

## Research Article

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# A nondestructive leaf-disk assay for rapid diagnosis of weed resistance to multiple herbicides

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**Abstract**

Weed resistance surveys that monitor the spread of resistant weeds have mainly been conducted through time-consuming, labor-intensive, and destructive greenhouse herbicide screens. As an alternative, we introduce here a nondestructive leaf-disk assay based on chlorophyll fluorescence ( $F_v/F_m$  values that measure photosynthetic efficiency) that allows the detection of resistance to both systemic and contact herbicides within ~48 h. The current study validated the assay for detecting resistance to fomesafen, glyphosate, and dicamba in Palmer amaranth (*Amaranthus palmeri* S. Watson), waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], kochia [*Bassia scoparia* (L.) A.J. Scott], and goosegrass [*Eleusine indica* (L.) Gaertn.]. Negative correlation between  $F_v/F_m$  values and spray injury levels was observed in all herbicide–weed combinations at the discriminating doses, except for glyphosate in *Amaranthus*. The correlation coefficients were  $-0.41$  for fomesafen (10  $\mu\text{M}$ ,  $P < 0.0001$ ) in *Amaranthus*,  $-0.92$  for glyphosate in *E. indica* (250  $\mu\text{M}$ ,  $P < 0.0001$ ), and  $-0.44$  for dicamba in *B. scoparia* (800  $\mu\text{M}$ ,  $P = 0.0023$ ). At the population level, the assay clearly separated susceptible from highly resistant populations. However, the assay showed lower sensitivity in distinguishing populations of different resistance levels or separating populations with low resistance from susceptible populations. At the individual plant level, results from the leaf-disk assay and whole-plant spray tests were concordant in 85.5%, 92.3%, and 71.7% of the plants tested for fomesafen–*Amaranthus*, glyphosate–*Eleusine*, and dicamba–*Bassia*, respectively. The assay yielded 1% to 15% false-positive and 6% to 13% false-negative results across herbicides. The current study demonstrated that the leaf-disk assay is a useful tool to identify weed resistance. Optimization is needed to improve its sensitivities and expand its usage to more diverse herbicide–weed species combinations.

**Introduction**

Rapid diagnosis is critical to monitoring herbicide-resistant weeds, allowing a proactive response before the dissemination of the resistance alleles. A common practice to evaluate the spread of herbicide resistance is to do routine and massive surveys of weed populations from different geographic locations (Evans et al. 2016; González-Torralva et al. 2020; Westra et al. 2019). These surveys have been done solely through time-consuming and labor-intensive greenhouse herbicide efficacy trials or a combination of greenhouse trials with molecular marker assays (Chatham et al. 2015; Salas-Perez et al. 2017; Schultz et al. 2015; Varanasi et al. 2018; Wu et al. 2020a). A more rapid assay requiring fewer technical skills (e.g., molecular assays) will enable more weed science labs to conduct weed resistance surveys regularly and more efficiently. Furthermore, the destructive nature of the greenhouse herbicide screens requires researchers to spray separate sets of plants, generate progeny, or create clones for other research activities, such as testing for multiple resistance or investigating resistance mechanisms. In contrast, a nondestructive herbicide-resistance diagnostic assay will give weed scientists more flexibility in weed resistance research and will simplify the process for weed surveys for multiple resistance.

Multiple replacements for the traditional greenhouse whole-plant assay have been explored, such as seed germination assays, agar-based seedling assays, pollen germination tests, and DNA-based assays (Burgos et al. 2013). A rapid nondestructive assay with excised leaf disks was also used by previous researchers but was limited to herbicides with well-characterized modes of action and involved complicated quantification methods such as chloroplast extraction to measure enzymatic activities (Gerwick et al. 1993; Hanson et al. 2009; Shaner et al. 2005). To identify simpler and more cost-effective methods with lower technical requirements, we propose the use of photosynthetic efficiency (chlorophyll fluorescence) in quantitative characterization of herbicide response through a single weed leaf disk (Juneau et al. 2007; Lakra et al. 2015). This assay is based on a commonly used plant stress measurement: the  $F_v/F_m$  test (Maxwell and Johnson 2000; Oxborough and Baker 1997), where variable fluorescence ( $F_v$ ) is the difference between maximum fluorescence ( $F_m$ ) and minimum fluorescence ( $F_0$ ) as plants are exposed to different

levels of modulated light during measurement.  $F_v/F_m$  ratio represents the maximum quantum yield, which indicates the probability that the light energy absorbed by plant leaves will be used for photosynthesis (Baker 2008; Oxborough and Baker 1997). The optimal  $F_v/F_m$  values for many plant species range from 0.79 to 0.84, and the greater the plant stress, the lower the  $F_v/F_m$  ratios are, as well as the leaf area that is actively photosynthesizing (Maxwell and Johnson 2000). To quantify herbicide stress using the  $F_v/F_m$  test, leaf disks of actively growing weed seedlings are excised and incubated in different doses of herbicide solutions on microplates. The plates are then scanned within 48 h in a chlorophyll fluorescence imager that returns both imaging and quantification data (e.g., leaf areas that are actively photosynthesizing,  $F_v/F_m$  values). Our hypothesis is that leaf disks from resistant plants are less affected by herbicide challenge and are thus able to maintain higher photosynthetic efficiency (e.g., higher  $F_v/F_m$  values and bigger leaf areas with open photosystem II [PSII] centers) compared with susceptible plants.

The goal of the current study is to validate the feasibility of using chlorophyll fluorescence ( $F_v/F_m$  ratios) to determine the resistance status of a plant and compare its prediction accuracies with the results from a whole-plant spray test. In the current study, we validated the assay on both contact and systemic herbicides from three herbicide modes of action: (1) fomesafen (protoporphyrinogen oxidase [PPO] inhibitor); (2) glyphosate (5-enolpyruvylshikimate-3-phosphate synthase inhibitor); and (3) dicamba (synthetic auxins). The assay was conducted on multiple populations of four weed species: waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], Palmer amaranth (*Amaranthus palmeri* S. Watson), goosegrass [*Eleusine indica* (L.) Gaertn.], and kochia [*Bassia scoparia* (L.) A.J. Scott]. We investigated the following key questions: (1) Is the assay able to detect resistance to both contact and systemic herbicides, including herbicides that do not directly target plant photosynthesis? (2) What are the discriminating doses that distinguish resistant plants from susceptible plants for each herbicide? (3) Is the assay able to distinguish resistant and susceptible plants at both population and individual-plant levels? Results from the validation studies and suggested usage and optimization strategies will also be discussed.

## Materials and Methods

### Plant Materials

About 250 plants from 23 *Amaranthus* spp. populations (19 *A. palmeri* and 4 *A. tuberculatus*) with varied fomesafen resistance levels (eight high-, eight moderate-, four low-resistance and three sensitive populations) were collected from eight U.S. states (TN, AR, OK, MS, AR, IL, IA, and MO) and used to validate the leaf-disk assay for fomesafen (4 to 16 plants per population). Forty-five plants from four glyphosate-resistant or glyphosate-sensitive *A. palmeri* or *A. tuberculatus* populations were used to validate the glyphosate leaf-disk assays on broadleaf weeds (Table 1). Details of the populations are listed in Supplementary Table S1. Eighty plants from five previously characterized glyphosate-resistant or glyphosate-sensitive *E. indica* populations from Malaysia and Missouri, USA (Baerson et al. 2002; Yu et al. 2015), were used to validate the glyphosate leaf-disk assays for grass weeds. Forty-six plants from two previously characterized *B. scoparia* lines (9425 and WT) (Wu et al. 2020b) were used to validate the leaf-disk assay for dicamba. The segregating 9425 *B. scoparia* line has an IAA16 mutation that endows

dicamba resistance resulting in three genotypes: homozygous-resistant (RR), heterozygous-resistant (RS), and homozygous-susceptible (SS). *Bassia scoparia* plants were genotyped using the method described in LeClere et al. (2018). Our rationales for sample size calculation are listed in Supplementary Table S2.

### Resistance Characterization through Whole-Plant Spray Tests

Plants were grown at the Bayer CropScience glasshouse in St Louis, MO (29/26 ± 3 C day/night temperatures, 14/10 h day/night photoperiods, supplemental lighting to maintain ≥1,050 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity). Plants at 8- to 10-cm height were sprayed with 560 g ha<sup>-1</sup> of dicamba (XtendiMax®, Bayer CropScience, 700 Chesterfield Parkway W, St Louis, MO 63017, USA), 420 g ha<sup>-1</sup> of fomesafen (Flexstar®, Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419, USA) with 1% v/v crop oil concentrate (COC), or 1,260 g ha<sup>-1</sup> of glyphosate (Roundup PowerMax®, Bayer CropScience). Herbicides were applied using a research track sprayer calibrated to deliver 140 L ha<sup>-1</sup> of herbicide solution at 276 kPa, moving at 2.57 km h<sup>-1</sup>. Dicamba was sprayed with a TTI spray nozzle (TeeJet® spray nozzles, TeeJet Technologies, 204 Kilcote Ct. Louisville, KY 40245, USA), while fomesafen and glyphosate were sprayed with a flat-fan nozzle (XR9501E, TeeJet®). Plant visual injury was evaluated at 14 or 21 d after treatment (DAT) using the scale of 0% to 100%, with 0% indicating no visual injury compared with untreated plants and 100% indicating plant death.

### Resistance Characterization through Leaf-Disk Assays

Analytical reference standards of fomesafen (CAS no. 72178-02-0, 250 mg) and glyphosate (CAS no. 1071-83-6, 100 mg) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Analytical standards of dicamba were produced in house at Bayer CropScience. Stock solutions (10 mM) of different herbicides were dissolved in deionized water (glyphosate and dicamba) or acetone (fomesafen). These stock solutions were diluted in leaf-disk assay buffer for desirable doses (the final concentration of acetone in 100 μM fomesafen solution was 1%). The leaf-disk buffer was made up with 1 mM MES and 1% sucrose with a pH of 6.5 (CAS no. 1266615-59-1; CAS no. 57-50-1, Sigma-Aldrich, St. Louis, MO, USA) for desirable doses. The doses used for each herbicide are listed in Table 1.

At 24 h before herbicide was sprayed, leaf disks were excised from the youngest fully unfolded green leaves and used for the leaf-disk assay. Leaf punches were taken using disposable 4-mm (*E. indica*) and 5-mm (broadleaf) biopsy punches (4 mm: 33-34-P/25; 5 mm: 33-35; Integra™ Miltex®, Integra LifeSciences, 1100 Campus Road, Princeton, NJ 08540, USA). Only the 4-mm punch comes with plunger system, so a thin metal wire was used to gently remove the 5-mm leaf disks from the punch. A tweezer was used to gently place individual leaf disks onto 96-well microplates (cat. no. 92096, 96F, TPP Techno Plastic Products AG, Zollstraße 7, 8219 Trasadingen, Switzerland) prefilled with 200 μl per well of leaf assay buffer or herbicide solution. The leaf disks were allowed to float on the surface of the buffer or herbicide solution with the adaxial surface facing upward.

The leaf disks were then placed into a growth chamber programmed as follows: day/night temperature: 14/10 h; 25/24 C; humidity: 50%; and light intensity: 180 to 250 μmol m<sup>-2</sup> s<sup>-1</sup>. Before data collection, the plates were wrapped in aluminum foil and dark adapted for 20 min. Photosynthetic efficiency indicators such as  $F_v/F_m$  values and leaf area of active PSII reaction center

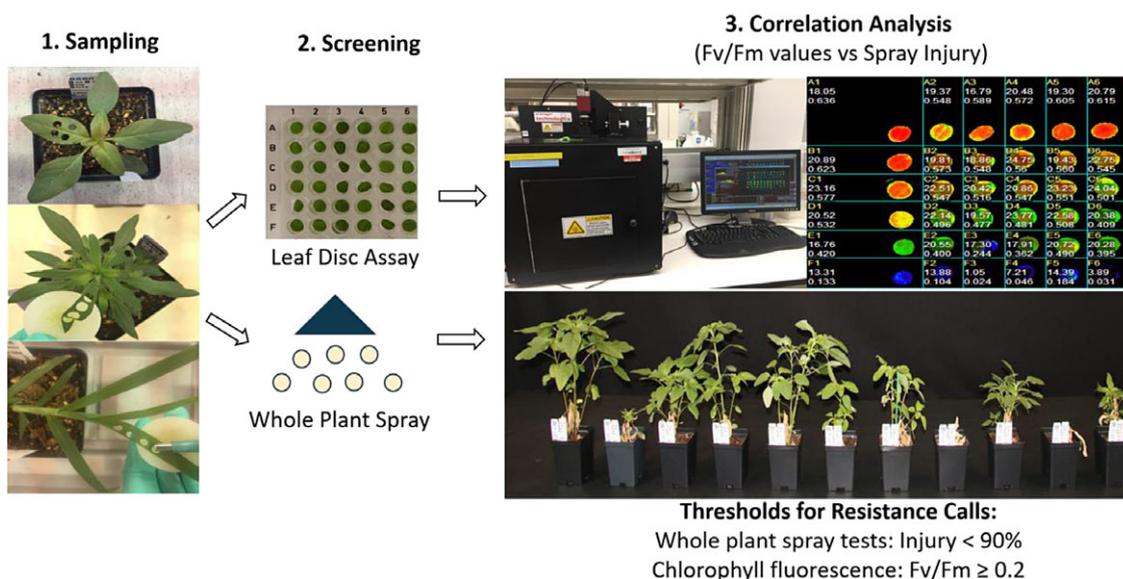
**Table 1.** Comparison of the results from the leaf-disk assays and the whole-plant spray tests on four weed species across three herbicide sites of action.

Chemistry	Weed species	Populations tested	Plants tested	Dose	LD assay vs. WPS <sup>a</sup>		
					Concordant <sup>b</sup>	False positive	False negative
Fomesafen	<i>Amaranthus tuberculatus</i> , <i>Amaranthus palmeri</i>	23	<i>N</i>	$\mu\text{M}$			
			<b>241</b>	<b>10<sup>c</sup></b>	<b>85.5%</b>	<b>7.9%</b>	<b>6.6%</b>
			232	50	65.5%	6.5%	28.0%
Glyphosate	<i>Eleusine indica</i>	5	<b>78</b>	<b>250</b>	<b>92.3%</b>	<b>1.3%</b>	<b>6.4%</b>
			77	500	81.8%	1.3%	16.9%
			74	1,000	73.0%	8.1%	18.9%
Glyphosate	<i>A. tuberculatus</i> , <i>A. palmeri</i>	4	<b>45</b>	<b>500</b>	<b>71.1%</b>	<b>20.0%</b>	<b>8.9%</b>
			36	1,000	66.7%	11.1%	22.2%
			45	2,500	42.2%	6.7%	51.1%
Dicamba	<i>Bassia scoparia</i>	4	46	250	69.6%	30.4%	0.0%
			46	500	69.6%	30.4%	0.0%
			<b>46</b>	<b>800</b>	<b>71.7%</b>	<b>15.2%</b>	<b>13.0%</b>

<sup>a</sup>LD, leaf-disk assay, plants were considered resistant if  $F_v/F_m \geq 0.2$ ; WPS, whole-plant spray test, plants were considered resistant if spray injury  $< 90\%$ .

<sup>b</sup>Results from the two methods were considered concordant when the same resistance results were reported by both methods (e.g., a sprayed plant with  $< 90\%$  visual injury also had an  $F_v/F_m$  value  $\geq 0.2$ ).

<sup>c</sup>Highlighted in bold are discriminating doses with the most negative relationship between  $F_v/F_m$  values and herbicide spray injury data and lowest false-positive/false-negative results.



**Figure 1.** Overview of the study to validate the leaf-disk assay through the comparison with the whole-plant spray test. Two data points for each leaf disk were exported from the chlorophyll fluorescence imager: leaf area ( $\text{mm}^2$ ) and  $F_v/F_m$  values (0–0.8) (e.g., Cell A1, leaf area = 18.05  $\text{mm}^2$ ,  $F_v/F_m = 0.636$ ).

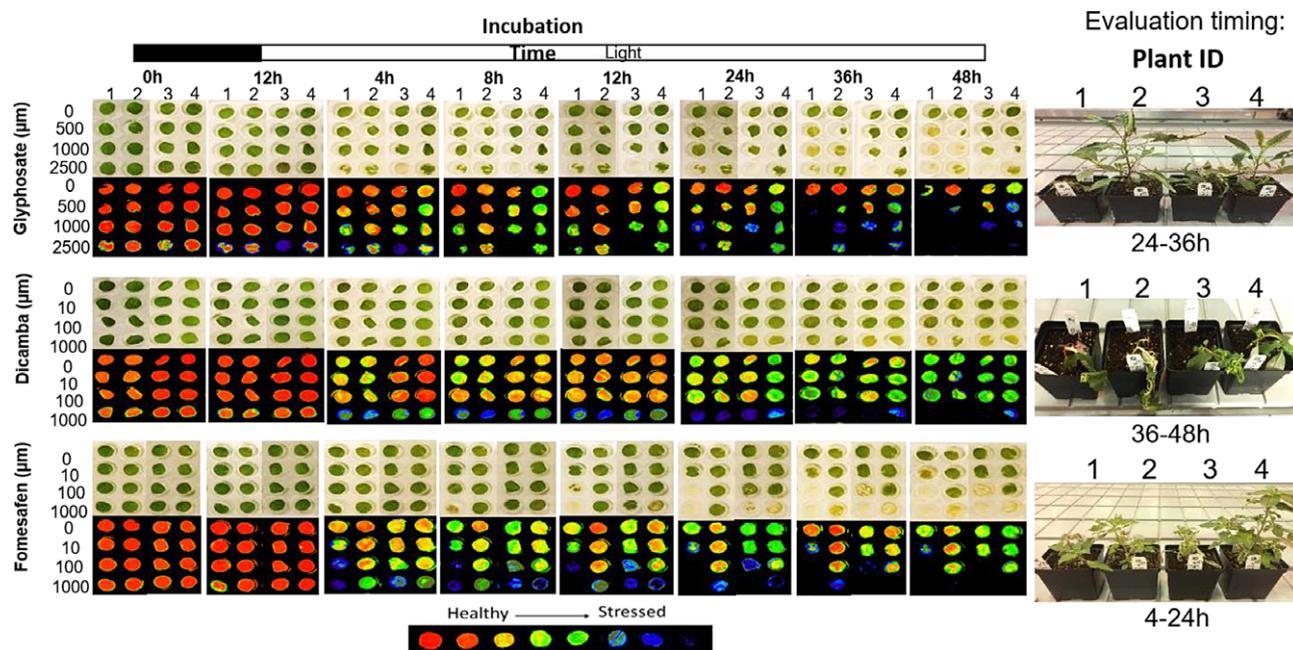
were measured through a chlorophyll fluorescence imager (Technologica CF Imager, Technologica Ltd, FOURWAYS Bromley Road, Frating, Colchester, Essex CO7 7DP, UK) (Figure 1). Data were exported from the CF imager and analyzed in Excel. To determine the best evaluation timing for different herbicides, we first did a time-course study to incubate and collect chlorophyll fluorescence data at multiple time points for up to 48 h. All dose–response studies were repeated, with majorities of the populations included in the repeated studies.

#### Data Visualization and Statistical Analysis

The data from the whole-plant spray tests and the leaf-disk assays were compared at both the individual plant and population levels (Figure 1). Scatter plots and regression lines showing the correlation between  $F_v/F_m$  values or leaf areas and visual injury from the spray tests were generated in R (v. 3.5.1, R Foundation for Statistical Computing, Vienna, Austria) using the GGPlot2

package. Pearson's correlation coefficients were calculated from the regression lines for each herbicide dose. Violin plots and box plots were generated for fomesafen, glyphosate (*E. indica*), and dicamba data to show the variance of the  $F_v/F_m$  values both before and after herbicide incubation. Bar charts showing means of  $F_v/F_m$ , spray injury, and leaf area for each *Amaranthus* population were also generated to compare the two herbicide diagnostic methods at the population level for fomesafen.

Phenotypic results from the leaf-disk assay were compared with whole-plant spray tests at discriminating doses at the individual plant level using the following standards: plants were identified as resistant if visual injury was  $< 90\%$ ,  $F_v/F_m$  raw values were  $\geq 0.2$ , or  $F_v/F_m$  values of the treated plants expressed as a percentage of the untreated control were  $\geq 25\%$ . Results from the two methods were considered concordant when the same resistance results were reported by both methods (e.g., a sprayed plant with  $< 90\%$  visual injury also had an  $F_v/F_m$  value  $\geq 0.2$ ).



**Figure 2.** Forty-eight-hour time course images of leaf-disk chlorophyll fluorescence and photos of plants sprayed with glyphosate, dicamba, and fomesafen. One leaf disk was sampled from each of the four sprayed plants (1–4) and chlorophyll fluorescence images were taken 12 h after dark incubation and 4, 8, 12, 24, 36, and 48 h after light incubation. The best evaluation timing for each herbicide was determined when distinguishable chlorophyll fluorescence signals produced by plants aligned with herbicide sensitivities determined through whole-plant spray tests.

## Results and Discussion

### Evaluation Timing for Fomesafen, Glyphosate, and Dicamba

The time course study that measured leaf-disk chlorophyll fluorescence at different time points during a 48-h incubation time suggests that the best incubation or evaluation timings for fomesafen, glyphosate, and dicamba were 4 to 24 h, 24 to 48 h, and 36 to 48 h, respectively (Figure 2). For a fast-acting herbicide like fomesafen, sensitive plants lost chlorophyll fluorescence signals rapidly. The separation among resistant and susceptible plants was observed as early as 4 h, while it took at least 24 to 36 h for glyphosate and dicamba to produce distinguishable symptoms. The order of how fast the herbicide symptoms developed for the leaf-disk assay was consistent with the whole-plant spray tests: fomesafen > glyphosate > dicamba. For PPO herbicides that directly target plant photosynthesis and inhibit the biosynthesis of chlorophyll (Beale and Weinstein 1990), the leaf disks of sensitive plants can be bleached due to chlorophyll degradation. This indicates that visual ratings might be conducted without subjecting the plates to CF imager scanning (Figure 3). However, the leaf disks of glyphosate- or dicamba-sensitive plants largely stayed green, even though chlorophyll fluorescence was absent (Figure 4; Supplementary Figures S1 and S2). These results also indicate that although the leaf-disk assay was built upon the photochemistry of the PSII system (Murchie and Lawson 2013), it showed promise in detecting resistance to systemic herbicides that do not directly target plant photosynthesis.

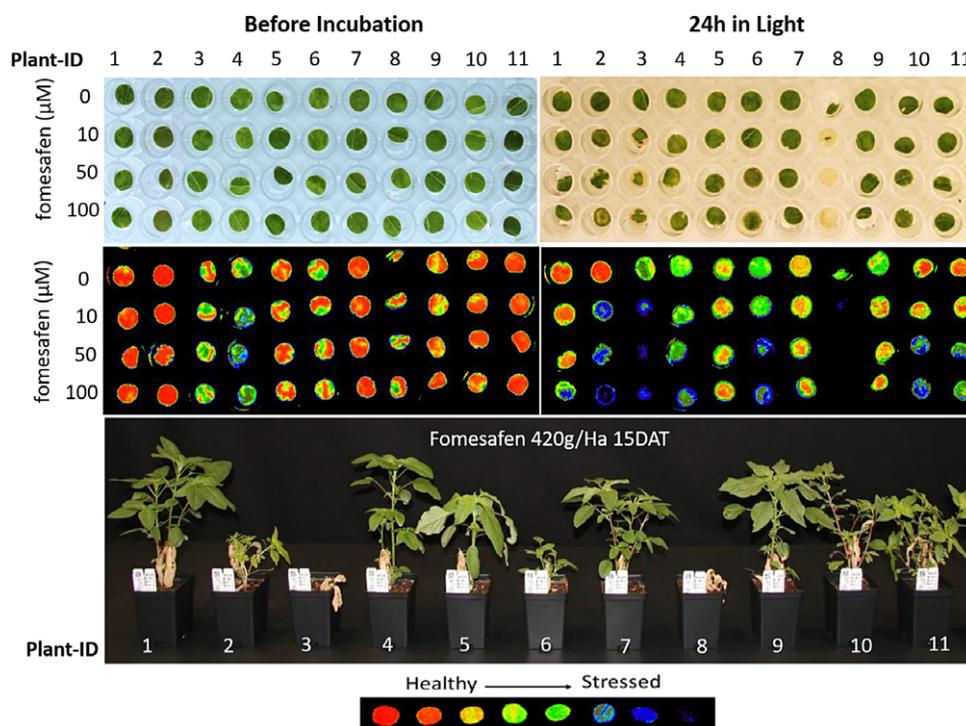
### Effective Doses for Fomesafen, Glyphosate, and Dicamba

Effective doses for different herbicides varied. For fomesafen, sensitive plants were unable to survive the lowest dose tested: the leaf disks of the resistant plants still had strong fluorescence at a dose up to 100  $\mu\text{M}$ , while the leaf disks of the sensitive plants had weak

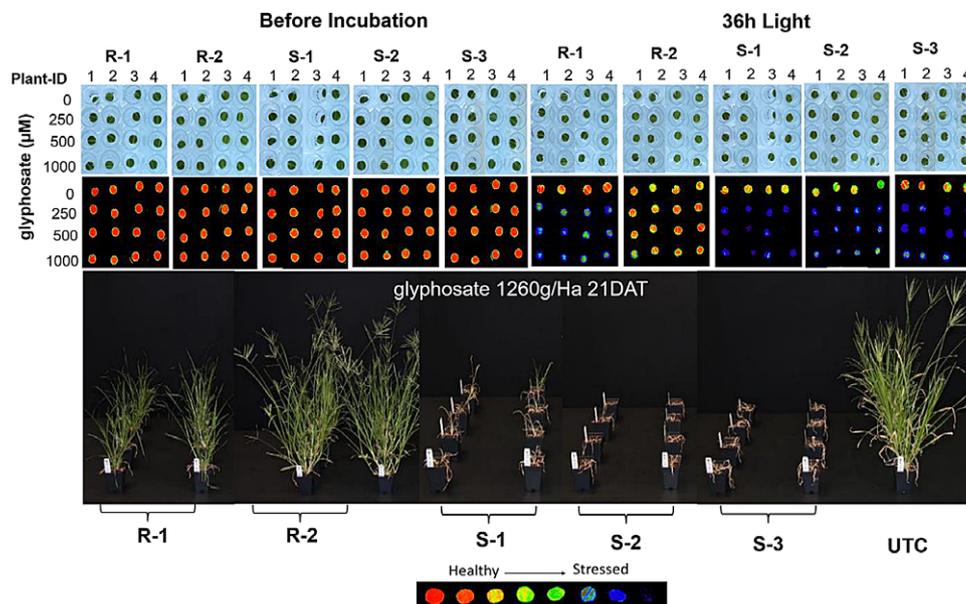
fluorescence signals at the lowest dose of 10  $\mu\text{M}$  (e.g., plants 3 and 8 in Figure 3). Results from the leaf-disk assay largely agreed with the phenotypes of sprayed plants determined 14 d after herbicide treatment (Figure 3). For glyphosate, strong correlation between the two methods was only observed for *E. indica* but not *Amaranthus* (Figure 4; Supplementary Figures S1 and S3). Glyphosate at 250  $\mu\text{M}$  clearly separated the two resistant *E. indica* populations from the three sensitive populations. The less-resistant R-1 line also yielded weaker chlorophyll fluorescence signals compared with the R-2 line, indicating the sensitivity of the glyphosate leaf-disk assay to distinguish *E. indica* populations with varying levels of resistance (Figure 4). For dicamba, no difference was observed at 250  $\mu\text{M}$ . All the RR plants and some RS plants survived the highest dose of 800  $\mu\text{M}$ , while none of the SS or WT plants produced chlorophyll fluorescence signals, suggesting 800  $\mu\text{M}$  can be used as a discriminating dose for dicamba-resistant and dicamba-susceptible *B. scoparia* plants with the known IAA16 mutation (Supplementary Figure S2). However, more fine-tuned dose–response studies might be needed to identify a dose that distinguishes between RR and RS plants.

### Correlation between $F_v/F_m$ Values and Herbicide Spray Injuries

Regression analysis (Figure 5) confirmed that there were significant negative correlations between the  $F_v/F_m$  values and the herbicide injuries for fomesafen, glyphosate (*E. indica*), and dicamba. For fomesafen (Figure 5A), the slopes of the regression lines were significant across all doses, with 10  $\mu\text{M}$  having the strongest negative correlation ( $R = -0.41$ ,  $P < 0.0001$ ). For dicamba (Figure 5B), the slopes of the regression lines were only significant for the highest dose of 800  $\mu\text{M}$ , with a negative slope value of  $-0.44$  ( $P = 0.0023$ ). For glyphosate, none of the regression lines for *Amaranthus* were significant (Figure 5C), indicating the response



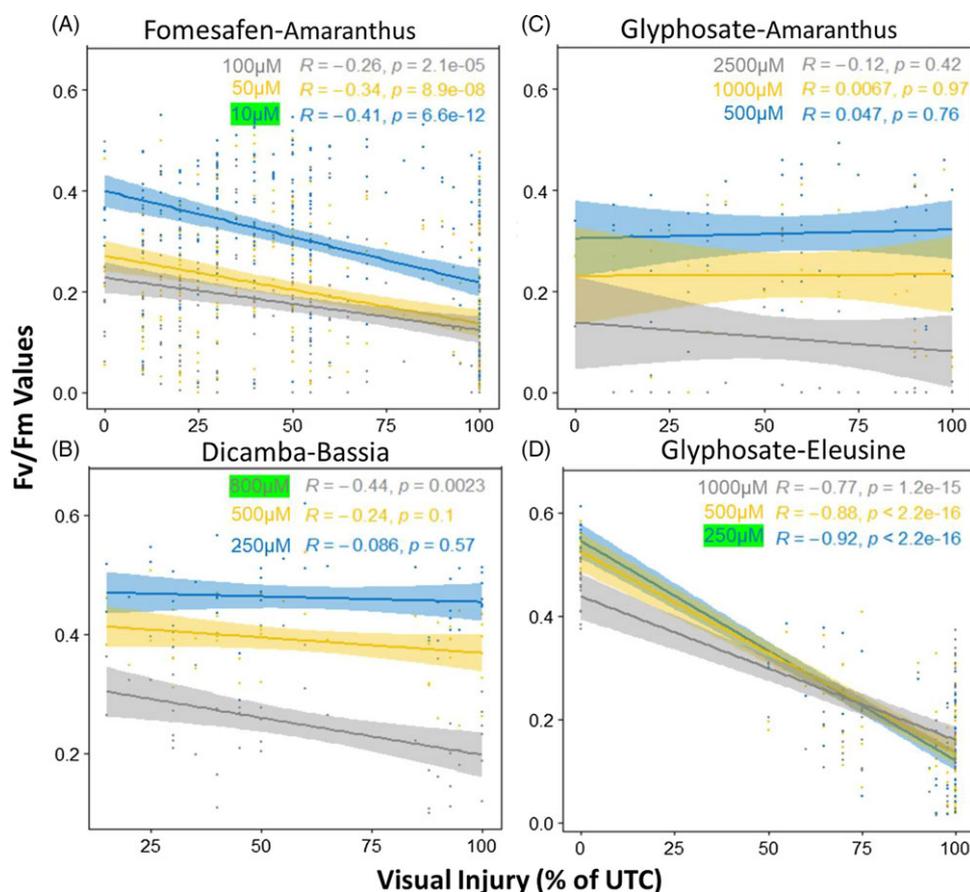
**Figure 3.** Leaf-disk chlorophyll fluorescence images and photos of *Amaranthus* plants sprayed with fomesafen. Plants 1–11 represent individual plants from 11 different *Amaranthus* populations. The top panels are photos of the leaf disks taken before incubation and 24 h after being incubated in herbicide solutions under light. The middle panels are corresponding chlorophyll images for leaf disks in the top panel. The bottom panel shows photos of sprayed plants from which the leaf disks were sampled (4 leaf punches per plant).



**Figure 4.** Leaf-disk chlorophyll fluorescence images and photos of *Eleusine indica* populations sprayed with glyphosate. R-1, R-2, and S1–S3 represent two glyphosate-resistant and three sensitive *E. indica* populations, respectively; 1–4 represent leaf disks from four individual plants from each *E. indica* population. The top panels are photos of the leaf disks taken before incubation and 36 h after being incubated in herbicide solutions under light. The middle panels are corresponding chlorophyll images for the leaf disks in the top panel. The bottom panel shows photos of sprayed plants from which the leaf disks were sampled (4 leaf punches per plant).

of the leaf disks did not align with the response of the whole plant in the spray tests. In contrast, significant regression relations were observed for *E. indica* with slopes ranging from  $-0.77$  to  $-0.92$  (Figure 5D) and 250  $\mu\text{M}$  having the most negative slope ( $P < 0.0001$ ). Similar results were observed for  $F_v/F_m$  expressed

as percentage of untreated plants, as well as leaf area data (Supplementary Figure S3). The 10, 250, and 800  $\mu\text{M}$  doses were thus used as discriminating doses for fomesafen, glyphosate (*E. indica*), and dicamba, respectively, for our down-stream analysis at both the individual plant and population levels.



**Figure 5.** Regression lines showing the correlation between the  $F_v/F_m$  values with spray visual injuries. (A) Fomesafen–*Amaranthus* doses: 0, 10, 50, 100  $\mu\text{M}$ ; (B) dicamba–*Bassia scoparia* doses: 0, 250, 500, 800  $\mu\text{M}$ ; (C) glyphosate–*Amaranthus* doses: 0, 500, 1,000, 2,500  $\mu\text{M}$ ; (D) glyphosate–*Eleusine indica* doses: 0, 250, 500, 1,000  $\mu\text{M}$ . In each figure, different-colored regression lines indicate doses from low to high: blue, yellow, and gray.

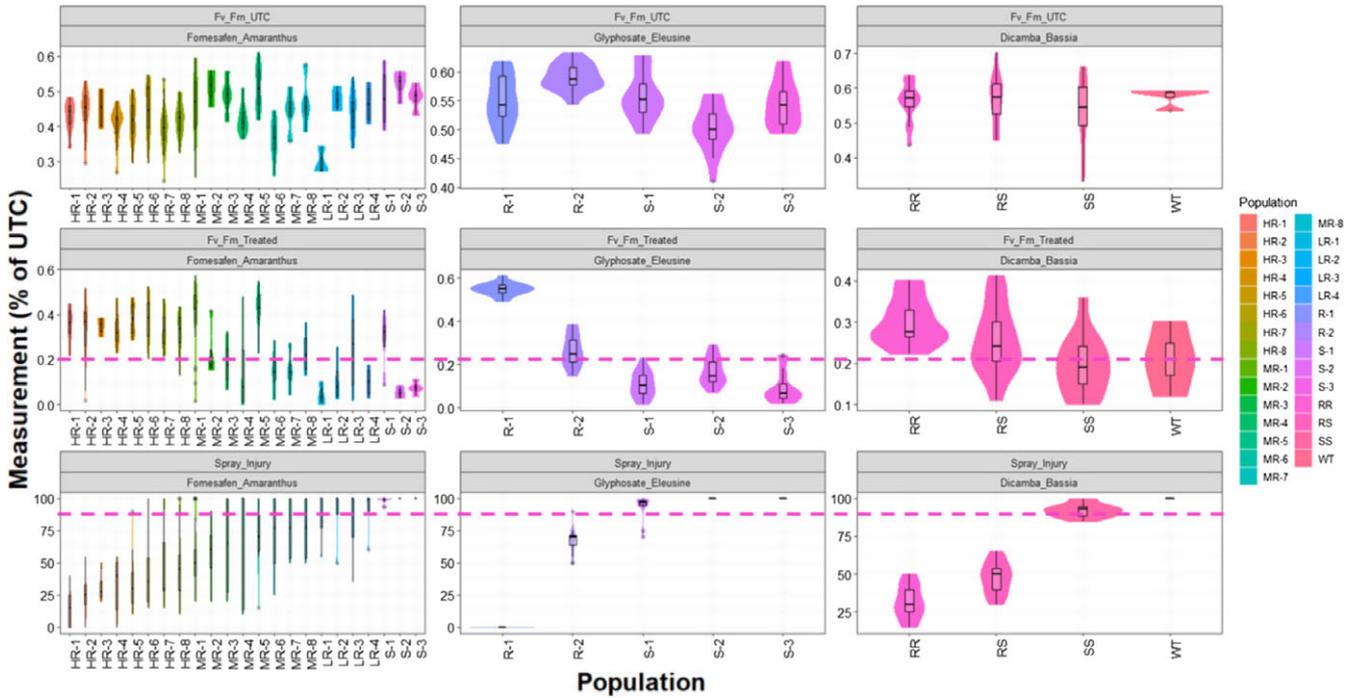
The differences between the glyphosate leaf-disk assay on *E. indica* and *Amaranthus* might be related to different genetic diversity and biological response to glyphosate in different species. For example, Cañero et al. (2011) showed that glyphosate treatment had no effect on photosystem II photochemistry of young olive (*Olea europaea* L.) plants and observed no reduction in  $F_v/F_m$  values. However, Vivancos et al. (2011) observed rapidly inhibited photosynthesis and decreased  $F_v/F_m$  values in glyphosate-sensitive soybean [*Glycine max* (L.) Merr.]. Furthermore, the lower genetic diversity in a monoecious grass weeds compared with outcrossing dicot weeds like *Amaranthus* (Chandi et al. 2013; Shukla et al. 2018), might also contribute to the more uniform leaf-disk herbicide response we observed.

It is also possible that as a simplified testing system that focuses on the target organelle of the plants (chloroplast), the leaf-disk assay is more effective in detecting target site-based resistance (TR) than non-target site based resistance (NTR). Interestingly, for the *E. indica*, *B. scoparia*, and *Amaranthus* populations we tested for glyphosate, dicamba, and fomesafen, target-site mutations were the main underlying resistance mechanism (Baerson et al. 2002; LeClere et al. 2018; Wu et al. 2020a, 2020b; Yu et al. 2015). If NTR mechanisms such as reduced uptake and translocation are involved (Heap and Duke 2018; Sammons and Gaines 2014), the response of the resistant and sensitive plants at the chloroplast/PSII system level might be similar and thus prevent the leaf-disk assay from capturing their differences.

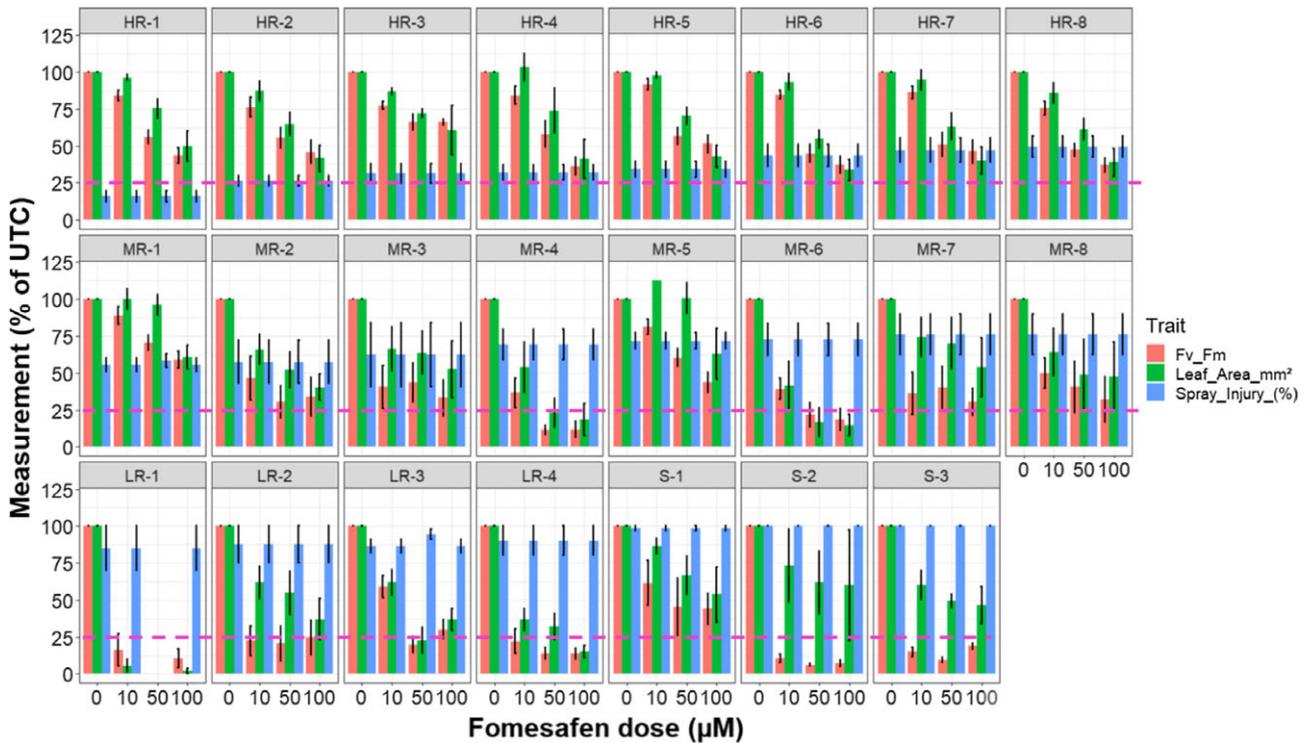
### The Sensitivities of the Leaf-Disk Assay in Distinguishing Resistance at the Population Level

To determine whether the leaf-disk assay can effectively distinguish resistant and sensitive weed populations, violin and box plots were projected to show the distribution and changes of the  $F_v/F_m$  values both before and after the incubation of herbicides at the discriminating doses (Figure 6). For the 23 *Amaranthus* populations tested for fomesafen, the majority of the populations had starting  $F_v/F_m$  values ranging from 0.4 to 0.6, with a few populations having lower  $F_v/F_m$  values ( $\sim 0.3$ ), indicating these populations might be associated with a lower fitness. Higher  $F_v/F_m$  values were observed for *E. indica* (0.45 to 0.7) and *B. scoparia* (0.5 to 0.7) in general. This indicates that converting final  $F_v/F_m$  values of treated plants to a percentage of the untreated plants might be necessary to account for the variance of the starting values of  $F_v/F_m$  in different weed populations, or different threshold values might be set to distinguish resistant versus susceptible plants from different populations.

Herbicide incubation significantly reduced  $F_v/F_m$  values. For fomesafen, low resistance and sensitive populations were consistently associated with lower  $F_v/F_m$  values, while highly and moderately resistant populations were able to maintain  $F_v/F_m$  values around 0.2 to 0.5 after herbicide treatment (Figure 6; Supplementary Figure S4). We also calculated the means of  $F_v/F_m$  and spray injuries across all 23 *Amaranthus* populations we tested (Figure 7). Based on different resistance levels, the 23



**Figure 6.** Violin and box plots showing  $F_v/F_m$  values before and after herbicide treatment and spray injuries across different weed–herbicide combinations. “ $F_v/F_m$ \_treated” represent the  $F_v/F_m$  values of distinguishing dose: fomesafen (*Amaranthus*) 10  $\mu\text{M}$ , glyphosate (*Eleusine indica*) 250  $\mu\text{M}$  and dicamba (*Bassia scoparia*) 800  $\mu\text{M}$ . Twenty-three *Amaranthus* populations were included for the fomesafen assay: eight high- (HR), eight moderate- (MR), and four low-resistance populations (LR) and three sensitive (S) populations. Five *E. indica* populations were included for glyphosate, two resistant (R-1, R-2) and three sensitive populations (S-1, S-2, S-3). Four *B. scoparia* genotypes with or without the resistance-endowing mutation were included for dicamba: homozygous- (RR), heterozygous-resistant (RS), homozygous sensitive (SS), and WT. UTC, untreated control.



**Figure 7.** Bar charts showing means of  $F_v/F_m$  values, leaf areas, and spray injuries across different fomesafen doses and *Amaranthus* populations. Each figure represents means of  $F_v/F_m$ , actively photosynthesizing leaf area expressed as percentage of untreated leaf, and spray injury data across different fomesafen doses (10, 50, 100  $\mu\text{M}$ ) for 23 *Amaranthus* populations: eight high- (HR), eight moderate- (MR), and four low-resistance populations (LR) and three sensitive populations (S). The dashed lines indicate the threshold values that were used to distinguish resistant vs. susceptible plants: 25% spray visual injury or  $F_v/F_m$  values expressed as percentages of untreated plants. UTC, untreated control.



**Figure 8.** Leaf-disk chlorophyll fluorescence images and visual injuries of individual *Amaranthus* plants sprayed with fomesafen. Plants 1–12 in each panel represent an individual plant from 12 different *Amaranthus* populations (4 replications  $\times$  12 populations = 48 plants in total). For each plant, four leaf disks were challenged with different doses of fomesafen (0, 10, 50, 100  $\mu$ M) and chlorophyll images were taken 24 h after being incubated in herbicide solutions under light. Visual injuries of each plant evaluated 14 d after application of 1X rate of fomesafen are indicated at the top of each panel and colored coded based on resistance levels. The panels on the right are the same images as those in the left panels but with the quantitative data. False-negative (low  $F_v/F_m$  values for resistant plants) and false-positive (high  $F_v/F_m$  values for susceptible plants) results from the leaf-disk assay are highlighted in cyan and magenta rectangles, respectively.

populations were divided into four categories: high- (HR-1 to HR-8, <50% spray injury), moderate- (MR-1 to MR-8, 50% to 80% spray injury), and low-resistance (LR-1 to LR-4, 80% to 90% spray injury) and sensitive (S-1 to S-3, >90% spray injury). Using a sustained 25%  $F_v/F_m$  signal (that of an untreated leaf) as our threshold value, the fomesafen leaf-disk assay (10  $\mu$ M dose) clearly separated all the high-resistance and most moderate-resistance populations (Figure 7). However, the two methods yielded different results for the low-resistance and sensitive populations, with some susceptible populations having higher  $F_v/F_m$  than observed for the resistant plants (e.g., LR-3, S-1)

Similar trends were observed for glyphosate in *E. indica* and dicamba in *B. scoparia*. The  $F_v/F_m$  values were two to three times higher in the two resistant *E. indica* populations compared with the three sensitive populations (Figure 6). Slightly more overlaps were observed for different dicamba-resistant and dicamba-sensitive *B. scoparia* populations (Figure 6). Because only *B. scoparia* populations with TR were used in the current study, more research is needed to expand the application of this assay to more diverse *B. scoparia* field populations. Overall, our results indicate that the leaf-disk assay was effective in distinguishing resistant and sensitive populations for all the three herbicides tested.

### The Sensitivities of the Leaf-Disk Assay in Distinguishing Resistance at the Individual Plant Level

Further comparison of the two diagnostic methods for resistance showed similar results at the individual plant level compared with the population-level characterization, but with a higher level of variance (Table 1). For the 241 plants sprayed with fomesafen, results from the two methods aligned with each other for 85.5% of the plants (10  $\mu$ M). Among the remaining 14.5%, 7.9% could

be attributed to false-positive results from the leaf-disk assay (e.g., plants with >90% spray injury [identified as sensitive], but  $F_v/F_m$  values >0.2 [identified as resistant]) and 6.6% could be attributed to false-negative results (Table 1; Figure 8; Supplementary Figures S5–S7). For the 80 *E. indica* plants sprayed with glyphosate, the two methods aligned with each other in 92.3% of the plants (250  $\mu$ M), and of the remaining 7.7%, 1.3% could be attributed to false-positive results from the leaf-disk assay, and 6.4% could be attributed to false-negative results (Table 1; Supplementary Figure S8). Higher discrepancies were observed for the dicamba assay in dicamba–*B. scoparia* and glyphosate–*Amaranthus* combinations, for which the two assays only aligned in 71.7% (800  $\mu$ M) and 71.1% (500  $\mu$ M) of the cases, respectively (Table 1). Slightly lower concordant rates were observed for  $F_v/F_m$  expressed as percentage of untreated plants (Supplementary Table S3). Comparing concordant rates of data collected at different time points also indicates that evaluation timing and reliable resistant and susceptible controls are critical and will influence the accuracies of the assay (Supplementary Figure S9). Longer incubation time might be needed for some susceptible populations to fully responded to herbicide challenges (i.e., plants with higher leaf chlorophyll concentrations or leaves not in good contact with the herbicide solution).

The comparison of two resistance diagnostic methods through regression models, as well as concordant rates at both population and individual levels, suggests more work is needed to improve and optimize the leaf-disk assay for higher sensitivity and prediction accuracies, especially for systemic herbicides such as glyphosate and dicamba. Though characterization of plant stress response through chlorophyll fluorescence emission is a powerful tool, studies have pointed out the potential pitfalls for interpreting the chlorophyll fluorescence data, especially when focusing on a single

parameter (Juneau et al. 2007). Logan et al. (2007) have suggested using it cautiously as a rapid, preliminary method of assessing plant responses to stress in conjunction with other characterization methods. In the case of a herbicide-resistance survey, an initial screen of all weed accessions with the leaf-disk assay followed by whole-plant spray tests or molecular marker assays could be an intermediate approach to leverage the advantages of this rapid method. Follow-up studies on the resistant accessions identified by the leaf-disk assay can effectively exclude false-positive results.

### Future Research Directions on Optimizing the Assay

In the current study, we report a nondestructive leaf-disk assay based on chlorophyll fluorescence emission to enable the detection of resistance to both systemic and contact herbicides within ~48 h. We demonstrated the feasibility of the assay to rapidly detect resistance to glyphosate, dicamba, and fomesafen in both broadleaf and grass weeds and showed good correlation between data from the greenhouse spray test and the leaf-disk assay. However, the current concordant rates could be improved by optimizing the assay in multiple ways: (1) conduct more fine-tuned dose–response studies across diverse field weed populations to identify more robust discriminating doses for each herbicide (e.g., dicamba–*B. scoparia*); (2) determine whether dose response is a better proxy than a single dose in making the correct calls for resistance (Supplementary Figure S7, top left panel, plant 9); (3) conduct the assays on resistant weeds with both TR and NTR mechanisms to confirm whether this assay's applicability is limited to resistant weeds with a TR mechanism; and (4) investigate whether better algorithms could be used to convert the CF images into  $F_v/F_m$  values and leaf area data, as suggested by previous researchers (Rousseau et al. 2013). Furthermore, future research could also focus on validating the assay on other herbicide sites of action as well as herbicide mixtures. Nevertheless, the current study demonstrated the promise of the leaf-disk assay as a rapid tool to survey herbicide-resistant weeds, and it is worthy of more optimization to improve its sensitivity.

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### References

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, GM Dill (2002) Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Plant Physiol* 129: 1265–1275

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu Rev Plant Biol* 59:89–113

Beale SI, Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. Pages 287–391 in Dailey HA, ed. *Biosynthesis of Heme and Chlorophylls*. New York: McGraw-Hill

Burgos N, Tranel PG, Strebjg JC, Davis VM, Shaner D, Norsworthy JK, Ritz C (2013) Review: confirmation of resistance to herbicides and evaluation of resistance levels. *Weed Sci* 61:4–20

Cañero AI, Cox L, Redondo-Gómez S, Mateos-Naranjo E, Hermosin MC, Cornejo J (2011) Effect of the herbicides terbuthylazine and glyphosate on photosystem II photochemistry of young olive (*Olea europaea*) plants. *J Agric Food Chem* 59:5528–5534

Chandi A, Milla-Lewis SR, Jordan DL, York AC, Burton JD, Zuleta MC, Whitaker JR, Culpepper AS (2013) Use of AFLP markers to assess genetic diversity in Palmer amaranth (*Amaranthus palmeri*) populations from North Carolina and Georgia. *Weed Sci* 61:136–145

Chatham LA, Wu C, Riggins CW, Hager AG, Young BG, Roskamp GK, Tranel PJ (2015) EPSPS gene amplification is present in the majority of glyphosate-resistant Illinois waterhemp (*Amaranthus tuberculatus*) populations. *Weed Technol* 29:48–55

Evans JA, Tranel PJ, Hager AG, Davis AS, Schutte B, Wu C, Chatham LA, Davis AS (2016) Managing the evolution of herbicide resistance. *Pest Manag Sci* 72:74–80

Gerwick BC, Mireles LC, Eilers RJ (1993). Rapid diagnosis of ALS/AHAS-resistant weeds. *Weed Technol* 7:519–524

González-Torralva F, Norsworthy JK, Piveta LB, Varanasi VK, Barber T, Brabham C (2020) Susceptibility of Arkansas Palmer amaranth accessions to common herbicide sites of action. *Weed Technol* 34:770–775

Hanson B, Shrestha A, Shaner D (2009) Distribution of glyphosate-resistant horseweed (*Conyza canadensis*) and relationship to cropping systems in the Central Valley of California. *Weed Sci* 57:48–53

Heap I, Duke SO (2018) Overview of glyphosate-resistant weeds worldwide. *Pest Manag Sci* 74:1040–1049

Juneau P, Qiu B, Deblois CP (2007) Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review. *Toxicol Environ Chem* 89:609–625

Lakra N, Nutan KK, Das P, Anwar K, Singla-Pareek SL, Pareek A (2015) A nuclear-localized histone-gene binding protein from rice (OsHBP1b) functions in salinity and drought stress tolerance by maintaining chlorophyll content and improving the antioxidant machinery. *J Plant Physiol* 176:36–46

LeClere S, Wu C, Westra P, Sammons RD (2018) Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Bassia scoparia* is endowed by a mutation in an *AUX/IAA* gene. *Proc Natl Acad Sci USA* 115:2911–2920

Logan BA, Adams WW III, Demmig-Adams B (2007) Avoiding common pitfalls of chlorophyll fluorescence analysis under field conditions. *Funct Plant Biol* 34:853–859

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. *J Exp Bot* 51:659–668

Murchie EH, Lawson T (2013) Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *J Exp Bot* 64: 3983–3998

Oxborough K, Baker NR (1997) Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components—calculation of  $qP$  and  $F_v'/F_m'$  without measuring  $F_o'$ . *Photosynth Res* 54:135–142

Rousseau C, Belin E, Bove E, Rousseau D, Fabre F, Berruyer R, Guillaumès J, Manceau C, Jacques MA, Boureau T (2013) High throughput quantitative phenotyping of plant resistance using chlorophyll fluorescence image analysis. *Plant Methods* 9:17

Salas-Perez RA, Burgos NR, Rangani G, Singh S, Refatti JP, Pivetam L, Tranel PJ, Mauromoustakos A, Scott RC (2017) Frequency of Gly-210 deletion mutation among protoporphyrinogen oxidase inhibitor-resistant Palmer amaranth (*Amaranthus palmeri*) populations. *Weed Sci* 65:718–731

Sammons RD, Gaines TA (2014) Glyphosate resistance: state of knowledge. *Pest Manag Sci* 70:1367–1377

Schultz JL, Chatham LA, Riggins CW, Tranel PJ, Bradley KW (2015) Distribution of herbicide resistances and molecular mechanisms conferring resistance in Missouri waterhemp (*Amaranthus rudis* Sauer) populations. *Weed Sci* 63:336–345

Shaner DL, Nadler-Hassar T, Henry B, Koger C (2005) A rapid in vivo shikimate accumulation assay with excised leaf discs. *Weed Sci* 53: 769–774

Shukla A, Srivastava N, Suneja P, Yadav SK, Hussain Z, Rana JC, Yadav S (2018) Untapped amaranth (*Amaranthus* spp.) genetic diversity with potential for nutritional enhancement. *Genet Resour Crop Evol* 65:243–253

Varanasi VK, Brabham C, Norsworthy JK, Nie H, Young BG, Houston M, Barber T, Scott RC (2018) A statewide survey of PPO-inhibitor resistance

- and the prevalent target-site mechanisms in Palmer amaranth (*Amaranthus palmeri*) accessions from Arkansas. *Weed Sci* 66:149–158
- Westra EP, Nissen SJ, Getts TJ, Westra P, Gaines TA (2019) Survey reveals frequency of multiple resistance to glyphosate and dicamba in kochia (*Bassia scoparia*). *Weed Technol* 33:664–672
- Wu C, Goldsmith R, Pawlak J, Feng P, Smith S, Navarro S, Perez-Jones A (2020a) Differences in efficacy, resistance mechanism and target protein interaction between two PPO inhibitors in Palmer amaranth (*Amaranthus palmeri*). *Weed Sci* 68:105–115
- Wu, C, LeClere S, Liu K, Paciorek M, Perez-Jones A, Westra P, Sammons RD (2020b) A dicamba resistance endowing IAA16 mutation leads to significant vegetative growth defects and impaired competitiveness in kochia (*Bassia scoparia*). *Pest Manag Sci* 77:795–804
- Vivancos PD, Driscoll SP, Bulman CA, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH (2011) Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiol* 157:256–268
- Yu Q, Jalaludin A, Han H, Chen M, Sammons RD, Powles SB (2015) Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. *Plant Physiol* 167:1440–1447