sodium. Studies were conducted to determine if resistance to acetolactate synthase (ALS)-inhibiting herbicides in these biotypes is target-site or non-target-site based. Sequencing and analysis of a 1701 base pair ALS coding sequence revealed Ala_{122} to Val and Ala_{122} to Thr substitutions in AR1 and AR2, respectively. The imazamox concentrations required for 50% inhibition of ALS enzyme activity in vitro of AR1 and AR2 were 2.0 and 5.8 times, respectively, greater than the susceptible biotype. Absorption of ^{14}C-bispyribac-sodium, -imazamox, and -penoxsulam was similar in all biotypes. ^{14}C-Penoxsulam translocation out of the treated leaf (≤2%) was similar among all biotypes. ^{14}C-Bispyribac-treated AR1 and MS1 translocated 31− 43% less radioactivity to aboveground tissue below the treated leaf compared to the susceptible biotype. ^{14}C-Imazamox-treated AR1 plants translocated 39% less radioactivity above the treated leaf and aboveground tissue below the treated leaf, and MS1 translocated 54 and 18% less radioactivity to aboveground tissue above and below the treated leaf, respectively, compared to the susceptible biotype. Phosphorimaging results further corroborated the above results. This study shows that altered target site is a mechanism of resistance to imazamox in AR2 and probably in AR1. Additionally, reduced translocation, which may be a result of metabolism, could contribute to imazamox and bispyribac-sodium resistance in AR1 and MS1.

KEYWORDS: *absorption, herbicide resistance mechanism, noxious weed, radioactivity, translocation*

INTRODUCTION

The use of herbicides that inhibit acetolactate synthase (ALS) (EC 4.1.3.18) has increased tremendously since the commercialization of chlorsulfuron in 1982 because of low field application rates, residual soil activity, and broad-spectrum weed control.[1,2] So far, five structurally distinct chemical classes of ALS-inhibiting herbicides have been commercialized: imidazolinones (IMI), pyrimidinylthiobenzoates (PTB), sulfonylureas (SU), sulfonylamino-carbonyl-triazolinones (SCT), and triazolopyrimidines (TP). These herbicides inhibit the ALS enzyme that catalyzes the biosynthesis of branched-chain amino acids valine, leucine, and isoleucine.[3] Utilization of ALS-inhibiting herbicides increased further after the introduction of IMI-resistant (Clearfield) corn (*Zea mays* L.), rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.).

In the Midsouth United States, the area under IMI-resistant rice has increased to 64% by 2011 since its introduction in 2002 with 11% of the fields not rotated from IMI-resistant rice over the past 5 years.[4] This increase was primarily for two reasons. First, control of red rice (*O. sativa* L.), before evolution of ALS inhibitor-resistant red rice biotypes, was easy with sequential applications of imazethapyr (IMI herbicide) on IMI-resistant rice. Second, barnyardgrass, the most important weed of dry-seeded rice in the Midsouth United States, has evolved resistance to the most commonly used herbicides of rice, including propanil,[5] quinclorac,[6] and clomazone [7] in Arkansas; and to propanil and quinclorac in Mississippi.[8] Propanil-, quinclorac-, and clomazone-resistant barnyardgrass led growers to shift to IMI-resistant rice or to include bispyribac-sodium (PTB herbicide) and penoxsulam (TP herbicide) in herbicide programs for conventional rice.[9]

Unfortunately, the chance of evolution of resistance to ALS-inhibiting herbicides in weeds is very high. Just 5 years after the introduction of the first SU herbicide, prickly lettuce (*Lactuca serriola* L.) in Idaho[10] and kochia [*Kochia scoparia* (L.) Shrad] in Kansas[11] evolved resistance to ALS-inhibiting herbicides. To date, 126 weed species worldwide have been reported to be resistant to herbicides from at least one of the five chemical

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families of ALS-inhibiting herbicides.[12] After extensive use of ALS-inhibiting herbicides, resistance also evolved in rice weeds in the Midsouth United States. Two populations from northeastern Arkansas (AR1 and AR2 from Greene and Prairie Counties, respectively) and one from Mississippi (MS1 from Sunflower County) with various levels of cross-resistance to ALS-inhibiting herbicides have been found in rice fields.[13] Dose response studies revealed that AR1, AR2, and MS1 were >94, >94, and 3.3 times, respectively, more resistant to imazamox and >94, 30, and 9.4 times, respectively, more resistant to penoxsulam compared to a known susceptible biotype.[13] Additionally, AR1 and MS1 were 15 and 7.2 times more resistant to bispyribac-sodium compared to a susceptible biotype, whereas AR2 was susceptible to bispyribac-sodium.

Several studies have been conducted to determine if reduced absorption and translocation are the mechanism of resistance in ALS inhibitor-resistant weed species. Reduced absorption of ALS-inhibiting herbicides was reported as the partial mechanism of resistance in common sunflower (*Helianthus annuus* L.)[14] and shattercane [*Sorghum bicolor* (L.) Moench].[15] However, differential translocation has not been reported as the mechanism of resistance in any ALS inhibitor-resistant weed species.[14,16−21] Enhanced metabolism by cytochrome P450 monooxygenases (CYP) and an altered ALS gene are the primary mechanisms of resistance in ALS inhibitor-resistant weed species.[22] Herbicide metabolism catalyzed by CYP has been shown to be inhibited in the presence of organophosphate insecticides such as malathion.[23,24] In our previous studies, addition of malathion to penoxsulam reduced dry weight up to 96% and increased mortality up to 90% of penoxsulam-resistant barnyardgrass biotypes (AR2 and MS1) compared to penoxsulam applied alone.[13]

Target-site-based resistance, the most common resistance mechanism in ALS inhibitor-resistant weed species, is caused by amino acid substitutions in the conserved region of the ALS enzyme. Eight naturally occurring amino acid substitutions in the ALS gene of resistant weeds are Ala_{122} to Thr; Pro_{197} to several amino acids; Ala_{205} to Val; Asp_{376} to Glu; Arg_{377} to His; Trp_{574} to Arg, Leu, and Gly; Ser_{653} to Thr, Asn, and Ile; and Gly_{654} to Asp and Glu.[25,26] Recently, two new substitutions, Ala_{122} to Tyr and Ala_{122} to Val, have been reported in wild radish (*Raphanus raphanistrum* L.)[27] and silky windgrass [*Apera spica-venti* (L.) Beauv.],[28] respectively. Three other substitutions, Ser_{186} to Pro, Lys_{416} to Glu, and Leu_{662} to Pro, were reported in ALS inhibitor-resistant red rice hybrids.[29]

Substitution of Ala_{122} to Thr causes resistance to IMI herbicides only (not determined for SCT herbicides yet), whereas substitution from Ala_{122} to Tyr imparts a high level of resistance to IMI, SU, and TP herbicides (not determined for PTB and SCT herbicides)[26,27] and Ala_{122} to Val imparts partial resistance to some SU herbicides (not determined for IMI, PTB, and TP herbicides).[28] Substitutions at Ala_{205}, Pro_{197}, Asp_{376}, Arg_{377}, and Ser_{653} codons can result in cross-resistance to one or more IMI, PTB, SCT, SU, and TP herbicides.[22,26] Amino acid substitution at Gly_{654} caused cross-resistance to SU, IMI, and SCT herbicides in green foxtail [*Setaria viridis* (L.) Beauv.].[30]

Studies were conducted with the objective of determining if reduced absorption and translocation or an altered ALS gene is the mechanism of resistance in barnyardgrass biotypes resistant to ALS-inhibiting herbicides in Arkansas and Mississippi.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Single representative plants of ALS inhibitor-resistant (AR1 and AR2 from Arkansas and MS1 from Mississippi) and -susceptible barnyardgrass populations[13] were selfed separately for one more generation to increase homozygosity of seeds. Seeds of resistant and susceptible biotypes were sown in individual 55.5 × 26.5 × 5.5-cm^3 greenhouse flats containing commercial potting media (Professional growing mix, LC1Mix, Sun Gro Horticulture Distribution Inc., Bellevue, WA, USA). Plants were maintained in the greenhouse under conditions of 30/20 ± 3 °C day/night temperature and 16 h photoperiod.

^{14}C-Herbicide Solution Preparation. Stock solutions of technical grade ^{14}C-labeled bispyribac-sodium (specific activity = 3.1 MBq mg^{-1}; Valent U.S.A. Corp., Walnut Creek, CA, USA), ^{14}C-labeled imazamox (specific activity = 6.65 MBq mg^{-1}; BASF Agricultural Center Limburgerhof, Germany), and ^{14}C-labeled penoxsulam (specific activity = 2.24 MBq mg^{-1}; Dow Agrosciences LLC, Indianapolis, IN, USA) were prepared by dissolving them in a 500 μL acetonitrile and water (2:1 by vol) solution, 300 μL of water, and 200 μL of acetone and water (95:5 by vol) solution, respectively. Nonradiolabeled, formulated bispyribac-sodium (Regiment, 22.5 g ai ha^{-1}, Valent U.S.A. Corp.), imazamox (Beyond, 35 g ai ha^{-1}, BASF Corp., Research Triangle Park, NC, USA), and penoxsulam (Grasp SC, 35 g ai ha^{-1}, Dow AgroSciences LLC) solutions were spiked with aliquots from respective stock solutions to make ^{14}C-labeled bispyribac-sodium, imazamox, and penoxsulam working solutions with specific activities of 1.67, 1.67, and 2.2 kBq μL^{-1}, respectively. A spray adjuvant and deposition aid (Dyne-A-Pak, Helena Chemical Co., Collierville, TN, USA) at 2.5% v/v was added to bispyribac-sodium working solution, and a nonionic surfactant (NIS) (Induce, Helena Chemical Co.) at 0.25% v/v was added to imazamox and penoxsulam working solutions.

Absorption and Translocation of ^{14}C-Bispyribac-sodium, -Imazamox, and -Penoxsulam. Plants of all biotypes at the 1−2-leaf stage were transplanted to individual 15 cm diameter plastic pots filled with potting mediaum. Two days before treatment, plants were moved to a growth chamber with 30/20 ± 3 °C day/night temperature, 16 h photoperiod, and 500 μmol m^{-2} s^{-1} photon flux density. Plants of all four biotypes at the4−5-leaf stage were treated with nonradiolabeled bispyribac-sodium, imazamox, and penoxsulam at 22.5, 35, and 35 g ha^{-1}, respectively. Adjuvants were added to the herbicide solutions based on label recommendations. Herbicide applications were made using an automated sprayer chamber with a boom containing two flat-fan 80067 nozzles calibrated to deliver 192 L ha^{-1}. After application of nonradiolabeled bispyribac-sodium, imazamox, or penoxsulam, the middle marked portion of the second youngest fully opened leaf of each plant was treated on the adaxial side with 1 μL of working solution of ^{14}C-bispyribac-sodium, -imazamox, and -penoxsulam, respectively. A 25 μL microsyringe (Microliter syringe, Hamilton Co., Reno, NV, USA) equipped with a repeating dispenser to deliver 0.25 μL droplets was used to apply ^{14}C-bispyribac-sodium, -imazamox, or -penoxsulam at 1.67, 1.67, or 2.2 kBq $plant^{-1}$, respectively. A nontreated control for each radioactive herbicide−biotype combination was included.

Plants were harvested at 16 and 96 h after treatment (HAT) and sectioned into four parts including treated leaf, tissue above treated leaf, aboveground tissue below treated leaf, and roots. The marked portion of the treated leaf of each plant was rinsed for 15 s in 1 mL of methanol and water (1:1 by vol) solution containing NIS at 0.25% v/v to remove nonabsorbed radioactivity. The rinsate with nonabsorbed radioactivity from each plant was mixed individually with 10 mL of scintillation cocktail (Ultima Gold, PerkinElmer Inc., Waltham, MA, USA) and radioassayed using a liquid scintillation spectrometer (LSS) (Packard Tri-Carb 2100TR Liquid Scintillation Spectrometer, Packard Instrument Co., Downers Grove, IL, USA). Absorption of ^{14}C-herbicides by plants was calculated by subtracting the amount of radioactivity in leaf washes from the total applied. Absorption data were expressed as percentage of applied ^{14}C-bispyribac-sodium, -imazamox, or -penoxsulam. All plant parts were dried at 50 °C for

Table 1. Primers Used for Amplification and Genotyping of ALS Gene of Barnyardgrass

primer	sequence (5′−3′)	amplicon size (bp)	annealing temp (°C)
BYG_F12	GCAAGGGCGCCGACATCCT	874	66
BYG_R12	CCTGCTTGCAAAAGCCTCAAT		60
BYG_F22	ATTGAGGCTTTTGCAAGCAGG	864	60
BYG_R22	ATACACAGTCCTGCCATCACC		60
BYG_F31	AAGGACATCCAGCAGCAGAT	756	60
BYG_R31	TGAAGACAACCACTGCCTTG		60

48 h and oxidized individually in a biological oxidizer (OX500, R. J. Harvey Instrument Corp., Tappan, NY, USA) to determine the amount of radioactivity transported to different plant parts. Evolved CO_2 was trapped in a scintillation vial containing 15 mL of scintillation cocktail (Carbon-14 Cocktail, R. J. Harvey Instrument Corp.) and radioassayed using LSS. Translocation data were expressed as percentage of recovered radioactivity.

The experiment was arranged as a randomized complete block design with a three (herbicides: ^{14}C-bispyribac-sodium, -imazamox, and −penoxsulam) by two (harvest intervals: 16 and 96 HAT) by four (biotypes: AR1, AR2, MS1, and susceptible) factorial arrangement of treatments. All treatments were replicated four times, and the experiment was repeated. Percentage absorption and translocation data were tested for normality using PROC UNIVARIATE in SAS, version 9.1.3 (SAS Institute Inc., Cary, NC, USA), and data were subjected to arcsine square root transformation before analysis. Transformed data were subjected to ANOVA using PROC MIXED in SAS. There were herbicide−harvest interval−biotype interactions; thus, percentage absorption and translocation data for each herbicide are presented separately. Experiments were considered to be random, and percentage absorption and translocation data for two experiments were pooled for each herbicide because of nonsignificant experiment−harvest interval, experiment−biotype, or experiment−harvest interval−biotype interactions. Means were separated using Fisher's Protected LSD at $\alpha = 0.05$.

Autoradiography. The marked portion of the second youngest fully expanded leaf of ALS inhibitor-resistant (AR1, AR2, and MS1) and -susceptible barnyardgrass plants was treated with ^{14}C-bispyribac-soidum, -imazamox, or -penoxsulam at 5.0, 5.0, or 6.6 kBq plant^{-1}, respectively, as described for the absorption and translocation experiment. Entire plants were harvested at 96 HAT. Soil was removed from the roots by washing with distilled water with minimal root loss. Nonabsorbed radioactivity was removed by rinsing the treated leaf with 10 mL of methanol and water (1:1 by vol) solution containing NIS at 0.25% v/v. Plants were immediately mounted on a 22 × 28 cm^2 sheet of plain copier/printer paper, spread carefully to avoid the treated leaf contacting other plant parts, covered with another sheet of paper, pressed in wooden press boards, and stored at −20 C. After 1 week, plants were taken out of the press, wrapped in plastic film, and stored again at −20 °C. Wrapped plants were exposed to a phosphorimaging plate for 24 h and then scanned using a Storm 820 PhophorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Nontreated plants were used as control.

ALS Gene Sequencing. *Genomic DNA Extraction and Quantification.* DNA was extracted from the leaf tissue of ALS inhibitor-resistant and -susceptible barnyardgrass biotypes using a modified cetyl trimethyl ammonium bromide (CTAB) method.[31] The genomic DNA of each biotype was dissolved in 100 μL of autoclaved water and stored at −20 °C until used. The DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies Inc., Wilmington, DE, USA) and was diluted after quantification with autoclaved water to 25 ng L^{-1} concentration.

Primer Synthesis. Primers for barnyardgrass ALS gene sequencing were not available. To develop primers for barnyardgrass ALS gene sequencing, first the nucleotide sequences of the ALS coding region of Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], corn, rice, and wheat with GenBank (National Center for Biotechnology Information, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/) accession numbers AF310684, X63553, AY885675, and AY210408, respectively, were aligned using ClustalW2 multiple-sequence alignment program (available online at http://www.ebi.ac.uk/Tools/msa/clustalw2/). After sequence alignment, forward and reverse primers intended to amplify the partial coding sequence of barnyardgrass ALS were developed from the conserved regions of Italian ryegrass, corn, rice, and wheat ALS coding sequences. Primers were designed using Oligo Calculator, version 3.26, software (available online at http://www.basic.northwestern.edu/biotools/OligoCalc.html) and were synthesized by Eurofins MWG Operon (Eurofins MWG Operon, Huntsville, AL, USA). Of the several primers that were synthesized and tested, functional primers used for sequencing barnyardgrass ALS coding sequence are listed in Table 1.

DNA Amplification. Three primer pairs were used to amplify the ALS coding sequence from genomic DNA of four barnyardgrass biotypes (Table 1 and Figure 1). Polymerase chain reaction (PCR)

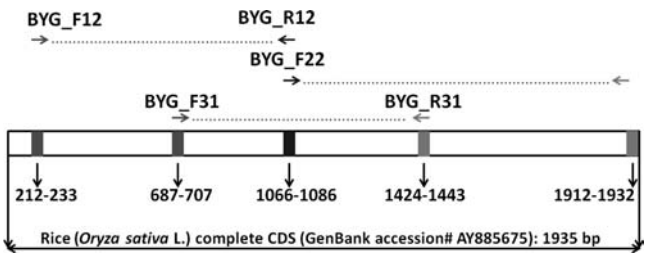

Figure 1. Putative locations [using rice ALS coding sequence (CDS) as reference] of three primer pairs used to amplify and sequence ALS gene from genomic DNA of ALS inhibitor-resistant and -susceptible barnyardgrass biotypes.

amplification for each primer set was conducted in a 50 μL mixture of 10 μL of 5× PCR buffer, 5 μL of 2 mM dNTPs, 3 μL of 25 mM $MgCl_2$, 2 μL each of 10 μM forward and reverse primers, 2 μL of 25 ng μL^{-1} DNA, 1 μL of Go Taq Flexi DNA Polymerase (Promega Corp., Madison, WI, USA), and 25 μL of autoclaved water using a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The conditions for the PCR reaction were as follows: initial denaturation at 95 °C for 5 min; followed by 40 cycles of 94 °C for 1 min, 64 °C (for BYG_F12 and BYG_R12 primer pair), or 60 °C (for the other two primer pairs) for 1 min, and 72 °C for 1 min; followed by final extension cycle at 72 °C for 5 min. The PCR amplification products were resolved on a Agarose gel with 0.3× solution of GelRed Nucleic Acid Gel Stain (0.8%, w/v) (VWR International, LLC, Radnor, PA, USA), using a 1000 base pair (bp) DNA ladder (Promega Corp.) as reference.

DNA Sequencing and Sequence Analysis. The amplified fragments of all reactions were purified from gel with a GENECLEAN Spin kit (MP Biomedicals, LLC, Solon, OH, USA) following the manufacturer's instructions. The purified PCR products were used directly for sequencing at the University of Arkansas DNA Sequencing Core Facility. Both strands of DNA amplicon were sequenced using the respective reverse and forward primers to confirm accuracy. Chromatograms for each sequence were analyzed using Applied

Biosystems Sequence Scanner, version 1.0 (Life Technologies Corp., Carlsbad, CA, USA) and FinchTV, version 1.4.0 (Geospiza Inc., Seattle, WA, USA) software. For each barnyardgrass biotype, top strand nucleotide sequences (corrected and manipulated on the basis of the complementary bottom strand sequences) were arranged in a unified contig by aligning sequences using the BLASTN algorithm (National Center for Biotechnology Information, Bethesda, MD, USA;http://www.ncbi.nlm.nih.gov/). The redundant overlapping segments of sequences were removed manually to re-enact the continuous ALS coding region sequence. The single continuous sequences thus obtained were compared with the GenBank database for nonredundant and reference protein sequences to validate similarity of sequences with previously characterized ALS genes and to obtain respective ALS amino acid sequences for each barnyardgrass biotype. ALS nucleotide and amino acid sequences of all barnyardgrass biotypes were aligned using ClustalW2 software and were compared to *Arabidopsis thaliana* ALS sequence available at GenBank (accession number NM114714).

ALS Enzyme Assay. ALS inhibitor-resistant and -susceptible barnyardgrass plants were grown as before. ALS enzyme activity from 2−3-leaf plants was assayed in vitro using procedures similar to previous descriptions.[1,32] Herbicide concentrations used were 0, 0.1, 1, 10, 100, and 1000 μM for imazamox (BASF) and penoxsulam (Dow AgroSciences) and 0, 0.1, 1, 10, and 100 μM for bispyribac-sodium (Valent U.S.A. Corp.). This assay measured acetoin that was formed from acid decarboxylation of acetolactate. Background acetoin sources were included as controls. There were three replications per treatment, and each experiment was conducted three times.

All data were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.2, SAS Institute Inc.). No significant experiment effect was observed in repeated experiments; therefore, data from experiments were pooled. Nonlinear regression analysis was applied to define a four-parametric log−logistic curve of the form

$$y = c + (d - c)/(1 + (x/x_0) \wedge (-b)) \quad (1)$$

to relate the effect of herbicide concentration (x) on ALS activity (y), where c and d are mean responses at lowest and highest herbicide concentrations, respectively, x_0 is the herbicide concentration required for 50% inhibition of enzyme activity (I_{50}), and b is the slope of the curve around x_0. Equation parameters were computed using SigmaPlot (version 11.0, Systat Software Inc., San Jose, CA, USA). Herbicide concentration values, required to inhibit ALS activity by 50%, were separated using Fisher's protected LSD test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Absorption and Translocation of ^{14}C-Bispyribac-sodium, -Imazamox, and -Penoxsulam. Recovery of applied radioactivity was >90% across harvest intervals and biotypes. Percentage absorption data for each herbicide were pooled over harvest intervals because there was no harvest interval−biotype interaction. The absorption of ^{14}C-bispyribac-sodium (94−96%), -imazamox (56−65%), and -penoxsulam (63−74%) across experiments and harvest intervals (16 and 96 HAT) was similar among all barnyardgrass biotypes (data not shown). A similar level of absorption was reported for ^{14}C-bispyribac-sodium plus spray adjuvant and deposition aid in barnyardgrass;[33] ^{14}C-penoxsulam in alligatorweed [*Alternanthera philoxeroides* (Mart.) Griseb.];[34] and ^{14}C-imazamox plus NIS in feral rye (*Secale cereale* L.).[35]

Data for percentage translocation of ^{14}C-imazamox are presented by harvest intervals because of significant harvest interval−biotype interactions (Table 2). All biotypes retained 75−80% radioactivity in the treated leaf, and no difference among biotypes was observed for ^{14}C translocation at 16 HAT. Total translocation out of the treated leaf at 96 HAT in AR1 and MS1, respectively, was approximately 16% (36.3% translocated) and 20% (34.6% translocated), less than translocation of the susceptible biotype (43.4%). AR1 translocated 39% less (20.1%) radioactivity to aboveground tissue below the treated leaf compared to the susceptible biotype (28.3%). Similarly, MS1 translocated 54% (3.2%) and 18% (23.3%) less radioactivity to aboveground tissue above and below the treated leaf, respectively, compared to aboveground tissue above (6.9%) and below (28.3%) the treated leaf of the susceptible biotype.

Table 2. Translocation of ^{14}C-Imazamox (Pooled over Experiments) in Different Plant Parts of ALS Inhibitor-Resistant (AR1, AR2, and MS1) and -Susceptible (SUS) Barnyardgrass Biotypes at 16 and 96 h after Treatment[a]

		% of recovered ^{14}C			
HI	biotype	TL	ATL	BTL	roots
16 h	AR1	78.2 a	3.93 c	14.4 cd	3.49 c
	AR2	79.7 a	3.15 c	12.9 d	4.23 c
	MS1	78.5 a	2.30 c	15.1 cd	4.09 c
	SUS	75.2 a	3.37 c	16.1 cd	5.30 bc
96 h	AR1	63.7 bc	8.04 b	20.1 bc	8.17 ab
	AR2	60.4 bcd	12.3 a	16.9 c	10.4 a
	MS1	65.4 b	3.23 c	23.2 b	8.12 ab
	SUS	56.6 d	6.86 b	28.3 a	8.26 ab

[a]Abbreviations: ATL, tissue above treated leaf; BTL, aboveground tissue below treated leaf; HI, harvest interval; TL, treated leaf. All treatments contained nonionic surfactant at 0.25% v/v. Means within a column followed by the same letters are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$).

For ^{14}C-bispyribac-sodium and -penoxsulam translocation, the harvest interval−biotype interaction was not significant; thus, data for 16 and 96 HAT were pooled. ^{14}C-Bispyribac-sodium-treated plants of all biotypes retained ≥86% radioactivity in the treated leaf; however, total ^{14}C translocation out of the treated leaf was 34−46% less in AR1 (7.3% translocated) and MS1 (8.3% translocated) compared to AR2 (13.6%) and susceptible (12.6%) biotypes (Table 3). Additionally, translocation of radioactivity was 30−43% less in aboveground tissue below treated leaf of AR1 (3.9% translocated) and MS1 (4.7% translocated) compared to AR2 (6.7% translocated) and the susceptible (6.8%) biotype. ^{14}C-Penoxsulam-treated plants of all biotypes retained >98% radioactivity in the treated leaf, and translocation of radioactivity to different plant parts did not differ across harvest intervals and biotypes (Table 3).

Bispyribac-sodium, imazamox, and penoxsulam movement in plants is via the phloem and xylem.[36,37] However, the solubility of penoxsulam in water is only 0.41 g L^{-1} compared to 73.3 and 626 g L^{-1} of bispyribac-sodium and imazamox,[37,38] respectively, at pH 7. The solubility of penoxsulam in water is pH dependent and decreases to 0.0057 g L^{-1} at pH 5. The pH of cytosol and apoplast in general is 7.5 and 5.5, respectively.[39] Therefore, the low solubility of penoxsulam in water might be the reason for reduced translocation (≤2%) of penoxsulam out of the treated leaf in all biotypes (Figure 2 and Table 3). Interestingly, total translocation out of the treated leaf at 96 HAT for bispyribac-sodium (7−14%) and imazamox (35−43%) also correlated with the solubility of these herbicides in water (Tables 2 and 3).

Translocation at 96 HAT was also assessed qualitatively by scanning phosphorimaging plates exposed for 24 h to plants individually treated with ^{14}C-bispyribac-sodium, -imazamox, or -penoxsulam (Figure 2). Autoradiography results supported the

Table 3. Translocation of ^{14}C-Bispyribac-sodium, -Imazamox, and -Penoxsulam (Pooled over Harvest Intervals and Experiments) in Different Plant Parts of ALS Inhibitor-Resistant (AR1, AR2, and MS1) and -Susceptible (SUS) Barnyardgrass Biotypes[a]

	% of recovered ^{14}C							
	^{14}C-bispyribac-sodium[b]				^{14}C-penoxsulam[c]			
biotype	TL	ATL	BTL	roots	TL	ATL	BTL	roots
AR1	92.7 a	2.38 a	3.86 b	1.06 a	98.8 a	0.61 a	0.40 a	0.19 a
AR2	86.4 b	5.48 a	6.74 a	1.40 a	98.4 a	0.68 a	0.65 a	0.23 a
MS1	91.7 a	2.35 a	4.73 b	1.17 a	99.2 a	0.35 a	0.35 a	0.12 a
SUS	87.4 b	4.35 a	6.83 a	1.38 a	98.1 a	0.81 a	0.91 a	0.20 a

[a]Abbreviations: ATL, tissue above treated leaf; BTL, aboveground tissue below treated leaf; TL, treated leaf. Means within a column followed by the same letters are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$). [b] ^{14}C-Bispyribac-sodium treatments contained a spray adjuvant and deposition aid at 2.5% v/v. [c] ^{14}C-Penoxsulam treatments contained nonionic surfactant at 0.25% v/v.

findings of the translocation study and showed less ^{14}C-bispyribac-sodium and -imazamox translocation out of the treated leaf in AR1 and MS1 compared to AR2 and susceptible biotypes. In general, radioactivity was more evenly distributed across whole plants of AR2 and susceptible biotypes compared to AR1 and MS1 biotypes; however, reduced translocation to tissue above and below the treated leaf was more clearly visible for ^{14}C-bispyribac-sodium in AR1 and ^{14}C-imazamox in MS1. Furthermore, as observed in the translocation study, most of the applied ^{14}C-penoxsulam remained in the treated leaf with minimal movement out of the treated leaf in all biotypes.

On the basis of the results of the ^{14}C translocation and autoradiography studies, reduced translocation appears to be partly responsible for bispyribac-sodium and imazamox resistance in AR1 and MS1, but not for penoxsulam resistance in all biotypes and for bispyribac-sodium and imazamox resistance in AR2. To date, reduced translocation has not been documented as a mechanism of resistance to ALS-inhibiting herbicides in any weed species. It is very likely that a part or a significant portion of the translocated radioactivity in the AR1 and MS2 biotypes could be metabolites of the said parent herbicide. In fact, metabolism, especially by CYP, has been implicated[40−45] as one of the two major mechanisms of resistance to ALS-inhibiting herbicides, the other being altered target site. Our recent studies with bispyribac-sodium, imazethapyr, and penoxsulam in mixture with malathion, a known inhibitor of CYP, have indicated that increased herbicide metabolism by CYP is partially involved in the mechanism of resistance to penoxsulam in all resistant biotypes and probably to bispyribac-sodium and imazethapyr in AR1 and MS1, respectively.[13] Addition of malathion to penoxsulam reduced dry weight (40−96%) of all biotypes and increased mortality (up to 90%) of AR2 and MS1 biotypes compared to penoxsulam applied alone. Addition of malathion to imazethapyr and bispyribac-sodium increased the mortality of MS1 and AR1 biotypes, respectively, compared to treatments with imazethapyr and bispyribac-sodium applied alone.[13] Thus, pending confirmation, or lack thereof, of herbicide metabolism, we cautiously speculate that differential herbicide translocation may have a role in explaining resistance to bispyribac and imazamox in AR1 and MS1.

ALS Gene Sequencing. Sequences covering a 1701 bp coding region of the ALS gene were sequenced from the PCR amplified DNA fragments of each ALS inhibitor-resistant (AR1, AR2, and MS1) and -susceptible barnyardgrass biotype (Figure 3). These sequences have been submitted to GenBank as accession numbers JX415268 for AR1, JX415269 for AR2, JX415270 for MS1, and JX415271 for the susceptible biotype. The partial ALS gene sequences of these four barnyardgrass biotypes spanned over all five highly conserved domains of the ALS gene and contained eight of the most common sites of ALS gene mutation that cause ALS inhibitor resistance in plants: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp574, Ser_{653}, and Gly_{654}. There were no introns in the sequenced region of the ALS gene of all four biotypes. Alignment of ALS sequences of four barnyardgrass biotypes showed 99% similarity among sequences. However, single nucleotide mutations from GCC to GTC and from GCC to ACC resulted in Ala_{122} to Val substitution in AR1 and Ala_{122} to Thr substitution in AR2, respectively (Figures 3 and 4). The sequenced region of the ALS gene of MS1 was similar to that of the susceptible biotype, and no amino acid substitutions were detected.

Analysis of barnyardgrass ALS sequence chromatograms revealed that along with the GTC (for Val) and ACC (for Thr) codon in AR1 and AR2, respectively, the GCC codon for Ala_{122} was also present in both of these biotypes (Figure 4). All AR1 and AR2 plants showed a high level of resistance (>94 times) compared to the susceptible biotype to imazamox application in previous studies,[13] ruling out the possibility of heterozygosity in the population. Barnyardgrass is a hexaploid species with $2n = 6X = 54$.[46,47] Hexaploid species usually have multiple copies of most genes. Therefore, the presence of codons for both Ala and Val in AR1 and Ala and Thr in AR2 at the Ala_{122} codon site might be due to multiple copies of the ALS gene in barnyardgrass. Two codons coding for the same amino acid were present at Val_{196}, Ile_{228}, Val_{238}, Pro_{281}, Ser_{299}, Leu_{349}, Arg_{373}, Phe_{374}, Val_{415}, Phe_{458}, Pro_{518}, Leu_{568}, Gly_{607}, Ala_{612}, Pro_{652}, and Ser_{653} sites in all biotypes (data not shown). Additionally, two or more codons were present at Ala_{311}/Ser_{311} (GCT/TCT) and $Ile_{235}/Met_{235}/Val_{235}$ (NTN, where N is A or G) (data not shown). These multiple codons coding for more than one amino acid were present in all biotypes including susceptible biotypes, revealing that amino acid substitutions at these sites were not the cause of ALS inhibitor resistance. Of the multiple codons (coding for same or different amino acids), codons with the strongest signal in the DNA sequence chromatograms were included in the ALS gene sequence shown in Figure 3.

Substitution of Ala_{122} to Thr has been reported to cause a high level of resistance to IMI herbicides in common cocklebur (*Xanthium strumarium* L.),[48] smooth pigweed (*Amaranthus hybridus* L.),[49,50] eastern black nightshade (*Solanum ptycanthum* Dun.),[51] redroot pigweed (*Amaranthus retroflexus* L.), and Powell amaranth (*Amaranthus powellii* S. Wats.).[52] Recently, the Ala_{122} to Val substitution was reported in one of several ALS inhibitor-resistant silky windgrass biotypes from Poland.[28]

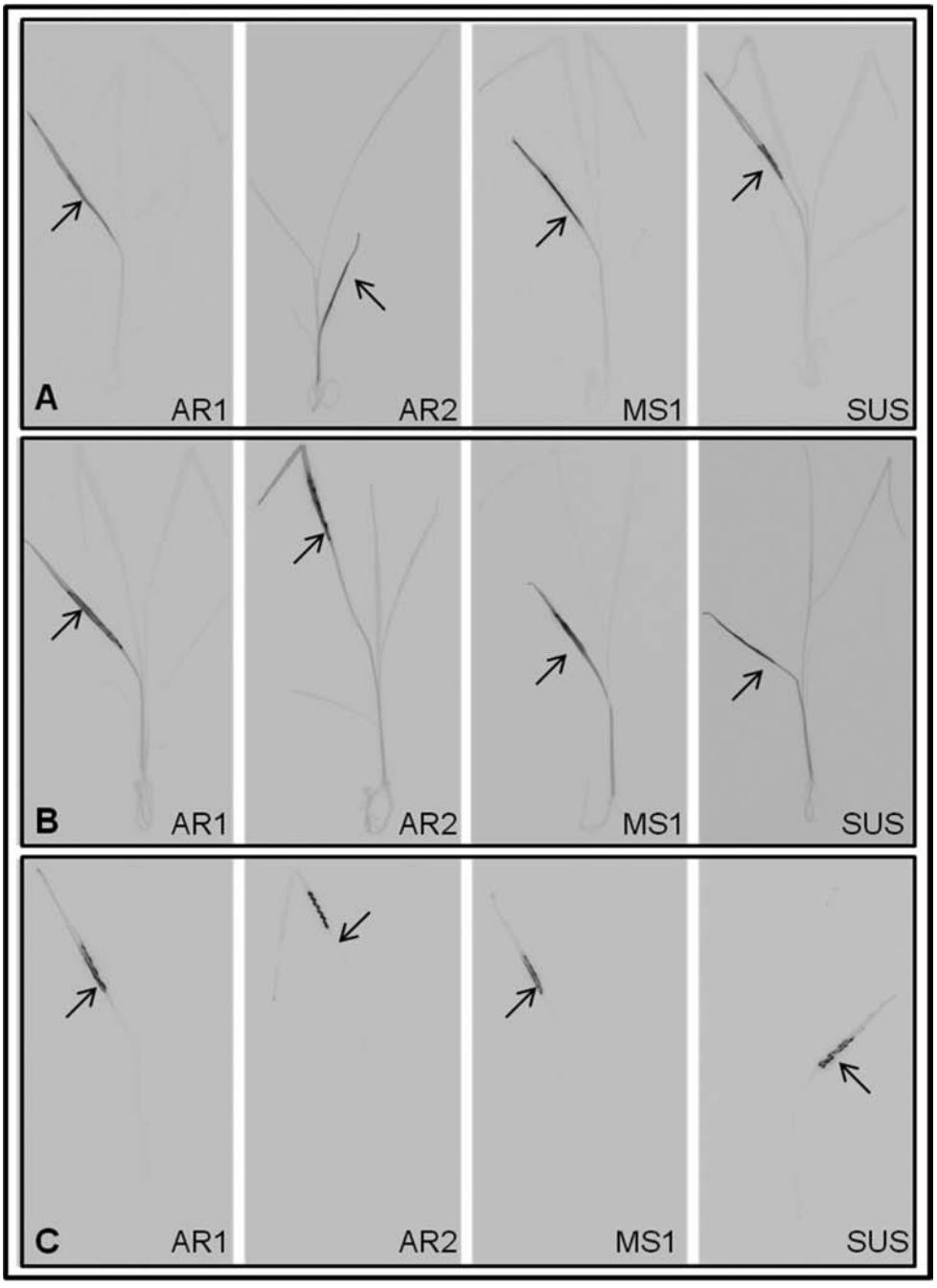

Figure 2. Autoradiographs of ALS inhibitor-resistant (AR1, AR2, and MS1) and -susceptible (SUS) biotypes treated with (A) ^{14}C-bispyribac-sodium, (B) ^{14}C-imazamox, and (C) ^{14}C-penoxsulam at 96 h after treatment. Arrow denotes treated leaf.

The silky windgrass biotype with only Ala_{122} to Val substitution in the sequenced region of the ALS gene (domain A with Ala_{122}, Pro_{197}, and Ala_{205}; and domain B with Trp_{574} and Ser_{653}) had resistance to 3 times the recommended field rate of chlorsulfuron and 2 times the recommended field rate of mesosulfuron plus iodosulfuron; however, it was susceptible to the recommended field rate of sulfosulfuron and propoxycarbazone-sodium. Another recently reported novel substitution is Ala_{122} to Tyr in wild radish that imparts high-level resistance to chlorsulfuron, imazamox, and metosulam and moderate resistance to imazapyr.[27]

Although both AR1 and AR2 were >32 times more resistant to imazamox compared to the susceptible biotype based on the lethal dose required to kill 50% of plants (LD_{50}), the morphological response to even a field rate application of imazamox was different in these biotypes.[13] After application of imazamox, there was little to no reduction in plant dry weight in AR2; however, there was a decrease in dry weight and increase in tillering, a typical response of ALS-inhibiting herbicides, in AR1.[13] Ala_{122} to Val and Thr substitution in AR1 and AR2, respectively, justifies the high resistance level to imazamox with differential morphological response in these biotypes. Resistance to herbicides of SU and SCT families (the only herbicides tested) was found in the silky windgrass biotype (12/1) with Ala_{122} to Val substitution.[28] Additionally, cross-resistance to imazamox (high), penoxsulam (high), and bispyribac-sodium (medium) occurs in AR1.[13] Complete ALS gene sequencing of barnyardgrass (AR1 biotype) and silky

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AR1     GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGTCTCC 60
                                                              Val

AR2     GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCACCTCC 60
                                                              Thr

MS1     GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCCTCC 60
SUS     GTCGAGGCCCTCGAGCGCTGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCCTCC 60
        ************************************************************

                                                          Ala122

AR1     ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC 12C
AR2     ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC 12C
MS1     ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC 12C
SUS     ATGGAGATCCACCAGGCGCTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCAC 12C
        ************************************************************

AR1     GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC 18C
AR2     GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC 18C
MS1     GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC 18C
SUS     GAGCAGGGGGAGGCCTTCGCCGCCTCCGGGTTCGCGCGCTCGTCCGGCCGCGTCGGCGTC 18C
        ************************************************************

AR1     TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG 24C
AR2     TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG 24C
MS1     TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG 24C
SUS     TGCGTCGCCACCTCGGGCCCCGGCGCCACCAACCTCGTCTCCGCGCTCGCCGACGCGCTG 24C
        ************************************************************

AR1     CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCGCCGCATGATCGGCACC 30C
AR2     CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCGCCGCATGATCGGCACC 30C
MS1     CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCGCCGCATGATCGGCACC 30C
SUS     CTCGACTCCATCCCCATGGTCGCCATCACCGGCCAGGTGCCCGCCGCATGATCGGCACC 30C
        ************************************************************

                                                  Pro197

AR1     GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC 36C
AR2     GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC 36C
MS1     GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC 36C
SUS     GACGCCTTCCAGGAGACGCCAATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTAC 36C
        ************************************************************

           Ala205

AR1     CTCGTCCTCGACATCGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC 42C
AR2     CTCGTCCTCGACATCGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC 42C
MS1     CTCGTCCTCGACATCGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC 42C
SUS     CTCGTCCTCGACATCGACGACATCCCCCGCGTCATACAGGAGGCGTTCTTCCTCGCCTCC 42C
        ************************************************************

AR1     TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC 48C
AR2     TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC 48C
MS1     TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC 48C
SUS     TCTGGCCGGCCGGGGCCGGTGCTCGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCC 48C
        ************************************************************

AR1     GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT 54C
AR2     GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT 54C
MS1     GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT 54C
SUS     GTGCCGGTCTGGAACACGCCCATGAGTCTGCCGGGGTACATTGCGCGCCTGCCCAAGCCT 54C
        ************************************************************

AR1     CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT 60C
AR2     CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT 60C
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Figure 3. continued

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MS1     CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT 600
SUS     CCGGCAACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAGTCGCGGCGCCCTGTT 600
        ************************************************************

AR1     CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG 660
AR2     CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG 660
MS1     CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG 660
SUS     CTTTATGTTGGTGGTGGTTGCGCTGCATCCGGTGAGGAGCTGCGCCGCTTTGTGGAGATG 660
        ************************************************************

AR1     ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA 720
AR2     ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA 720
MS1     ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA 720
SUS     ACCGGAATCCCAGTGACAACTACTCTGATGGGCCTTGGCAACTTCCCCAGTGATGACCCA 720
        ************************************************************

AR1     CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG 780
AR2     CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG 780
MS1     CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG 780
SUS     CTGTCTCTGCGCATGCTCGGTATGCACGGTACTGTATATGCAAATTATGCAGTGGATAAG 780
        ************************************************************

AR1     GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG 840
AR2     GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG 840
MS1     GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG 840
SUS     GCCGACCTGTTGCTGGCATTTGGTGTGCGGTTCGATGATCGTGTGACAGGGAAAATTGAG 840
        ************************************************************
                                          Asp376|Arg377

AR1     GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG 900
AR2     GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG 900
MS1     GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG 900
SUS     GCTTTTGCAAGCAGGGCCAAGATTGTGCACATTGATATTGATCCAGCTGAGATTGGCAAG 900
        ************************************************************

AR1     AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT 960
AR2     AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT 960
MS1     AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT 960
SUS     AACAAGCAGCCACATGTGTCCATCTGTGCGGATGTCAAGCTTGCTTTGCAGGGCATGAAT 960
        ************************************************************

AR1     GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG 102C
AR2     GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG 102C
MS1     GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG 102C
SUS     GCTCTTCTGGAAGGAATCATATCAAAGAAGAGTTTTGACTTTGGCTCATGGCAGGATGAG 102C
        ************************************************************

AR1     TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG 108C
AR2     TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG 108C
MS1     TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG 108C
SUS     TTGGATCAGCAGAAGAGGGAATTCCCCCTGGGGTACAAAACTTTCGATGAGGAGATTCAG 108C
        ************************************************************

AR1     CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT 114C
AR2     CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT 114C
MS1     CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT 114C
SUS     CCACAGTATGCTATCCAGGTTCTGGATGAGCTGACGAAAGGGGAGGCCATCATTGCCACT 114C
        ************************************************************

AR1     GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG 120C
AR2     GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG 120C
MS1     GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG 120C
SUS     GGTGTTGGGCAACACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGACCAAGGCAG 120C
```

Figure 3. continued

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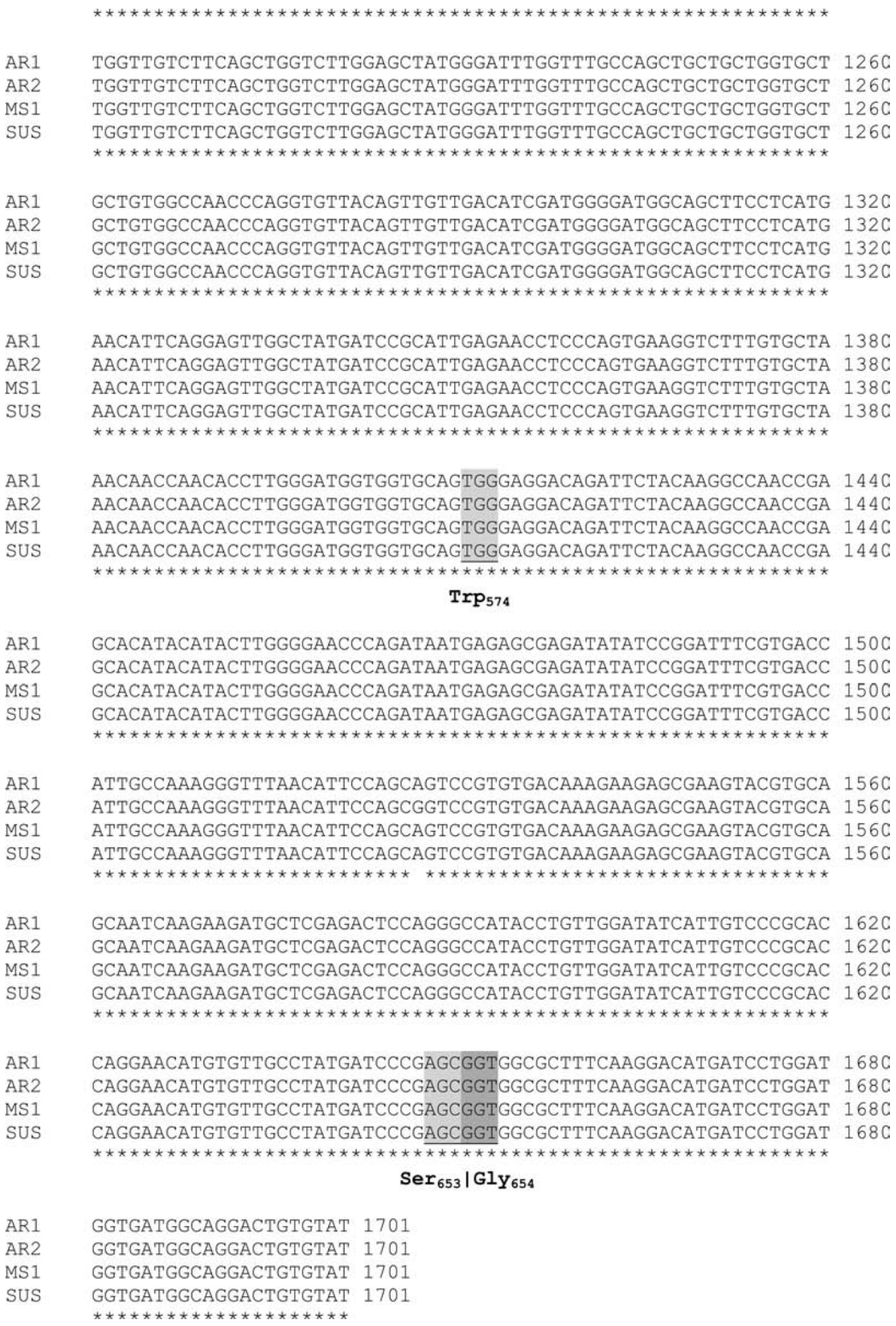

Figure 3. ALS nucleotide sequence alignment of ALS inhibitor-resistant (AR1, AR2, and MS1) and -susceptible (SUS) barnyardgrass biotypes showing Ala_{122} to Val and Ala_{122} to Thr substitutions in AR1 and AR2, respectively. The seven other most common sites for naturally occurring amino acid substitutions causing ALS-inhibiting herbicide resistance in plants are also shown.

windgrass is required to confirm that no additional substitutions contribute to resistance.

ALS Enzyme Assay. The pattern of inhibition of ALS in the resistant biotypes by imazamox, bispyribac-sodium, and penoxsulam (Table 4) mostly correlated with the ALS sequence data, except for AR1 with imazamox and AR2 with imazamox and penoxsulam. The I_{50} values for the resistant biotypes, AR1, AR2, and MS1, were 2.6, 7.5, and 1.8 μM imazamox, respectively, resulting in resistance indices (RI, ratio of resistant and susceptible I_{50} values) of 2.0, 5.8, and 1.4, respectively, when compared to the susceptible biotype that had an I_{50} value of 1.3 μM imazamox. The I_{50} values with

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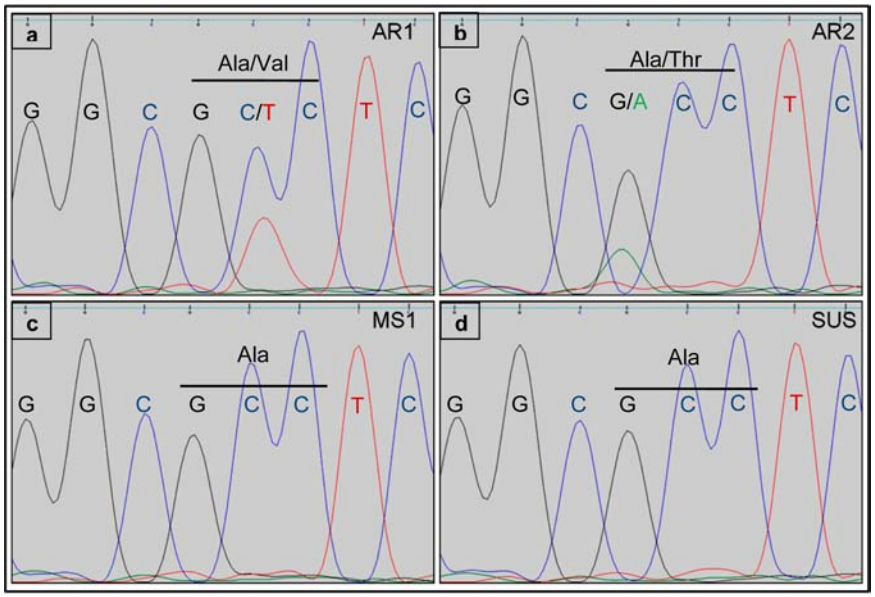

Figure 4. ALS sequence chromatogram showing (a) GCC/GTC codons for Ala_{122}/Val_{122} in AR1, (b) GCC/ACC codons for Ala_{122}/Thr_{122} in AR2, and (c, d) GCC codon for Ala_{122} in MS1 and susceptible (SUS) biotypes.

Table 4. Log-Logistic Equation Parameters and Herbicide Concentrations Required for 50% Inhibition of Acetolactate Synthase (ALS) Enzyme in Vitro and Corresponding Resistance Index Values of ALS Inhibitor-Resistant (AR1, AR2, and MS1) and -Susceptible (SUS) Barnyardgrass Biotypes[a]

		regression equation parameters[b]					
biotype	herbicide	c	d	b	R^2	I_{50}[c] (μM)	RI[d]
AR1	imazamox	17.6	99	−1.04	0.99	2.6 b	2.0
AR2	imazamox	17.7	101	−0.44	0.99	7.5 a	5.8
MS1	imazamox	10.7	100	−0.97	0.99	1.8 c	1.4
SUS	imazamox	12.1	100	−1.14	0.99	1.3 c	
AR1	bispyribac-sodium	8.3	100	−0.86	0.99	0.009 b	0.60
AR2	bispyribac-sodium	5.2	100	−0.29	0.99	0.011 b	0.73
MS1	bispyribac-sodium	23.7	100	−1.14	0.99	0.023 a	1.53
SUS	bispyribac-sodium	22.6	100	−0.37	0.99	0.015 b	
AR1	penoxsulam	8.2	100	−0.4	0.99	0.01 c	0.5
AR2	penoxsulam	5.5	100	−0.32	0.99	0.07 a	3.5
MS1	penoxsulam	24.1	100	−0.42	0.99	0.02 b	1.0
SUS	penoxsulam	20.6	100	−0.59	0.99	0.02 b	

[a]Abbreviations: I_{50}, herbicide concentration required to cause a 50% inhibition of ALS enzyme activity in vitro; RI, resistance index. [b]Regression equation parameters were generated by fitting a nonlinear regression equation of the form $y = c + (d - c)/(1 + (x/x_0)/\backslash(-b))$ to response of ALS enzyme to herbicide concentration. Details are provided in the text. [c]Values followed by the same lower case letter are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$) within each herbicide. [d]Values represent ratios of I_{50} values of resistant to susceptible biotypes within each herbicide.

bispyribac-sodium were 0.009, 0.011, 0.023, and 0.015 μM for the AR1, AR2, MS1, and susceptible biotypes, respectively; thus, bispyribac-sodium RI values for the AR1, AR2, and MS1 biotypes were 0.60, 0.73, and 1.53, respectively. These values are similar to the bispyribac-sodium I_{50} value of 0.009 μM reported for a resistant *Echinochloa phyllopogon* accession.[53] The I_{50} values for AR1, AR2, MS1, and the susceptible biotypes for penoxsulam were 0.01, 0.07, 0.02, and 0.02 μM, respectively, resulting in RI estimates of 0.5, 3.5, and 1.0 for AR1, AR2, and MS1 biotypes, respectively.

Sensitivity of ALS from the AR1 and AR2 biotypes to imazamox did not correspond to point mutations determined through ALS sequence analysis. Whereas resistance of the AR1 biotype to imazamox could be partially explained by herbicide retention in treated leaf or metabolism (not investigated), the high level of resistance (>94-fold) reported at the whole-plant level[13] for both AR1 and AR2 may involve other mechanisms in addition to altered target site. Amplification of the *EPSPS* gene was determined as the mechanism of resistance to glyphosate in a glyphosate-resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.) biotype.[54] Studies need to be conducted in the future to determine if amplification of the resistant ALS gene plays a role in imparting ALS inhibitor resistance.

As discussed previously, barnyardgrass is hexaploid in nature and may possess multiple copies of each gene. Differential expression of various copies of the ALS enzyme in resistant

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biotype can be another reason for less than expected imazamox I_{50} of AR1 and AR2. It is also possible that the ALS enzyme could have been phosphorylated under in vitro conditions, resulting in reduced activity. A different approach could be the separation of multiple forms of the ALS enzyme using 2-D gel electrophoresis followed by analysis of the amino acid sequence. Studies on the nature of inheritance of the ALS resistance trait could shed light onto the genetics of resistance with regard to number of genes involved and their contribution toward expression. In the future, there is a need to sequence the complete ALS gene. RNA-seq technology can be used to analyze the whole transcriptome of the resistant and susceptible biotypes to gain a better understanding of the findings from this research. Additionally, there is a need to determine differential metabolism and CYP expression to completely understand the mechanism of resistance to ALS-inhibiting herbicides. Better understanding of mechanisms of resistance will aid development of future strategies to control barnyardgrass populations resistant to the ALS-inhibiting herbicides.

AUTHOR INFORMATION

Corresponding Author

*Phone: 1 (479) 575-6244. E-mail: driar@uark.edu.

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Notes

The authors declare no competing financial interest.

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