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Detecting the effect of ACCase-targeting herbicides on ACCase activity utilizing a malachite green colorimetric functional assay

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Research Article

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Abstract

Research was conducted using a functional malachite green colorimetric assay to evaluate acetyl-coenzyme A carboxylase (ACCase) activity previously identified as resistant to sethoxydim and select aryloxyphenoxypropionate (FOPs) herbicides, fenoxaprop, and fluazifop. Two resistant southern crabgrass [*Digitaria ciliaris* (Retz.) Koeler] biotypes, R1 and R2, containing an Ile-1781-Leu amino acid substitution and previously identified as resistant to sethoxydim, pinoxaden, and fluazifop but not clethodim was utilized as the resistant chloroplastic ACCase source compared with known susceptible (S) ACCase. Dose-response studies with sethoxydim, clethodim, fluazifop-*p*-butyl, and pinoxaden (0.6 to 40 µM) were conducted to compare the ACCase–herbicide interactions of R1, R2, and S using the malachite green functional assay. Assay results indicated that R biotypes required more ACCase-targeting herbicides to inhibit ACCase activity compared with S. IC_{50} values of all four herbicides for R biotypes were consistently an order of magnitude greater than those of S. No sequencing differences in the carboxyltransferase domain was observed for R1 and R2; however, R2 IC_{50} values were greater across all herbicides. These results indicate the malachite green functional assay is effective in evaluating ACCase activity of R and S biotypes in the presence of ACCase-targeting herbicides, which can be used as a replacement for the ^{14}C-based radiometric functional assays.

Introduction

Acetyl-coenzyme A carboxylase (ACCase or ACCs; EC.6.4.1.2) is an essential enzyme that catalyzes the formation of malonyl-CoA. The reaction product, malonyl-CoA, is involved in the biosynthesis of de novo fatty acids in plastids and the elongation of long-chain fatty and secondary metabolites that are crucial for energy storage, cell or organelle biomembrane structure composition, and hormonal regulation (Délye et al. 2011; Harwood, 1988; Keereetaweep et al. 2018; Konishi et al. 1996; Ohlrogge and Browse 1995; Petit et al. 2010; Podkowinski et al. 2003; Yang et al. 2018; Ye et al. 2018; Yu et al. 2007). ACCase-targeting herbicides inhibit chloroplastic ACCase activity in grasses of the Poaceae family, resulting in a decrease in fatty-acid production (Lancaster et al. 2018; Powles 2005). These herbicides are commonly applied postemergence to control grass weeds in both crop and turf systems.

Herbicide resistance to ACCase-targeting herbicides has developed via target-site (TSR) and non–target-site resistance (NTSR) mechanisms. The TSR amino acid substitutions associated with ACCase-targeting herbicides occurring in the carboxyl transferase domain have been reported as follows: Gln-1756-Glu, Ile-1781-Leu, Thr-1805-Ser, Lys-1930-Arg, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala (Beckie and Tardif 2012; Collavo et al. 2011; Délye 2005; Kaundun 2010; Kaundun et al. 2012, 2013; Powles and Yu 2010). NTSR encompasses a range of processes, including increased enzyme expression, increased enzyme abundance, enhanced metabolism of the herbicides, herbicide detoxification, reduced herbicide uptake, penetration, and impaired translocation. Different enzymes are involved in the development of NTSR, including, but not limited to, cytochrome P450 monooxygenases, glutathione-*S*-transferases, glycosyl-transferases, hydrolases, oxidases, and peroxidases (Cocker et al. 1999; Kaundun 2014; Kukorelli et al. 2013; Powles and Yu 2010; Preston 2003; Yuan et al. 2007).

A research component in herbicide-resistance discovery is a functional assay evaluating the interaction of the target site with herbicide. Functional assays measure the target-site activity of

suspected resistance biotypes compared with the known susceptible biotypes. Functional assays assess enzymatic activity in the presence of herbicides to determine whether amino acid changes affect the enzyme–herbicide interaction. The most commonly used functional assay to assess ACCase activity is a ^{14}C-based radiometric assay (Cocker et al. 1999; Cruz-Hipolito et al. 2011; De Prado et al. 2004; Secor and Cséke 1988; Seefeldt et al. 1996; Yang et al. 2007). The ^{14}C-based radiometric assay, however, is expensive and requires special ^{14}C-detection equipment and handling of radioactive materials, and the enzyme can be insoluble in scintillation mixtures. Howard and Ridley (1990) found a similar inhibition concentration value comparing the ^{14}C-based radiometric assay and a malachite green colorimetric assay in the maize (*Zea mays* L.) ACCase–fluazifop-*p*-butyl interaction test. Compared with the ^{14}C-based assay, the malachite green assay offers several advantages, including simplicity, specificity for inorganic orthophosphate, accuracy, high sensitivity, stability of the reagents, and lower cost, because nonradioactive materials are used for the labeling of the enzyme-substrate (Baykov et al. 1988; Carter and Karl 1982; Geladopoulos et al. 1991; Van Veldhoven and Mannaerts 1987).

Our research objective was to develop the methodology and evaluate the malachite green colorimetric assay as a functional assay for evaluating ACCase–herbicide interaction. To our knowledge, the malachite green functional assay has not been utilized to assess ACCase herbicide resistance, as the majority of studies have used the ^{14}C functional assay. Furthermore, our research will be the first to report ACCase activity for all three families of ACCase-targeting herbicides. Two resistant biotypes with the Ile-1781-Leu amino acid substitution and a known susceptible biotype of southern crabgrass [*Digitaria ciliaris* (Retz.) Koeler] were used as a model for evaluating the effectiveness of the malachite green assay in ACCase-resistance detection. Previous research quantified the TSR mechanism and rate response screen to these biotypes (Basak et al. 2019; Yu et al. 2017).

Materials and Methods

Seed Sample Collection and Growth Conditions

Previously collected seeds of two resistant biotypes (R1 and R2) of *D. ciliaris* with confirmed resistance to select ACCase-targeting herbicides and one susceptible (S) biotype were included in this study (Basak et al. 2019; Yu et al. 2017). To generate green plant material for enzyme extraction, seeds of resistant and susceptible *D. ciliaris* biotypes were sown in separate plastic flats containing commercial potting soil and peat moss (2:1 v/v). The plastic flats were placed for 2 wk in a greenhouse set for 32/25 C (day/night). Ambient lighting was used throughout the experiment with no supplemental light added. Relative humidity levels alternated between 65% during the day and 75% during the night. No supplementary fertility was provided because of the quick turnaround and no signs of nutrient stress were observed. Plastic flats were irrigated three times daily (around 0.2 cm cycle^{-1}) to provide adequate moisture.

Malachite Green Colorimetric Assay

Research was conducted in the Department of Chemistry and Biochemistry, Auburn University, Auburn, AL, USA. ACCase extraction and activity bioassay were performed as described by Howard and Ridley (1990) with some modifications (Supplementary Data 1). Enzymes were extracted in the cold chamber at 4 C from healthy plants of three *D. ciliaris* biotypes: S, R1, and R2. Approximately 10 g of fresh leaf tissues were harvested and ground in liquid nitrogen with a mortar and pestle and then suspended in 40 ml of ice-cold enzyme extraction buffer (100 mM Tricine, pH 8.0, 5mM dithiothreitol, 10 mM $MgCl_2.6H_2O$, 1 mM Na_2EDTA, 0.5% [w/w] polyvinylpyrrolidone, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was stirred for 30 min on ice and then filtered through four layers of cheesecloth. The solution was kept on ice until being centrifuged at 22,000 × *g* (Optima XE-90 Ultracentrifuge, Beckman Coulter, Inc. Brea, CA, USA) for 30 min to remove cell debris. The pellet was discarded, and the supernatant was collected and adjusted to 30% ammonium sulfate saturation with solid ammonium sulfate. After being stirred for 20 min, the solution was centrifuged at 22,000 × *g* for 30 min. The supernatant was decanted, adjusted to 60% ammonium sulfate saturation, and centrifuged to allow protein precipitation as previously described. The final pellet after the 60% precipitation was resuspended in 2 ml elution buffer I (10 mM Tris, 20 mM mercaptoethanol, 1 mM Na_2EDTA, 1 mM benzamidine, 10 mM $MgCl_2.6H_2O$, and 20% glycerol). The enzyme extract was desalted on a Sephadex G-25 column (SIGMA@ Chemical Company, St. Louis, MO, USA) equilibrated with elution buffer II solution (10 mM Tris, 20 mM mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, 10 mM $MgCl_2.6H_2O$, and 10% glycerol). The enzyme extracts were frozen at −80°C and assayed within a week of extraction.

The enzyme concentration in the enzyme extracts was measured using a Bradford assay (Bradford 1976) with bovine serum albumin (BSA) as a standard. The concentration of enzyme extract used for all biotypes was 5.3 µM after standardization with BSA. Using SDS-PAGE (superior protein separation−polyacrylamide gel electrophoresis) analysis, the enzyme extracts from S, R1, and R2 were separated and compared with maize. The assay was performed as three independent extractions, and each treatment was replicated three times per ACCase-targeting herbicide dose such as sethoxydim (Segment®, BASF, Research Triangle Park, NC), clethodim (Envoy®, Valent, Walnut Creek, CA), fluazifop-*p*-butyl (Fusillade®, Syngenta, Greensboro, NC), and pinoxaden (Axial®, Syngenta, Greensboro, NC). The assay was carried out using 96-well plates (TECAN®, Morrisville, NC), where each well contained a total of 250 µl of the reaction mixture. Each reaction mixture contained 25 µl of enzyme extract (final concentration of enzyme for each biotype in each tube was maintained to 0.53 µM by adding 25 µl of enzyme extracts), 25 µl of ACCase-targeting herbicide at a series of concentrations (0, 0.6, 1.2, 2.5, 5.0, 10, 20, and 40 µM), and 150 µl of the enzyme assay buffer (0.1 M Tricine, pH 8.0, 15 mM KCl, 3 mM $MgCl_2.6H_2O$ 1 mM dithiothreitol, 0.01 BSA, 120 mM $NaHCO_3$, and 25 mM ATP). Then 25 µl of acetyl CoA (lithium salt, final concentration 4.5 mM) was added to start the reaction. All the reaction mixtures were incubated at 30 C for 20 min before the addition of malachite green to stop the reaction. The reaction was terminated by the addition of 25 µl of malachite green termination solution. The malachite green stock solution was prepared using 72.9 mg of malachite green dissolved in 3.31 ml of 12.1 M HCl with molecular-grade deionized water added for a final volume of 200 ml. The solution was filtered through a 0.45-µm PTFE filter. The malachite green termination solution was prepared with a 5 ml malachite green stock solution

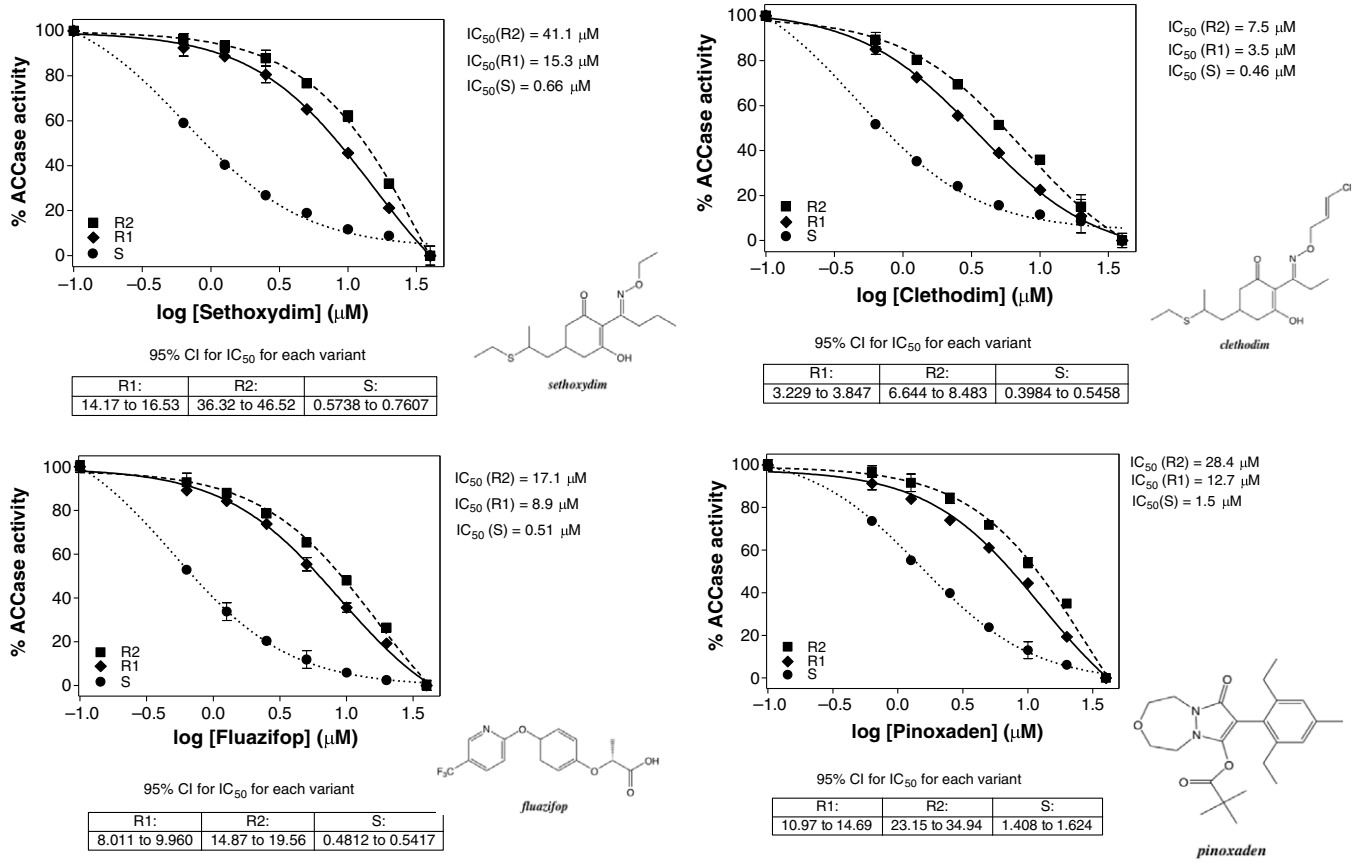

Figure 1. Response curves for percent acetyl-coenzyme A carboxylase (ACCase) activities of resistant and susceptible *Digitaria ciliaris* biotypes in response to the increasing concentrations of the ACCase-targeting herbicides, sethoxydim, clethodim, fluazifop-*p*-butyl, and pinoxaden. The response was modeled based on the log rate of ACCase-targeting herbicides to create equal spacing between rates using least-squares fit regression of ACCase activity to the non-treated check. Means are represented by differing symbols for each biotype, and regression equation models are represented by differing line types for each biotype. Vertical bars represent the standard errors of the means ($n = 6$). *Digitaria ciliaris* biotypes: R1 and R2, resistant; S, susceptible. The concentration of ACCase-targeting herbicides required to cause 50% inhibition of ACCase activity (IC_{50}) was calculated from concentration-response curves. CI, confidence interval.

mixed with 1.44 ml of 8.5 mM ammonium molybdate and 0.104 ml of 10% Triton-X.

Standard curves were generated with inorganic phosphate-containing non-treated control at 1.2, 2.5, 0.5, 1.0, 2.0, and 4.0 μM concentrations dissolved in water and added to the wells before the addition of 25 μl of malachite green solution. The absorbance of ACCase enzyme activity was taken at 630 nm colorimetrically on a microplate photometer (TECAN®) and was expressed as a percentage of the nonherbicidal control (Supplementary Data 1).

The design of the experiment was replicated twice in time as a completely randomized design with three replications. An ANOVA using PROC GLM in SAS v. 9.4 (SAS Institute Inc. Cary, NC, USA) was performed on all data to detect the significant differences among the herbicide concentrations and biotypes. The linear model was developed with herbicide treatment, herbicide rates, biotypes, replication, and experiments repeated in time as main effects. Experimental run by herbicide treatment by biotype was evaluated as an indicator of differences over experimental runs. The data were pooled overruns for subsequent analysis, as differences between the data of the two experimental runs were not detected in the ANOVA at the 0.05 probability level. Regression models were developed using Prism v. 5.0 (GraphPad Software, La Jolla, CA). ACCase-targeting herbicide concentrations causing 50% inhibition of the ACCase activity (IC_{50}) values were estimated using nonlinear regression models. The following nonlinear regression analysis was used to calculate the IC_{50} value in the enzymatic experiment:

$$Y = bottom + (top - bottom)/(1 + 10^{(X - logIC50)}) \quad [1]$$

where Y is the enzyme response (%), X is log-transformed ACCase-targeting herbicide concentration (μM), top and bottom are the plateaus in the units of the y axis, and $logIC_{50}$ is the log-transformed ACCase-targeting herbicide concentration (μM). The 95% confidence intervals ($\alpha = 0.05$) 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 with that of the non-treated for each biotype and each ACCase-targeting herbicide. The IC_{50} and R/S values (ratio of R to S IC_{50} values) were determined for each resistant biotype versus the susceptible biotype. Percent ACCase activity relative to the non-treated response to ACCase-targeting herbicide was modeled for all three biotypes using the least-squares fit model to allow for calculation of IC_{50} values (Figure 1) presented in Table 1.

Results and Discussion

Herbicide treatment by biotype by experimental run interactions was nonsignificant ($P > 0.05$) for ACCase enzyme activity;

Table 1. Comparison of resistant and susceptible *Digitaria ciliaris* biotypes for percent of acetyl-coenzyme A carboxylase (ACCase) activity to increasing ACCase-targeting herbicide concentration relative to the non-treated control measured with the least-squares fit model.

Herbicide[a]	Biotype[b]	Equation[c]	Parameter estimates and confidence intervals[d]					
		$Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10\text{^}(X - \log IC_{50}))$	Bottom	95% CI	Top	95% CI	Log IC_{50}	95% CI
Sethoxydim	R1	$Y = -38.3 + (99.5 + 38.3)/(1 + 10\text{^}(X - 1.2))$	−38.3	(−42.4, −34.2)	99.5	(98.6, 100.5)	1.1	(1.1, 1.2)
	R2	$Y = -103.5 + (99.7 + 103.5)/(1 + 10\text{^}(X - 1.6))$	−103.5	(−117.7, −89.3)	99.7	(98.8, 100.6)	1.6	(1.6, 1.7)
	S	$Y = -3.3 + (114.2 + 3.3)/(1 + 10\text{^}(X + 0.2))$	3.3	(1.4, 5.2)	114.2	(109.4,118.9)	−0.1	(−0.2, −0.1)
Clethodim	R1	$Y = -6.9 + (102.1 + 6.9)/(1 + 10\text{^}(X - 0.5))$	−6.9	(−9.0, −4.8)	102.1	(100.3, 103.9)	0.5	(0.5, 0.6)
	R2	$Y = -17.1 + (99.1 + 17.1)/(1 + 10\text{^}(X - 0.9))$	−17.1	(−21.2, −13)	99.1	(97.2, 101.1)	0.9	(0.8, 0.9)
	S	$Y = 4.2 + (120.1 - 4.2)/(1 + 10\text{^}(X + 0.3))$	4.2	(2.3, 6.1)	120.1	(114.2, 125.9)	−0.3	(−0.4, −0.3)
Fluazifop-*p*-butyl	R1	$Y = -20.4 + (99.4 + 20.4)/(1 + 10\text{^}(X - 0.9))$	−20.4	(−24.5, −16.4)	99.4	(97.7, 101)	0.9	(0.9, 1.0)
	R2	$Y = -39.8 + (98.2 + 39.8)/(1 + 10\text{^}(X - 1.2))$	−39.8	(−47.4, −32.3)	98.2	(96.7, 99.8)	1.2	(1.1, 1.3)
	S	$Y = -0.4 + (119.5 + 0.4)/(1 + 10\text{^}(X + 0.3))$	−0.4	(−1.2, 0.4)	119.5	(117.3, 121.8)	−0.2	(−0.3, −0.3)
Pinoxaden	R1	$Y = -30.2 + (97.8 + 30.2)/(1 + 10\text{^}(X - 1.1))$	−30.2	(−36.8, −23.6)	97.8	(95.9, 99.7)	1.1	(1.0, 1.2)
	R2	$Y = -67.7 + (99.1 + 67.7)/(1 + 10\text{^}(X - 1.5))$	−67.7	(−84.5, −50.8)	99.1	(97.3, 100.9)	1.5	(1.4, 1.5)
	S	$Y = -2.1 + (106.1 + 2.1)/(1 + 10\text{^}(X - 0.2))$	−2.1	(−3.4, −0.8)	106.1	(104.1, 108)	0.2	(0.1, 0.2)

[a]ACCase-targeting herbicides: sethoxydim, clethodim, fluazifop-*p*-butyl, and pinoxaden.
[b]*D. ciliaris* biotypes: R1 and R2, resistant biotypes; S, susceptible biotype.
[c]In the least-squares fit equation, *X* represents the concentration of ACCase-targeting herbicide, *Y* represents the response variable of ACCase activity.
[d]Parameter estimates and parameter estimate 95% confidence intervals (CI) are presented as means of model comparison.

therefore, data pooled over the experimental runs. Herbicide treatment by biotype was significant ($P < 0.05$) for all four herbicides tested. Data presented will focus on biotype response to increasing concentrations of the four herbicides tested. ACCase activity in the presence of a given herbicide concentration was expressed as a percentage of enzyme activity reduction relative to no herbicide. In general, 40 µM of all four herbicides resulted in completely diminished ACCase activity for all three biotypes. IC_{50} values were, however, consistently higher for ACCase activity from R1 and R2 biotypes relative to the S biotype (Figure 1). Depending on the herbicide under evaluation, IC_{50} for R1 ACCase was 7.6- to 21.9-fold higher than for S ACCase, and IC_{50} for R2 ACCase was 16.3- to 58.7-fold higher than for S ACCase (Table 1).

R1 and R2 had higher ACCase activity in response to sethoxydim compared with S (Figure 1). For example, sethoxydim at 0.63 µM inhibited S ACCase activity 41%, while R1 and R2 ACCase activity was inhibited 7.5% and 3.7%, respectively. Sethoxydim at 1.3, 2.5, and 5 µM inhibited S ACCase enzyme activity 59.5%, 73.2%, and 81.1%, respectively, while R1 activity was inhibited 11.2%, 19.4%, and 34.9%, respectively, and the R2 biotype activity was inhibited 6.9%, 12.1%, 23.4%, respectively. Sethoxydim at 10 and 20 µM inhibited S biotype ACCase activity 88.3% and 91.2%, respectively, whereas R1 was inhibited 68% and 78.8%, respectively, and R2 was inhibited 38.1% and 54.3%, respectively. R1 and R2 IC_{50} values were 15.3 and 41.1 µM, respectively, compared with 0.7 µM for S, which was 21.9-fold higher than S for R1 and 58.7-fold higher than S for R2 (Figure 1).

Previous research reported R1 and R2 were less resistant to clethodim when foliar applied compared with other ACCase-targeting herbicides (Yu et al. 2017), which was confirmed with the assay. S was relatively more sensitive to clethodim than R1 and R2 (Figure 1). For example, clethodim at 0.63, 1.3, and 2.5 µM inhibited S ACCase activity 48.3%, 64.8%, and 75.9%, respectively, while R1 was inhibited 14.9%, 27.4%, and 44.5%, respectively, and R2 10.7%, 19.7%, and 30.6%, respectively. R1 and R2 IC_{50} values for clethodim were 3.5 and 7.5 µM, respectively, compared with 0.46 µM for S, which was 7.6- and 16.3-fold higher for R1 and R2, respectively, than S. The assay was sensitive enough to detect a difference in the inhibition of R1 and R2 by clethodim, which was unexpected based on previous postemergence applications (Yu et al. 2017). R1 and R2 ACCase activities were lower in the presence of clethodim relative to sethoxydim, fluazifop-*p*-butyl, and pinoxaden, which may explain the difference in whole-plant response observed previously.

Similar to sethoxydim, fluazifop-*p*-butyl and pinoxaden inhibited ACCase activity of S more than that of R1 and R2. Fluazifop-*p*-butyl at 0.63 to 10 µM inhibited S biotype ACCase activity 47.1% to 94.2%, while the enzyme activity of the R1 and R2 biotypes was inhibited 10.8% to 64.4% and 7.1% to 52%, respectively. R1 and R2 IC_{50} values for fluazifop-*p*-butyl were 8.9 and 17.1 µM compared with 0.5 µM for S, which was 17.8- and 34.2-fold higher for R1 and R2, respectively, than S. Pinoxaden at 0.63 to 10 µM inhibited S ACCase activity 26.4% to 87%, respectively, while R1 activity was inhibited 8.6% to 55.4%, respectively, and R2 activity was inhibited 3.2% to 45.9%, respectively. R1 and R2 IC_{50} values for pinoxaden were 12.7 and 28.4 µM, respectively, compared with 1.5 µM for S, which was 8.5- and 18.9-fold higher for R1 and R2, respectively, than S (Figure 1).

Research Implications

While the ultimate research purpose was to develop the malachite green assay and test its effectiveness, unique results were acquired from this research. The development of the malachite green functional assay was conducted using previously researched *D. ciliaris* populations. Understanding previous research is necessary to interpret the assay results. Both R1 and R2 *D. ciliaris* were first identified as sethoxydim resistant in centipede grass [*Eremochloa ophiuroides* (Munro) Hack.] sod production fields in Georgia, USA, by Yu et al. (2017). R1 and R2 were determined to be >64 times more resistant than the susceptible population. R1 and R2 had similar cross-resistance to fenoxaprop and fluazifop, but less resistance to clethodim. Clethodim at 290 g ha^{-1} controlled *D. ciliaris* 83% at 4 wk after treatment. Clethodim reduced susceptible shoot dry biomass to 21% of the non-treated, while R1 and R2 were reduced to 47% and 46%, respectively, of the non-treated. Additional research by Basak et al. (2019) identified R1 and R2 as resistant to pinoxaden and a known target-site mutation, Ile-1781-Leu.

We theorized that R1 and R2 would respond similarly to herbicides using the malachite green assay. However, one unanticipated result was observed for this assay. ACCase activity of R1 and R2 biotypes was inhibited to different extents for each herbicide. This result can only be because of differential interaction with the ACCase substrate and the tested ACCase-targeting herbicide, as no absorption, translocation, or metabolism is at play as would be the case when screening whole plants. The ACCase carboxyl transferase domains were sequenced and reported for S, R1, and R2 previously (Basak et al. 2019). No other amino acid substitutions except Ile-1781-Leu were observed between R1 or R2 that would explain the difference between these biotypes (Supplementary Data 2). Therefore, the specific mechanisms behind lower herbicide inhibition of ACCase from the R2 biotype compared with R1 and S remain unknown. We theorize that R2 expresses more resistant chloroplastic ACCase homologues containing Ile-1781-Leu compared with non-resistant homologues; however, such is only a theory at this time and was not a focus of our research. *Digitaria ciliaris* is a polyploid species with ACCase encoded on separate progenitor genomes (Adoukonou-Sagbadja et al. 2007; Bennett et al. 2000). Enzyme extraction for this procedure provides a bulk sample of all translated ACCase, resulting in a mixture of resistant and susceptible chloroplastic ACCase isoforms in the extract. While the total amount of enzyme is the same in each sample, it is unknown what the ratio of resistant to susceptible isoforms is in an extracted enzyme sample. This unexpected result is seen as a positive outcome, as it indicates the sensitivity of the assay in detecting subtle differences in ACCase to ACCase-inhibiting herbicide interactions that will be beneficial for determining mechanisms of resistance in the future.

The primary purpose of this research was to evaluate the effectiveness of the malachite green assay to assess plastidic ACCase–herbicide interactions. Based on these results, we conclude that the malachite green assay is a highly sensitive assay for measuring ACCase activity as well as a functional assay for ACCase-targeting herbicide resistance. While only the Ile-1781-Leu amino acid substitution was evaluated, we see no reason that other known mutations could not be evaluated in the same system. Utilization of the malachite green assay in the future will eliminate the need for a ^{14}C-based radiometric assay and may uncover other unknown subtle differences in ACCase to herbicide interactions that are still unknown.

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