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Discrimination of ACCase-inhibiting herbicides-resistant *Digitaria ciliaris* populations with three diagnostic bioassays

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Abstract: Background: Diagnostic bioassays are used to screen the suspected R population. They are conducted at a single herbicide dose and evaluated at a specific time after treatment that can differentiate resistant from susceptible population.

Objective: Three different bioassays were evaluated to assess the detection of acetyl CoA carboxylase-inhibiting herbicides resistance in *D. ciliaris*.

Method: Increasing herbicide rates were used to evaluate the three bioassays for differentiating R from S populations.

Results: R1 and R2 differed from S in all employed bioassays. In the Agar-based gel box box assay, the S biotype had greater plant damage at the lower herbicide concentration relative to the R biotypes 3 DAT but differences between R and S decreased over time. In the leaf flotation assay, R biotypes floated at the lower concentration on the surface, whereas the leaves of S biotypes failed to float. For the electrical conductivity assay, the S biotype contained high electrical conductivity due to the high leaching of electrolyte into the water across all four herbicides tested than the R biotypes.

Conclusion: While these assays were able to separate R and S biotypes, the level of resistance difference for any assay was no greater than 40% depending on rating data and exposure dose. While a statistical separation could be achieved using a rate response regression analysis for these bioassays, our data highlights the challenges associated whether these methods could provide an obvious difference at any single rate or rating data to be used as a consistent, effective first-phase resistance screen.

Keywords: Herbicide resistance test, Sethoxydim; Fluazifop-p-butyl; Clethodim; Pinoxaden; Agar-based gel box assay; Leaf flotation assay; Electrical conductivity assay

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1. Introduction

Herbicides inhibiting acetyl-CoA carboxylase (ACCase) are unique selective herbicides utilized as a post-emergence (POST) to control weeds in a variety of field crops (Délye, 2005; Powles, Yu, 2010). More than 50% of grass crops are treated with these herbicides at some point during a crop rotation (Kaundun, 2011). Three distinct chemical classes of herbicides, cyclohexanediones (CHD or DIMs), aryloxyphenoxypropionates (AOPP or FOPs), and pyrazolines (DENs) are included in ACCase-inhibiting herbicides (Hochberg et al., 2009; Powles, Yu, 2010). Typically, these herbicides control grass weeds by inhibiting the enzyme ACCase, which catalyzes the carboxylation of acetyl-CoA to malonyl Co-A in de novo fatty acid biosynthesis. When susceptible plants are treated with ACCase-inhibiting herbicides, ACCase blocks the biosynthesis of *de novo* fatty acid (Cronan, Waldrop, 2002; Délye et al., 2005). Resistance to ACCase-inhibiting herbicides can be caused to target-site resistance (TSR) or non-target-site resistance (NTSR). TSR is generally initiated by gene duplication (Laforest et al., 2017) or mutation (s) in the gene, where a mutation alters the protein structure at the herbicide's binding site rendering this protein insensitive to the active ingredient. NTSR is initiated without alteration to the target site. This resistance encompasses a range of diverse mechanisms including reduced herbicide uptake, penetration, impaired translocation, and enhanced metabolism of herbicides. Both target and nontarget resistance mechanisms can exist in a single population (Burnet et al., 1994; Délye et al., 2011; Powles, Yu, 2010).

Researchers have established resistance testing procedures for various weed and herbicide combinations (Rüegg et al., 2007). Agar-based testing has been described as simple, robust, cost-effective, quick, and successful for detecting both target and nontarget site resistance in an array of broadleaf and grass weeds of the cropping system. For example, Kaundun et al. (2011) developed a rapid in-season quick test (RISQ) for resistance screening of *Lolium* spp. (ryegrass) and *Alopecurus myosuroides* Huds. (blackgrass) to acetolactate synthase (ALS) and ACCase-inhibiting herbicides in the growing season. Brosnan et al. (2017) used the agar-based quick test for determining the resistance of glyphosate and ALS-inhibiting herbicides in *Poa annua* L. (annual bluegrass) within less

than two weeks. Similarly, Shaner et al. (2005) tested with excised leaf discs for identifying EPSPS-resistant *Glycine max* (soybean), *Brassica napus* (canola), and *Zea mays* (corn). The accumulation of shikimate rate of soybean and canola leaf discs was greater with 250 µM glyphosate than 500 µM at 48 h. Thus, various alternative procedures have been developed to determine herbicide resistance in whole plants (Boutsalis, 2001; Kaundun et al., 2014), seedlings (Letouzé, Gasquez, 1999), seeds (Bourgeois et al., 1997; Kim et al., 2000; Tal et al., 2000), and pollen bioassays (Letouzé, Gsquez, 2000) conducted in a greenhouse environment. For laboratory experiments, tests include plantlet evaluations, seed germination percentage with hypocotyl, radicle length (Abdurruhman et al., 2018), pollen germination (Richter, Powles, 1993), and chlorophyll fluorescence (Norsworthy et al., 1998; Van Oorschot, Van Leeuwen, 1992).

Petri dish bioassay contains a test for seed germination resistance in herbicide-saturated media (Murray et al., 1996; Abdurruhman et al., 2018). A major limitation associated with the seed-petri dish bioassay is the time required for seed production, development of seed dormancy, and seed ripening, followed by uniform germination of seed for evaluation (Cutulle et al., 2009). The whole plant assay, however, is the most common technique for detecting resistance among others (Beckie et al., 2000). Conventionally, the characterization of weed resistance has mainly relied on the greenhouse. Seeds from plants surviving herbicide treatments in the field are collected, grown in the greenhouse, and sprayed at specific rates. The collected seeds then are grown in a controlled environment in a glasshouse or a growth chamber. New individuals are treated with either PRE or POST control herbicides and compared their response to herbicide treatment with that of confirmed herbicide-susceptible individuals in the same manner (Burgos et al., 2013). The conventional techniques to diagnose herbicide-resistance weeds are tedious, laborious, time-consuming, and require more space.

Our research objective was to evaluate the three rapid bioassays with ACCase resistance in two biotypes (R1 and R2) of *Digitaria ciliaris* previously confirmed resistant to sethoxydim and selected aryloxyphenoxypropionate (FOPs) herbicide with a mutant allele, Ile-1781-Leu amino acid substitution in ACCase gene, and greater ACCase enzyme activity. The rapid detection of weed resistance is important for efficient resistant weed management. We hypothesize that the leaves from resistant biotypes will be able to minimize the ACCase herbicidal effects and thus float. Further based on the pattern of resistance, we also hypothesized that the resistant biotypes would release fewer electrolytes into the water previously associated with ACCase herbicides resistance. No reports have been published for detecting ACCase resistance in *D. ciliaris* with the different bioassays, an agar-based gel box assay, a leaf flotation assay, and an electrical conductivity assay. The primary goal of our research, therefore, was to determine the resistance level of *D. ciliaris* to ACCase-inhibiting herbicides through the rapid bioassay for the field plant population.

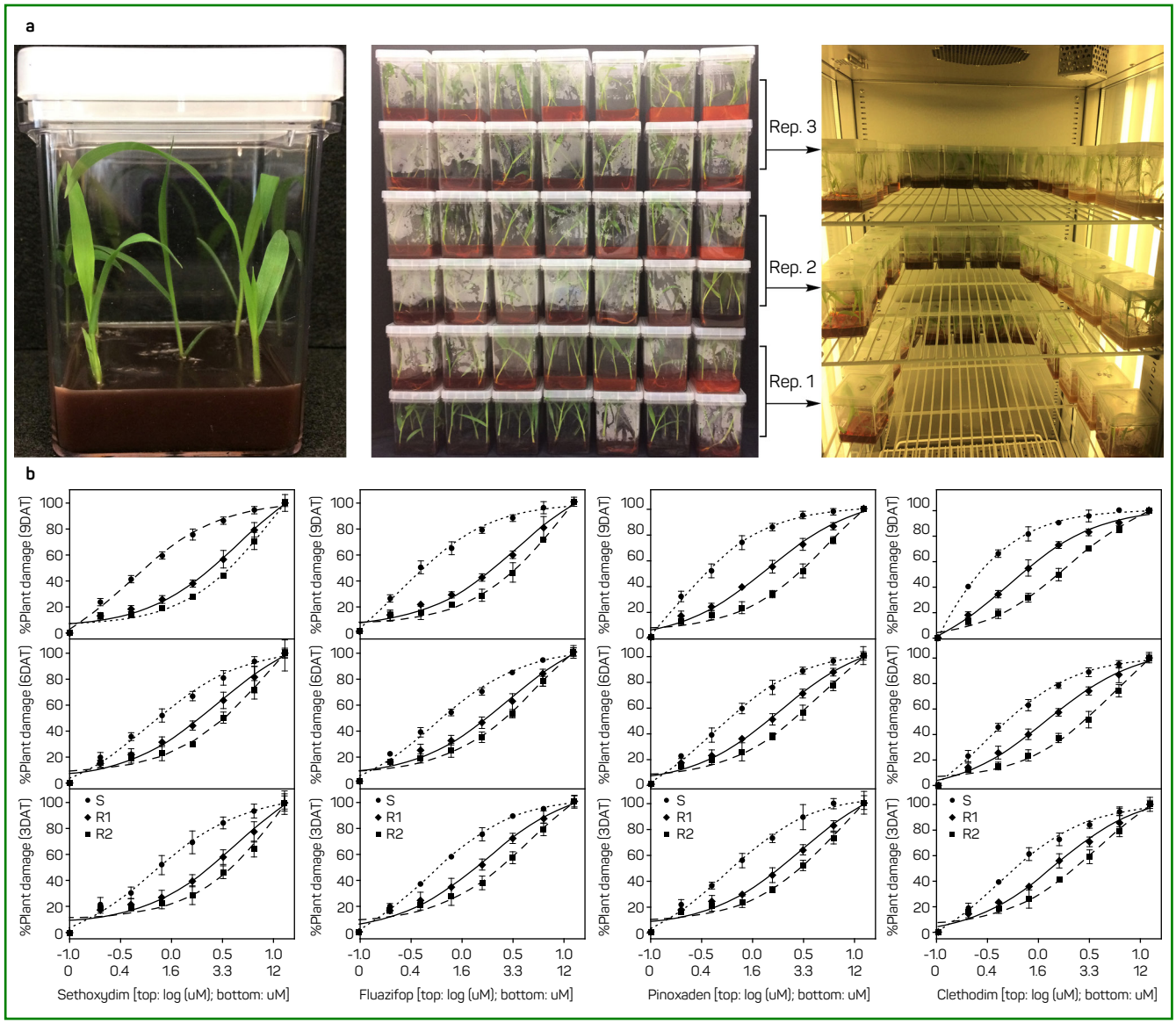
2. Materials and Methods

Experiments were conducted in the Department of Crop, Soil and Environmental Sciences, Auburn, AL, USA. Two resistant biotypes (R1 and R2) of *D. ciliaris* with confirmed resistance to select ACCase herbicides (Yu et al., 2017; Basak et al., 2019; Basak et al., 2021), and one susceptible biotype (S), a total of 360 plantlets from each biotype was included in this study. Seeds of the test plant *D. ciliaris* were grown in separate plastic flats containing commercial potting soil and peat moss (2:1 v/v) under greenhouse conditions. Daily temperatures in the greenhouse were maintained between a low of 28 °C and a high of 32 °C (night/day) (+/- 3C) throughout the study. Relative humidity levels were alternated between 65/75% (day/night). Ambient lighting was used throughout the experiment with no supplemental light added. Irrigation for plastic flats three times daily (around 0.2 cm per cycle) was provided as needed to maintain a moist soil condition. The three bioassays, agar-based gel box assay, leaf flotation assay, and electrical conductivity assay were used to evaluate the responses of *D. ciliaris* resistant and susceptible biotypes from a rate titration of sethoxydim (Segment®, BASF Corp, Research Triangle Park, NC), fluazifop-P-butyl (Fusilade®, Syngenta Crop Protection, Greensboro, NC), pinoxaden (Axial®, Syngenta Crop Protection, Greensboro, NC) and clethodim (Envoy®, Valent U.S.A. Corp., Walnut Creek, CA).

Agar-Based Gel Box Assay. Seedlings of each biotype were carefully uprooted from > 5 tiller plants with each tiller. Roots were washed under tap water to remove any growing media. The seedlings then were dissected into a single tiller with approximately 7 cm of the shoot and 5 cm of root and washed with distilled water. Polycarbonate plant culture boxes (Magenta GA-7, Bioworld, Dublin, OH) were used to determine if the agar-based gel box bioassay was useful in detecting the level of ACCase-inhibiting herbicides resistance in *D. ciliaris*. The agar-based resistance test assay was previously reported by Kaundun et al. (2011) and Brosnan et al. (2017).

MS (Murashige, Skoog, 1962) basal medium was prepared as described by Brosnan et al. (2017) and added to the solution at a dose of 4.43g L^{-1}, and the pH of the medium was adjusted to 6.5 with a pH meter with the help of 0.1 or 10 N sodium hydroxide/ hydrochloric acid solution, whichever was necessary. After adjusting the pH, 5.5 g agar powder (Plant Agar, Duchefa Biochemie, Haarlem, Netherlands) was dissolved in a 1 L MS medium to solidify the medium. This amended solution was sterilized properly at 121 °C for 20 minutes at 1.5 kg

cm^{-2} pressure using an autoclave machine (Vaccum Steam Sterilizer, Getinge, Inc, Wayne, NJ). Three milliliters of a rifampicin antibiotic and 15 µL azoxystrobin (Heritage TL, Syngenta Professional Products, Greensboro, NC) were used in the autoclaved agar medium to avoid fungal and bacterial contamination. A total of 70 mL MS media was poured into the polycarbonate plant tissue culture boxes of 10.2 cm x 7.6 cm x 7.6 cm. Prior to the agar solidifying, the commercial herbicide solution was added to the liquid agar in the tissue culture boxes. Each biotype was treated with each herbicide at a large range of concentrations, i.e., nontreated control, 0.2, 0.4, 0.8, 1.6, 3.3, 6.5, and 12 µM. Five single tiller plantlets then were embedded on top of the agar culture in each plant tissue culture box using a sterilized scalpel and forceps represented in Figure 1a. The roots below the growing point were then gently pushed into the agar, ensuring that the remaining roots were in contact with the agar. The plant tissue culture boxes then were covered with lids and incubated in the growth chamber configured to provide a constant 30°C air temperature, 60% relative humidity, and two photoperiods 10 and 18 h of light. Plant damage data were recorded at 3, 6, and 9 days after treatment (DAT) on a 0 to 100 percent scale in which 0%



Source: Photographed using a personel Canon EW-83M camera (a) Prism v. 5.0 (GraphPad Software, La Jolla, CA) (b)

Figure 1 - Single tiller plantlet of *D. ciliaris* was inserted into agar medium containing different concentrations of ACCase-inhibiting herbicides, sethoxydim, fluazifop, pinoxaden, and clethodim inside polycarbonate plant tissue culture box and the boxes were incubated into the growth chamber (a).Percent plant damage response relative to non-treated resistant and susceptible *D. ciliaris* biotypes with increasing concentrations of ACCase-inhibiting herbicides at 3, 6, and 9 days after treatment (DAT). Vertical bars represent the standard errors of the means (n=6). The response was modeled based on the log rate of ACCase-inhibiting herbicides to create equal spacing between rates using least-squares fit. *D. ciliaris* biotypes: R1 and R2, Resistant biotypes and S, susceptible biotype. (b)

corresponded to no damage and 100% corresponded to complete plant death or desiccation.

Leaf Flotation Assay. A leaf flotation test was conducted using polypropylene centrifuge 50 mL tubes containing herbicide solution to determine if the time of leaf flotation could be correlated with resistance. The solution was prepared by adding commercial herbicides and surfactants and adjusted to pH 6.5 using 0.1 or 10 N sodium hydroxide/ hydrochloric acid solution as necessary. Surfactant (2 µL; Induce, Helena® Chemical Company, Collierville, TN) was added to the solution to reduce the surface tension. A total of 40 mL solution was poured into the sterile polypropylene centrifuge tube. Each biotype was treated with each herbicide at six ranges of concentrations, i.e., nontreated control, 0.6, 1.2, 2.4, 4.8, and 9.6 µM. The leaf was cut into a single 2.5-cm long leaf segment and immediately transferred to deionized water. Three leaves then were embedded horizontally on top of the solution in each tube using sterilized forceps represented in Figure 2a. The tubes then were covered with lids and incubated at 25 °C temperature. At 8, 16, and 32 hours after treatment (HAT), leaf flotation or sinking data was recorded on a 0 or 100 scale in which 0 corresponded to sink and 100 corresponded to float (Hensley, 1981). In fact, the leaves from the susceptible biotype were highly affected by the ACCase herbicide test and sunk in the herbicidal solution.

Electrical Conductivity Assay. The electrical conductivity test was performed on the Extech EC150 conductivity meter (FLIR Systems, Wilsonville, Oregon, USA) according to the manufacturer's instructions. The 2.5-cm long leaf of each biotype collected from the 2-3 leaf stage plant was first placed in a solution containing nontreated control, 0.5, 0.9, 1.9, 3.8, and 7.5 µM of each herbicide and incubated for 24 h at room temperature. The treated leaves were removed from the herbicide solution, washed with distilled water, placed into another polypropylene centrifuge tube containing 50 ml of distilled water, and measured the electrical conductivity prior to incubate into boiling water. All the seven tubes containing treated leaves then were placed into a glass beaker containing tap water and warmed at 100 °C for 3-5 minutes with intermittent shaking of the tubes (Whitlow et al., 1992). Then, the leaves were discarded from tubes and the solution was cooled down until reaching room temperature. The conductivity meter was placed horizontally into the tubes measuring the values of electrical conductivity for each biotype. The electrical conductivity was monitored with a conductivity meter with a sensor cap represented in Figure 3a and the conductivity (µS/cm) was expressed as a percentage of the nonherbicidal control.

Experimental Design and Data Analysis. All experiments were established as a complete randomized factorial design and repeated once. All treatments were replicated three times on five individual plantlets per biotype for the gel box assay, three leaves per biotype from three individuals for the leaf flotation assay, and three leaves per biotype electrical conductivity test assays. All statistical test was carried out with the PROC GLM procedure through SAS (SAS version 9.4, SAS Institute Inc., Cary, NC). Fisher's protected LSD (P = 0.05) was used to compare the difference among S, R2, and R1. Since differences between the data of the two experimental runs were not detected in the analysis of variance at the 0.05 probability level, the data were pooled overruns for subsequent analysis. Each herbicide rates were log-transformed to produce equal spacing among treatments prior to regression analysis. A model was characterized that the relationship of the response curves with herbicide rate after plotting treatment means. All measurements relative to nontreated were used for the regression model.

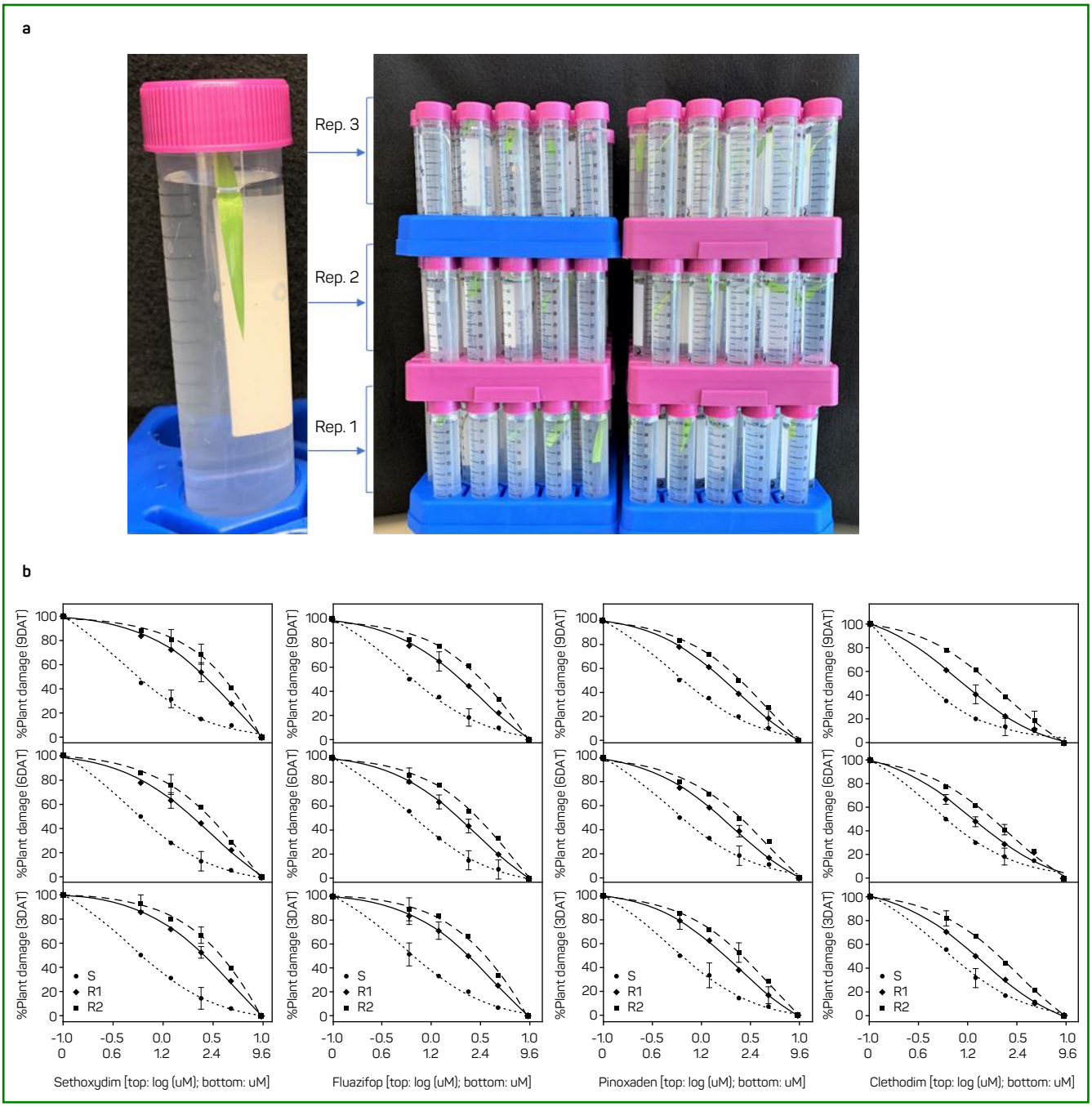
Regression models were developed using Prism (GraphPad Software, version 5.0, Inc., La Jolla, CA). ACCase-inhibiting herbicide concentrations causing 50% plant damage, leaf flotation, and electrical conductivity (IC50) values were estimated using nonlinear regression models. The following non-linear regression analysis was used to calculate the IC50 value in the experiments:

$$Y=Bottom + (Top-Bottom)/(1+10^{((X-LogIC_{50}))})$$

Y represents the response (%) of *D. ciliaris*, x is the log-transformed ACCase-inhibiting herbicides concentration (µM), Top and bottom are the plateaus in the units of the Y-axis, and LogIC50 is the log-transformed ACCase-inhibiting herbicides concentration (µM). 95% confidence intervals (α = 0.05) for the estimates were calculated for nonlinear-regression model parameters. Regression equations were used to calculate inhibition concentration values at 50% (referred to as IC_{50} values) compared to that of the nontreated for each biotype and each ACCase-inhibiting herbicide. The IC_{50} and R/S values were determined for each resistant biotype versus susceptible biotype. Percent of plant damage from agar-based gel box assay, percent of leaf flotation from leaf flotation assay, and percent data of electrical conductivity from electroconductivity assay relative to the nontreated response to ACCase-inhibiting herbicides were modeled for all three biotypes using the least-squares fit model, Figure 1b, 2b, and 3b, respectively. The response curves from each biotype were allowed for calculation of $IC_{50's}$ and R:S presented in Tables 1 and the complete tables were listed as supplementary data 1, 2, and 3.

3. Results and Discussion

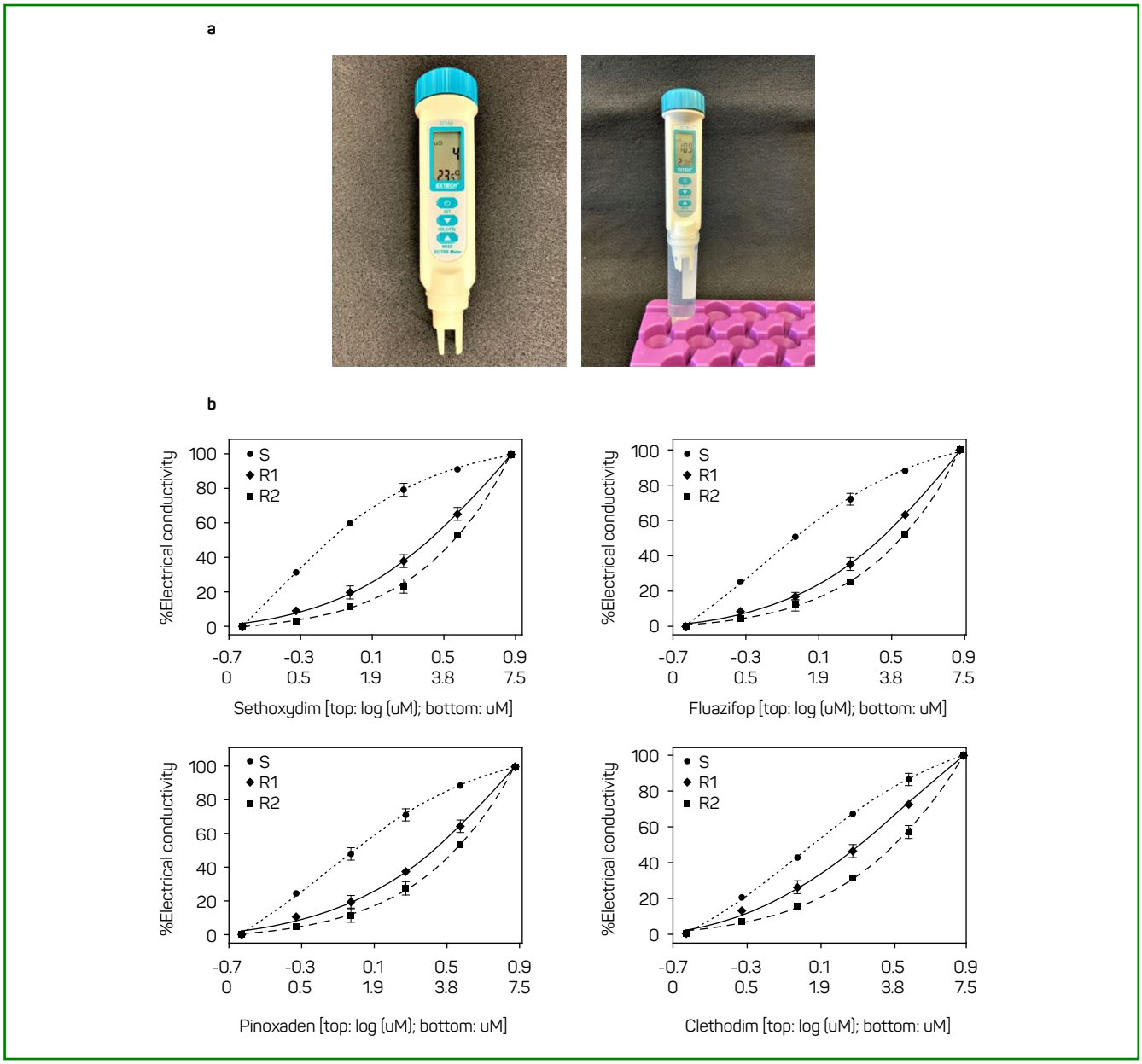
Agar-Based Gel Box Assay. The resistant and susceptible biotypes were easily discriminated against using an agar-herbicide test. Greater plant damage was observed for the S biotype compared to the R biotypes for all the herbicides tested in this study. ACCase-inhibiting herbicides at 1.6 uM produced the largest vertical differences in plant damage between dose-response curves of R and S biotypes (Figure 1b). Sethoxydim at 1.6 µM induced 56% plant damage in the S biotype, and 16.0% and 24.6% plant damage for the R2 and R1 biotypes, respectively, at 3 DAT



Source: Photographed using a personel Canon EW-83M camera (a) Prism v. 5.0 (GraphPad Software, La Jolla, CA) (b)

Figure 2 - Response of leaves flotation of susceptible and resistant *D. ciliaris* biotypes tested into polypropylene centrifuge tubes containing different concentrations of ACCase-inhibiting herbicides, sethoxydim, fluazifop, pinoxaden, and clethodim from leaf flotation assay (a). Percent leaf flotation response relative to non-treated of *D. ciliaris* biotypes with increasing concentrations of ACCase-inhibiting herbicides at 8, 16, and 32 hours after treatment (HAT). Vertical bars represent the standard errors of the means (n=6). The response was modeled based on the log rate of ACCase-inhibiting herbicides to create equal spacing between rates using least-squares fit. *D. ciliaris* biotypes: R1 and R2, Resistant biotypes and S, susceptible biotype (b)

(Days After Transplanting). Differences in phytotoxicity between S, R2, and R1 were observed at 6 and 9 DAT. For example, sethoxydim at 1.6 µM induced 63.3-74.6% plant damage at 6 and 9 DAT in the S biotype and only ≤ 20.6-36.0% for the R biotypes (Supplementary data 1). IC_{50} values of the S biotype at 3, 6, and 9 DAT were 0.7, 0.6, and 0.4 µM, respectively, compared to 13.9, 8.0, and 10.7 µM, respectively, for the R2 biotype and 4.2, 2.7, and 5.8 µM, respectively, for the R1 biotype. Sethoxydim was 19.9, 13.3, and 26.8 times more plant damage to S than R2 and 6.0, 4.5, and 14.5 times more plant damage to S than R1 at 3, 6, and 9 DAT, respectively, presented in Table 1.



Source: Photographed using a personel Canon EW-83M camera (a) Prism v. 5.0 (GraphPad Software, La Jolla, CA) (b)

Figure 3 - Response of susceptible and resistant *D. ciliaris* biotypes for the leached electrolytes from leaves samples measured with an electroconductivity meter after 24 hours ACCase-inhibiting herbicides, sethoxydim, fluazifop, pinoxaden, and clethodim treatment in electrical conductivity assay (a). Percent electrical conductivity response relative to non-treated of D. ciliaris biotypes with increasing concentrations of ACCase-inhibiting herbicides at 24 hours after treatment (HAT). Vertical bars represent the standard errors of the means (n=6). The response was modeled based on the log rate of ACCase-inhibiting herbicides to create equal spacing between rates using least-squares fit. *D. ciliaris* biotypes: R1 and R2, Resistant biotypes and S, susceptible biotype (b)

Similar to sethoxydim, fluazifop-p-butyl, and pinoxaden induced plant damage in the S more than that of R biotypes. Fluazifop-p-butyl at 1.6 µM induced 61.3% plant damage in the S biotype, and 22% and 34% plant damage for the R2 and R1 biotypes, respectively, at 3 DAT. Differences in plant damage between S, R2, and R1 were observed at 6 and 9 DAT. For example, fluazifop-p-butyl at 0.8 µM induced 50.7-64.0% plant damage at 6 and 9 DAT in the S biotype and only ≤ 17.3-28.0% for the R biotypes. IC_{50} values of the S biotype at 3, 6, and 9 DAT were 0.6, 0.5, and 0.3 µM, respectively, compared to 4.6, 5.9, and 9.8 µM, respectively, for the R2 biotype and 1.8, 2.8, and 3.4 µM, respectively, for the R1 biotype. Fluazifop-p-butyl was 7.7, 11.8, and 32.7 times more plant damage to S than R2 and 3.0, 5.6, and 11.3 times more plant damage to S than R1 at 3, 6, and 9 DAT, respectively. In the pinoxaden response evaluation, pinoxaden at 1.6 µM induced 64.7% plant damage in the S biotype, and 20.6% and 31.3% plant

Table 1 - Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing concentrations of ACCase-inhibiting herbicides relative to the nontreated control measured with least squares fit model for percent of plant damage from agar-based gel box assay, percent of leaf flotation from leaf flotation assay, and percent of electrical conductivity from electrical conductivity assay. The required concentration of ACCase-inhibiting herbicides (IC_{50}) to cause 50% in vitro inhibition of plant damage at 3, 6, and 9 (DAT), leaf flotation at 8, 16, and 32 (HAT), and electrical conductivity at 24 (HAT) were calculated from concentration-response curves. The values of IC_{50} and R:S ratio are presented as means of model comparison

Biotype[a]	ACCase-inhibiting herbicides[b]	Agar-based gel box assay			Leaf flotation assay			Electrical conductivity assay		
		Time[c]	IC_{50} (μM)[d]	R:S[e]	Time[c]	IC_{50} (μM)[d]	R:S[e]	Time[c]	IC_{50} (μM)[d]	R:S[e]
R1	Sethoxydim	3DAT	4.2	6.0	8HAT	5.1	10.2	24HAT	11.2	28
R2			13.9	19.9		16.1	32.2		31.5	78.8
S			0.7			0.5			0.4	
R1		6DAT	2.7	4.5	16HAT	2.9	7.3			
R2			8	13.3		6.7	16.8			
S			0.6			0.4				
R1		9DAT	5.8	14.5	32HAT	5.4	18			
R2			10.7	26.8		22.4	74.6			
S			0.4			0.3				
R1	Fluazifop-p-butyl	3DAT	1.8	3.0	8HAT	4.4	7.3	24HAT	8.7	14.5
R2			4.6	7.7		12.7	21.2		46.7	77.8
S			0.6			0.6			0.6	
R1		6DAT	2.8	5.6	16HAT	2.8	5.6			
R2			5.9	11.8		7.5	15			
S			0.5			0.5				
R1		9DAT	3.4	11.3	32HAT	3.1	7.8			
R2			9.8	32.7		9.7	24.3			
S			0.3			0.4				
R1	Pinoxaden	3DAT	3.0	5.0	8HAT	2.5	5.0	24HAT	7.6	10.9
R2			7.1	14.8		5.1	10.2		43.4	62
S			0.6			0.5			0.7	
R1		6DAT	1.9	4.8	16HAT	1.3	5.3			
R2			4.7	11.7		4.7	11.8			
S			0.4			0.4				
R1		9DAT	1.5	7.5	32HAT	2.3	7.7			
R2			6.1	30.5		5.4	18			
S			0.2			0.3				
R1	Clethodim	3DAT	1.5	3.8	8HAT	1.4	1.6	24HAT	3.5	3.9
R2			3.8	9.5		3.2	3.6		15.7	17.4
S			0.4			0.9			0.9	
R1		6DAT	1.2	4.0	16HAT	1.1	5.5			
R2			5.5	18.3		2.5	12.5			
S			0.3			0.2				
R1		9DAT	0.5	5	32HAT	0.8	8.0			
R2			2.0	20		2.3	23			
S			0.1			0.1				

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype; [b]ACCase-inhibiting herbicides: sethoxydim, fluazifop-p-butyl, pinoxaden, and clethodim; [c] Abbreviations: Days After Transplanting (DAT) and Hours After Treatment (HAT); [d]IC_{50}: The required concentration of ACCase-inhibiting herbicides was calculated by 50% based on regression curve to fit in the concentration response inhibition equation, and [e]R:S: resistant/susceptible ratios Source: The value of IC50 was generated from the Prism v. 5.0 (GraphPad Software, La Jolla, CA). Based on IC50 value, the R/S ratio was calculated for each biotypes.

damage for the R2 and R1 biotype, respectively, at 3 DAT. Differences in plant damage between S, R2, and R1 were observed at 6 and 9 DAT. For example, pinoxaden at 3.3 µM induced 88.3-94.7% plant damage at 6 and 9 DAT in the S biotype and only ≤ 42.6-71.3% plant damage for both R biotypes. IC_{50} values of the S biotype at 3, 6, and 9 DAT were 0.6, 0.4, and 0.2 µM, respectively, compared to 7.1, 4.7, and 6.1 µM, respectively, for the R2 biotype and 3.0, 1.9, and 1.5 µM, respectively, for the R1 biotype. Pinoxaden was 14.8, 11.7, and 30.5 times more plant damage to S than R2 and 5.0, 4.8, and 7.5 times more plant damage to S than R1 at 3, 6, and 9 DAT, respectively.

Clethodim induced greater plant damage in S biotype than in both resistant biotypes. Clethodim at 1.6 µM induced 72.0% plant damage in the S biotype, and 30.6% and 48.0% plant damage for the R2 and R1 biotypes, respectively, at 3 DAT. Differences in plant damage between S, R2, and R1 were observed at 6 and 9 DAT. For example, clethodim at 1.6 µM induced 78.0-90.0% plant damage at 6 and 9 DAT in the S biotype and only ≤ 36.0-72.6% for the R biotypes, indicating the R biotypes had a degree of resistance to clethodim. Clethodim induced greater plant damage in both resistant biotypes than other ACCase-inhibiting herbicides. For instance, clethodim at 0.8 µM produced 22.7-35.3% plant damage of resistant biotypes at 6 DAT, while other ACCase-inhibiting herbicides, sethoxydim, fluazifop, and pinoxaden induced 16.0-19.3%, 17.3-22.5%, and 18.6-24.7%, respectively in R biotypes. IC_{50} values of the S biotype at 3, 6, and 9 DAT were 0.4, 0.3, and 0.1 µM, respectively, compared to 3.8, 5.5, and 2.0 µM, respectively, for the R2 biotype and 1.5, 1.2, and 0.5 µM, respectively, for the R1 biotype. Clethodim was 9.5, 18.3, and 20 times more plant damage to S than R2 and 3.8, 4.0, and 5.0 times more plant damage to S than R1 at 3, 6, and 9 DAT, respectively. For the most part, agar-based rapid bioassays utilizing seedlings have been used to identify EPSPS, ALS, ACCase (Brosnan et al., 2017; Kaundun et al., 2011; Perez, Kogan, 2003; Tal et al., 2000), and auxinic MCPA (Polit et al., 2014) inhibiting herbicides resistances. This bioassay has the advantage of a quicker response than whole plant or pot bioassays, which can be completed by inexperienced personnel. Overall, this bioassay's findings showed that the ACCase herbicide resistance in *D. ciliaris* could be distinguished utilizing a gel box bioassay with agar media.

Leaf Flotation Assay. R biotype leaves had a greater flotation tendency at lower concentrations than S. ACCase-inhibiting herbicides at 1.2 uM produced the largest vertical differences in leaf flotation between dose-response curves of R and S biotypes (Figure 2b). At 8 HAT (Hours After Treatment), sethoxydim at 1.2 µM inhibited S biotype leaf flotation by 68.7%, while R2 and R1 biotypes inhibited flotation by 20.0% and 28.6%, respectively. Inhibition of leaf flotation was observed at 16 and 32 HAT for all biotypes. For example, sethoxydim at 2.4 µM inhibited S biotype leaf flotation by 78.4-85.0% at 16 and 32 HAT, while the R2 biotype inhibited flotation by 11.6-31.4%, respectively, and R1 inhibited flotation at 16.7-55.6%, respectively (Supplementary data 2). IC_{50} values of the S biotype at 8, 16, and 32 HAT were 0.5, 0.4, and 0.3 µM, respectively, compared to 16.1, 6.7, and 22.4 µM, respectively, for the R2 biotype and 5.1, 2.9, and 5.4 µM, respectively, for the R1 biotype. Sethoxydim was 32.2, 16.8, and 74.6 times more inhibited in flotation to S than R2 and 10.2, 7.3, and 18 times more inhibited to S than R1 at 8, 16, and 32 HAT, respectively, presented in Table 1.

Similarly, fluazifop-p-butyl and pinoxaden inhibited S biotype leaf flotation greater across all concentrations than both R biotypes. At 8 HAT, fluazifop-p-butyl at 1.2 µM inhibited S biotype leaf flotation by 66.7%, while the R2 and R1 biotypes inhibited flotation by only 16.7% and 29.2%, respectively. Inhibition of leaf flotation was observed at 16 and 32 HAT for all biotypes. For example, fluazifop-p-butyl at 4.8 µM inhibited S biotype leaf flotation by 90-92.6% at 16 and 32 HAT, while the R2 biotype inhibited flotation by 55.0-66.7%, respectively, and R1 inhibited flotation by 70.0-77.0%, respectively. IC_{50} values of the S biotype at 8, 16, and 32 HAT were 0.6, 0.5, and 0.4 µM, respectively, compared to 12.7, 7.5, and 9.7 µM, respectively, for the R2 biotype and 4.4, 2.8, and 3.1 µM, respectively, for the R1 biotype. Fluazifop-p-butyl was 21.2, 15, and 24.3 times more inhibited in flotation to S than R2 and 7.3, 5.6, and 7.8 times more inhibited to S than R1 at 8, 16, and 32 HAT, respectively. At 8 HAT, pinoxaden at 1.2 µM inhibited S biotype leaf flotation by 60.8%, while R2 and R1 biotypes inhibited flotation by 28.6% and 37.5%, respectively. Inhibition of leaf flotation was observed at 16 and 32 HAT for all biotypes. For example, pinoxaden at 0.6 µM inhibited S biotype leaf flotation by 48.0-50.0% at 16 and 32 HAT, while R2 inhibited flotation by 16.7-20.0%, respectively, and R1 biotype inhibited flotation by 22.2-25.0%, respectively. IC_{50} values of the S biotype at 8, 16, and 32 HAT were 0.5, 0.4, and 0.3 µM, respectively, compared to 5.1, 4.7, and 5.4 µM, respectively, for the R2 biotype and 2.5, 1.3, and 2.3 µM, respectively, for the R1 biotype. Pinoxaden was 10.2, 11.8, and 18 times more inhibited in flotation to S than R2 and 5.0, 5.3, and 7.7 times more inhibited to S than R1 at 8, 16, and 32 HAT, respectively.

The S biotype was relatively more sensitive to clethodim herbicide than the R biotype. At 8 HAT, clethodim at 1.2 µM inhibited S biotype leaf flotation 68.5%, while R2 and R1 biotypes inhibited 33.4% and 50.0% flotation, respectively. Inhibition of leaf flotation was observed at 16 and 32 HAT for all biotypes. For example, clethodim at 2.4 µM inhibited S biotype leaf flotation by 81.7-86.7% at 16 and 32 HAT, while the R2 biotype was inhibited by 58.9-61.1% flotation, respectively, and R1 biotype by inhibited 71.4-77.8% flotation, respectively. IC_{50} values of the S biotype at 8, 16, and 32 HAT were 0.9, 0.2, and 0.1 µM of clethodim, respectively, compared to 3.2, 2.5, and 2.3 µM, respectively, for the R2 biotype and 1.4, 1.1,

and 0.8 µM, respectively, for the R1 biotype. Clethodim was 3.6, 12.5, and 23 times more inhibited in flotation to S than R2 and 1.6, 5.5, and 8 times more inhibited to S than R1 at 8, 16, and 32 HAT, respectively. Our finding of ACCase herbicide resistance in *D. ciliaris* is consistent with other herbicides resistance in most of the literature on the subject in resistance to triazine in *Senecio vulgaris* L. (common groundsel), *Chenopodium album* L. (common lambs-quarters), and *Amaranthus hybridus* L. (smooth pigweed) (Hensley, 1981); resistance to EPSPS in *Glycine max* (soybean), *Brassica napus* (canola), and *Zea mays* (corn) (Shaner et al., 2005), and resistance to fomesafen, glyphosate, and dicamba in *Amaranthus palmeri* S. Watson (Palmer amaranth), *Amaranthus tuberculatus* (Moq.) Sauer (water hemp), *Bassia scoparia* L. (kochia), and *Eleusine indica* (L.) Gaertn. goosegrass (Wu et al., 2021). This leaf flotation bioassay's benefits allow for the detection of herbicide resistance in numerous plants without destroying any plant parts or restricting their ability to reproduce. The results of this bioassay indicated that it may have been possible to detect *D. ciliaris* ACCase herbicide resistance by using a leaf flotation bioassay with the herbicidal solution.

Electrical conductivity Assay. The S biotype produced a greater electrical conductivity for leaching electrolytes into the water to the ACCase-inhibiting herbicides relative to the R biotypes. ACCase-inhibiting herbicides at 2.0 uM produced the largest vertical differences in electrical conductivity between dose-response curves of R and S biotypes (Figure 3b). Sethoxydim at 0.9-3.8 µM produced 59.7-90.7% electrical conductivity in S, respectively, 11.7-53.3% electrical conductivity in R2, respectively, and 19.7-65.2% electrical conductivity in R1, respectively (Supplementary data 3). The IC_{50} value of the S biotype was 0.4 µM compared to 31.5 and 11.2 µM for the R2 and R1 biotypes, respectively. Sethoxydim was 79 and 28 times more leaching electrolyte to S than R2 and R1, respectively, implying that sethoxydim efficiently caused greater leaching electrolyte into the water from the S presented in Table 1.

Like sethoxydim, fluazifop-P-butyl and pinoxaden produced electrical conductivity in the S more than that of R biotypes. Fluazifop-p-butyl at 0.9-3.8 µM produced 47.8-88.4% electrical conductivity in S, respectively, 12.6-50.4% electrical conductivity in R2, respectively, and 17.1-59.0% electrical conductivity in R1, respectively. The IC_{50} value of the S biotype was 0.6 µM compared to 46.7 and 8.7 µM for the R2 and R1 biotypes, respectively. Fluazifop-p-butyl was 77 and 15 times more leaching electrolyte to S than R2 and R1, respectively. Pinoxaden at 0.9-3.8 µM produced 47.8-86.3% electrical conductivity in S, respectively, 11.2-53.2%, and 19.4-64.2% electrical conductivity in R2 and R1, respectively. The IC_{50} value of the S biotype was 0.7 µM compared to 43.4 and 7.6 µM for the R2 and R1 biotypes, respectively. Pinoxaden was 62 and 11 times more leaching electrolyte to S than R2 and R1, respectively.

Clethodim resulted in a significant increase in electrical conductivity for all biotypes comparing all four herbicides used. However, the S biotype had relatively greater clethodim electrical conductivity than the R biotype. Clethodim at 0.9-3.8 µM produced 50.7-84.5% electrical conductivity in the S biotype, respectively, 15.6-57.1% electrical conductivity in R2, respectively, and 26.1-68.3% electrical conductivity in R1, respectively. The IC_{50} value of the S biotype was 0.97 µM compared to 15.7 and 3.5 µM for the R2 and R1 biotypes, respectively. Clethodim was 17 and 4 times more leaching electrolyte to S than R2 and R1, respectively. Previous research also reported the R1 and R2 biotypes had differential resistance to clethodim when foliar was applied (Yu et al., 2017) and comparatively lower ACCase enzyme activity to clethodim than the other inhibitors sethoxydim, pinoxaden, and fluazifop (Basak et al., 2019; Basak et al., 2021). Using the electrical conductivity assay, the viability of seeds from a variety of crops, including *Brassica* spp. (cabbage), *Pisum sativum* (garden pea), *G. max* (soybean), and *Phaseolus vulgaris* (common bean), was evaluated (Matthews et al., 2009). Our results from electrical conductivity indicated that compared to the other two bioassays, the electrical conductivity bioassay with leaching electrolyte into the water was highly sensitive, accurate, rapid, and required only 24h for discriminating between R and S biotypes. This assay can be useful tool in determination of herbicide resistant weed.

4. Conclusion

Research Implication. Timely detection of herbicide resistance is essential to aid end-users to identify alternative solutions for managing herbicide-resistant weeds. Traditionally, the characterization of grass weed resistance has mainly relied on greenhouse screening, which required considerable time and labor. Only the electrical conductivity bioassay could be considered a rapid assay per se as initial injury from a greenhouse screen could likely be observed in the same timeline as the other bioassays evaluated. However, the development of a rapid bioassay was not our objective. Rather our objective was simply to evaluate the utility of these bioassays for ACCase resistance in *D. ciliaris* as the ACCase carboxyl transferase domains of the R and S biotypes have already been sequenced and characterized (Basak et al., 2019). While our evaluation may have broader implications than this specific mode of action and this specific species, we also acknowledge that the positive and negative implications of our findings may be restricted to this mode of action and species. In fact, it is our opinion that the mode of action and species bioassay specificity should be the rule rather than the exception.

While differences were observed between resistant and susceptible biotypes, we would also admit that the bioassay methods were not without complications. Susceptible biotypes had greater plant damage to ACCase-

inhibiting herbicides in the gel-box assay after 9 days than the R biotypes. Although plant damage was significantly greater in the susceptible biotype compared to R biotypes, plant damage development also was found in R biotypes in the gel box assay. We realized that plantlets not only were inhibited by ACCase herbicide treatment but also experienced wilting and plant damage, possibly from simple transplant shock. Also, this visual assessment test assay has required the researcher to create a lot of clones, sacrifice the clones, and need to wait at least 9 days to investigate the ACCase herbicides resistance of the suspected *D. ciliaris* plants.

The leaf flotation assay required less time and an easier setup than the agar-based gel box assay. This assay allowed to separate between R and S biotypes in visual evaluations of ACCase-inhibiting herbicides within 32 h by avoiding transpiration. The leaf flotation assay also can be used to determine the resistance to ACCase-inhibiting herbicides in large numbers of plants without destroying the whole plant or preventing reproduction. The rate of leaves sinking was always significantly higher in the susceptible biotype compared to the R biotypes. However, the tendency of leaves sinking was found for both susceptible and resistant biotypes. This unpredictable result indicated that the leaf flotation assay may be more suitable for photosynthesis-inhibiting herbicides rather than ALS and ACCase-inhibiting herbicides, as has been demonstrated in the past (Hensley, 1981). Likewise, the electrical conductivity assay identified a separation between R and S biotypes 24 h after treatment. However, the difference in electrical conductivity between R and S was less than 45% across all herbicides and concentrations. We question whether a single rate could provide the necessary clear difference to consistently diagnose resistance.

The IC_{50} values for resistant biotypes across all four herbicides were consistently greater than the susceptible biotypes in the three different assays, indicating that the resistant biotypes are resistant to ACCase-inhibiting herbicides. All the rapid bioassays showed similar trends in resistance. To our knowledge. this is the first report of rapid bioassay with three different assays from *D. ciliaris* to the ACCase-inhibiting herbicides resistance. While these bioassays effectively distinguished between previously diagnosed resistant populations, we question if a single rate at a single rating data could be used to diagnose ACCase resistance. After conducting this extensive evaluation, we are skeptical of their utility and would recommend traditional herbicide spray exposure to evaluate de novo resistance until these methodologies have been more thoroughly vetted.

Authors' contributions

All authors reviewed and agreed to the published version of the manuscript. SB and JSM: Conceptualization of the manuscript and development of the methodology. SB, BB, CGG, and QL: Data collection and curation. SB and JDP: data analysis. SB and JSM: data interpretation. JSM and PEM: funding acquisition and resources. SB and JSM: project administration. JSM: supervision. SB: writing the original draft of the manuscript. SB and JSM: writing, review and editing.

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