Confirmation and differential metabolism associated with quinclorac resistance in smooth crabgrass (*Digitaria ischaemum*)

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Abstract

Quinclorac controls crabgrass (*Digitaria* spp.) in cool- and warm-season turfgrass species. Herbicide-resistant smooth crabgrass [*Digitaria ischaemum* (Schreb.) Schreb. ex Muhl.] biotypes have evolved due to recurrent usage of quinclorac. Two Mississippi populations (MSU1 and MSU2) of *D. ischaemum* were characterized using standard greenhouse dose–response screens to assess their resistance relative to known susceptible populations. Subsequent investigations explored mechanisms of resistance, including examining cyanide accumulation, glutathione *S*-transferase (GST) activity, and the potential involvement of cytochrome P450s in MSU1, MSU2, and a susceptible (SMT2). Resistant populations MSU1 and MSU2 required 80 and 5 times more quinclorac, respectively, to reach 50% biomass reduction than susceptible populations. The SMT2 biotype accumulated three times more cyanide than the resistant MSU1 and MSU2 populations. GST activity was elevated in resistant MSU1 and MSU2 populations. Furthermore, quinclorac concentrations in treated resistant populations were elevated when plants were pretreated with the P450 inhibitor malathion. These findings suggest a non–target site based mechanism of resistance involving the accumulation of cyanide. This may provide a scientific basis for understanding the occurrence of quinclorac-resistant *D. ischaemum*, although further research is needed to investigate potential target-site mechanisms of resistance.

Introduction

Various *Digitaria* species are often undesirable turfgrass weeds (Gannon et al. 2015). Smooth crabgrass [*Digitaria ischaemum* (Schreb.) Schreb. ex Muhl.] causes aesthetic and functional issues in maintained turfgrass settings (Masin et al. 2006) and has become increasingly challenging due to the development of resistance to commonly used herbicides (Abdallah et al. 2006; Derr 2002). The quinolinecarboxylic acid quinclorac (3,7-dichloro-8-quinolinecarboxylic acid, BAS 514H) belongs to a class of highly selective auxin-mimicking herbicides (Grossmann 1998; Grossmann and Kwiatkowski 1995, 2000) developed for control of annual grasses in rice (*Oryza sativa* L.) (Yasuor et al. 2012), small grains (barley [*Hordeum vulgare* L.] and wheat [*Triticum aestivum* L.]) (Franetovich and Peeper 1995; Manthey et al. 1990), grain sorghum [*Sorghum bicolor* (L.) Moench] (Bararpour et al. 2019), and turfgrass (Neal 1990). Quinclorac was commercially introduced for use in turfgrass in 1992 (Malik et al. 2010) to control *D. ischaemum* in various cool- and warm-season turfgrass scenarios (Dernoeden et al. 2003; Enache and Ilnicki 1991). Extensive use of quinclorac has resulted in the evolution of resistant populations of certain species, including false cleavers (*Galium spurium* L.) (Hall et al. 1998), late watergrass [*Echinochloa oryzicola* (Vasinger) Vasinger] (Yasuor et al. 2012), and barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] (Lopez-Martinez et al. 1997).

Research on quinclorac's biokinetic properties indicates that selectivity is not primarily governed by differences in compound uptake, distribution, or metabolism (Chism et al. 1991; Peng et al. 2019). Quinclorac's mechanism of action is not well understood (Rangani et al. 2022; Shaner 2014; Van Eerd et al. 2004). The target process of auxin-mimicking herbicides in some dicot species entails the induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in ethylene biosynthesis (Grossmann 2000a; Hansen and Grossmann 2000; Wei et al. 2000). The ACC is enzymatically converted by 1-aminocyclopropane-1-carboxilic acid oxidase (ACO), leading to the production of ethylene, CO_2, and cyanide (Fipke and Vidal 2016). Cyanide is produced in stoichiometrically equal amounts to ethylene in its biosynthetic pathway (Peiser

et al. 1984). Slowed shoot and root growth, followed by chlorosis of the leaves, is caused by the rapid accumulation of cyanide (Grossmann 1998; Grossmann and Kwiatkowski 1993).

Studies of quinclorac-susceptible grass species have suggested that they are unable to quickly degrade cyanide, resulting in phytotoxic effects from cyanide accumulation in the shoot tissues (Abdallah et al. 2006; Grossmann 2010; Grossmann and Kwiatkowski 2000). However, in quinclorac-resistant grass biotypes, ACC synthase activities are not induced, and there are no significant changes in the cyanide levels (Grossmann and Kwiatkowski 2000), suggesting that enzymatic insensitivity to quinclorac contributes to plant resistance (Fipke and Vidal 2016). An alternative resistance mechanism is possibly related to enhanced herbicide detoxification (Kreuz et al. 1996). Detoxification processes involve different enzymatic families in distinct phases, such as degradation by cytochrome P450 mono-oxygenases (P450s) (Bartholomew et al. 2002; Yuan et al. 2007), conjugation by glutathione *S*-transferases (GSTs) (Bartholomew et al. 2002; Bowles et al. 2005; Reade et al. 2004), and transport into the vacuole or extracellular space by ATP-binding cassette transporters (Bartholomew et al. 2002; Gaines et al. 2020; Yuan et al. 2007).

P450 enzymes are key detoxification agents attributed to non–target site related quinclorac resistance (Chayapakdee et al. 2020; Rangani et al. 2022). Since the late 1980s, the involvement of P450 in metabolic resistance has been documented at the plant level (Kemp and Caseley 1987), and utilizing P450 inhibitors allows understanding whether common P450s are implicated in cross-resistance to distinct sites of action (Preston et al. 1996). A second well-established non–target site resistance gene family is *GST* (Yuan et al. 2007). Several *GST* genes have been identified in quinclorac-resistant *E. crus-galli* (Li et al. 2013) as well as atrazine-tolerant fall panicum (*Panicum dichotomiflorum* Michx.) (Deprado et al. 1995) and velvetleaf (*Abutilon theophrasti* Medik.) (Anderson and Gronwald 1991; Gray et al. 1996).

Aside from resistance mechanisms due to enhanced metabolism, reports of resistance to other auxinic herbicides have occurred due to target-site resistance (TSR). Mutations have been identified in the degron region of the auxin/indole-3-acetic acid (AUX/IAA) protein of multiple auxin-resistant broadleaf species (de Figueiredo et al. 2022; LeClere et al. 2018). AUX/IAAs are a group of repressor proteins that regulate the transcription of auxin-responsive genes due to the level of auxin present in the plant (Taiz and Zeiger 2006). Thus far, no target-site mutations for quinclorac resistance have been identified in any grass species.

Quinclorac-resistant crabgrass (*Digitaria* spp.) is not frequently reported, nor is a resistance mechanism known. This may be because of quinclorac's inconsistent control of crabgrass (*Digitaria* spp.) for a variety of reasons, including environment, but also plant growth stage (Dernoeden et al. 2003; Johnson 1993, 1994). Despite the potential of TSR, its exploration in the context of quinclorac resistance in *Digitaria* spp. has been limited. Research presented herein evaluated two suspected quinclorac-resistant *D. ischaemum* populations in order to characterize resistance and to better understand their resistance mechanism(s). Dose–response experiments were performed to assess the sensitivity of two suspected quinclorac-resistant *D. ischaemum* populations relative to known susceptible populations. Target-site mutations as well as accumulation of cyanide and enhanced metabolism were examined as mechanisms of resistance.

Materials and Methods

Confirmation of Quinclorac Resistance

A greenhouse dose–response study was conducted at Mississippi State University (MSU), near Starkville, MS (33.4533°N, 88.2029°W) to confirm quinclorac resistance in *D. ischaemum*. The study was conducted as a completely randomized design, with four replications repeated twice in the summer of 2021. Two *D. ischaemum* populations suspected of quinclorac resistance (MSU1 and MSU2) were evaluated against known susceptible populations (three *D. ischaemum* populations and one large crabgrass [*Digitaria sanguinalis* (L.) Scop.] population––SMT1, SMT2, SMT3, and LRG, respectively).

Seeds of these *Digitaria* spp. populations (200) were sown in greenhouse flats (50 by 25 cm) filled with Sunshine Professional Growing Mix (SunGro® Horticulture Sunshine Mix No. 2 Basic, Colombier, Quebec, Canada). All flats were maintained under conditions of natural light, average daily temperatures of 25/18 C (day/night), and daily irrigation. When plants reached the 2-leaf stage, they were transplanted into plastic pots (10-cm diameter) filled with native Marietta silt-loam soil (fine-loamy, siliceous, active Fluvaquentic Eutrudepts) with a pH of 6.4 and an organic matter content of 0.4%. Plants were fertilized biweekly with a water-soluble complete fertilizer at 29.4 kg N ha^{-1} (Miracle-Gro® Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Marysville, OH; 24-8-16) and were watered as needed to maintain adequate soil moisture.

Quinclorac treatments of 0.21, 0.42, 0.84, 2.52, 7.56, and 22.7 kg ai ha^{-1} were applied at the 3-leaf stage of growth with a commercial water-based dimethylamine salt of quinclorac (Drive® XLR8, BASF, Research Triangle Park, NC) using an enclosed spray chamber (Generation III track sprayer, DeVries Manufacturing, Hollandale, MN) delivering 374 L ha^{-1}. Methylated seed oil (MSO) was included with all applications (0.5% v/v). The spray chamber was equipped with two nozzles (TeeJet® AIXR 11003 flat-fan nozzles, TeeJet Spraying Systems, Glendale Heights, IL), spaced 48 cm apart, with 240-kPa pressure operated at 3.5 km h^{-1}. Plants were placed 50 cm below the nozzles and were returned to the greenhouse 2 h after treatment (HAT). A nontreated control was included.

Plant injury was visually assessed on a 0% to 100% scale (0% = no injury; 100% = plant death), 28 d after treatment, and the aboveground biomass was harvested, oven-dried at 60 C for 72 h, and weighed. Dry mass data were normalized as a percentage reduction relative to the nontreated control for each population. Herbicide rates were log transformed to facilitate analysis using nonlinear regression. Data were regressed using a log-logistic model within GraphPad Prism v. 9.4.1 (GraphPad Software, San Diego, CA) using the following equation:

$$Y = C + \{(D - C)/(1 + 1^{(\log \text{IC50} - X) * B)})\} \qquad [1]$$

where Y is the dry mass expressed as a percentage of the nontreated control; D and C are the coefficients corresponding to the upper and lower asymptotes; B is the slope of the curve around $\log \text{IC}_{50}$; and IC_{50} gives a response at 50% of the regressed y scale. The value of IC_{50} corresponds to the dose that causes a 50% response (GR_{50}).

Target-Site Sequence Analysis

RNA was extracted from MSU1, MSU2, and SMT2 using the Direct-zol RNA MiniPrep Plus Kit (Zymo Research, Irvine,

CA). Tissue was freeze-dried with liquid nitrogen and ground with a mortar and pestle before following the standard protocol in the kit. After extraction, RNA samples were quality checked on a NanoDrop™ One spectrophotometer (Thermo Scientific™, Waltham, MA) before Illumina sequencing (Novogene, Sacramento, CA). Due to the lack of a *D. ischaemum* genome and without a true understanding of the mechanism of quinclorac resistance, sequence analysis was limited to searching for known target-site mutations within the degron region of *AUX/IAA*s. FASTQ sequences were assembled using Trinity (https://github.com/trinityrnaseq/trinityrnaseq.git) de novo transcriptome assembly and were subsequently annotated using Trinotate (https://github.com/Trinotate/Trinotate.git). Reads were then extracted based on gene (*AUX/IAA*) annotations and aligned to search for mutations within the degron. Reads for resistant MSU1 and MSU2 and the known susceptible SMT2 can be found on the National Center for Biotechnology Information (NCBI) website with accession numbers SAMN39243124, SAMN39243125, and SAMN39243126, respectively.

Plant Material and Growing Conditions for Metabolism Studies

Greenhouse research was conducted to assess cyanide production, GST activity, and quinclorac metabolism (using the P450 inhibitor malathion) in *D. ischaemum*. Studies were conducted as a completely randomized design, with four replications repeated twice in the spring of 2022 for cyanide accumulation, summer of 2022 for GST activity, and spring of 2023 for P450 inhibition. Quinclorac-resistant populations MSU1 and MSU2 were evaluated against SMT2. Plants were sown from seed in greenhouse flats (50 by 25 cm) filled with Sunshine Professional Growing Mix. When plants reached the 2-leaf stage of growth, individuals were transplanted into plastic pots (10-cm diameter) with commercial potting mix (Promix BX, BX general purpose, Premier Tech Horticulture, Quakertown, PA) and were maintained in conditions similar to those previously described.

Plants were treated with quinclorac at the 3-leaf stage of growth for a total of five treatments for cyanide studies (0, 0.42, 0.84, 2.52, and 7.56 kg ai ha^{-1}) and three treatments for the GST assay (0, 0.84, and 7.56 kg ai ha^{-1}). Quinclorac metabolism studies using malathion as a P450 inhibitor were conducted with four treatments, including 0.84 kg quinclorac ha^{-1} alone, 2 kg malathion ha^{-1} alone, 2 kg malathion ha^{-1} followed by 0.84 kg quinclorac ha^{-1} (malathion was sprayed 2 h before quinclorac), and a nontreated control. All quinclorac applications included MSO (0.5% v/v). Treatments were applied in a water carrier volume of 374 L ha^{-1} using an enclosed spray chamber, as previously described. The study explores different quinclorac treatments at varying dose rates to unravel the mechanisms of toxicity. Lower doses were employed to understand subtle cellular reactions, while higher doses were used to reveal more severe effects. This approach was intended to comprehensively examine quinclorac's impact across a range of concentrations.

Cyanide Determination Studies

Three days after treatment, plant foliage was collected for cyanide determination using a modification of the method developed by Grossmann and Kwiatkowski (1993). Aboveground foliage in individual pots was harvested, bagged, and refrigerated (3 C) before analysis. Samples were thawed and macerated into small pieces using scissors before 1.0 g of foliar tissue was transferred to a reagent tube (Nunc 50-ml conical centrifuge tube, Thermo Fisher

Scientific, Fair Lawn, NJ). One hundred microliters of sodium hydroxide (NaOH, 1.5 N) was applied to a filter paper (Whatmann Qualitative Filter Paper Grade 1, Millipore Sigma, Burlington, MA) attached to the bottom of a tube cap, where it remained suspended above the plant material. Three milliliters of H$_2$SO$_4$ (0.92 M) were injected into each sample, and the mixture was stirred at 20 C for 20 h to allow the evolved cyanide to become trapped in the NaOH-saturated filter paper. The filter was eluted with 3 ml NaOH (0.1 N). A 200-μl aliquot of the eluent was colorimetrically analyzed at 580-nm absorbance using a NanoDrop spectrophotometer as previously described. Plant tissue cyanide content was based on a standard curve composed of six serial dilutions of potassium cyanide.

Data were expressed as percentage change relative to the nontreated control of each population and were subjected to an ANOVA ($\alpha = 0.05$) using SAS PROC GLM v. 9.4 (SAS Institute, Cary, NC). A nonlinear regression model (one-phase association) and pairwise *F*-test comparisons, variable slope regression curves ($\alpha = 0.05$) were conducted to analyze the data in GraphPad Prism v. 9.4.1, as follows:

$$Y = Y_0 + a(1 - e^{-k*x}) \qquad [2]$$

where *Y* represents the percentage increase in cyanide production, *a* and *K* are constants generated by the analysis, Y_0 is the *y* intercept, and *X* is the quinclorac application rate. Cyanide production was compared using Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$) to determine whether the populations differed in response to various treatments.

Glutathione S-Transferase Studies

Foliage was collected similarly and weighed for a total of 1.0 g foliage at 0, 72, and 168 HAT before being wrapped in aluminum foil and frozen in liquid nitrogen. Samples were stored at −80 C. Tissue extracts were prepared by homogenizing leaf tissue in liquid nitrogen. Samples were ground to a powder using a mortar and pestle before a 1-ml extraction buffer containing 0.2 M Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, and 10% polyvinylpolypyrrolidone was added. The homogenates were vortexed and centrifuged for 20 min at 12,000 rpm and then filtered. A 10-μl aliquot of filtered supernatant was placed in a 96-well plate before 190 μl of a solution containing Dulbecco's phosphate buffer, reduced glutathione, and 1-chloro-2,4-dinitrobenzene (CDNB) from the GST assay kit (Sigma-Aldrich CS0410, St Louis, MO) was added. Enzyme activity was determined spectrophotometrically (Agilent BioTek Synergy LX Multi-Mode Reader, Fisher Scientific Company, Pittsburgh, PA) by measuring the conjugation of L-glutathione (GSH) to CDNB. Conjugation was accompanied by an increase in absorbance at 340 nm that is directly proportional to the GST activity in the sample. An ANOVA, followed by a pairwise-comparisons test using SAS PROC GLM v. 9.4 software, was conducted to determine the differences in GST-specific activity among treatment rates for each population. GST-specific activity of each biotype was also compared with respective control treatments expressed as percentage change relative to the nontreated control of each population.

Quinclorac Metabolism Studies with P450 Inhibitor

Aboveground foliage was harvested at 72 and 168 HAT and 1.0-g samples were placed in 2-ml tubes along with five ceramic beads per tube (Thermo Fisher Scientific, Waltham, MA). Samples were

then homogenized for 1 min using Precellys® bead-beating homogenizer solution (Bertin Instruments, Montigny-le-Bretonneux, France) before 900 µl of methanol was added and samples were homogenized for another 1-min period. All samples were centrifuged at 13,000 rpm for 10 min, and 1.5 ml of supernatant was filtered using a 13-mm syringe (Biomed Scientific International SFPTFE013022, https://www.biomedscientific.com). Filtered samples were added into glass vials and were stored at 4 C until analysis.

Standard solutions of quinclorac were prepared by serial dilution to create working solutions at: 0.07, 0.16, 0.313, 0.625, 1.25, and 2.5 mg L^{-1}. Solutions at these concentrations were further used to generate the calibration curve with a correlation coefficient (R^2) value of >99.8%. Analysis of standard solutions and samples was performed via an Agilent 1100 series HPLC equipped with degasser, auto-sampler, quaternary pump (QuatPump), thermo-statted column compartment, fluorescence detector, and diode array detector (DAD). Data were processed using software from Agilent Chemstation (v. A.10.02) with a spectral module (Agilent Technologies, Wilmington, DE).

The sampling column was an Alltech Adsorbsphere C18 column: 150 mm by 4.6 mm with a particle size of 3 µm. The injection volume was set to 10 µl with column temperature set at 30 C and DAD detection at 240 nm. Flow rate was 0.5 ml min^{-1} with a stop time of 10 min and a post time of 2 min. The eluent comprised 60% molecular-grade water with 0.2% acetic acid and 40% acetonitrile. The peak intensities of the standard solutions were used for creating the calibration curve and identifying the peaks in the plant samples.

An ANOVA, followed by a means-separation test using SAS PROC GLM v. 9.4 software ($\alpha = 0.05$), was conducted to determine differences in quinclorac concentration among treatments for each population. Quinclorac concentration was compared by Fisher's protected LSD test ($\alpha = 0.05$) within SAS PROC GLM v. 9.4 software to determine whether populations differed in response to various treatments.

Results and Discussion

Confirmation of Resistance to Quinclorac

Digitaria ischaemum populations that survived field applications of quinclorac were confirmed resistant in greenhouse dose–response screens. Data were similar between experimental runs and were pooled. Visual control of resistant and susceptible crabgrass *D. ischaemum* populations varied in response to increasing concentrations of quinclorac (Figure 1). Quinclorac at 8.5 and 0.6 kg ha^{-1} injured MSU1 and MSU2 50%, respectively (Table 1). Quinclorac at 0.84 kg ha^{-1} controlled susceptible populations greater than 98% (Figure 1), which was consistent with dry mass reduction results.

Dry mass of the suspected resistant populations, MSU1 and MSU2, was reduced 50% by 8 and 0.5 kg quinclorac ha^{-1}, respectively (Table 1). The MSU1 population, required 80 times the amount of quinclorac to achieve similar levels of dry mass reduction as the susceptible populations (Figure 2). Although MSU2 presented lower resistance than MSU1, based on the GR_{50} value, this population still required five times the amount of herbicide to reduce dry mass relative to susceptible populations.

Pairwise *F*-tests of the regressed model confirmed that MSU1 and MSU2 differed in their response to quinclorac relative to susceptible populations. Based on the calculated GR_{50} value and resistance ratio, *D. ischaemum* MSU1, MSU2, and SMT2 were selected for further experimentation.
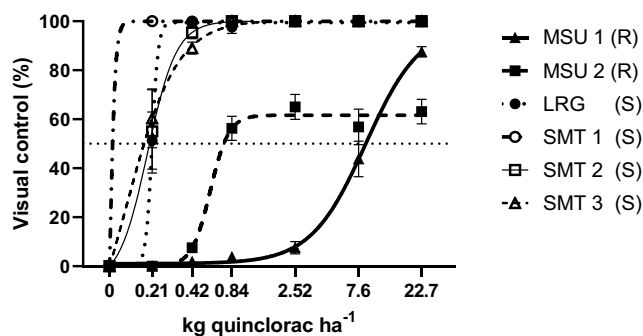

Figure 1. Visual control of susceptible and resistant *Digitaria ischaemum* crabgrass at 28 d after treatment. The crabgrass was at the three-leaf stage of growth when herbicide was applied. Control was visually assessed on a 0%–100% scale (0% = no plant death; 100% = complete plant death). The data were normalized relative to the nontreated control. Error bars show the standard error of the mean. R, resistant; S, susceptible.

Target-Site Sequence Analysis

Reported resistance does not appear to be due to TSR. Analysis focused on the degron region of the AUX/IAA. Comparative analyses between susceptible and resistant biotypes did not elucidate any mutations within the AUX/IAA proteins.

Cyanide Accumulation in Quinclorac-Resistant Digitaria ischaemum

Cyanide accumulation was similar between study runs; thus, data were pooled. Cyanide content in all populations increased in response to quinclorac rates (Figure 3). The susceptible population had a greater increase in cyanide accumulation than the resistant MSU1 and MSU2 populations.

Application of 7.56 kg quinclorac ha^{-1} caused the susceptible population to exhibit a peak in cyanide content greater than 600% relative to untreated plants from the same population (Figure 3). The MSU1 and MSU2 populations, however, produced 200% and 230% less cyanide at 7.56 kg quinclorac ha^{-1} treatment, respectively, relative to the susceptible population. Studies of quinclorac-resistant *Echinochloa*, *Digitaria*, *Brachiaria*, and *Setaria* reported similarly that the ACC synthase activity and cyanide accumulation were unaffected by the quinclorac treatments (Abdallah et al. 2006; Grossmann 2000a; Lopez-Martines et al. 1997; Yasuor et al. 2012).

Shoots from the SMT2 population exhibited greater damage than did those of the MSU1 and MSU2 populations. Shoots of the resistant populations were undamaged, and arrested growth was only observed at the highest quinclorac rate. Previous studies (Grossmann and Kwiatkowski 1995) have documented heightened ACC synthase activity in the roots of susceptible plants, such as *E. crus-galli*, upon exposure to quinclorac. Grossmann and Scheltrup (1997) suggested that the accumulated ACC from the increased ACC synthase activity in roots is transported acropetally to shoots, where it acts as a signal, stimulating ACC synthase activity, and finally triggers the release of cyanide, along with the cyanide produced by oxidation. Our findings suggest that quinclorac toxicity in the susceptible population was primarily caused by cyanide accumulation in the shoot tissues.

Cyanide levels rise due to increased ethylene biosynthesis (Grossmann 1998; Grossmann and Kwiatkowski 1993, 2000; Grossmann and Scheltrup 1997). Ethylene is a plant hormone that is responsible for several physiological reactions, and its presence is a normal stress response in various plants experiencing

Table 1. Effects of quinclorac applied at the 3three-leaf stage of growth in greenhouse dose–response screens conducted at Mississippi State University.[a]

Population[b]	Latitude, longitude	Dry mass reduction				Visual control			
		GR$_{50}$[c]	95% CI[d]	R^2	R/S[e]	GR$_{50}$[c]	95% CI[d]	R^2	R/S[e]
MSU1 (R)	33.47211° N, −88.77739° W	8.02	6.388 to undefined	0.48	92.54	8.59	7.076 to 19.03	0.93	60.17
MSU2 (R)	33.46645° N, −88.78133° W	0.49	0.004 to 1.123	0.44	5.65	0.58	NA	0.85	4.04
SMT1 (S)	33.21797° N, −87.54164° W	0.09	NA	0.75	1.04	0.10	NA	0.99	0.70
SMT2 (S)	33.48564° N, −88.79139° W	0.08	Undefined to 0.260	0.54	0.92	0.19	0.0172 to 0.227	0.81	1.36
SMT3 (S)	33.48525° N, −88.79722° W	0.09	2.441 to undefined	0.81	1.04	0.13	Undefined to 0.199	0.89	0.94
LRG (S)	33.4533° N, −88.7943° W	0.14	NA	0.72	1.62	0.21	0.177 to 0.223	0.90	1.46

[a]Research was replicated twice in time. Data were pooled for analysis.
[b]R, resistant; S, susceptible.
[c]GR$_{50}$, the dose required (kg ai ha^{-1}) to inhibit the growth of *Digitaria* populations by 50%.
[d]Undefined: When representing the upper limit in this manner, it signifies a 95% confidence that the parameter exceeds the lower limit, without providing insight into the potential magnitude of the parameter. Conversely, when presenting the lower limit in this manner, there is a 95% confidence that the parameter is less than the upper limit, yet without indicating the potential smallness of the parameter. CI, confidence interval; NA, not available due to poor fit to the log-logistic model.
[e]R/S ratio is the ratio of the GR$_{50}$ value tested biotype to the average of GR$_{50}$ value of the SMT populations.
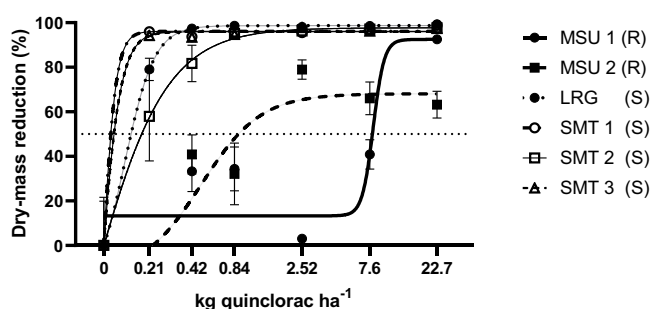

Figure 2. Aboveground dry mass of susceptible and resistant *Digitaria ischaemum* at 28 d after treatment. The *D. ischaemum* was at the 3-leaf stage of growth when herbicide was applied. Data were expressed as a percentage decrease of the mean dry mass of the nontreated control. Error bars show the standard error of the mean. R, resistant; S, susceptible.

Figure 3. Cyanide content of susceptible and resistant *Digitaria ischaemum* at 3 d after treatment. Data were expressed as a percentage increase of the mean control. Error bars indicate the standard error of the mean. R, resistant; S, susceptible.

unfavorable conditions (Morgan and Drew 1997). In addition to the hydrolysis of cyanogenic glycosides, the oxidation of ACC by ACO to produce ethylene generates cyanide as a by-product (Tittle et al. 1990; Yip and Yang 1988). This process is the primary source of cyanide in many plant tissues (Goudey et al. 1989). While the current data set provides evidence supporting cyanide as the primary driver of injury in the susceptible population, it is essential to acknowledge the absence of specific ethylene data in this study. Without direct measurements of ethylene accumulation, a conclusive determination of its role in toxicity remains challenging. Even though it has been widely documented that ACC stimulates ethylene synthesis in response to auxin herbicides, endogenous cyanide was more likely to be the key factor in quinclorac's phytotoxic effect (Grossmann and Kwiatkowski 1995). Peiser et al. (1984) studied the formation of cyanide from ACC synthase and found that the ACC transformation produced an equivalent amount of cyanide to ethylene. This has been demonstrated by treating a detached shoot with ACC, which resulted in the simultaneous formation of ethylene and cyanide. Recently, Song et al. (2022) demonstrated that ACC, ethylene, and cyanide content in shoot tissue increased when cyanide was applied through the grass roots.

Glutathione S-Transferase in Quinclorac-Resistant Digitaria ischaemum

The GST-specific activity was similar between runs; thus, data were pooled. Overall, in the presence of various quinclorac rates, at 0, 72, and 168 HAT, the GST-s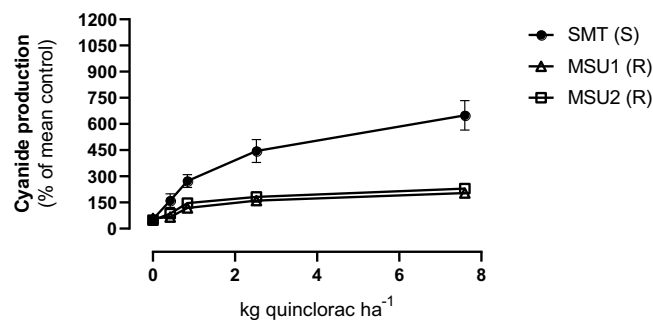pecific activity in the MSU1 and MSU2 populations was higher than in the SMT2 population (Table 2). Pairwise comparison tests ($\alpha = 0.05$) confirmed that the GST-specific activity differed between the susceptible and two resistant populations but did not differ due to rate.

In the absence of quinclorac, there was a significant difference in GST-specific activity between the two resistant MSU populations and the susceptible SMT2 population (Table 2). Regardless of harvest time, MSU1 and MSU2 exhibited more GST-specific activity than SMT2. The nontreated MSU1 population had GST-specific activity of 0.0121, 0.0177, and 0.0147 µmol min^{-1} ml^{-1} protein at 0, 72, and 168 HAT, respectively (Table 2). At 0, 72, and 168 HAT, MSU2 had 0.0105, 0.0174, and 0.0145 µmol min^{-1} ml^{-1} protein, respectively (Table 2). However, SMT2 had only 0.0059, 0.0097, and 0.0085 µmol min^{-1} ml^{-1} protein at 0, 72, and 168 HAT, respectively (Table 2). The presence of elevated GST activity in resistant populations, even at 0 kg quinclorac ha^{-1}, suggests that GST may be a non–target site mechanism of resistance. After conducting a gene expression analysis in the absence of herbicide treatment, Wright et al. (2018) reported that GST was expressed in herbicide-resistant junglerice [*Echinochloa colona* (L.) Link] populations. Similarly, Cummins et al. (2013) reported that increased expression of GST was involved in herbicide resistance in select populations of blackgrass (*Alopecurus myosuroides* Huds.) and annual ryegrass (*Lolium rigidum* Gaudin).

A key role played by GSTs is their ability to deactivate toxic compounds (Marrs 1996). After quinclorac treatment at 0.84 and 7.56 kg ai ha^{-1}, the GST-specific activity was elevated in MSU1 and MSU2 relative to SMT2, which suggests that quinclorac resistance in *D. ischaemum* may be due to enhanced GST activity, resulting in an enhanced capacity to detoxify the herbicide via GSH

Table 2. Specific glutathione *S*-transferase (GST) activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in *Digitaria ischaemum* with quinclorac treatments.

| | | Specific GST (CDNB) activity[c] | | |
| | | μmol min^{-1} ml^{-1} protein | | |
Population[a]	HAT[b]	Nontreated	0.84 kg quinclorac ha^{-1}	7.56 kg quinclorac ha^{-1}
MSU1	0	0.0121 ± 0.0009	0.0126 ± 0.0009	0.0132 ± 0.0009
MSU2		0.0105 ± 0.0009	0.0117 ± 0.0009	0.0125 ± 0.0009
SMT2		0.0059 ± 0.0009	0.0055 ± 0.0009	0.0055 ± 0.0009
MSU1	72	0.0177 ± 0.0019	0.0207 ± 0.0019	0.0225 ± 0.0019
MSU2		0.0174 ± 0.0019	0.0192 ± 0.0019	0.0205 ± 0.0019
SMT2		0.0097 ± 0.0019	0.0079 ± 0.0019	0.0066 ± 0.0019
MSU1	168	0.0147 ± 0.0014	0.0163 ± 0.0014	0.0171 ± 0.0014
MSU2		0.0145 ± 0.0014	0.0159 ± 0.0014	0.0166 ± 0.0014
SMT2		0.0085 ± 0.0014	0.0089 ± 0.0014	0.0077 ± 0.0014

[a]MSU1 and MSU2 are resistant to quinclorac. SMT2 is susceptible.
[b]HAT, hours after treatment.
[c]Data were pooled across the two runs of the experiment. Values are presented as mean ± SE.

conjugation (Cummins et al. 2011; Edwards et al. 2000; Kreuz et al. 1996), possibly aiding in its transport into the vacuole, away from its site of action. As a result, GSH could support herbicide compartmentalization in resistant biotypes (Reade et al. 2004). The first GST activity caused by GSH conjugation was observed in atrazine-resistant sorghum (*Sorghum vulgare* Pers. 'North Dakota 104') and triazine-resistant maize (*Zea mays* L.) (Frear and Swanson 1970; Lamoureux et al. 1970). Various herbicide-induced GSTs have since been isolated and characterized for their roles in herbicide tolerance, selectivity, and resistance across a wide range of crop and weed species (Marrs 1996). Xu et al. (2015) adapted gene ontology analysis to obtain an overview of quinclorac-response genes in rice, finding that *GST* was significantly increased among all quinclorac-response genes. Numerous instances of herbicide resistance and detoxification have been documented (Dixon et al. 2003; Karavangeli et al. 2005; Labrou et al. 2005; Marcacci et al. 2005). Nakka et al. (2017) revealed rapid atrazine detoxification in Palmer amaranth (*Amaranthus palmeri* S. Watson) due to GSH conjugation. GSTs have also been associated with herbicide resistance in broadleaf (Gronwald et al. 1989; Ma et al. 2013) and grassy weeds (Bakkali et al. 2007).

The changes in GST activity following quinclorac treatment varied chronologically when expressed as a percentage change compared with the nontreated control (Figure 4). GST activity in SMT2 decreased by 8% and 11% after 0.84 kg quinclorac ha^{-1} at 0 and 72 HAT, respectively. However, the activity increased by about 25% at 168 HAT. This result suggests that GST as an antioxidant enzyme increases in response to a rise of reactive oxygen species (ROS) production caused by the high rates of quinclorac (Fernández et al. 2010). GST activity in SMT2 decreased to about 20% after 7.56 kg quinclorac ha^{-1} treatment at 72 HAT, while the GST activity in MSU1 increased by more than 25% at 72 HAT, but decreased at 168 HAT for all quinclorac rates. GST activity in MSU2 increased the most at 0 HAT, followed by a 72 and 68 HAT decrease in all quinclorac treatments. These results suggest that variation in changes in GST activity across all populations at all harvesting times is considered a marker for plant response to multiple environmental stresses, including but not limited to different quinclorac rates (Edwards et al. 2000; Mars 1996).

Quinclorac Metabolism Studies with P450 Inhibitor

When malathion was applied 2 h before quinclorac, quinclorac concentration within foliage was higher than in plants treated with
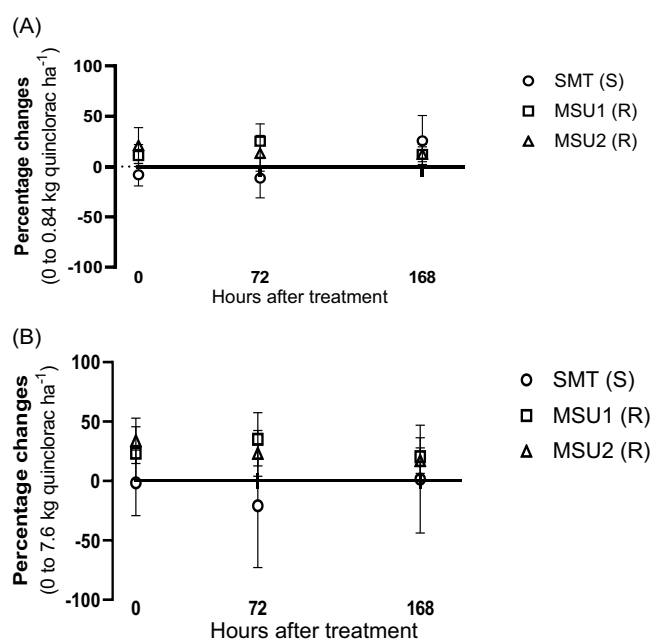

Figure 4. Specific glutathione *S*-transferase activity changes toward 1-chloro-2,4-dinitrobenzene in *Digitaria ischaemum* tissues after (A) 0.84 kg quinclorac ha^{-1} or (B) 7.56 kg quinclorac ha^{-1}. Data were expressed as a percentage change of the nontreated. Values are presented as mean ± SE. Error bars indicate the standard error of the mean. R, resistant; S, susceptible.

quinclorac alone for all populations (Figure 5) during both harvest times. The SMT2 populations exhibited higher quinclorac concentrations than MSU1 and MSU2 when quinclorac was applied alone. However, the application of malathion alone did not affect all populations equally (Figure 5).

Quinclorac concentration was higher in the susceptible SMT2 population treated with malathion plus quinclorac relative to those treated with quinclorac alone at 168 HAT. Conversely, no difference in quinclorac concentration was observed between SMT2 treated with malathion plus quinclorac and quinclorac alone at 72 HAT. These findings suggest a time-dependent influence, indicating that the presence of malathion may affect quinclorac concentrations in the susceptible SMT2 population, with the difference becoming more pronounced at later time points. According to Huang et al. (2017), this extended persistence can be due to quinclorac's high stability and moderate degradation rate.
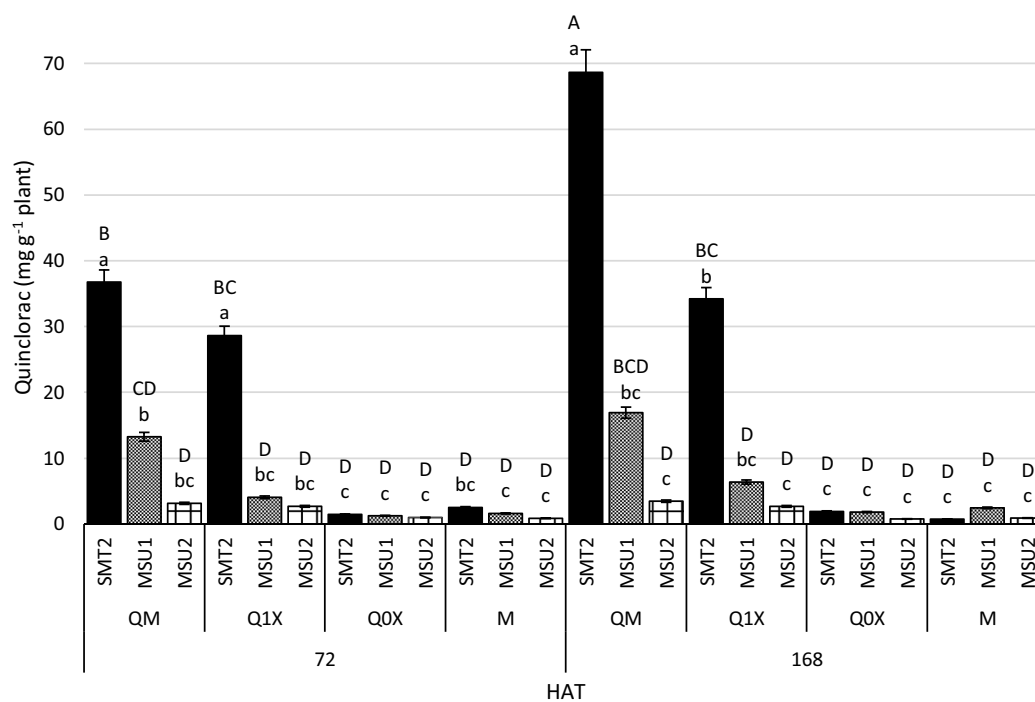
Figure 5. Amount of quinclorac (mg g^{-1} plant, mean ± SE) detected by liquid chromatography–diode array detector (DAD) in extracts of plants (aerial parts) from susceptible (SMT2) and two resistant (MSU1 and MSU2) *Digitaria ischaemum* populations, with previous application of 2 kg malathion ha^{-1} followed by 0.84 kg quinclorac ha^{-1} (QM), 0.84 kg quinclorac ha^{-1} (Q1X), no application of quinclorac or malathion (Q0X), and application of 2 kg malathion ha^{-1} alone (M), evaluated at 72 and 168 h after treatment (HAT). Means within an evaluation time and treatment with the same capital letter are not significantly different at the 5% level as determined by Fisher's protected least significant difference (LSD) test; means for the same population and evaluation time with the same letter are not significantly different at the 5% level as determined by the LSD test.

Recovered quinclorac concentrations from resistant MSU1 and MSU2 populations were similar, regardless of whether malathion was applied prior or quinclorac was applied alone. However, when the combination of malathion and quinclorac was applied, quinclorac concentrations were consistently higher in both MSU1 and MSU2 compared with when quinclorac was applied alone. This suggests that the combined application of malathion and quinclorac results in elevated quinclorac concentrations in both resistant MSU1 and MSU2 populations, highlighting a unique interaction caused by malathion and quinclorac under these conditions. The MSU1 population treated with malathion followed by quinclorac had higher concentrations of quinclorac than plants treated with quinclorac alone, with 13.26 and 4.06 mg g^{-1} plant, respectively, 72 HAT. At the same harvest time, MSU2 treated with the combination of malathion and quinclorac had a greater quinclorac concentration than plants treated with quinclorac alone (3.14 and 2.69 mg g^{-1} plant, respectively). Results suggest that the metabolism of quinclorac mediated by malathion-induced P450 enzymes enhances quinclorac sensitivity and improved quinclorac efficacy across all populations (Yasuor et al. 2012).

Quinclorac's Mechanisms of Action and Metabolism

Xenobiotic metabolism, such as herbicide metabolism in weeds, can be greatly aided by detoxification of plant endogenous enzymes such as GST, glucosyltransferases, and/or cytochrome P450 (Ghanizadeh and Harrington 2017). Similarly, Wright et al. (2018) found that combining quinclorac with malathion lowered the resistance level of quinclorac-resistant *E. colona*.

Results presented herein demonstrate enhanced metabolism in two quinclorac-resistant *D. ischaemum* populations, MSU1 and

MSU2. Excess cyanide concentrations have been shown to harm antioxidant systems (Rai et al. 2020). Because these antioxidant systems play a critical role in quenching ROS generated in plants under various abiotic stresses, their disruption can disrupt their redox homeostatic mechanisms (Kebeish et al. 2017). As a result, a synchronous overexpression of genes encoding a cyanide-degrading enzyme and an oxidative stress–relieving protein, such as GST, could improve the cyanide assimilation capacity in resistant plants by preserving the redox homeostasis (Kebeish et al. 2017).

Results suggest that the resistant populations MSU1 and MSU2 had higher GST-specific activity than the susceptible population, implying that GST family enzymes are involved in cyanide degradation in resistant populations. These findings are supported by previous research on the overexpression of *GST* genes in transgenic tobacco (*Nicotiana* L.), which allows the tobacco to grow normally, even when exposed to high levels of cyanide (Kebeish et al. 2017). In this case, the GST appears to act synergistically in plants, stimulating the antioxidant-scavenging machinery to deal with the high level of ROS production (Kebeish et al. 2017). Other authors (Rangani et al. 2022) have found that several detoxification genes, including cytochrome P450 and GST, were elevated in *E. colona* resistant to quinclorac and propanil.

The discovery that the P450 inhibitor malathion improved quinclorac sensitivity in the resistant plants also supports the participation of P450-mediated metabolism (Yasuor et al. 2012). However, metabolism may not play an essential role in quinclorac sensitivity, as no obvious association between it and herbicide sensitivity has been found among the plant species studied (Grossmann 2000b). Multiple mechanisms may be responsible for quinclorac resistance in *D. ischaemum*. The lack of cyanide biosynthesis in resistant populations treated with quinclorac

suggests that the observed resistance is due to a lack of activation of the auxin response pathway (Abdallah et al. 2006). This could be caused by mutations in certain auxin receptors, such as AFB5, which has already been found to interact with quinclorac (Lee et al. 2014); however, preliminary genomic studies of MSU1, MSU2, and SMT2 failed to identify known or novel mutations within the degron region, as all potential novel mutations were present in all three populations. Although alternative mutational events remain plausible, until further research is done to confirm the mechanism of resistance to quinclorac, it will be difficult to confidently identify point mutations as the source of resistance. In essence, the situation is complex, and while some aspects have been explored, a comprehensive understanding requires further investigation.

Failed field-scale control of *D. ischaemum* is common when quinclorac is used as a postemergence herbicide. The mechanisms of resistance in quinclorac-resistant *D. ischaemum* have only been reported once in California (Abdallah et al. 2006). Due to the possibility of a non–target site based mechanism of resistance being present in plants that also possess target-site mutations, the true prevalence of non–target site resistance in *D. ischaemum* may be underreported. Future research should investigate the potential target-site mechanism in resistant populations, provide an overview of the transcription map for *D. ischaemum* under quinclorac treatment for the response genes, and, most importantly, obtain a number of candidate genes––especially the cytochrome P450 and *GST* genes that may act as markers for potential quinclorac resistance.

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