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RESEARCH ARTICLE

Target-site mutations Ile1781Leu and Ile2041Asn in the *ACCase2* gene confer resistance to fluazifop-p-butyl and pinoxaden herbicides in a johnsongrass accession from Arkansas, USA

Fidel González-Torralva | **Jason K. Norsworthy**

Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA

Correspondence
Fidel González-Torralva, Postdoctoral Fellow, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA.
Email: fg013@uark.edu

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Abstract

Johnsongrass [*Sorghum halepense* (L.) Pers.] is a troublesome weed species in different agricultural and non-agricultural areas. Because of its biology, reproductive system, and seed production, effective management is challenging. An accession with low susceptibility to the acetyl-CoA carboxylase (ACCase)-inhibiting herbicides fluazifop-p-butyl (fluazifop) and pinoxaden was collected in eastern Arkansas. In this research, the molecular mechanisms responsible for ACCase resistance were investigated. Dose–response experiments showed a resistance factor of 181 and 133 for fluazifop and pinoxaden, respectively. Molecular analysis of both *ACCase1* and *ACCase2* genes was researched. Nucleotide comparison of *ACCase1* between resistant and susceptible accessions showed no single nucleotide polymorphisms. Nonetheless, analysis of *ACCase2* in fluazifop-resistant johnsongrass plants revealed the Ile1781Leu target-site mutation was dominant (nearly 75%), whereas the majority of pinoxaden-resistant johnsongrass plants had the Ile2041Asn (60%). Not all sequenced johnsongrass plants displayed a target-site mutation, suggesting the presence of additional resistance mechanisms. Amplification of *ACCase1* and *ACCase2* was not responsible for resistance because of the similar values obtained in both resistant and susceptible accessions. Experiments with malathion and NBD-Cl suggest the presence of herbicide metabolism. Outcomes of this research demonstrated that fluazifop- and pinoxaden-resistant johnsongrass plants displayed a target-site mutation in *ACCase2*, but also that non-target-site resistance mechanisms would be involved and require a detailed study.

KEYWORDS
ACCase; herbicide resistance; johnsongrass, target-site mutation

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1 | INTRODUCTION

Johnsongrass is a difficult-to-control weed species; it has been found affecting different cropping systems, urban areas, and abandoned lands (Klein & Smith, 2021). In the mid-south US, it is considered a noxious weed in cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] fields. Furthermore, johnsongrass serves as a host for a diverse range of pests and diseases that eventually affect different crops (Klein & Smith, 2021). Johnsongrass is a tetraploid ($2n = 40$) weed species that emerged from a natural crossing of sorghums, *Sorghum bicolor* (L.) Moench and *Sorghum propinquum* (Kunth) Hitchc. (Kong et al., 2013; Paterson et al., 2020). The reproduction based on seeds and rhizomes has made johnsongrass an invasive weed species (Klein & Smith, 2021). Estimates of seed production have determined that johnsongrass can produce up to 80,000 seeds per plant in a single season; additionally, these seeds may be viable for up to 10 years in the soil (McWhorter, 1961; Ryder et al., 2018). Crop yield losses due to johnsongrass plants vary according to the specific situation. For example, in soybean, reports indicate that johnsongrass competition can reduce soybean yields by up to 88% (McWhorter, 1991; Williams & Hayes, 1984). In cotton, reports indicate that yield can be reduced by up to 70% depending on johnsongrass infestation (Bridges & Chandler, 1987). Other reports have estimated that a single johnsongrass plant can reduce cotton lint yield by approximately 7% (Uludag et al., 2007).

Herbicides are the most common way to suppress weeds in different situations, and within these, the ACCase-inhibiting herbicides are a very effective chemical tool for controlling johnsongrass. Different reports have described two structurally different ACCase in plants: heteromeric and homomeric, the first one located in the plastids, where *the novo* fatty acids are built up and the second one located in the cytosol, where among others very long-chain fatty acids (VLCFA) are produced. These ACCase, are commonly referred to as plastidic and cytosolic ACCase, respectively. Poaceae members have a homomeric plastidic ACCase (Konishi & Sasaki, 1994; Sasaki & Nagano, 2004; Yu et al., 2007). Each ACCase is constituted by three different domains namely biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyl transferase (CT) (Nikolau et al., 2003; Sasaki & Nagano, 2004). Several reports have indicated that the CT domain is crucial for the sensitivity of ACCase-inhibiting herbicides (Yu et al., 2007; Yu et al., 2010; Zhang et al., 2004). However, given the interactions between the ACCase herbicides and the specific position of residues in the CT domain these can alter the affinity and efficacy. Then, any change in the nucleotide sequence of *ACCase* gene could produce an amino acid replacement conveying resistance to such herbicides (Takano et al., 2021). Malonyl CoA, a crucial component of *the novo* fatty acid biosynthesis is affected with the application of ACCase-inhibiting herbicides. In ACCase-susceptible plants, that metabolic disruption causes an imbalance in the integrity of the cell membrane, followed by metabolite leakage and finally cell death (Délye et al., 2005; Kaundun, 2014). ACCase herbicides comprise three chemically different groups that differ in their binding site positions within the CT domain in the *ACCase* gene: aryloxyphenoxypropionate (APP), cyclohexanedione (CHD), and phenylpyrazoline (DEN). Among others, fluazifop belongs to the APP herbicide group, whereas clethodim and pinoxaden belong to the CHD and DEN groups, respectively (Burton et al., 1989; Muehlebach et al., 2009; Rendina et al., 1990). APP and CHD herbicides have been in use since the late 1970s; however, DEN herbicides were introduced into the market in 2006 (Muehlebach et al., 2009; Yu et al., 2010).

The prevalent and overreliance use of ACCase-inhibiting herbicides has provoked the appearance of resistant accessions. Worldwide, resistance to ACCase-inhibiting herbicides has been reported in approximately 265 accessions of different grass weed species. *Avena* spp., *Lolium* spp., and *Echinochloa* spp. are clearly the dominant genus with evolved resistance to ACCase-inhibiting herbicides (Heap, 2023). There are only twelve reported cases of resistance to ACCase-inhibiting herbicides in johnsongrass; of these, almost 50% are reported in the US (Heap, 2023). Additionally, accessions of johnsongrass have also evolved resistance to acetolactate synthase (ALS), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), and microtubule assembly-inhibiting herbicides (Heap, 2023; Hernández et al., 2015; Vila-Aiub et al., 2007). Resistance to herbicides can be of two types: a) target-site resistance or b) non-target-site resistance. Target-site resistance involves modifications in the structure of the herbicide target protein, which causes a reduction in herbicide binding and a higher amount of protein target generated by higher gene expression or gene amplification, whereas non-target-site resistance comprises resistance mechanisms (e.g., absorption and translocation, herbicide metabolism or degradation, vacuolar sequestration, etc.) different from target-site resistance (Délye et al., 2015; Gaines et al., 2020; Powles & Yu, 2010).

In this work, a further characterization of a putative fluazifop-resistant johnsongrass accession that survived the commercial field rate of fluazifop ($1\times = 210$ g ai ha^{-1}) was carried out. The objectives of this research were: a) to assess cross-resistance in this putative fluazifop-resistant accession; b) to characterize the resistance level of a putative fluazifop-resistant johnsongrass accession; c) to evaluate molecular mechanisms (target-site and gene amplification) for endowing ACCase resistance; and d) to elucidate the role of malathion and NBD-Cl on the resistance mechanism of this putative fluazifop-resistant johnsongrass accession.

2 | MATERIAL AND METHODS

2.1 | Plant material and growing conditions

During a herbicide susceptibility screening with different johnsongrass accessions collected across Arkansas, Kansas, Texas, and Oklahoma, a johnsongrass accession that survived the fluazifop recommended field rate was detected (Fleming et al., 2021). The putative fluazifop-resistant johnsongrass accession was collected in eastern Arkansas (N34° 57.725, W090° 18.141) and was used to perform all the experiments described below. A susceptible johnsongrass accession that has not been treated with herbicides was also used for comparison. Seeds

were germinated in square pots (2,542 cm^3) filled with growing medium (Promix, LP15, Premier Horticulture Inc., PA, USA) and transplanted into 10 cm-diameter plastic pots containing the same substrate when seedlings reached the one-leaf growth stage. Seedlings were maintained under greenhouse conditions at 35/25 °C day/night temperature and a 16-h photoperiod.

2.2 | Response to fluazifop, clethodim, and pinoxaden field application rates

Putative-resistant and susceptible johnsongrass plants were treated at the three- to four-leaf growth stage with the commercial field rates of fluazifop (210 g ai ha^{-1}), clethodim (110 g ai ha^{-1}), and pinoxaden (70 g ai ha^{-1}) (Supplemental Table S1). Fluazifop and clethodim treatments included 1% v/v of crop oil concentrate, whereas pinoxaden included 1% v/v methylated seed oil. Herbicides were applied with an automatic sprayer chamber calibrated to spray 187 L ha^{-1} using 1100067 nozzles. At 21 days after treatment (DAT), alive plants were counted; an alive plant was considered a plant with active green growing tissue. Per herbicide, 30 putative fluazifop-resistant johnsongrass plants along with 10 susceptible johnsongrass plants were sprayed. Nontreated control plants were maintained for comparison. Results were presented in plant survival percentages (%).

2.3 | Whole plant dose–response assays

Johnsongrass plants at the three- to four-leaf growth stage were treated with fluazifop and pinoxaden. Rates for the putative fluazifop-resistant accession ranged from 0 to 32× the commercial field rate, whereas for susceptible plants, rates ranged from 0 to 2×. Herbicides included the respective surfactant and were sprayed as described earlier. Plants were cut at ground level, and fresh weight was measured at 21 DAT. The experiment was duplicated, and each treatment rate had three replicates, each of which contained a single johnsongrass plant ($n = 3$).

Data obtained in dose–response studies were subjected to a nonlinear, log-logistic regression model as follows:

$$Y = c + \left\{(d-c)/\left[1+(x/g)^b\right]\right\}$$

where Y represents the fresh weight; c and d correspond to the lower and upper asymptotes, respectively; x represents the herbicide rate (independent variable); the GR_{50}, which is the herbicide rate that inhibits growth by 50%, is represented by g; and b corresponds to the slope of the line (Seefeldt et al., 1995). SigmaPlot v.14.5 software (Systat Software Inc., CA, USA) was used to perform the regression analysis and plot the dose–response curves.

2.4 | *ACCase* gene sequencing

Plant tissue (approx. 100 mg) from plants that had survived the commercial field rate of fluazifop and pinoxaden was collected and placed in Eppendorf tubes, which were maintained at −80 °C until further processing. Genomic DNA was extracted using the E.Z.N.A.® Plant DNA kit (Omega Bio-Tek, GA, USA), which includes an RNase A step treatment. Assessment of the quality and quantity of extracted DNA was carried out spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Waltham, MA, USA), and DNA concentrations were set at 10 ng μl^{-1} to be used in further experiments. A set of gene-specific *ACCase* primers was designed to amplify the *ACCase1* and *ACCase2* gene sequences of johnsongrass. Thus, GenBank MK492464 and MK492465 nucleotide sequences were used to partially amplify the *ACCase1* and *ACCase2* genes in johnsongrass. Primers were designed to cover the region where target-site mutations have been correlated with ACCase resistance in other grass weed species (Jang et al., 2013). The design of primers was carried out using the freely available Primer3plus software (Untergasser et al., 2007). Thus, the forward 116F 5′ ACGAGCTGCAACTAGAAAATGG 3′ and the reverse 116R 5′ TCAGCAAGATGCGAGAACCAG 3′, along with the forward F1 5′ TGCAGCTAGATAGCGGTGAA 3′ and the reverse R1 5′ TTATCAACTCGGGGTCAAGC 3′ primers, were used to partially amplify the *ACCase1* and *ACCase2* genes in johnsongrass, respectively.

Polymerase Chain Reactions (PCRs) were run in a T100 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using 25 µl total volume reactions and comprising the following components: 1× Colorless GoTaq® Flexi Buffer (Promega Corp., Madison, WI, USA), 1.5 mM $MgCl_2$, .2 mM dNTP's, .2 µM each forward and reverse primer, .625 units GoTaq® Hot Start Polymerase (Promega Corp., Madison, WI, USA), 50 ng genomic DNA, and 11.8 µl deionized water. *ACCase1* cycling conditions were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1:35 min, and finally a cycle of 72 °C for 5 min. *ACCase2* cycling conditions were similar to those used in *ACCase1* except for the annealing temperature, which was set to 57 °C for 30 s, and the extension time, which was set to 72 °C for 1:05 min.

PCR products (5 µl) were loaded onto 1.2% agarose gel, and electrophoresis was run at 85 v for 30 min using 1× tris-borate-EDTA-pH 8.0 buffer to corroborate correct amplification. After electrophoresis, PCR products were cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA). Then, samples were sent for Sanger sequencing, and raw sequences were managed and aligned using BioEdit (Hall, 1999) and Multalin (Corpet, 1988), freely available software. At least three biological samples per accession and herbicide were Sanger sequenced and analyzed.

TABLE 1 Primer sequences used in quantitative PCR to quantify the *ACCase1* and *ACCase2* genes in fluazifop- and pinoxaden-resistant johnsongrass plants.

Gene[a]	5′ → 3′ sequence	Amplicon (bp)	Efficiency (%)
ACCase1	119F AGGAACTGGAAGATTGCATGCTA 119R CCGAGATGCTGGCATTTTGT	96	104.0
ACCase2	121F GCTTGATTCCCATGAGCGATCC 121R GCCAGGATAAACAGAGGCAATCC	123	106.0
CCR	117F GTCCTGACCTCGTCCATCG 117R CCAGTTCTTGGTCTTCTTGCAG	114	105.4
PPAN	103F CCGTCATTACTCCATCAAGCTC 103R CCTAAGGTCTGGCACTTGATTG	88	99.7

[a]*ACCase*, *acetyl-CoA carboxylase*; *CCR*, *Cinnamoyl-CoA reductase*; *PPAN*, *peter Pan-like*. F, forward; R, reverse.

2.5 | *ACCase* gene amplification using quantitative PCR (qPCR)

Genomic DNA isolated for *ACCase* gene sequencing was used to estimate the relative gene amplification of *ACCase1* and *ACCase2* genes in resistant and susceptible johnsongrass accessions. Thus, a qPCR approach was utilized, and relative gene amplification was estimated using *Cinnamoyl-CoA reductase* (*CCR*) and *peter Pan-like* (*PPAN*) as reference genes (Table 1). Such genes have been used to estimate the relative gene amplification of target genes in either monocot and dicot plant species (González-Torralva & Norsworthy, 2021; McInnes et al., 2002; Salas et al., 2012; The Arabidopsis Information Resource, 2022). qPCR methodology was adhered to MIQE guideline suggestions (Bustin et al., 2009). Primer design was performed as described in the previous section (Table 1).

A CFX Connect Real Time System (Bio-Rad Laboratories Inc., Hercules, CA, USA) was utilized to run qPCR reactions. Each of them comprised 1× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), .3 µM each forward and reverse primers, 15 ng genomic DNA, and 2.9 µl deionized water in a 10 µl final volume. On each run, an extra reaction was included in each primer set, whereby the genomic DNA template was replaced by deionized water to serve as a non-template control. Cycling conditions were as follows: a cycle of 98 °C for 3 min, followed by 40 cycles of 98 °C for 10 s, and 61 °C for 30 s. In addition, melting curves were generated at the end of the run in steps of .5 °C each 5 s by increasing the temperature from 65 °C to 95 °C. Quantification cycles were generated automatically by CFX Maestro software (Bio-Rad Laboratories Inc., Hercules, CA, USA), and relative gene amplification was calculated as $2^{-\Delta\Delta Cq}$ (Livak & Schmittgen, 2001). Experiments consisted of four different plants per accession ($n = 4$), which were considered biological replications, and each of them ran with two technical replicates per gene. Between accessions, a Student *t*-test was used to detect differences in gene amplification relative to reference genes.

2.6 | Inhibition of metabolism experiments using field seeds

Plants of putative fluazifop-resistant and -susceptible johnsongrass plants were treated at the three- to four-leaf growth stage with malathion, a known P450-inhibiting insecticide, or NBD-Cl, a known GST inhibitor, to evaluate if the metabolism of fluazifop is involved in the resistance mechanism. Treatments comprised a nontreated control (T1), fluazifop at 210 g ai ha^{-1} (T2), fluazifop at 210 g ai ha^{-1} + 2000 g ha^{-1} malathion (T3), and fluazifop at 210 g ai ha^{-1} + 80 g ha^{-1} NBD-Cl (T4). Treatment with fluazifop + malathion was mixed and sprayed at the same time, whereas in fluazifop + NBD-Cl, the latter was sprayed 48 h before fluazifop treatment. Malathion and NBD-Cl rates have shown to cause no phytotoxicity in many different experiments and plant species, so treatments of these chemicals alone were not included in this research (Cummins et al., 2013; Liu et al., 2018; Varanasi et al., 2018; Wright et al., 2016). Herbicide treatments were applied under the same conditions as described earlier. Each treatment had eleven replicates, and each replicate had one single plant per pot ($n = 11$). At 21 DAT, plants were clipped at ground level, placed in a paper bag, and dried until constant weight. A Student *t*-test was performed to detect significant differences among treatments.

3 | RESULTS AND DISCUSSION

3.1 | Response to fluazifop, clethodim, and pinoxaden field application rates

A total of 30 individual johnsongrass plants were sprayed at the three to four leaf growth stage with 1× field application rate to assess the survival percentage. At 21 DAT, resistance to fluazifop was corroborated. Out of the 30 plants treated, only eight were dead, showing that approximately 75% of plants survived the fluazifop treatment. Most surviving plants displayed green tissue and new leaf growth; however, differences in size were observed, meaning that resistance

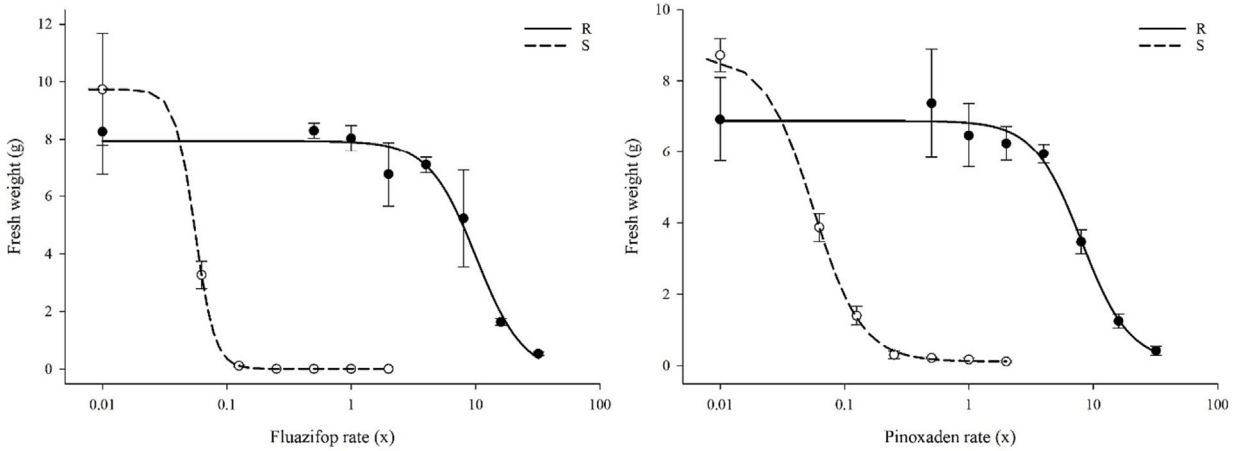

FIGURE 1 Dose–response curves of resistant (R) and susceptible (S) johnsongrass plants treated with fluazifop (left) and pinoxaden (right). Vertical bars represent ± standard deviation of the mean.

to fluazifop was not homogeneous. All susceptible plants (100%) treated with the 1× commercial field rate were dead in the same period. Additionally, fluazifop-resistant plants treated with pinoxaden displayed 83% survival, whereas in the susceptible accession all pinoxaden-treated plants were dead at the same period. Clethodim showed 100% efficacy in controlling this fluazifop-resistant accession (Supplemental Figure 1).

3.2 | Whole plant dose–response assays

The susceptible accession of johnsongrass treated with fluazifop and pinoxaden was completely controlled with lower than recommended field rates (e.g., .1×), whereas in the resistant accession, even 32× the recommended field rate was not good enough to adequately control all resistant plants. Using fluazifop, dose–response curves displayed GR_{50} values of 10 and .055× for resistant and susceptible accessions, respectively (Figure 1). In addition, dose–response curves using pinoxaden showed GR_{50} values of 8 and .06× for resistant and susceptible accessions, respectively (Figure 1). Resistant factors found were 181 and 133 for fluazifop and pinoxaden, respectively. Even though seeds of fluazifop-resistant and -susceptible accessions were equally processed in time and space, the fresh weight was slightly lower in the fluazifop-resistant accession compared with the susceptible one. The latter would suggest a fitness penalty in the fluazifop-resistant accession (Figure 1). Fitness penalty in johnsongrass accessions and other grass weeds resistant to ACCase-inhibiting herbicides has been reported before (Panozzo & Sattin, 2021; Wang et al., 2010). In northern Italy, four johnsongrass accessions highly resistant to fluazifop were reported by Scarabel et al. (2014). Resistance indexes reported for fluazifop were higher (>600) than those obtained in this research (Scarabel et al., 2014). Similarly, in a johnsongrass accession resistant to fluazifop, quizalofop, and sethoxydim, resistance factors reported were higher than 388, 15, and approximately 3.4, respectively (Smeda et al., 1997). An accession collected in Virginia, USA, displayed 17-fold more resistance to quizalofop than the susceptible check used for comparison; this accession was cross-resistant to sethoxydim with a resistance value of approximately six-fold and 29.5-fold more resistant to fluazifop (Bradley & Hagood, 2001). Both resistant and susceptible johnsongrass accessions were controlled effectively with clethodim (Bradley & Hagood, 2001). Similarly, a johnsongrass accession collected in Mississippi, USA, showed cross-resistance to clethodim (resistance index 11-fold), fluazifop, and sethoxydim (resistance index approx. six-fold) (Burke et al., 2006). Even with several accessions of johnsongrass having evolved resistance to ACCase-inhibiting herbicides, it is interesting to note that so far there are no publicly available reports of pinoxaden-resistant johnsongrass (Heap, 2023). Resistance to pinoxaden in other grass weed species has been reported. For instance, in a pinoxaden-resistant perennial ryegrass (*Lolium perenne* L.) accession from Australia, the resistance level was found to be 41.4-fold higher than the susceptible accession used for comparison (Ghanizadeh et al., 2022). Additionally, that pinoxaden-resistant ryegrass accession was successfully controlled by clethodim. In our study, similar outcomes were attained by applying clethodim to johnsongrass plants that were fluazifop- and pinoxaden-resistant (Supplemental Figure 1). The fact that clethodim controlled satisfactory the resistant johnsongrass accession may be explained by the binding site of clethodim within the CT domain. It has been shown for instance, that in *Phalaris minor* Retz. different amino acids residues are involved in the binding of aryloxyphenoxypropionate herbicides while a different set of amino acids are involved in cyclohexanedione herbicides (Rani et al., 2019).

3.3 | *ACCase* gene sequencing

Mutations in the target site of the *ACCase* gene have been linked to confer resistance to ACCase-inhibiting herbicides (Délye et al., 2005; Yu et al., 2007). Point mutations described so far in conferring resistance to ACCase-inhibiting herbicides include a single exon in the CT

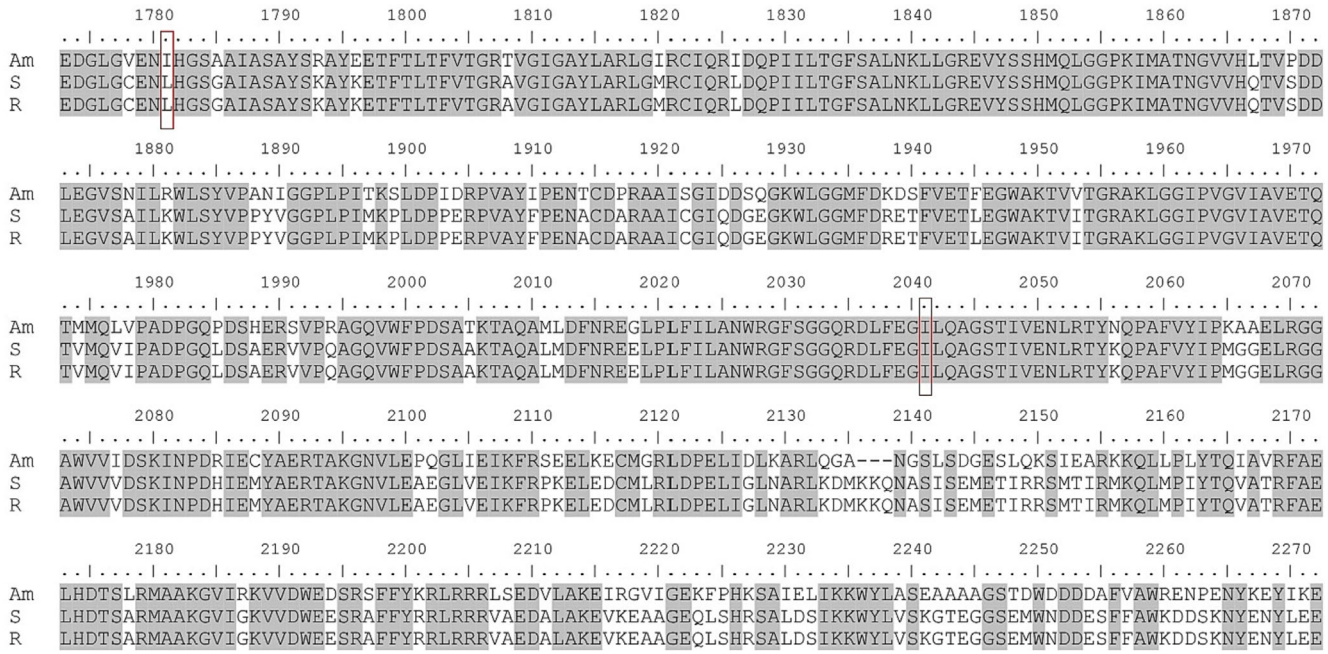

FIGURE 2 Partial ACCase protein sequence alignments of *Alopecurus myosuroides* (am) (GenBank accession CAC84161) and *ACCase1* of susceptible (S) and resistant (R) johnsongrass accessions. Highlighting indicates similarity among the aligned sequences. Boxes show the amino acid positions described in this research.

domain of the *ACCase* gene and cover punctual amino acid changes from positions 1781 to 2097, numbered relative to the blackgrass (*Alopecurus myosuroides* Huds) ACCase sequence (GenBank accession AJ310767.1). ACCase target-site mutations have been described, for example, in barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], where an Asp2078Glu mutation was reported (Fang et al., 2020); in a fluazifop-resistant johnsongrass accession, an Ile2041Asn was found in the ACCase-resistant accession (Scarabel et al., 2014); in *Hordeum* species, the mutations Ile1781Leu and Gly2096Ala have been described (Shergill et al., 2015). The mutation Asn2097Asp was described in a fluazifop-resistant accession of goosegrass [*Eleusine indica* (L.) Gaertn.] (Cha et al., 2014).

However, in other ACCase-resistant accessions, a target-site mutation has not been found. For instance, in a junglerice [*Echinochloa colona* (L.) Link] accession resistant to fenoxaprop-p-ethyl, no mutations were reported (Wright et al., 2016). No mutations were also described in a quizalofop-p-ethyl-resistant accession of Asia minor bluegrass (*Polypogon fugax* Nees ex Steud.) collected in Chinese canola fields (Chen et al., 2020) or a barnyardgrass accession resistant to cyhalofop-p-butyl, fenoxaprop-ethyl, and quizalofop-ethyl in Arkansas (Hwang et al., 2022).

In this research, 1,501 bp and 1,022 bp of the *ACCase1* and *ACCase2*, respectively, were sequenced. Nucleotide sequences were searched using BLAST (Basic Local Alignment Search Tool) available at https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on May 24, 2022), to ensure correct gene sequencing. Nucleotide searching of *ACCase1* displayed high similarity with other *ACCase1* nucleotide sequences. Thus, *expect* values of .0 were observed with *S. halepense* (GenBank accession MK492464.1) and *S. bicolor* (GenBank accession XM_021446242.1). Conversely, sequenced *ACCase2* was similar to many *ACCase2* sequences, including *S. halepense* and *S. bicolor* (GenBank accessions MK492465.1 and XM_002446133.2, respectively), with *expect* values of .0.

Open reading frames were obtained using the Open Reading Frame Finder tool available at https://www.ncbi.nlm.nih.gov/orffinder/ (accessed on May 24, 2022). Protein BLAST of *ACCase1* showed 100% identity with *ACCase1* of *S. bicolor* (GenBank accession XP_021301917.1) and 100% identity with *ACCase1* of *S. halepense* (GenBank accession QEG99492.1). Similarly, protein BLAST of *ACCase2* displayed 100% identity with that of *S. halepense* and *S. bicolor* protein sequences (GenBank accessions QEG99493.1 and XP_002446178.1, respectively) with *expect* values of .0. Comparison of sequences obtained in *ACCase1* between resistant and susceptible johnsongrass accessions did not display any amino acid substitution (Figure 2). However, in *ACCase2*, an Ile1781Leu amino acid substitution in the fluazifop-resistant johnsongrass plants was dominant (nearly 75%) (Figure 3). Additionally, the majority of sequences obtained in pinoxaden-resistant plants displayed an Ile2041Asn amino acid substitution (60%) (Figure 4). No resistant plant was detected harboring both alleles, the Ile1781Leu and the Ile2041Asn target site-mutations. However, in other studies, such as in a pinoxaden-resistant *Lolium* spp., the presence of double ACCase mutations in the same plant has been reported (Scarabel et al., 2011). Research into the molecular resistance mechanisms to ACCase-inhibiting herbicides in downy brome (*Bromus tectorum* L.), has shown the presence of two different mutations, and authors have suggested a multiple evolutionary origin of ACCase resistance. Mutations described correspond to Ile2041Thr and Gly2096Ala which rendered a different pattern of ACCase-resistance (Ribeiro et al., 2023).

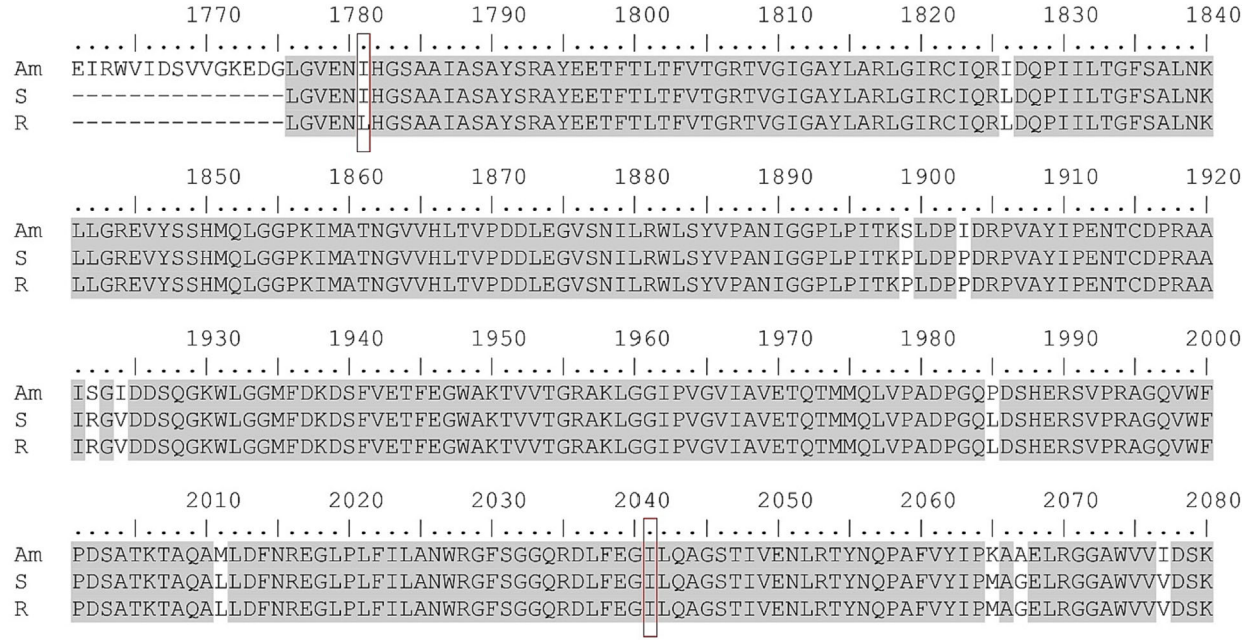

FIGURE 3 Partial ACCase protein alignments of *Alopecurus myosuroides* (Am) (GenBank accession CAC84161) and *ACCase2* of fluazifop-susceptible (S) and -resistant (R) johnsongrass plants. Protein sequences display a target-site substitution at 1781 residue between johnsongrass accessions. Highlighting indicates similarity among the aligned protein sequences. Boxes show the amino acid positions described in this research.

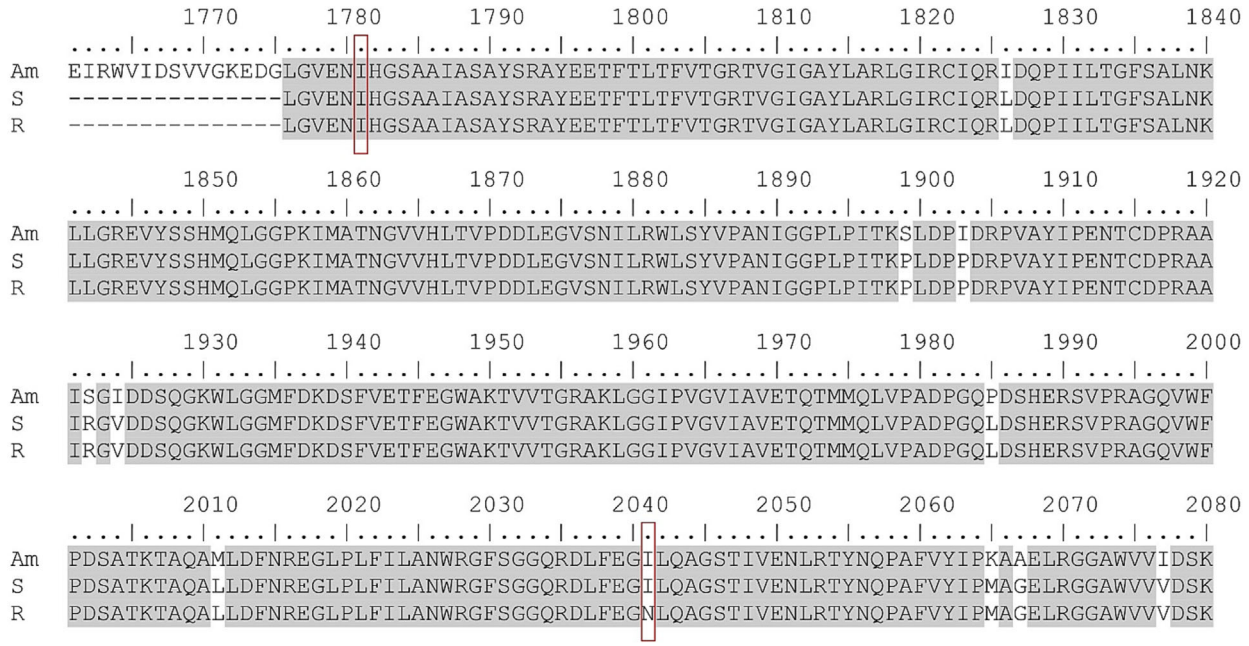

FIGURE 4 Partial ACCase protein sequence alignments of *Alopecurus myosuroides* (Am) (GenBank accession CAC84161) and *ACCase2* of pinoxaden-susceptible (S) and -resistant (R) johnsongrass plants. Protein sequences display a target-site substitution at 2041 residue between johnsongrass accessions. Highlighting indicates similarity among the aligned sequences. Boxes show the amino acid positions described in this research.

Additionally, double mutations within the same plant have been reported in different glyphosate-resistant plant species, which confer a higher degree of herbicide resistance compared with a single point mutation (reviewed by Gaines et al., 2020). In other ACCase-resistant plant species, such as *E. indica* from Malaysia, the target-site mutation Trp2027Cys was found in the resistant accessions; however, the authors also reported the target-site mutation Asn2097Asp in one resistant accession (Cha et al., 2014). Trp2027Cys was also reported in sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] and American sloughgrass (*Beckmannia syzigachne* Steud.) resistant to

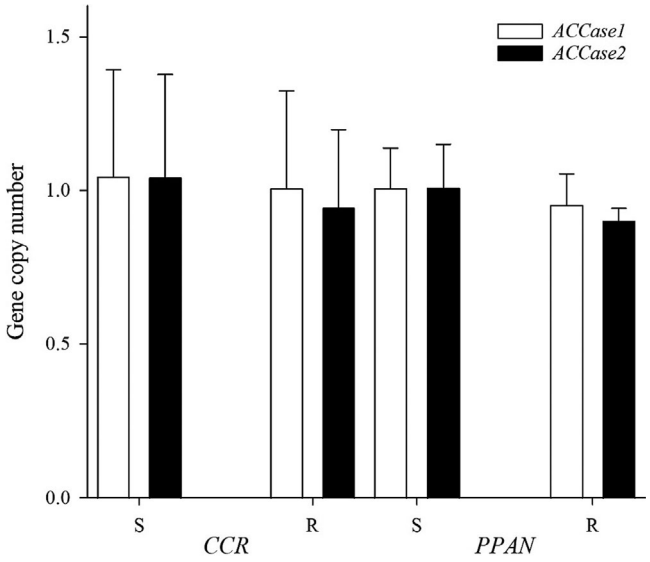

FIGURE 5 Relative gene amplification of *ACCase1* and *ACCase2* in susceptible (S) and resistant (R) johnsongrass accessions. *CCR* and *PPAN* correspond to the reference genes used. Bars ± standard deviation of the mean ($n = 4$).

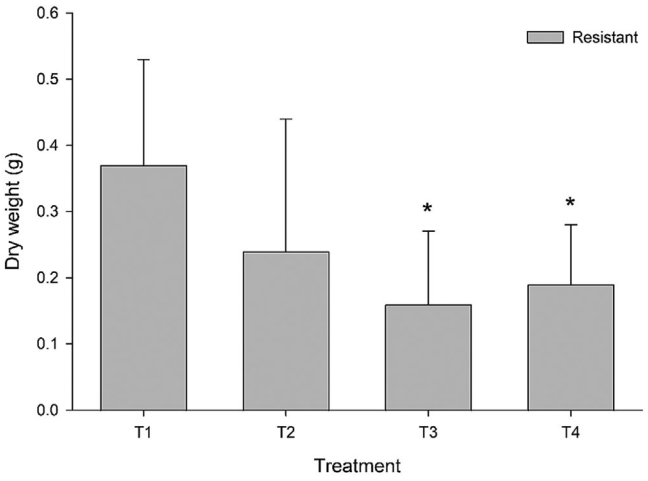

FIGURE 6 Dry weight of johnsongrass plants under different treatments. T1, nontreated control; T2, fluazifop at 210 g ai ha^{-1}; T3, fluazifop at 210 g ai ha^{-1} + 2000 g ha^{-1} malathion; and T4, fluazifop at 210 g ai ha^{-1} + 80 g ha^{-1} NBD-Cl, which was sprayed 48 h before fluazifop. Bars ± standard deviation of the mean ($n = 11$). Significant differences ($p \leq .05$) between the nontreated control (T1) and the rest of the treatments (T2, T3, and T4) are indicated by an asterisk. Field seeds were used to perform this experiment.

ACCase-inhibiting herbicides (Li et al., 2014; Takano et al., 2020). Additionally, in perennial ryegrass resistant to pinoxaden, the target-site mutation Ile2041Val was described (Ghanizadeh et al., 2022). In a southern crabgrass [*Digitaria ciliaris* var. *chrysoblephara* (Retz.) Koeler] accession resistant to metamifop, cyhalofop-butyl, fenoxaprop-p-ethyl, haloxyfop-p-methyl, clethodim, sethoxydim, and pinoxaden, the Ile1781Leu target-site mutation was reported to confer resistance to these herbicides; but additionally, non-target site resistance mechanisms were involved (Yang et al., 2023).

3.4 | *ACCase* gene amplification using qPCR

In this research, we explored the amplification of *ACCase1* and *ACCase2* genes as a potential target-site resistance mechanism. In an herbicide-resistant accession, gene copy number variation or gene amplification means that the target gene is being produced in more quantities than in an herbicide-susceptible accession to avoid herbicide phytotoxicity (Gaines et al., 2020; Powles, 2010). Results demonstrated that genomic DNA quantities in the reference genes (*CCR* and *PPAN*) were lower than those in target genes, and then values were standardized against the susceptible accession. The comparison of both *ACCase1* and *ACCase2* displayed no significant differences between susceptible and resistant accessions, regardless of *ACCase* isoform and reference gene. Results obtained suggest that gene amplification of *ACCase*, either *ACCase1* or *ACCase2*, is not involved in the observed resistance to fluazifop and pinoxaden (Figure 5). These outcomes are different from those reported in an ACCase-resistant accession of large crabgrass [*Digitaria sanguinalis* (L.) Scop.], where authors determined that the *ACCase* amplification in the resistant accession was found to be between five and seven-fold higher compared with the susceptible population (Laforest et al., 2017). Other than that, gene amplification as a resistance mechanism has not been reported in other ACCase-resistant grass weed species or gene amplification experiments have not been discarded from the resistance mechanism. For example, in an ACCase-resistant accession (fenoxaprop-p-ethyl and pinoxaden) of Japanese foxtail (*Alopecurus japonicus* Steud.), no difference in *ACCase* gene amplification was reported between resistant and susceptible accessions (Xu et al., 2014). Gene amplification of the target gene has been widely described in weeds that are glyphosate-resistant, for instance, Palmer amaranth (*Amaranthus palmeri* S. Watson) or ryegrass (*Lolium* spp.), but also in glufosinate-resistant Palmer amaranth (Carvalho-Moore et al., 2022; Gaines et al., 2010; Salas et al., 2012).

3.5 | Inhibition of metabolism experiments using field seeds

In this research, we assessed the response of fluazifop-resistant johnsongrass plants to malathion and NBD-Cl, P450- and GST-inhibiting molecules, respectively. The rationale of this experiment is because the presence of either molecule, if involved in the resistance mechanism, will have an inhibition effect on the activity of P450 and GSTs, and as a consequence, the fluazifop herbicide will have a "normal" phytotoxicity effect, as happened in the susceptible accession (Gaines et al., 2020; González-Torralva & Norsworthy, 2023). That P450 and GSTs activity inhibition will have an impact in the dry weight of the treated plants, having lower dry weight accumulation. At 21 DAT, no significant differences were found between nontreated control plants (T1) and fluazifop treatment (T2) ($p = .098$). However, the dry weight

of plants sprayed with fluazifop + malathion (T3) was reduced by more than half compared with nontreated control plants (T1) ($p = .001$). Additionally, the use of fluazifop + NBD-Cl also reduced the dry weight of treated plants ($p = .004$) compared with nontreated control plants (T1) (Figure 6). Use of malathion and NBD-Cl along with fluazifop significantly reduced the dry weight of resistant johnsongrass plants compared with nontreated plants, and even though no differences were detected against fluazifop alone, these results suggest the involvement of non-target-site resistance mechanisms in this fluazifop-resistant johnsongrass accession. Furthermore, at 21 DAT, some fluazifop-resistant plants in T3 and T4 were severely injured, and some others were dead. The fact that no significant differences were detected between T3 and T4 compared with fluazifop alone (T2) may be explained by the presence of "susceptible" plants in the original seed batch, which can be further verified by the dispersion of the data (Figure 6). Data obtained in cross-resistance further corroborate that this accession is not homogeneous. On the other hand, susceptible johnsongrass plants were dead in all treatments except for the nontreated control plants, and as consequence, they were neither further analyzed nor included in Figure 6. As both fluazifop-resistant and pinoxaden-resistant plants were derived from the same accession, the fact that the fluazifop-resistant plants may have herbicide metabolism as a resistance mechanism is also suggestive of pinoxaden-resistant plants. Herbicide metabolism of ACCase-inhibiting herbicides has been widely described as a non-target resistance mechanism. Thus, different accessions resistant to ACCase due to herbicide metabolism have been reported. For instance, in a barnyardgrass population resistant to ALS and ACCase-inhibiting herbicides, it was corroborated that metabolic resistance is conferred by a cytochrome P450 gene (Pan et al., 2022). In a perennial ryegrass accession resistant to pinoxaden, both target and non-target site resistance mechanisms were reported. Authors demonstrated that the addition of NBD-Cl had no effect on pinoxaden resistance; however, metabolic resistance was mediated by cytochrome P450s (Ghanizadeh et al., 2022). Studies on Asia minor bluegrass have suggested that several "metabolic" genes were involved in the resistance mechanism to fenoxaprop-p-ethyl, including P450s, GSTs, UDP-glucosyltransferase, and adenosine triphosphate (ATP)-binding cassette transporters (Zhao et al., 2022).

Despite the fact that this fluazifop-resistant accession is confined to a specific area in eastern Arkansas, preventive measures must be taken to reduce its dispersion. Depending on the selection pressure exerted, it would not be rare to visualize in the coming years a johnsongrass accession with multiple target-site mutations within a single plant (as has occurred with *Lolium* spp.) or an accession resistant to different site of action herbicides via "metabolic degradation" with no presence of target-site mutations. Thus, different countermeasures should be taken to have satisfactory johnsongrass control. Our results demonstrated that clethodim still remains a viable chemical tool for its management since it showed excellent control over both fluazifop- and pinoxaden-resistant plants. In summary, the fluazifop-resistant johnsongrass accession, which was also cross-resistant to pinoxaden, deployed target-site mechanisms to surpass herbicide phytotoxicity. The Ile1781Leu and Ile2041Asn target-site mutations play a crucial role in the resistance to fluazifop and pinoxaden herbicides. Additionally, our results also suggest, to a lesser extent, the involvement of herbicide metabolism; nonetheless, this assumption needs to be further corroborated.

AUTHOR CONTRIBUTIONS

F.G.T. and J.K.N., conceptualized and designed the research. F.G.T., performed the experimental work, curated the data, and carried out the formal analysis. J.K.N., obtained funding for this research. F.G.T., provided the original draft of this manuscript. F.G.T. and J.K.N. revised, edited, and approved the final version of this manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest related to this research.

DATA AVAILABILITY STATEMENT

All data are presented in this manuscript.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

ORCID

Fidel González-Torralva https://orcid.org/0000-0001-8222-9949
Jason K. Norsworthy https://orcid.org/0000-0002-7379-6201

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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