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Discrimination between protoporphyrinogen oxidase–inhibiting herbicide-resistant and herbicide-susceptible redroot pigweed (*Amaranthus retroflexus*) with spectral reflectance

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Research Article

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Abstract

The current assays to confirm herbicide resistance can be time- and labor-intensive (dose–response) or require a skill set/technical equipment (genetic sequencing). Stakeholders could benefit from a rapid assay to confirm herbicide-resistant weeds to ensure sustainable crop production. Because protoporphyrinogen oxidase (PPO)-inhibiting herbicides rapidly interfere with chlorophyll production/integrity; we propose a new, rapid assay utilizing spectral reflectance to confirm resistance. Leaf disks were excised from two PPO-inhibiting herbicide-resistant (target-site [TSR] and non–target site [NTSR]) and herbicide-susceptible redroot pigweed (*Amaranthus retroflexus* L.) populations and placed into a 24-well plate containing different concentrations (0 to 10 mM) of fomesafen for 48 h. A multispectral sensor captured images from the red (668 nm), green (560 nm), blue (475 nm), and red edge (717 nm) wavebands after a 48-h incubation period. The green leaf index (GLI) was utilized to determine spectral reflectance ratios of the treated leaf disks. Clear differences of spectral reflectance were observed in the red edge waveband for all populations treated with the 10 mM concentration in the dose–response assays. Differences of spectral reflectance were observed for the NTSR population compared with the TSR and susceptible populations treated with the 10 mM concentration in the green waveband and the GLI in the dose–response assay. Leaf disks from the aforementioned *A. retroflexus* populations and two additional susceptible populations were subjected to a similar assay with the discriminating concentration (10 mM). Spectral reflectance was different between the PPO-inhibiting herbicide-resistant and herbicide-susceptible populations in the red, blue, and green wavebands. Spectral reflectance was not distinctive between the populations in the red edge waveband and the GLI. The results provide a basis for rapidly (~48 h) detecting PPO-inhibiting herbicide-resistant *A. retroflexus* via spectral reflectance. Discrimination between TSR and NTSR populations was possible only in the dose–response assay, but the assay still has utility in distinguishing herbicide-resistant plants from herbicide-susceptible plants.

Introduction

Redroot pigweed (*Amaranthus retroflexus* L.) is a globally pervasive weed species (Costea et al. 2004; Weaver and McWilliams 1980). Despite being pervasive, the species has historically been easy to control with herbicides (Mayo et al. 1995; Oliveira et al. 2017). Control difficulty is exacerbated in crops where the chemical control options are limited (Miranda et al. 2022; Owen and Zelaya 2005). Acetolactate synthase (ALS; EC 2.2.1.6; Group 2) and protoporphyrinogen oxidase (PPO; EC 1.3.3.4; Group 14) are herbicides used in soybean [*Glycine max* (L.) Merr.] to control many weeds and are efficacious on *Amaranthus* spp.; thus, herbicide-resistant weeds have been selected with recurrent use and overuse of these herbicides (Gressel et al. 2017; Hinz and Owen 1997; Shoup et al. 2003). One hundred and sixty-nine and fourteen species have evolved resistance to the ALS- and PPO-inhibiting herbicides, respectively, including *A. retroflexus* (Heap 2022). While ALS-inhibiting herbicide resistance has been well characterized in *A. retroflexus* (Ferguson et al. 2001; McNaughton et al. 2005), recent reports of populations

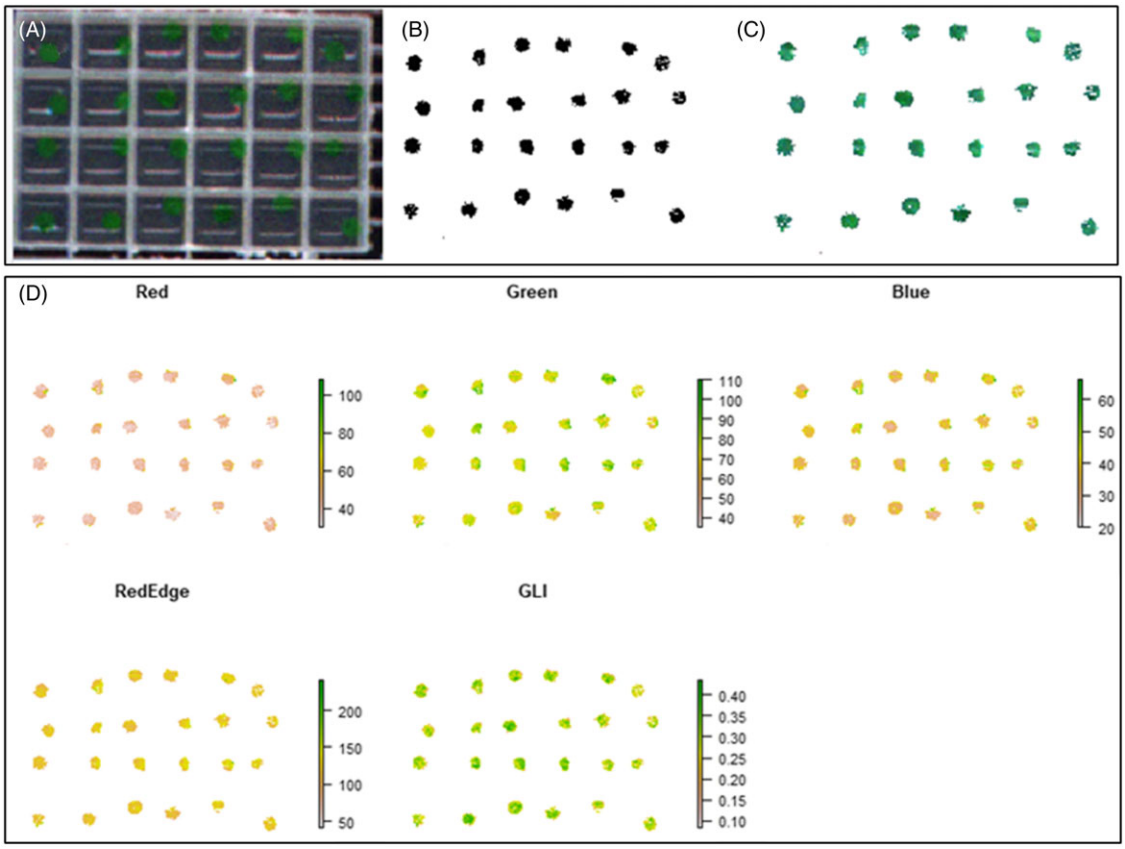

Figure 1. Flowchart of how the image of the leaf disks in the 24-well plates containing various fomesafen concentrations (A) has the background pixels removed; (B) then only the green leaf disks remain (C), and the different digital number values are extracted from the wavebands and the green leaf index (GLI) (D).

resistant to ALS- and PPO-inhibiting herbicides are very concerning (Cao et al. 2021; Jones et al. 2023a; Wang et al. 2019). Stakeholders could benefit from a rapid assay to determine whether putative PPO-inhibiting herbicide-resistant *A. retroflexus* individuals are present so effective control can be implemented before the biotypes become widespread (Burgos et al. 2013; Squires et al. 2021).

Protoporphyrin oxidase–inhibiting herbicides cease the conversion of protoporphyrinogen IX (protogen) into protoporphyrin IX (proto) in chloroplasts (Beale and Weinstein 1990). Under light conditions, proto generates singlet oxygens that extract hydrogens from lipids, disrupting cell membrane integrity relatively quickly (Beale and Weinstein 1990). Chloroplast integrity is compromised, and vegetative tissue loses green pigment (Carter 1993; Major et al. 2003). Chlorophyll content and spectral reflectance are correlated; thus, PPO-inhibiting herbicide-treated plants could be imaged and analyzed to detect differences in spectral reflectance and thus confirm resistance (Gitelson et al. 2003a; Major et al. 2003; Paril and Fournier-Level 2019). Previous research has demonstrated that spectral reflectance can discriminate between herbicide-treated plants, weed species, and herbicide-resistant weed biotypes (Everman et al. 2008; Reddy et al. 2014; Sanders et al. 2021; Zhao et al. 2014). In many instances of PPO-inhibiting herbicide resistance, the mechanism of resistance is facilitated by a mutation in the *PPX2* gene (Mendes et al. 2020; Montgomery et al. 2021). However, some PPO-inhibiting herbicide resistance is facilitated by non–target site mechanisms (Jones et al. 2023a; Varanasi et al. 2018). Because both target-site resistance and non–target site resistance to PPO-inhibiting herbicides have been documented in *A. retroflexus*, knowledge of spectral reflectance profiles between populations possessing the different mechanisms would be beneficial in developing a rapid detection assay.

While dose–response assays are a proven way to document differential susceptibility to herbicides, the results are not procured in time to implement effective control in the growing season and require a lot of labor, time, and space (Burgos 2015; Burgos et al. 2013; Squires et al. 2021). Genetic sequencing can expedite the time to procure results (on the magnitude of hours to days), but the technical knowledge and equipment needed represent a barrier to entry (Burgos et al. 2013; Squires et al. 2021). Thus, the objectives of this research were to determine whether PPO-inhibiting herbicide-resistant and herbicide-susceptible *A. retroflexus* populations could be successfully discriminated with multispectral reflectance and whether a single discriminating herbicide concentration could be adapted into a rapid detection assay.

Materials and Methods

Plant Material

Five *A. retroflexus* populations were utilized in the experiments. Three herbicide-susceptible *A. retroflexus* populations were collected from Wake and Yadkin (A and B) counties, North Carolina, in 2019. Two ALS- and PPO-inhibiting herbicide-resistant *A. retroflexus* populations were collected from Camden and Pasquotank counties, North Carolina, in 2019 and 2020, respectively. The Camden County population carries a Arg-98-Gly mutation in the *PPX2* gene, while the Pasquotank County population

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exhibited no mutation in the *PPX2* gene (Jones et al. 2023a). The Camden County and Pasquotank County populations are also resistant to ALS-inhibiting herbicides and carry a Trp-574-Leu and Pro-197-His mutation in the *ALS* gene, respectively.

Dose–Response Assay

Seeds of the Camden County, Pasquotank County, and Wake County populations were sown into separate 21 cm by 28 cm flats containing a 4:1 soil mixture and 5 g of pellet fertilizer (14-14-14). Plants were transplanted at 5 cm in height (2- to 4-leaf) into 10-cm pots containing a 4:1 soil mixture with 1 g of pellet fertilizer (14-14-14). When the plants reached approximately 7.6 to 10 cm in height (4- to 6-leaf), leaf disks were excised using a 6-mm hole puncher from the youngest completely unfolded leaves. Fomesafen (290 g ai L^{-1}) was diluted to various concentrations (mM) using de-ionized water. The tested fomesafen concentrations were 0.01, 0.0316, 0.1, 0.316, 1, 3.16, and 10 mM. A nontreated leaf disk was included as well. These concentrations were selected based on similar previously conducted research (Jones et al. 2023b; Wu et al. 2021). Treatments were arranged as a randomized complete block design with three replications (each replication was a plant), and the experiment was conducted twice. Twenty-four-well plates (Spex Sample Prep, Metuchen, NJ) were cut in half to a depth of 1.25 cm and used as the vessel to hold the leaf disk and herbicide aliquot. One-milliliter aliquots of the various fomesafen concentrations were added to the corresponding well, and leaf disks were placed in the well with forceps. The leaf disks were submerged into the aliquot and allowed to resurface with the adaxial side facing up. The 24-well plates containing the herbicide aliquots and leaf disks were placed into a photography box and subjected to continuous light from an LED light (12 lumens m^{-2}) at 22 C with 50% relative humidity.

Multispectral images were acquired using a MicaSense RedEdge multispectral sensor (MicaSense, Seattle, WA) every 2 h for 48 h. The multispectral sensor measures reflected energy in five discrete regions: red (668 to 682 nm), green (560 to 587 nm), blue (475 to 507nm), red edge (717 to 729 nm), and near infrared (842 to 899). Limited radiance output by the LED light source in the near infrared resulted in underexposed images at this wavelength and was not included in the analysis. The multispectral sensor has a focal length of 5.5 mm, a horizontal field of view of 47.2°, and an image resolution of 1,280 × 960 pixels. Ground spatial resolution for the multispectral sensor is 0.7 mm per pixel at 1 m above ground level. The multispectral sensor was placed approximately 1 m above the 24-well plates containing the leaf disks. The resulting images were combined into a single four-band, geometrically rectified image composite using custom Python (Python Software Foundation, Wilmington, DE) code and the SIFT (Scale-Invariant Feature Transform, https://docs.opencv.org/4.x/da/df5/tutorial_py_sift_intro.html) library available in OpenCV. The image composites were then used to determine reflected raw digital number (DN) values for each waveband using the FIELDIMAGER package in RStudio v. 4.1.1 (Matias et al. 2020; R Core Team 2020). Reflectance ratios were calculated as well for the green leaf index (GLI) in addition to the wavebands captured by the multispectral sensor in FIELDIMAGER and calculated using Equation 1.

$$\frac{(2*\text{red} - \text{green} - \text{blue})}{(2*\text{red} - \text{green} - \text{blue})} \quad [1]$$

Where red is the DN value from the red waveband, green is the DN value from the green waveband, and blue is the DN value from the blue waveband. The GLI was selected, as it is commonly used as a metric to measure chlorophyll content (Gitelson et al. 2003b). A flowchart of the process from the captured image to extracted DN is outlined in Figure 1.

Table 1. Parameter estimates from the regression models for the spectral reflectance of protoporphyrinogen oxidase–inhibiting herbicide-resistant and herbicide-susceptible *Amaranthus retroflexus* leaf disks treated with increasing fomesafen concentrations.

Waveband/ index	Population[a]	Model[b]	r^2
Red	TSR	$y = 47.7 + 5.0/1 + (x/-1.1)^{-18.1}$	0.67
	NTSR	$y = 49.9/1 + (x + 6.4)^{2.0}$	0.29
	Susceptible	$y = 46.5 + 3.4/1 + (x/0.3)^{-16.7}$	0.28
Green	TSR	$y = 73.0 + 5.0/1 + e^{-[(x - 0.1)/-0.003]}$	0.31
	NTSR	$y = 75.5 + 3.9/1 + e^{-[(x - 3.16)/0.1]}$	0.46
	Susceptible	$y = 58.7 + 19.4/1 + e^{-[(x + 4251.4)/0.2]}$	0.65
Blue	TSR	$y = 38.6 - 0.4 * x + 0.06 * x^2$	0.35
	Non-target site–resistant	$y = 38.9 + 0.07 * x - 0.0001 * x^2$	0.17
	Susceptible	$y = 38.0 + 1.6/1 + e^{-[(x + 0.2)/0.0045]}$	0.38
Red edge	TSR	$y = 115.0 - 2.0 * x + 0.3 * x^2$	0.3
	NTSR	$y = 117.0 + 9.0/1 + (x/-3.2)^{-18.1}$	0.6
	Susceptible	$y = 115.07 - 1.8 * x + 0.2 * x^2$	0.51
Green leaf index	TSR	$y = 0.2 + 0.03/1 + (x/0.2)^{2.6}$	0.52
	NTSR	$y = 0.3 - 0.0004 * x - 0.0006 * x^2$	0.38
	Susceptible	$y = 0.2 + 0.04/1 + e^{-[(x - 0.3)/16.5]}$	0.75

[a]NTSR, non–target site resistant; TSR, target-site resistant.
[b]Linear ($y = y_0 + ax$) or three-parameter sigmodal ($y = \frac{a}{1} + (x - x_0)^b$) equations were selected based on goodness of fit to the data.

Rapid Discrimination of PPO-inhibiting Herbicide Resistance across Populations

Two additional PPO-inhibiting herbicide-susceptible *A. retroflexus* populations (Yadkin County A and B) were utilized to determine whether the assay could discriminate across different populations. Seeds from all populations were sown and curated as described earlier. Leaf disks were excised and placed into the 24-well plate with the corresponding fomesafen concentrations and placed in a photography box as described earlier. Fomesafen concentrations were 0 mM and the discriminating concentration for the dose–response assay. Treatments were arranged as a randomized complete block design with four replications (each replication was a plant), and the experiment was conducted twice. The multispectral sensor collected imagery at 0, 24, and 48 h after treatment. The multispectral sensor was approximately 1 m above the 24-well plate containing the leaf disks, as described earlier. The images were mosaicked and reflected DN values from wavebands, and GLI values were extracted from the mosaicked images as described earlier.

Whole-Plant Response to Fomesafen

Plants that were sampled for the experiments described earlier were subsequently treated with fomesafen after the leaf disks were excised to ascertain whether the plants were resistant or susceptible. Fomesafen (290 g ai ha^{-1}) + crop oil (1% v/v) was applied 46 cm above the target height at an output of 140 L ha^{-1} using a CO_2-powered track-mounted sprayer equipped with a TeeJet® 8002EVS nozzle (TeeJet Technologies, Wheaton, IL). Plant survival was evaluated at 21 d after treatment on a binomial scale where 0 equaled plant death and 1 equaled plant survival.

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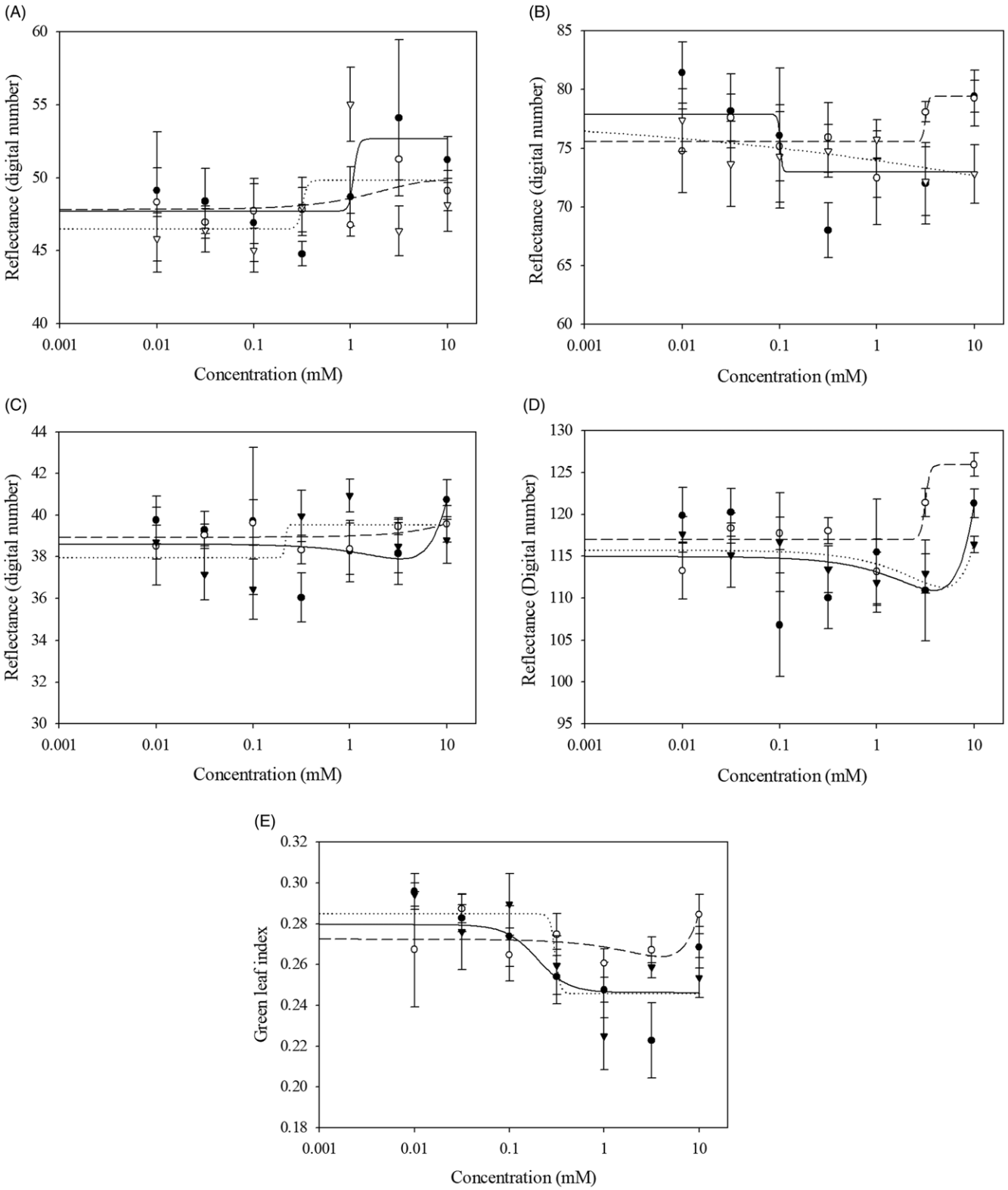

Figure 2. Spectral reflectance of protoporphyrinogen oxidase–inhibiting herbicide-resistant (target-site: black circle; non–target site: white circle) and herbicide-susceptible (triangle) *Amaranthus retroflexus* leaf disks treated with various concentrations of fomesafen. (A) Red (668) waveband; (B) green (560) waveband; (C) blue (475) waveband; (D) red edge (717 nm) waveband; (E) green leaf index. Error bars represent the standard errors of the mean.

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Statistical Analysis

Dose–Response Assay

Spectral reflectance data from all wavebands and the GLI were modeled across fomesafen concentrations using four-parameter log-logistic, three-parameter sigmoidal-logistic, four-parameter sigmoidal-logsitic, and quadratic equations in SigmaPlot v. 14.0 (Systat Software, Palo Alto, CA).

The four-parameter log-logistic model is described as

$$y = y_0 + a/\left(\frac{x}{x_0}\right)^b \quad [2]$$

where a is the upper asymptote, x is the fomesafen concentration, x_0 and y_0 equal inflection points, and b is the slope at x_0.

The three-parameter sigmoidal-logistic model is described as

$$\left(y = \frac{y_0}{1 + \exp\left\{-\left[\frac{(x+x_0)}{b}\right]\right\}}\right) \quad [3]$$

where x is the fomesafen concentration, x_0 and y_0 equal inflection points, and b is the slope at x_0.

The four-parameter sigmoidal-logistic model is described as

$$\left(y = y_0 + \frac{a}{1 + \exp\left\{-\left[\frac{(x+x_0)}{b}\right]\right\}}\right) \quad [4]$$

where y_0 is the upper asymptote, x is the fomesafen concentration, x_0 equals an inflection point, and b is the slope at x_0.

The quadratic model is described as

$$(y = y_0 + a{*}x + b{*}x^2) \quad [5]$$

where a is the upper asymptote, x is the fomesafen concentration, x_0 and y_0 equal inflection points, and b is the slope at x_0.

Rapid Discrimination of PPO-inhibiting Herbicide Resistance across Populations

Spectral reflectance data from all wavebands and GLI were subjected to ANOVA using PROC GLIMMIX in SAS v. 9.4 (Statistical Analysis Software, SAS Institute, Cary, NC), with fomesafen concentration, resistance trait, resistance trait (nested within population), and the interactions were considered fixed effects, while experimental run and replication were considered random effects. Treatment means were separated using Fisher's LSD ($\alpha \leq 0.1$).

Whole-Plant Assay

Plant survival data were subjected to ANOVA using PROC GLIMMIX in SAS v. 9.4 (Statistical Analysis Software, SAS Institute). *Amaranthus retroflexus* population was considered a fixed effect, while the experimental run and replication were considered random effects. Means were separated using Fisher's LSD ($\alpha \leq 0.05$).

Results and Discussion

Only the 48-h time point provided clear, significant differences of spectral reflectance; thus, all subsequent results will reflect data collected at 48 h after treatment.

Table 2. Survival of *Amaranthus retroflexus* treated with fomesafen (290 g ai ha^{-1}) after the leaf disks were excised for the dose–response assay.

	Survival[b]
Population[a]	%
Camden County	100 a
Pasquotank County	33 b
Wake County	0 c

[a]Herbicide-resistant populations: Camden County (target-site resistance); Pasquotank County (non–target site resistance). Herbicide-susceptible population: Wake County.
[b]Values that share the same letters are not statistically different based on Fisher's LSD ($P < 0.05$).

Dose–Response Assay

Red

Spectral reflectance of the *A. retroflexus* populations across fomesafen concentrations was best modeled with a three-parameter sigmoidal equation (Table 1; Figure 2). Spectral reflectance increased as the fomesafen concentration increased for all *A. retroflexus* populations (Figure 2). Largely, the spectral reflectance value of each *A. retroflexus* population was inseparable (Figure 2).

Green

Spectral reflectance of the *A. retroflexus* populations was best modeled with a linear equation (Table 1; Figure 2). Spectral reflectance values for the resistant (Camden County and Pasquotank County) and the susceptible (Wake County) *A. retroflexus* populations were inseparable until the 10 mM concentration (Figure 2). The spectral reflectance of both resistance populations increased, while the spectral reflectance decreased for the Wake County population (Figure 2; Table 1). The spectral reflectance values of the Camden County (target-site resistant [TSR]) and Pasquotank County (non–target site resistant [NTSR]) populations were no different at the 10 mM concentration (Figure 2).

Blue

Spectral reflectance of the *A. retroflexus* populations across fomesafen concentrations was best modeled with a linear equation (Table 1; Figure 2). The spectral reflectance values for each *A. retroflexus* population were inseparable across all fomesafen concentrations (Figure 2).

Red Edge

Spectral reflectance of the *A. retroflexus* populations across fomesafen concentrations was best modeled with a linear equation (Table 1; Figure 2). The spectral reflectance of the Wake County population remained constant across fomesafen concentrations; the spectral reflectance of the Camden County (TSR) and the Pasquotank County (NTSR) populations increased (Figure 2; Table 1). All *A. retroflexus* populations were spectrally distinct at the 10 mM concentration (Figure 2).

GLI

Spectral reflectance of the *A. retroflexus* across fomesafen concentrations was best modeled with a linear equation (Table 1; Figure 2). The spectral reflectance of the Camden County and Wake County populations decreased with increasing fomesafen concentrations, while the spectral reflectance of the Pasquotank County population increased with increasing fomesafen concentrations (Table 1; Figure 2). While the slopes of individual

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Table 3. Reflectance of protoporphyrinogen oxidase–inhibiting herbicide-resistant and herbicide-susceptible *Amaranthus retroflexus* leaf disks treated with 10 mM of fomesafen 48 h after treatment.

		Red	Green	Blue	Red edge	GLI[a]
Resistance profile	Concentration	Digital number (SE)[b]				
	—mM—					
Resistant	0	47.4 (10.2) c	78.8 (18.8) a	38.6 (9.1) b	119.5 (28.0) a	0.30 (0.08) a
	10	61.2 (13.0) a	76.2 (16.3) a	43.1 (9.8) a	110.0 (26.6) b	0.20 (0.04) b
Susceptible	0	47.5 (8.7) c	77.2 (15.5) a	38.3 (7.6) b	118.2 (23.9) a	0.29 (0.06) a
	10	54.2 (11.3) b	70.6 (14.5) b	39.5 (8.0) b	107.2 (23.2) b	0.20 (0.04) b

[a]GLI, green leaf index.
[b]Columns that share the same letters are not statistically different based on Fisher's LSD ($P < 0.1$).

Table 4. Survival of *Amaranthus retroflexus* treated with fomesafen (290 g ai ha^{-1}) after the leaf disks were excised for the rapid assay.

	Survival[b]
Population[a]	%
Camden County	100 a
Pasquotank County	50 b
Yadkin (A) County	12.5 c
Yadkin (B) County	0 c
Wake County	12.5 c

[a]Herbicide-resistant populations: Camden County (target-site resistance); Pasquotank County (non–target site resistance). Herbicide-susceptible populations: Wake County; Yadkin (A and B) County.
[b]Values that share the same letters are not statistically different based on Fisher's LSD ($P < 0.05$).

A. retroflexus populations showed different trends, the reflectance values were largely inseparable (Figure 2).

The concurrent whole-plant bioassay had concordant results where the Camden County (100%) and Pasquotank County (33%) population plants survived the fomesafen treatment, while the Wake County population had no surviving plants (Table 2).

Rapid Discrimination of PPO-inhibiting Herbicide Resistance across Populations

Amaranthus retroflexus population was not a significant effect ($P > 0.1$) on the spectral reflectance on the tested wavebands and GLI. However, the resistance profile was a significant effect ($P < 0.1$) on the spectral reflectance on the tested wavebands and GLI; thus, the spectral reflectance for each waveband and GLI was averaged across resistance profile.

Red

Spectral reflectance was not different between the nontreated resistant and susceptible populations (Table 3). The spectral reflectance was higher in the resistant populations compared was the susceptible populations when treated with 10 mM of fomesafen. This result is not concordant with the results from the dose–response assay (Figure 2; Table 3). While not concordant, the trends from the dose–response assay suggest that the reflectance increases as the concentration increases (Figure 2).

Green

Spectral reflectance was no different between the nontreated leaf disks and the 10 mM–treated resistant leaf disks; the spectral reflectance of the 10 mM–treated susceptible leaf disks was lower compared with the other leaf disks (Table 3). This result is concordant with the results from the dose–response assay (Figure 2; Table 3).

Blue

Spectral reflectance was no different between the nontreated leaf disks and the 10 mM–treated susceptible leaf disks; the spectral reflectance of the 10 mM–treated resistant leaf disks was higher compared with the other leaf disks (Table 3). This result is not concordant with the results from the dose–response assay (Figure 2; Table 3). The trends from the dose–response assay do not elucidate why spectral reflectance separation occurs; the increased sample size and inclusion of more populations may be a driving factor.

Red Edge

Spectral reflectance was no different between the nontreated and treated leaf disks (Table 3). This result is not concordant with the results from the dose–response assay (Figure 2; Table 3). When analyzed by population, the Wake County population had a lower reflectance value than the other *A. retroflexus* populations in the red edge waveband; this result may elucidate the differential responses at a population level (data not shown).

GLI

Spectral reflectance was no different between the nontreated and treated leaf disks (Table 3). This result is concordant with the results from the dose–response assay (Figure 2; Table 3).

The concurrent whole-plant bioassay had concordant results wherein the PPO-inhibiting herbicide-resistant populations had plant survival ranging from 50% to 100% and the herbicide-susceptible populations had plant survival ranging from 0% to 12.5% when treated with fomesafen (Table 4).

Results of the experiments provide evidence that spectral reflectance in combination with dose–response and rapid detection assays can discriminate between PPO-inhibiting herbicide-resistant and herbicide-susceptible *A. retroflexus* within 48 h. Because results showing the plants to be "resistant" or "susceptible" are procured rapidly, effective control tactics can be implemented before resistant individuals reproduce to cease the spread of this biotype in the agroecosystem, compared with whole-plant dose–response assays, in which results are procured much later after the growing season (Ervin et al. 2019; Norsworthy et al. 2012). This assay also has minimal barriers of entry (technical skill set and/or equipment) compared with genetic sequencing assays that can procure results in a similar time frame.

Both PPO-inhibiting herbicide-resistant populations could be distinguished from herbicide-susceptible populations with

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multispectral imaging in both assays; however, detection of target-site versus non–target site resistance was less clear (Figure 2; Table 3). While the mechanism of resistance was not clearly defined in either assay, the utility of these assays provides a methodology to rapidly confirm PPO-inhibiting herbicide-resistant *A. retroflexus*. Research utilizing chlorophyll fluorescence imaging successfully discriminated between weed populations possessing target-site and non–target site resistance (Kaiser et al. 2013). *Amaranthus retroflexus* is relatively genetically similar across individuals due to monoicy and plants being largely self-pollinated (Mandák et al. 2011; Weaver and McWilliams 1980). The distinct spectral response of the herbicide-resistant and herbicide-susceptible *A. retroflexus* populations to the 10-mM concentration of fomesafen may be due to the lack of differential genetic background (Bravo et al. 2017; Leon et al. 2021). These assays may need adjustments when implemented for very genetically diverse species such as waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Chandi et al. 2013; Lee et al. 2009). They may have utility in confirming/detecting herbicide-resistant weeds if the herbicide mode of action is comparable to that of fomesafen or inhibits chlorophyll/pigment production (Grossmann 2009; Hawkes 2014; Mitchell et al. 2001; Takano et. al 2019). Herbicide concentration may need to be adjusted based on herbicide activity and weed species.

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