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Physiological assessment of non–target site restistance in multiple-resistant junglerice (*Echinochloa colona*)

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Abstract

Herbicide-resistant *Echinochloa* species are among the most problematic weeds in agricultural crops globally. Recurring herbicide selection pressure in the absence of diverse management practices has resulted in greater than 20% of sampled *Echinochloa* populations from rice (*Oryza sativa* L.) fields demonstrating multiple resistance to herbicides in Arkansas, USA. We assessed the resistance profile and potential mechanisms of resistance in a multiple herbicide–resistant junglerice [*Echinochloa colona* (L.) Link] (ECO-R) population. Whole-plant and laboratory bioassays were conducted to identify the potential mechanisms of non–target site resistance in this population. ECO-R was highly resistant to propanil (>37,800 g ha^{-1}) and quinclorac (>17,920 g ha^{-1}) and had elevated tolerance to cyhalofop (R/S = 1.9) and glufosinate (R/S = 1.2) compared to the susceptible standard. The addition of glufosinate (590 g ha^{-1}) to cyhalofop (314 g ha^{-1}), propanil (4,500 g ha^{-1}), or quinclorac (560 g ha^{-1}) controlled ECO-R 100%. However, cyhalofop applied with propanil (48% control) or quinclorac (15% control) was antagonistic. The application of the known metabolic enzyme inhibitors malathion, carbaryl, and piperonyl butoxide increased control of ECO-R with propanil (>75%) but not with other herbicides. Neither absorption nor translocation of [^{14}C]cyhalofop or propanil was different between ECO-R and ECO-S. [^{14}C]Quinclorac absorption was also similar between ECO-R and ECO-S; however, translocation of quinclorac into tissues above the treated leaf of ECO-R was >20% higher than that in ECO-S. The abundance of metabolites was higher (~10%) in the treated leaves of ECO-R than in ECO-S beginning 48 h after treatment. The activity of β-cyanoalanine synthase, which detoxifies hydrogen cyanide, was not different between ECO-R and ECO-S following quinclorac treatment. Resistance to propanil was due to herbicide detoxification by metabolic enzymes. Resistance to quinclorac was due to a detoxification mechanism yet to be understood. The reduction in sensitivity to cyhalofop and glufosinate might be a secondary effect of the mechanisms conferring high resistance to propanil and quinclorac.

Introduction

Rice (*Oryza sativa* L.) is a global agricultural commodity, feeding more than 50% of the world's population and being produced across six continents (Prasad et al. 2017). Global trade is dominated by five exporter countries representing 74.7% of the net trade: India, Thailand, the United States, Pakistan, and Vietnam (Wailes and Chavez 2012). The United States accounts for 9.6% of the global export market (Workman 2018). Rice production in the United States arises mostly from the Midsouth region consisting of Arkansas, Mississippi, Missouri, and Louisiana. Maximizing yield within these regions is critical, and controlling weeds is of utmost importance, as they are the greatest yield-limiting biotic factor (Chauhan et al. 2017). Weedy species in rice production areas throughout the world are highly diverse because of the different environments and management systems employed within various countries. *Echinochloa* species are consistently ranked as the most common weeds impacting rice production and are the most common and troublesome weeds in several major cropping systems (Alarcón-Reverte 2013; Valverde et al. 2000; Van Wychen 2017). This global weed has a unique biology that originates from its early co-domestication with rice and its adaptive evolutionary traits, which allow for aggressive competition and phenotypic plasticity leading to crop mimics, making management difficult (Barrett 1983; Yang et al. 2015).

The most dominant *Echinochloa* species in rice-based cropping systems are junglerice [*Echinochloa colona* (L.) Link] and barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], which are most similar to each other morphologically and are considered the same for management purposes (Burgos et al. 2015; Rouse et al. 2018). In Arkansas, USA, herbicides supplemented with cultural management including permanent flooding and crop rotation to soybean [*Glycine max* (L.) Merr.] are the primary methods of weed management in rice production.

Herbicide-based strategies have been focused on *Echinochloa* management since the early 1950s, when propanil, a photosystem II–inhibiting herbicide (WSSA Group 7), was commercialized (Hoagland et al. 2004; Talbert and Burgos 2007). In the early 1990s, quinclorac, an auxinic herbicide (WSSA Group 4), and several acetyl CoA-carboxylase–inhibiting herbicides (WSSA Group 1) were commercialized. Starting in the early 2000s, the imidazolinones (i.e., imazamox, imazethapyr), which are among the acetolactate synthase–inhibiting herbicides (WSSA Group 2), were made available for use in rice with the Clearfield® rice technology. This was the first non-transgenic, herbicide-resistant rice. While each new herbicide provided excellent control of *Echinochloa* spp., their overuse resulted in rampant herbicide-resistant populations in Arkansas beginning in the 1990s (Carey et al. 1995; Norsworthy et al. 2012). This problem is not unique to Arkansas or the United States, as herbicide-resistant *Echinochloa* were first identified in 1986 and have been reported in 14 countries since (Heap 2019). This genus contains several of the most herbicide resistance–prone weeds in the world. Such status is attributed to the high degree of genetic diversity and adaptive abilities of *Echinochloa* (Heap 2014). While resistance to various single herbicides is a concern, populations with resistance to two or more herbicide modes of action are increasing (Rouse et al. 2018). To determine the cause of multiple resistance, comprehensive physiological and genomic studies of multiple-resistant populations are of the utmost importance.

Genetics and plant physiology play a significant role in herbicide-resistance evolution among weedy species. Two terms are often used to categorize the underlying mechanisms of resistance: target site (TSR) and non–target site (NTSR). TSR, arising from high-dose selection leading to selection of individuals with amino acid mutations at the herbicide binding domain, is the most prevalent and results in resistance to either only a single herbicide or cross-resistance to herbicides targeting the same site of action (Devine and Shukla 2000; Gardner et al. 1998). NTSR is a complex and polygenic adaptation to herbicide selection, involving several processes that limit the concentration of the herbicide at its target (Délye 2013). Although NTSR generally results from low-dose selection pressure, NTSR may also arise from intense, high-dose selection pressure. Such is the case with *Echinochloa* resistance to propanil, which is used at 4.48 kg ha^{-1} and has selected for populations with increased capability to detoxify the herbicide (Carey et al. 1997). NTSR is often due to the elevation in enzymes associated with one or more of the xenobiotic detoxification phases (Kreuz et al. 1996). The threat of multiple-resistance evolution is greater with NTSR mechanisms due to the substrate cross-reactivity of xenobiotic detoxification enzymes. Multiple resistance may be endowed by a single mechanism or by multiple independent mechanisms (Beckie and Tardif 2012). The threat of a single mechanism endowing multiple resistance is of great concern, as it would limit weed management options drastically. Multiple resistance may impact the biology of a weedy species or its fitness (Vila-Aiub et al. 2009). In some cases, multiple resistance via NTSR mechanisms may impact resilience to abiotic stressors, an even greater concern for management (Darmency et al. 2015). TSR and NTSR to a variety of herbicide modes of action have manifested in *Echinochloa* spp.; however, in-depth research on multiple-resistant populations is limited. This study aims to provide an understanding of NTSR mechanisms in multiple-resistant *E. colona* and to determine whether the same mechanism imparts resistance to propanil and quinclorac.

Materials and Methods

Plant Materials

Beginning in 2010, the University of Arkansas Weed Physiology research group conducted surveys of *Echinochloa* species demographics and herbicide resistance to assess the distribution and status of herbicide-resistant populations in Arkansas (Rouse et al. 2018). This research resulted in the characterization of approximately 200 populations of *Echinochloa* spp. collected from rice production areas of the state. Details of procedures can be found in Rouse et al. (2018). From this collection, two populations of *E. colona* (ECO-R and ECO-S) were selected for further characterization. The field source of ECO-R had been planted with rice for at least two decades and had been sprayed with propanil in all the rice years, and with quinclorac since its commercialization in 2000 (Talbert and Burgos 2007). These rice fields were also in rotation with, or in close proximity to, soybean fields. ECO-R was collected from Lincoln County and classified as putatively resistant to three rice herbicides, cyhalofop, propanil, and quinclorac, in the general resistance test. Response to glufosinate was also evaluated, because these populations may have been exposed to glufosinate in the soybean crop cycle, as farmers started planting glufosinate-resistant (LibertyLink®) soybean. ECO-R had elevated tolerance to glufosinate based on the moderate control and high level of recovery during the herbicide screen (data not shown). ECO-S was collected in 2011 from a field in Prairie County, AR, and was used as a susceptible counterpart to ECO-R based on the field history and results of the herbicide-resistance test. Single plants from ECO-R and ECO-S were grown in isolation to produce a generation of self-pollinated offspring. *Echinochloa colona* is predominantly self-pollinated; a single-selfed generation was deemed enough to produce near-homozygous individuals for further research. The F_1 plants were not affected by a 4X dose of propanil in the verification assay, just as the F_1 plants were not affected by a high dose of quinclorac. All research following this initial selection was conducted using these selected seed lines.

Herbicide Dose–Response Assay

ECO-R and ECO-S were grown in a greenhouse maintained at 30 to 35 C, with 14-h days. Natural light was supplemented with halide lamps. Approximately 10 to 20 seeds of either ECO-R or ECO-S were planted into square pots, 7.6-cm wide and 10.2-cm tall, filled with commercial potting soil (Sun Gro Horticulture, Agawam, MA). Approximately 1 wk after planting, seedlings were thinned to 1 plant per pot. Each treatment was replicated 6 to 9 times, with each experimental unit completely randomized. The final run had 20 plants per replication. A total of three runs for each herbicide were included. Plants were treated with herbicides at the 2- to 3-leaf stage. All replications were treated simultaneously in a spray chamber with a motorized boom calibrated to deliver 187 L ha^{-1}. Herbicides were applied based on the standard use rates for the four herbicides of interest: cyhalofop, 314 g ha^{-1}; propanil, 4,500 g ha^{-1}; quinclorac, 560 g ha^{-1}; and glufosinate, 590 g ha^{-1}. These were the 1X doses evaluated in all experiments. The specific doses used for the dose–response experiments are given in Table 1. For the initial run of the experiment, all herbicides were evaluated at eight doses from 0X to 16X for cyhalofop, glufosinate, and quinclorac, and 0X to 32X for propanil. Based on data from the first experiment, the dose range was expanded to produce a better response curve. Following herbicide treatment, plants were returned to the greenhouse and maintained for 3 wk. Plant control

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Table 1. Herbicides and respective doses used to evaluate the response of ECO-S and ECO-R populations.

Cyhalofop	Glufosinate	Propanil	Quinclorac
g ha^{-1}			
31.4	59	1,125	56
39.2	73.75	2,250	70
62.8	118	4,500	112
78.5	147.5	9,000	140
125.6	236	18,000	224
157.0	295	27,000	280
251.2	472	36,000	448
314.0	590	72,000	560
392.5	737.5	144,000	700
471.0	885		840
628.0	1,180		1,120
942.0	2,360		2,240
1,256.0	4,720		4,480
2,512.0	9,440		8,960
5,024.0			17,920

was evaluated visually 3 wk after treatment (WAT) on a scale of 0% to 100% (0 = no visible effect to 100 = dead). The plants were then harvested, and the fresh biomass was recorded. Fresh biomass was converted to a percentage of the nontreated control. Data across all runs of the experiment were combined for analysis, as the plants responded similarly to treatments across runs. The data were fit with a nonlinear logistic model. The ED_{50}, or effective dose resulting in 50% control, was inverse calculated from the model, and the resistance level of ECO-R (R/S ratio) was determined relative to the susceptible standard. The data were best fit with a four-parameter logistic model using the following formula, whereby *a* is growth rate, *b* is the inflection point, c is the lower asymptote, and d is the upper asymptote:

$$c + \frac{(d-c)}{1 + \mathrm{Exp}[-a \times (\mathrm{Rate} - b)]}. \quad (1)$$

Efficacy of Herbicide Mixtures

Approximately 25 seeds were germinated in square pots, 10.2-cm wide and 10.2-cm tall, containing commercial potting soil as described previously. One week after planting, the seedlings were thinned to 5 plants per pot. Treatments included the field application rate of all four herbicides and a tank mixture of each herbicide with another, for a total of 10 treatments. The experiment was established as a completely randomized design with four replications and three runs. The applications of herbicides in the sequential treatments were separated by 60 min. This was done because previous research has shown antagonism between graminicides and auxinic compounds in tank mixtures (Barnwell and Cobb 1994). All treatments were applied as described previously. The experiment was terminated at 3 WAT, and the same data were collected as in the dose–response experiments. Data across all runs were analyzed together. An ANOVA was conducted for both plant injury and biomass, with significant means separated using Fisher's protected LSD ($P \leq 0.05$). To test for herbicide interaction, a follow-up analysis using a modified Colby's method was conducted (Colby 1967; Flint et al. 1988).

Assessment of Xenobiotic Detoxification Enzyme Inhibitors

Seeds of ECO-R and ECO-S were germinated and grown as described previously. The plants were treated with known detoxifying enzyme inhibitors and herbicides at the 2- to 3-leaf stage. Three inhibitors were tested in the experiment: carbaryl (1.1 kg ha^{-1}), malathion (0.99 kg ha^{-1}), and piperonyl butoxide (PBO; 1.2 kg ha^{-1}). The enzyme inhibitors and herbicides were applied in the same manner, with the former applied 60 min ahead of the latter. Nontreated checks were included. The experiment was arranged as a completely randomized design, with four replications. Each experimental unit was 1 pot containing 5 plants. The experiment was conducted twice. Plant injury and fresh shoot biomass data were collected at 3 WAT. The analysis was conducted by herbicide, with the enzyme inhibitor as the single fixed factor. An ANOVA was conducted for both plant injury and biomass as a percent of the nontreated control, and significant means were separated using Fisher's protected LSD ($P \leq 0.05$).

Absorption and Translocation of Cyhalofop, Propanil, and Quinclorac

All experiments, regardless of herbicide, were conducted according to the procedures of Nandula and Vencill (2015), except when stated otherwise. ^{14}C-Radiolabeled herbicide was used to measure the absorption and translocation of cyhalofop, propanil, and quinclorac in ECO-R and ECO-S. Single plants were grown in separate pots, maintained, and treated as described in previous experiments. A total of 3 plants per herbicide were used. At the 3-leaf stage, the plants were sprayed with the field use rate of each herbicide. After the spray droplets had dried, the plants were moved into the laboratory for treatment with radiolabeled herbicides. A spotting solution containing 0.24 kBq µl^{-1} was formulated with an aliquot of the herbicide solution that was applied to the plants. Five 1-µl droplets were applied within a 2.54-cm area on the adaxial surface of the second fully expanded leaf. A total of 1.7 kBq of radiolabeled herbicide was applied on the treated leaf. For cyhalofop and propanil, plants were harvested at 6, 12, 24, 48, and 72 h after treatment (HAT). For quinclorac, plants were harvested at 24, 48, 72, 96, and 120 HAT. At each time point, the treated leaf was removed from the plant and rinsed in a vial containing 5 ml of deionized water (for cyhalofop and propanil) or 5 ml of 70% acetonitrile (for quinclorac). The vial was shaken gently to remove the unabsorbed ^{14}C-labeled herbicide, then the tissue was removed to dry. A 1-ml aliquot of the leaf wash was mixed with 15 ml of liquid scintillation cocktail (Ultima Gold^{TM}, Sigma Aldrich, MO, USA), and the radioactivity was counted using a liquid scintillation counter (Tri-Carb 2900TR, PerkinElmer Life and Analytical Sciences, CT, USA).

The remainder of each plant was lifted from the pot, and the roots were rinsed thoroughly with tap water. The plant was sectioned into three parts: above the treated leaf, below the treated leaf, and roots. The tissues were air-dried and oxidized using a biological oxidizer (OX700 Oxidizer, RJ Harvey Instruments, NY, USA). For data analysis, the quantity of radioactivity in each tissue section was converted to a percentage of the total absorbed ^{14}C-labeled herbicide. Data were analyzed as an average of the three plant replicates, by tissue and harvest timing. A *t*-test was performed to determine whether the total herbicide absorbed and the distribution of herbicide within the plant differed between ECO-S and ECO-R.

[^{14}C]Quinclorac Metabolism

ECO-R and ECO-S plants were grown as described in the absorption and translocation experiments. At the 3-leaf stage, the plants were spotted with approximately 14.3 kBq of [^{14}C]quinclorac without pretreatment of "cold" herbicide. The treated leaf was removed

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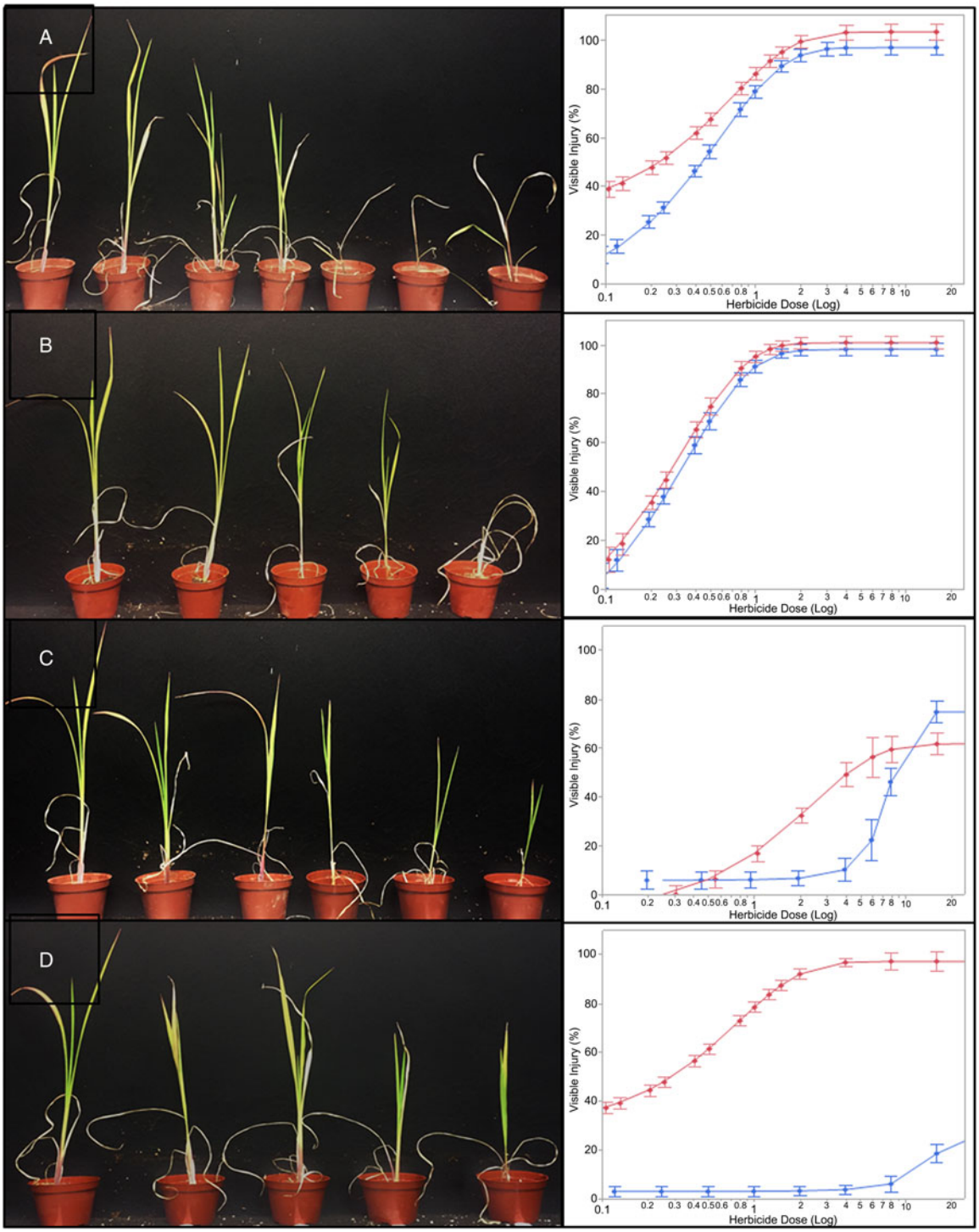

Figure 1. Nonlinear regression analysis of the herbicide dose (*x* axis) on a log scale and visible injury (*y* axis) with standard errors of the means for ECO-R (blue) and ECO-S (red). Photos depict ECO-R response to cyhalofop (A), glufosinate (B), propanil (C), and quinclorac (D) at 3 wk after application. The control plant is on the far left for each herbicide, with increasing doses to the right.

at 24, 48, 72, 96, and 120 HAT, washed, and placed in a tube for extraction. The tissue was homogenized with 3 ml of 70% acetonitrile to extract the [^{14}C]quinclorac. The homogenized tissue was then dried under a vacuum using a rotavaporator and resuspended in methanol:acetonitrile (40:60). A 100-µl aliquot was then analyzed using High-performance liquid chromatography (HPLC; Agilent Technologies, Germany). Samples were analyzed on a reverse-phase Kinetex 2.6-µm XB-C18 column (150 mm by 4.6 mm, Phenomenex, Germany) using the following solvents: (1) 20% (v/v) acetonitrile in water with 0.1% (v/v) formic acid and (2) 95% (v/v) acetonitrile in water with 0.1% (v/v) formic acid. The HPLC conditions involved a 20-min linear gradient of solvent B from 20% to 100%, followed by a 3-min linear gradient of solvent B from 100% to 20%, and a 4-min 20% solvent B isocratic hold (27 min total). The retention time of the

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parent compound, [^{14}C]quinclorac, was determined by injecting 50 µl of 150 dpm µl^{-1} [^{14}C]quinclorac diluted in 100% acetonitrile. The parent compound eluted at 11 min. Parent quinclorac and potential metabolites were detected with a radioflow detector (Berthold Technologies, Germany). The amount of remaining [^{14}C]quinclorac was quantified as a percentage of total extractable radioactivity. The quantity of parent molecules, or metabolites, was analyzed by harvest time. ECO-R and ECO-S means were compared using a *t*-test.

β-Cyanoalanine Synthase Enzyme Assessment

The activity of β-cyanoalanine synthase (β-CAS) was quantified using a colorimetric assay similar to Grossman and Kwiatkowski (1995) and Yasuor et al. (2012). Seeds of ECO-R and ECO-S were germinated in trays containing commercial potting medium (previously described) with two trays per accession, one treated and one nontreated. Plants were thinned approximately 1 wk after planting. At the 2-leaf stage, plants were treated with 560 g ha^{-1} quinclorac. Plants were harvested at 24 HAT; 5 plants were combined to produce enough tissue for a single composite replication, three replicates were used for the experiment. Harvested tissues were immediately frozen in liquid nitrogen. Plants were homogenized with liquid nitrogen and 100 mM Tris buffer (pH 8.5) using a mortar and pestle. Homogenized tissues were kept on ice. The samples were then centrifuged for 10 min at 6,708 × *g* and 4 C. The supernatant was transferred to a fresh, cold centrifuge tube. Fresh substrate was prepared by mixing 50 mM NaCN and 10 mM Tris-HCl (pH 8.5). Both the substrate mixture and supernatant were equilibrated at 30 C for 10 min. The reaction was started in a sealed test tube in which 0.5 ml of the crude enzyme extract (supernatant) was added to 4 ml of substrate mixture and incubated at 30 C for 60 min. The color was developed by adding a 1-ml aliquot of the color-developing mixture (30 mM $FeCl_3$ in 1.2 N HCL+ 40 mM *N,N*-dimethyl-phenylenediamine sulfate salt in 7.2 N HCl) to the substrate and enzyme mixture. The sample was vortexed and incubated in the dark at room temperature for 1 to 2 h to allow color to develop. The enzyme activity was quantified colorimetrically based on its reaction with cysteine (in the extract), which releases hydrogen sulfide during dark incubation, which then reduces methylene blue and changes the color of the mixture. The absorbance of each sample was measured at 650 nm using a Pharma Spec UV-100 (Shimadzu, Columbia, MD). The absorbance reading was converted to "M Na_2S" based on a standard curve. Treatment means were compared using a *t*-test.

Results and Discussion

Herbicide-Resistance Levels

The R/S values for ECO-R were 1.9 (Figure 1A) and 1.2 (Figure 1B) in response to cyhalofop and glufosinate, respectively. The 1X dose of cyhalofop controlled ECO-R less than 90%, which would be problematic in commercial fields, and the highest dose of 5,024 g ha^{-1} (16X) did not control ECO-R 100% (Figure 1A). Cyhalofop was significantly less effective on ECO-R than on ECO-S. The activity of glufosinate on ECO-R was consistently numerically lower than that on ECO-S and did not reach 100% at any of the doses tested. The level of control at the 1X dose was >90%, which is considered excellent in commercial fields. Therefore, the ECO-R field population had low-level resistance to cyhalofop and elevated tolerance to glufosinate. In the greenhouse, ECO-R responded to glufosinate similar to ECO-S (Figure 1B), because assessments were done at 3 WAT, which did not show the regeneration capacity of ECO-R. In the field, 100% of ECO-S plants would die after treatment with glufosinate, while several plants from the ECO-R population would recover and produce seed (CER and NRB, personal observation). The R/S value of 2 for ECO-R with propanil was deceptively low, because the reference population, ECO-S, was also resistant to propanil. The ED_{50} value for ECO-S (18,900 g ha^{-1}) was 4.2X the field dose of propanil, whereas the ED_{50} for ECO-R was approximately 8.3X the field dose, or 37,800 g ha^{-1}. The *E. colona* dose–response curve for quinclorac was different from those for the other herbicides. The ED_{50} for ECO-S (185 g ha^{-1}) was 0.33X of the recommended field dose for quinclorac. ECO-S could be controlled 100% by about 280 g ai ha^{-1} of quinclorac, which is around one-half of the field use rate; however, ECO-R was not controlled by the highest dose evaluated at 32X, or 17,920 g ha^{-1}. This dose caused less than 20% injury. Therefore, the ED_{50} value for ECO-R was >17,920 g ha^{-1}, and the R/S value could not be calculated.

Thus, the multiple-resistant *E. colona* population, ECO-R, has a unique resistance profile with high resistance to propanil and quinclorac, accompanied by low-level resistance to cyhalofop and elevated tolerance to glufosinate. Elevated tolerance to glufosinate was not apparent in the greenhouse at 3 WAT, but was noticeable in the field. This increased tolerance was enough to result in significant regrowth and eventual seed production of survivors in field experiments (unpublished data). The high levels of resistance to propanil and quinclorac are a concern to rice producers in the state, but the low resistance to cyhalofop and elevated tolerance to glufosinate could make the problem worse. High levels of resistance to propanil (Carey et al. 1995, 1997) and quinclorac (Lovelace et al. 2007; Malik et al. 2010) have been reported previously in Arkansas, but not to the level observed with this population. Other researchers reported "moderate" resistance to propanil at approximately 20 kg ha^{-1}, which was comparable to what was observed in ECO-S, but significantly less than the 37.8 kg ha^{-1} LD_{50} observed with ECO-R (Carey et al. 1995). Resistance to cyhalofop has been reported recently in Arkansas *Echinochloa* populations (Rouse et al. 2018); glufosinate resistance has not. Although ECO-R is still considered susceptible to glufosinate, the insignificant, but noticeably consistent reduction in efficacy on ECO-R cannot be overlooked. The low-level resistance to cyhalofop and elevated tolerance to glufosinate warrant attention, as this could aggravate the resistance problem.

Table 2. Control of multiple-resistant *Echinochloa colona* (ECO-R) as affected by the interaction of various pairs of four rice herbicides applied in tank mixtures at 3 wk after application.[a]

Herbicide	Cyhalofop	Glufosinate	Propanil	Quinclorac
	% injury			
Cyhalofop	—	70 (88)	48 (72)	15 (61)
Glufosinate	70 (88)	—	68 (77)	75 (72)
Propanil	48 (72)	68 (77)	—	28 (25)
Quinclorac	15 (61)	75 (72)	28 (25)	—

[a]Colors signify if the interaction was antagonistic (blue) or additive (yellow) according to Colby's method of assessing tank mixture interactions ($P \leq 0.05$). Observed values are given with expected values in parentheses.

Efficacy of Herbicide Mixtures

The field doses of propanil and quinclorac were ineffective, with 29% and 1% control of ECO-R, respectively (Figure 2A).

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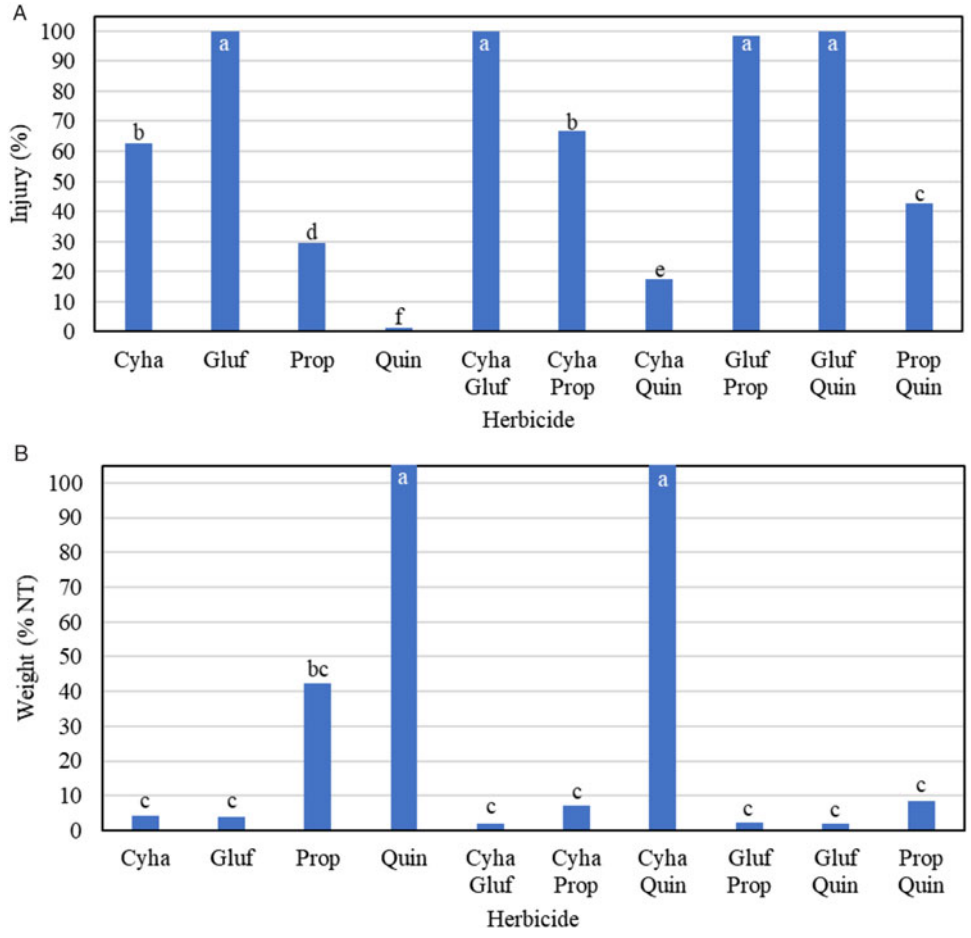

Figure 2. Visible injury (A) and fresh biomass (B) as a percentage of the no-herbicide control at 3 wk after treatment for the tank-mixture study conducted on multiple-resistant *Echinochloa colona* (ECO-R). Bars with the same letters are not different based on Fisher's protected LSD ($\alpha = 0.05$). Abbreviations: Cyha, cyhalofop; Gluf, glufosinate; Prop, propanil; Quin, quinclorac.

Cyhalofop had only moderate control (62%). In these experiments, glufosinate controlled ECO-R almost 100%. Cyhalofop applied with propanil increased the control of ECO-R by 5% relative to cyhalofop applied alone and by greater than 30% relative to propanil applied alone (Table 2). This increase was still not to a level that would be acceptable to producers. Cyhalofop + quinclorac reduced the control of *E. colona* to less than 20% compared with cyhalofop alone and only marginally increased *E. colona* control compared with quinclorac alone. Thus, quinclorac antagonized the activity of cyhalofop. The propanil and quinclorac tank mixture had an additive effect on ECO-R (42%). The quinclorac and cyhalofop + quinclorac applications resulted in more biomass of ECO-R than the nontreated control (Figure 2B). In other words, the application of quinclorac to ECO-R made the plants grow larger. Regardless of the tank-mixture companion, glufosinate application resulted in 100% control of ECO-R.

The use of tank mixtures to improve weed control is an effective recommendation to reduce the evolution of resistance (Norsworthy et al. 2012) and was thus evaluated in this research. Glufosinate is still an effective herbicide option for ECO-R in burndown or LibertyLink® soybean systems. Cyhalofop cannot be applied with propanil or quinclorac, because these mixtures are antagonistic and would reduce cyhalofop efficacy on grasses. Propanil and cyhalofop are known to antagonize each other, potentially due to reduced translocation of cyhalofop when mixed with propanil (Scherder et al. 2005). Antagonistic interactions between graminicides and auxinic herbicides are commonly observed (Barnwell and Cobb 1994). Herbicide mixtures are useful only if they will result in 100% control of weeds. Otherwise, weeds that are only partially affected are being subjected to suboptimal doses, which eventually results in selection for NTSR (Délye 2013).

Assessment of Xenobiotic Detoxification Enzyme Inhibitors

The use of known detoxifying enzyme inhibitors—malathion, carbaryl, and PBO—increased the efficacy of propanil (Figure 3A). Application of carbaryl before propanil increased plant injury to 93%, approximately 55% more than that of propanil applied alone. This was comparable to the effect of malathion (78%) but better than that of PBO (~60%). Without herbicide, PBO stunted the plants 7% and reduced shoot biomass to 60% relative to the no-inhibitor and no-herbicide controls. Applying the metabolic enzyme inhibitors before propanil reduced *E. colona* biomass by more than 90% compared with the nontreated control (Figure 3B). The presence of these compounds reduced the plants' ability to tolerate and/or recover from the herbicide treatment. Propanil activity was synergized by all three enzyme inhibitors tested, resulting in almost complete control of ECO-R. This indicates that resistance to propanil is due to herbicide detoxification by an oxidizing

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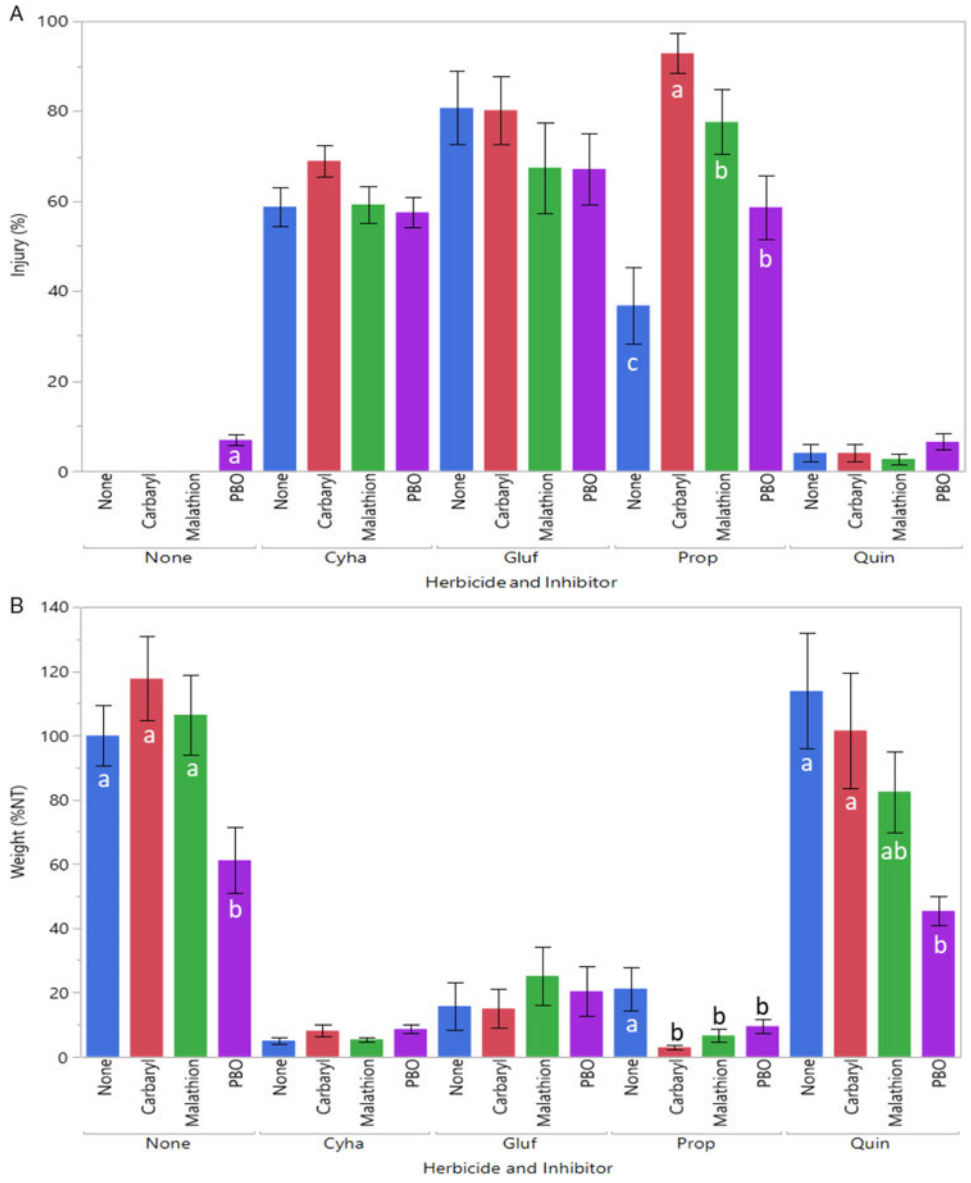

Figure 3. Visible injury (A) and fresh biomass (B) as a percentage of the no-herbicide and no-inhibitor control at 3 wk after treatment, with known detoxification enzyme inhibitors. Bars with the same letters are not different based on Fisher's protected LSD ($\alpha = 0.05$). PBO, piperonyl butoxide.

enzyme, most likely a cytochrome P450. The extremely high resistance to quinclorac and elevated tolerance to cyhalofop and glufosinate are enabled by other mechanisms not specifically affected by these inhibitors. With quinclorac, only malathion (83%) and PBO (45%) reduced biomass relative to quinclorac alone. However, quinclorac alone induced growth and resulted in 13% more biomass than the nontreated control plants. This had not been reported previously in any *Echinochloa* population response to quinclorac.

Enzyme inhibitors have been used to study metabolic-based resistance to ascertain the potential mechanisms of resistance in herbicide-resistant *Echinochloa* (Carey et al. 1995; Fischer et al. 2000; Yasuor et al. 2012). The efficacy of cyhalofop and glufosinate on ECO-R was not affected by any of the evaluated inhibitors, meaning the potential mechanisms imparting increased tolerance are most likely not metabolic in nature. Resistance to propanil and quinclorac has been reversed with the use of known detoxifying enzymatic inhibitors in previous research. Both malathion and carbaryl synergized the activity of propanil on ECO-R, which indicates that either one of these enzyme inhibitors blocked the reaction of metabolic enzymes with propanil, preventing herbicide detoxification, thus supporting the hypothesis that resistance to propanil is due to detoxification by at least one oxidizing enzyme. Previous researchers found that aryl acylamidase, the enzyme involved in propanil detoxification in rice and *Echinochloa*, is inhibited by malathion and carbaryl (Hoagland et al. 1974, 2004). Further research is required to determine which cytochrome P450s or other known detoxifying enzymes are involved in propanil degradation in ECO-R.

^{14}C-Labeled Herbicide Absorption and Translocation

Cyhalofop. Cyhalofop absorption was maximized at 72 HAT, at 68% in ECO-R and 78% in ECO-S (Table 3). The absorption

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Table 3. Cyhalofop and propanil absorption in multiple-resistant (ECO-R) and susceptible (ECO-S) *Echinochloa colona* as a percentage of the total applied ^{14}C-radiolabeled herbicides at five harvest times.

	Total cyhalofop absorbed[b]		Total propanil absorbed[b]	
Harvest time[a]	ECO-R	ECO-S	ECO-R	ECO-S
Hours	%			
6	35	57	14	13
12	43	41	*15*	*13*
24	53	55	20	20
48	60	52	*34*	*25*
72	*68*	*78*	42	32

[a]Hours after treatment.
[b]Means in italics indicate difference between ECO-R and ECO-S within the respective herbicides based on a *t*-test ($P \leq 0.05$).

Table 4. Quinclorac absorption in multiple-resistant (ECO-R) and susceptible (ECO-S) *Echinochloa colona* as a percentage of the total applied ^{14}C-radiolabeled herbicide at five harvest times.

	Total quinclorac absorbed[b]	
Harvest time[a]	ECO-R	ECO-S
Hours	%	
24	45	52
48	61	75
72	59	67
96	58	62
120	*73*	*64*

[a]Hours after treatment.
[b]Means in italics indicate difference between ECO-R and ECO-S based on a *t*-test ($P \leq 0.05$).

Table 5. Concentration of absorbed [^{14}C]quinclorac in the respective plant tissues of multiple-resistant (ECO-R) and susceptible (ECO-S) *Echinochloa colona* at five harvest times.

		Quinclorac[c]	
Harvest time[a]	Tissue[b]	ECO-R	ECO-S
Hours		%	
24	Trt	79	81
	AL	3	7
	BL	11	6
	RT	1	1
48	Trt	63	90
	AL	23	3
	BL	9	5
	RT	*2*	*1*
72	Trt	*58*	*89*
	AL	*25*	*3*
	BL	11	6
	RT	2	0
96	Trt	*57*	*85*
	AL	*24*	*6*
	BL	13	5
	RT	2	2
120	Trt	*43*	*83*
	AL	*41*	*7*
	BL	12	6
	RT	*2*	*1*

[a]Hours after treatment.
[b]Abbreviations: Trt, treated leaf; AL, tissues above the treated leaf; BL, tissues below the treated leaf; RT, roots.
[c]Means in italics indicate difference between ECO-R and ECO-S based on a *t*-test ($P \leq 0.05$).

was not different between R and S plants at any time, except at 72 HAT, when ECO-S absorbed more [^{14}C]cyhalofop than ECO-R. The majority of absorbed [^{14}C]cyhalofop remained in the treated leaf (>90%) (Supplemental Table 1) regardless of genotype. Within

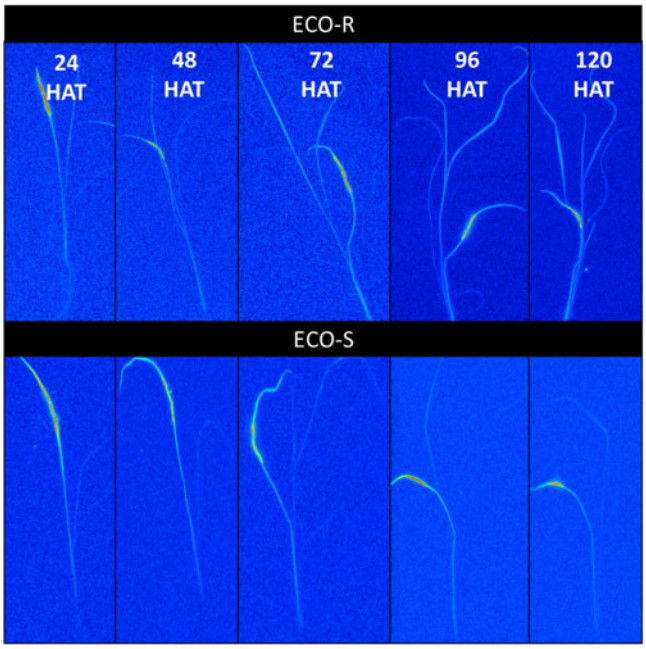

Figure 4. Phosphorimages depicting the relative quantities of [^{14}C]quinclorac and its distribution from the treated leaf throughout the plant at 24, 48, 72, 96, and 120 h after treatment (HAT). Red areas contain the highest concentration of [^{14}C]quinclorac.

12 HAT, minimal movement of herbicide (up to 4%) occurred in tissues above the treated leaf. By 24 HAT, a greater proportion of the herbicide moved to shoot tissues below the treated leaf than above the treated leaf. This distribution pattern remained the same at 72 HAT. Very little [^{14}C]cyhalofop (<1%) was translocated into the roots.

Propanil. Propanil absorption was greatest at 72 HAT, with ECO-R (42%) having a numerically higher concentration of [^{14}C]propanil than ECO-S (32%) (Table 3). Significant differences in absorption were observed only at 48 HAT, with absorption in ECO-R (34%) being greater than in ECO-S (25%). As with cyhalofop, the majority of propanil was retained in the treated leaf. From 12 to 72 HAT, the proportion of herbicide in the tissues below the treated leaf was less than 0.5% of the absorbed herbicide. Neither absorption nor translocation of [^{14}C]propanil differed between ECO-S and ECO-R at the termination of the study, but propanil was absorbed more rapidly in the resistant plants than in susceptible ones.

Quinclorac. Quinclorac absorption at 24, 48, 72, and 96 HAT was not different between ECO-R and ECO-S (Table 4). However, by 120 HAT, ECO-R had absorbed more quinclorac (72%) than ECO-S (64%). Beginning at 72 HAT, ECO-R had a lower concentration (<60%) of [^{14}C]quinclorac in the treated leaf but a higher concentration (>24%) in the tissues above the treated leaf than ECO-S (Table 5). Also, at 48 and 120 HAT, there was a greater concentration of the [^{14}C]quinclorac in the roots of ECO-R than in ECO-S. This was validated by phosphorimaging (Figure 4).

Results from the ^{14}C-labeled herbicide experiments provided more information on the behavior of quinclorac in the plant and, to a lesser extent, described the behavior of propanil and cyhalofop in the populations studied. Cyhalofop was distributed more within the plant than was propanil. Absorption and translocation of [^{14}C]cyhalofop was similar to what was reported in previous research in Arkansas *Echinochloa* populations (Scherder et al. 2005).

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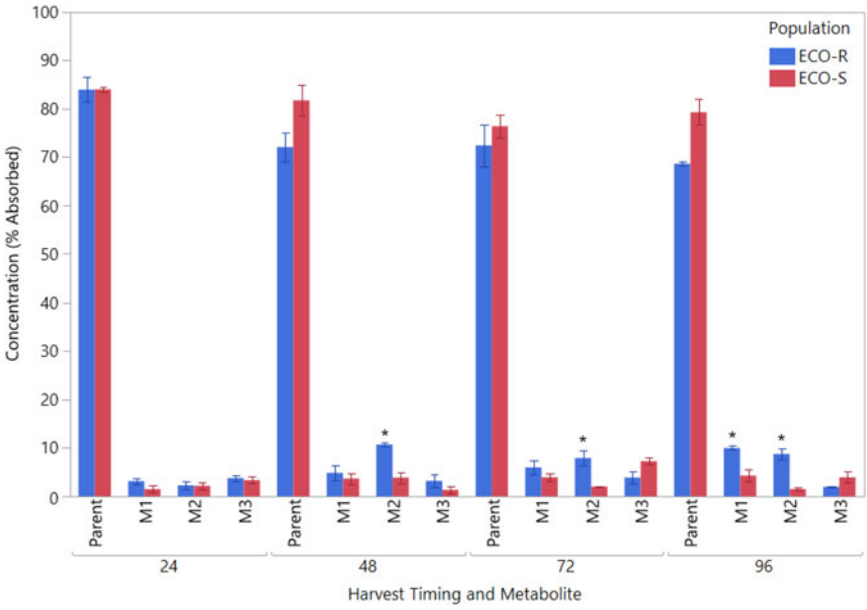

Figure 5. Concentration (%) of absorbed [^{14}C]quinclorac as the parent molecule and three unknown metabolites (M1–M3) in the treated leaves of ECO-R and ECO-S harvested at 24, 48, 72, and 96 h after treatment. Asterisks (*) indicate that the concentration of the molecule in ECO-R is different from that in ECO-S based on a *t*-test ($P \leq 0.05$).

For propanil, the observed absorption by ECO-R and ECO-S was greater than in previous experiments, but the translocation pattern was similar, with little being moved outside the treated leaf (Carey et al. 1995). The translocation pattern of [^{14}C]quinclorac has not been described previously in the literature. Quinclorac moved out of the treated leaf and accumulated in the new growth above the treated leaf, indicating translocation via phloem loading. The radioactive molecule can move either as the parent compound or as a polar metabolite, and more metabolites were detected in ECO-R than in ECO-S. Herbicide degradation occurring outside the treated leaf could create a gradient, allowing more parent compound to move out of the treated leaf. Given the high distribution of radioactivity throughout the plant by 120 HAT, herbicide metabolism must be involved in the high resistance of ECO-R to quinclorac. Quinclorac is highly mobile in the plant when applied to roots (Grossmann and Kwiatkowski 2000; Grossmann and Scheltrup 1997), but not to the extent observed from the foliar application in this experiment. Further research is needed to examine the identity of the metabolite and determine whether its polar nature has a role in the redistribution of the herbicide following treatment.

^{14}C-Labeled Herbicide Quinclorac Metabolism

Three unique metabolites were observed in both ECO-R and ECO-S. The metabolites were not identified, as no standards were available for comparison, but the relative quantities were analyzed. At all harvest times, the majority (>70%) of [^{14}C]quinclorac remained as the parent molecule (Figure 5). The amount of parent quinclorac molecule did not differ significantly between ECO-R and ECO-S but was numerically less in ECO-R than ECO-S beginning at 48 HAT. The parent molecule concentration in ECO-S remained relatively the same with time, approximately 75% to 80%, while in ECO-R it slowly decreased with time. Of the three metabolites, metabolite 2, was present in higher concentrations in ECO-R at 24, 48, and 72 HAT. At 72 HAT, metabolite 2 equated to about 9% of the absorbed parent molecule. Metabolite 1, also detected throughout the experiment, was significantly greater in ECO-R at 96 HAT (10%) than in ECO-S.

β-CAS Enzyme Activity

The β-CAS enzyme activity did not differ between quinclorac-treated ECO-R (0.3194 M Na_2S) and ECO-S (0.3192 M Na_2S). The enzyme activity was also the same between nontreated and treated plants of ECO-R or ECO-S. For ECO-R, β-CAS enzyme activity was the same between the nontreated (0.3194 M Na_2S) and quinclorac-treated (0.3194 M Na_2S) plants. The same was observed for ECO-S, with the quinclorac-treated β-CAS enzyme activity (0.3191 M Na_2S) being similar to that of the nontreated (0.319206 M Na_2S). These results are in contrast to previous research conducted with quinclorac-resistant rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.], wherein elevated β-CAS activity imparted the primary resistance mechanism for the population (Yasuor et al. 2012). The lack of β-CAS activity indicates a different mechanism of resistance to quinclorac in ECO-R.

In Arkansas (and the southern U.S. rice belt), *Echinochloa* populations with multiple resistance to propanil and quinclorac are increasing (Rouse et al. 2018). Multiple resistance is a serious concern. Historically, the use of highly effective grass herbicides in rice fields had not stopped the evolution of multiple resistance. Optimizing control of *Echinochloa* spp. (along with other grass weeds) must be prioritized in rice production. The efficacy of quinclorac was not improved by any of the enzymatic inhibitors, indicating that metabolic detoxification is most likely not the mechanism of resistance or that another family of cytochrome P450s is involved. While the metabolism inhibitors we tested interact with a range of xenobiotic detoxification enzymes, these inhibitors could not possibly cross-react with all potential quinclorac-detoxifying enzymes. The known HCN-detoxifying enzyme β-CAS, implicated in resistance to quinclorac, is inhibited by malathion (Yasuor et al. 2012). The lack of synergism between malathion and quinclorac, and the lack of induction in the β-CAS

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enzyme activity in this multiple-resistant population, led us to conclude that β-CAS does not have a role in extreme resistance to quinclorac in ECO-R.

It is also noteworthy that in several of these experiments, the application of quinclorac alone encouraged the growth and/or vigor of ECO-R following treatment. This has not been described previously and may implicate a NTSR mechanism that is not metabolic, but a component of a broader abiotic stress adaptation pathway (Délye 2013).

This research is the first comprehensive investigation of the physiological basis for multiple resistance in *E. colona*. While propanil- and quinclorac-resistant populations are widespread in Arkansas, we report extremely higher resistance to these two major rice herbicides than has been reported in previous research. This ECO-R population also had low-level resistance to cyhalofop and elevated tolerance to glufosinate, but it was still susceptible to the latter. Resistance to propanil and quinclorac in ECO-R is endowed by independent metabolic mechanisms. Resistance to propanil is most likely due to metabolic detoxification involving some cytochrome P450 enzyme(s) or other detoxifying proteins, unlike the commonly known detoxification pathway mediated by aryl acylamidase. Extreme resistance to quinclorac is facilitated by a mechanism yet to be revealed as well as some level of herbicide detoxification, as the low quantities of metabolites do not account for the high level of resistance. Follow-up studies are being conducted that use a global approach in analyzing gene expression and gene products to determine possible gene networks lending high resistance.

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Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2019.52

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