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Multiple-resistance evolution to ACCase inhibitors and glyphosate in sourgrass (*Digitaria insularis*) is attributed to diverse polymorphisms in the herbicide target sites

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Research Article

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Abstract

Sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] is considered the most troublesome weed in agronomic crops in South America. Overreliance on glyphosate has selected for resistant populations, although the resistance mechanisms remain unknown. Recently, populations were identified that exhibited multiple resistance to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetyl-CoA carboxylase (ACCase) inhibitors, posing a significant challenge due to the lack of alternative control options. This project aimed to identify the resistance patterns and levels to glyphosate and ACCase inhibitors of three suspected resistant populations (P1, P2, and P3), and elucidate the resistance mechanisms. We performed dose–response experiments with clethodim, fluazifop-P-butyl, glyphosate, and pinoxaden to identify the possibility of cross- and multiple resistance and to quantify the resistance levels. We sequenced the *ACCase* and *EPSPS* genes to test the hypothesis that target-site mutations were involved in the resistance mechanisms, given the resistance patterns observed. Our results indicated that two of the tested populations, P1 and P2, were multiple resistant to glyphosate and all ACCase-inhibitor classes, while P3 was resistant to glyphosate only. Resistance levels varied by herbicide, with resistance indices ranging from 2.7- to nearly 2,000-fold. We identified an amino acid substitution in ACCase at position 2078 (Asp-2078-Gly), homozygous for both P1 and P2, corroborating the resistance patterns observed. Interestingly, *EPSPS* sequencing identified multiple heterozygous DNA polymorphisms that resulted in amino acid substitutions at positions 106 (P1 and P2) or at both 102 and 106 (P3), indicating multiple evolutionary origins of glyphosate-resistance evolution. We show for the first time the genetic mechanisms of multiple resistance to glyphosate and ACCase in *D. insularis*, and provide a thorough discussion of the evolutionary and management implications of our work.

Introduction

Sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] is a troublesome grass species native to the Americas, introduced to Asia, Africa, and Oceania. It is a C_4, diploid plant that produces up to 40,000 seeds per plant per year that are equipped with trichomes that facilitates long-distance wind dispersal (Gemelli et al. 2012; Kissmann and Groth 1997; Lorenzi 2000). Additionally, *D. insularis* forms rhizomes during its initial growth, facilitating dispersal within fields with agricultural mechanical operations (Mitskas et al. 2003). The high seed production potential, low dormancy, and high germination rates of *D. insularis* (de Carvalho et al. 2011; de Mendonça et al. 2014) contribute to its persistence and replenishment of the weed seedbank in annual and perennial cropping systems (de Carvalho et al. 2011; Lacerda 2003).

The introduction of glyphosate-resistant (GR) crops in South America led to a swift and extensive increase in their cultivation, particularly in Brazil, Argentina, Paraguay, and Uruguay (Brookes and Barfoot 2020b). In 2018, for instance, GR soybean [*Glycine max* (L.) Merr.] was planted in 98% of Paraguay and Uruguay production areas (Brookes and Barfoot 2020a). In Argentina, the introduction of GR soybean dates back to 1996 (Finger et al. 2009), and within 4 yr, adoption rates increased to nearly 90% (Penna and Lema 2003). Brazil is the world's second-largest producer of transgenic crops (USDA 2024). Data from the USDA (2024) for the 2022 to 2023 season highlight Brazil's extensive use of GR soybean, with herbicide resistance being the predominant trait in more than 68 million ha of transgenic crops cultivated.

Adoption of no-tillage practices increased with GR crops, driven in part by the benefit of desiccating the vegetation before planting, as well as the benefits of using a nonselective herbicide such as glyphosate postemergence in the crop and weeds. Because *D. insularis* is a perennial species with deep rhizomes, nonchemical control options are inefficient. This scenario has encouraged overreliance on systemic herbicides as the primary method for *D. insularis* control, leading to evolution of glyphosate resistance. In South America, the first reports of GR *D. insularis* were in 2005 from Paraguay and in 2008 from Brazil (near the border with Paraguay; Lopez-Ovejero et al. 2017). Monitoring efforts over the years have described the increase in distribution of glyphosate resistance throughout South America in important soybean-growing regions (Lopez-Ovejero et al. 2017), likely facilitated by movement of agricultural equipment from the southern region northward (Gonçalves Netto et al. 2021).

The emergence of herbicide resistance often leads to the overreliance on another single, alternative herbicide. In a cropping system where soybeans are grown almost every year, the natural alternative chemistry to control *D. insularis* is the acetyl-CoA carboxylase (ACCase)-inhibiting mode of action. This is because most herbicides in this group are systemic and grass specific, while selective for soybean use. Not surprisingly, the first report of an ACCase inhibitor–resistant *D. insularis* was made in 2016 (Heap 2024) in soybean fields. A few years later, Takano et al. (2020) identified populations from a soybean–corn (*Zea mays* L.)–cotton (*Gossypium hirsutum* L.) cropping system in the Cerrado region of Brazil. An additional population has been identified more recently in Paraguay in 2020 in soybean fields with resistance to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and ACCase (Krzyzaniak et al. 2023).

The genetic mechanisms of glyphosate resistance in *D. insularis* remains largely unknown, and there is evidence that various resistance mechanisms exist in different populations. For example, de Carvalho et al. (2012) found that resistant populations had a combination of physiological alterations that resulted in reduced glyphosate damage. Those authors found less initial glyphosate absorption up to 12 h after treatment in resistant biotypes, as well as reduced translocation from the treated leaf to the rest of the plant, and enhanced herbicide metabolism to less toxic compounds. No amino acid substitutions were found in the residues known to confer resistance to glyphosate. In another study, da Costa et al. (2014) observed minor differences in initial glyphosate absorption and large differences in translocation and metabolism between susceptible and resistant biotypes (da Costa et al. 2014). Conversely, Melo et al. (2019) found no differences in absorption and translocation of glyphosate, as well as no mutations in the *EPSPS* gene in biotypes collected from agronomic fields in Mato Grosso, Minas Gerais, and São Paulo states, indicating that a novel resistance mechanism had evolved. Another study by Gazola et al. (2020) reported no translocation differences between susceptible and resistant biotypes. Taken together, these results suggest that various glyphosate-resistance mechanisms may have evolved in different populations, although the specific mechanisms have not been elucidated.

Herbicide alternatives to glyphosate, particularly those that inhibit ACCase, became key tools in *D. insularis* management. Given the overreliance on ACCase inhibitors, resistant populations were identified (Heap 2024). The mechanisms of resistance to ACCase-inhibiting herbicides have been studied by Takano et al. (2020). The authors studied a population collected in the Cerrado region of Brazil and observed that it was resistant to WSSA/HRAC Group 1 herbicides from the aryloxyphenoxypropionate (-fops) and phenylpyrazolin (-den) chemical families, but not cyclohexanedione (-dims). They identified an amino acid substitution in the carboxyltransferase (CT) domain of ACCase, where a tryptophan at position 2027 was substituted by a cysteine. The herbicide resistance pattern associated with this specific mutation has been observed in other species such as blackgrass (*Alopecurus myosuroides* Huds.) and Japanese foxtail (*Alopecurus japonicus* Steud.) (Kaundun 2014).

While the evolution of glyphosate and ACCase-inhibitor resistance has been individually observed in *D. insularis*, a detailed characterization of multiple-herbicide resistance at the phenotypic level is lacking. In addition, much remains unknown about the glyphosate resistance mechanisms in *D. insularis*. Multiple resistance to ACCase inhibitors and glyphosate poses additional challenges to *D. insularis* management, because other chemistries and methods are ineffective, and understanding the resistance mechanisms could help in understanding the evolutionary origins and design practices to limit resistance dispersal and improve management. In this project, we studied *D. insularis* populations that were recently identified in Paraguay that exhibited multiple resistance to EPSPS and ACCase inhibitors. The objective of this project was to characterize the resistance patterns and levels of three suspected resistant populations and elucidate their resistance mechanisms.

Materials and Methods

Source of Plant Material

Seeds of suspected glyphosate- and ACCase inhibitor–resistant *D. insularis* populations were collect from soybean fields in Paraguay at the end of the growing season and were labeled as P1 (Tucuru Puku, Paraguay, 25.34°S, 54.68°W), P2 (Paso Ita, Paraguay, 25.347°S, 54.68°W), and P3 (Hernandarias District, Paraguay, 25.33°S, 54.69°W). Seeds from 10 to 20 individuals were collected from each field and bulked. We included a susceptible population (P4) that has been characterized by Adegas et al. (2010), and population P1 has been included in a previous study by Krzyzaniak et al. (2023).

Quantification of Resistance Patterns and Levels

To determine the resistance levels of *D. insularis* populations to ACCase inhibitors and glyphosate, dose–response experiments were performed in a greenhouse of the Department of Plant Science, Pennsylvania State University, PA, USA. Seeds from the four populations were germinated in trays filled with commercial potting media (Pro-Mix® BX, PRO-MIX, Quakertown, PA, USA) and transplanted to larger pots (500 cm^3) at the seedling stage (BBCH 10-11; Hess et al. 1997). Greenhouse conditions were maintained at a constant temperature (25 C) with artificial illumination and irrigated three times per day using automatic sprinklers.

Dose–response experiments were performed for the ACCase inhibitors clethodim (SelectMax®, Valent U.S.A.), fluazifop-P-butyl (Fusilade® DX, Syngenta, San Ramon, CA, USA), and pinoxaden (Axial® XL, Syngenta, Greensboro, NC, USA), in addition to the EPSPS inhibitor glyphosate (Roundup PowerMax®, Bayer Crop Science, St. Louis, MO, USA). These herbicides were chosen based on their importance in South America for *D. insularis* control, except for pinoxaden. Although pinoxaden is not widely used, this herbicide can provide important information on cross-resistance patterns, as it is from a different

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Table 1. Herbicides rates for whole-plant dose–response curves tested in *Digitaria insularis* populations

Rates	ACCase inhibitors[a]			EPSPS inhibitor[b]
	Clethodim	Fluazifop-P-butyl	Pinoxaden	Glyphosate
	g ai ha^{-1}			g ae ha^{-1}
0×	0.0	0.0	0.0	0.0
0.125×	12.7	26.3	7.5	108.4
0.25×	25.5	52.5	15.1	216.7
0.5×	51.0	105.1	30.2	433.5
1×	101.9	210.2	60.3	866.9
2×	203.9	420.3	120.6	1,733.8
4×	407.7	840.6	241.3	3,467.6
8×	815.4	1,681.3	482.5	6,935.3

[a]Acetyl-CoA carboxylase (ACCase) inhibitors: clethodim (Select Max®, Valent U.S.A.), fluazifop-P-butyl (Fusilade® DX, Syngenta), and pinoxaden (Axial® XL, Syngenta).
[b]5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor: glyphosate (Roundup PowerMax®, Bayer Crop Science). Nonionic surfactant (Induce®, Helena Agri-Enterprises, Collierville, TN, USA) was included at 0.25% (v/v) for all treatments. NIS was included for every single herbicide treatment, not only glyphosate.

chemical group than clethodim and fluazifop-P-butyl. In addition, pinoxaden is labeled for use in wheat (*Triticum aestivum* L.), and understanding the response of *D. insularis* could help in decision-making processes, such as whether to include wheat as a rotational crop when and where possible. *Digitaria insularis* plants with three to four fully expanded leaves (BBCH 13-14; Hess et al. 1997) were sprayed using a commercial track sprayer (DeVries Manufacturing, Hollandale, MN, USA) equipped with an 8002EVS nozzle (TeeJet®, Spraying Systems, Denver, CO, USA) calibrated to deliver 187 L ha^{-1}. Herbicide rates varied from zero (nontreated control) to eight times the labeled rate (Table 1). Visual injury was assessed at 28 d after treatment (DAT), where 0% represented absence of visual injury, and 100% represented complete control. Plant material was collected at 28 DAT and placed in an oven at 60 C for 5 d to assess dry weight. Each treatment had four replications in a completely randomized design, and the experiment was repeated.

EPSPS and ACCase sequencing

Genomic DNA (gDNA) was extracted from six plants from each population. Young leaf tissue (approximately 50 mg) was collected, immediately frozen in liquid nitrogen, and ground to a fine powder with a Mixer Mill MM 400 (Retsch, Newtown, PA, USA). DNA was extracted from samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The concentration and quality of DNA were determined spectrophotometrically (NanoDrop OneC, Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were frozen and kept in a freezer at −80 C until further analysis.

Amino acid substitutions in ACCase have been extensively reported and associated with complex patterns of cross-resistance (reviewed by Kukorelli et al. 2013). Depending on the species, amino acid substitutions can be predictive of herbicide resistance patterns, with exceptions (Brunharo and Tranel 2023). We developed primers to cover all known single-nucleotide polymorphisms (SNPs) associated with ACCase-inhibitor resistance. First, we amplified a 1,714-bp fragment of *ACCase* that encoded the CT domain with primers CP1F: 5′-CAACTCTG GTGCTIGGATIGGCA-3′ from Délye and Michel (2005); and 2990R: 5′-CCAGCTGTCTCAGAAGCCAA-3′. Given the large size of this fragment, we used two primer sets for sequencing (set 1: CP1F and CP1R: 5′-GAACATAICTGAGCCACCTIAATA TATT-3′; set 2: 2208F: 5′-ACAGCCTGATTCCCATGAGC-3′; and 2672R: 5′-TCATGCTTTGCTCCCTGGAG-3′). These primers amplified a region of the ACCase containing the known resistance-endowing positions Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, and Gly-2096. Polymerase chain reactions (PCRs) were composed of 10 µl of OneTaq® Hot Start DNA Polymerase Master Mix (New England Biolabs, Ipswich, MA, USA), 0.4 µl of each primer (10 µM final concentration), 1 µl of DNA template, and 8.2 µl of ultrapure water to complete a 10-µl final volume. PCR cycling settings were: 1 cycle at 94 C for 2 min, 30 cycles at 94 C for 15 s, 57 C for 15 s, 68 C for 1 min, and a final extension at 68 C for 5 min. PCR product was analyzed on 1% agarose gel and visualized under ultraviolet light. Amplicons were purified using the Monarch® DNA Gel Extraction kit (New England Biolabs) and sequenced at the Genomics Core Facility at Penn State University, University Park, PA, USA.

The *EPSPS* gene was amplified and sequenced with primers AWF: 5′- AACAGTGAGGAYGTYCACTACATGCT-3′; and AWR:5′- CGAACAGGTGGGCAMTCAGTGCCAAG-3′ (Adu-Yeboah et al. 2014), targeting the nucleotides encoding positions 102 and 106 of the enzyme that have been previously reported to confer glyphosate resistance (Alarcón-Reverte et al. 2015; Takano et al. 2019). PCR reagents and concentrations, cycling, and assessment were performed as previously described (Brunharo and Hanson 2018).

Data Analysis

Three-parameter log-logistic models (Equation 1) were fit to dose–response data combined from both experiments (Knezevic et al. 2007):

$$Y = \frac{d}{1 + exp\ \{b[\log(x) - \log(e)]\}} \quad [1]$$

where Y is the response variable as injury or dry weight at 28 DAT, d is the upper limit, b is the slope of the curve, and e is the amount of herbicide that reduced the response variable by 50% or 80%. Confidence intervals were generated with the *predict* function in R, and plotted with *ggplot2*. The resistance index (RI) was calculated for clethodim, fluazifop-P-butyl, pinoxaden, and glyphosate by dividing the GR_{50} or GR_{80} of the resistant to the susceptible. Whenever our data lack fit for the three-parameter model, the Box-Cox transformation was implemented (Box and Cox 1964).

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Table 2. Dose–response analysis of *Digitaria insularis* resistant to glyphosate, clethodim, fluazifop-P-butyl, and pinoxaden[a]

	b	*d*	GR_{50}	GR_{80}	RI_{50}	RI_{80}
Population				Glyphosate		
P1	−0.9 ± 0.1	109.1 ± 7.3	573.9 ± 114.9	2,650.8 ± 1,020.3	2.3 ± 0.4 (<0.001)	6.9 ± 2.3 (0.01)
P2	−1.7 ± 0.2	107.2 ± 3.3	746.6 ± 54.0	1,703.2 ± 208.6	3.0 ± 0.2 (<0.001)	4.5 ± 0.6 (<0.001)
P3	−3.7 ± 1.3	46.7 ± 2.4	1076.1 ± 87.4	1,566.9 ± 250.2	4.3 ± 0.4 (<0.001)	4.1 ± 0.7 (<0.001)
P4	−3.3 ± 0.2	100.5 ± 0.9	250.3 ± 5.1	381.6 ± 14.4	—	—
				Clethodim		
P1	−2.7 ± 0.3	100.6 ± 1.4	27.9 ± 1.0	46.1 ± 3.0	2.7 ± 0.3 (<0.001)	3.0 ± 0.3 (<0.001)
P2	−1.2 ± 0.1	106.6 ± 3.7	54.6 ± 5.2	175.5 ± 31.6	5.3 ± 0.7 (<0.001)	11.4 ± 2.2 (<0.001)
P3	−3.0 ± 0.8	100.0 ± 1.1	9.3 ± 0.9	14.8 ± 1.1	0.9 ± 0.1 (0.48)	1.0 ± 0.1 (0.72)
P4	−3.5 ± 1.5	99.9 ± 1.7	10.3 ± 1.1	15.4 ± 1.2	—	—
				Fluazifop-P-butyl		
P1	−1.1 ± 0.2	102.6 ± 3.9	38.9 ± 5.4	137.2 ± 36.6	87.7 ± 19.0 (<0.001)	148.8 ± 34.9 (<0.001)
P2	−1.0 ± 0.2	95.8 ± 7.1	147.9 ± 30.1	566.4 ± 223.6	234.2 ± 47.3 (<0.001)	453.7 ± 97.0 (<0.001)
P3	−0.5 ± 0.2	106.6 ± 8.4	3.2 ± 2.6	60.6 ± 63.6	6.1 ± 1.6 (<0.001)	7.7 ± 2.2 (<0.001)
P4	−1.0 ± 0.3	100.2 ± 0.3	0.9 ± 0.9	3.5 ± 2.2	—	—
				Pinoxaden		
P1	−4.1 ± 0.6	96.8 ± 2.0	40.7 ± 1.6	57.3 ± 3.9	4.3 ± 0.3 (<0.001)	4.1 ± 0.5 (<0.001)
P2	−3.3 ± 0.5	95.4 ± 2.1	55.9 ± 2.2	84.6 ± 6.7	5.9 ± 0.5 (<0.001)	6.1 ± 0.9 (<0.001)
P3	−3.8 ± 1.1	100.0 ± 2.1	7.8 ± 0.4	11.3 ± 1.3	0.8 ± 0.1 (<0.001)	0.8 ± 0.1 (0.1)
P4	−3.6 ± 0.8	99.0 ± 2.7	9.5 ± 0.7	13.9 ± 1.7	—	—

[a]Visual injury assessed at 28 d after treatment fit to log-logistic model. Log-logistic equation: $Y = d/1 + \exp\{[b(\log x - \log GR_{50/80})]\}$, where d is the upper limit, b is the slope of the curve, and $GR_{50/80}$ are the herbicide rates that reduce the response variable by 50% or 80%. $RI_{50/80}$ are the resistance indices for clethodim, fluazifop-P-butyl, pinoxaden, or glyphosate calculated by dividing the GR_{50} or GR_{80} of the resistant population by that of the susceptible population (P4). Mean estimates are followed by their standard errors. P-values for the RIs are provided in parentheses.

Table 3. Dose–response analysis of *Digitaria insularis* resistant to glyphosate, clethodim, fluazifop-P-butyl, and pinoxaden[a]

	b	*d*	GR_{50}	GR_{80}	RI_{50}	RI_{80}
Population				Glyphosate		
P1	0.8 ± 0.1	99.9 ± 2.3	154.4 ± 19	964.8 ± 224.9	0.6 ± 0.1 (0.01)	2.4 ± 0.6 (0.02)
P2	2 ± 0.3	108.7 ± 4.4	709.8 ± 75.1	1,411.5 ± 138.7	2.9 ± 0.3 (<0.001)	3.5 ± 0.5 (<0.001)
P3	0.6 ± 0.1	98.8 ± 5.1	2,313.4 ± 673.1	23,079.3 ± 10,783.8	9.3 ± 2.3 (<0.001)	57.5 ± 27.6 (0.04)
P4	2.9 ± 0.3	103.8 ± 2.7	248.6 ± 11.7	401.2 ± 42.8	—	—
				Clethodim		
P1	2.5 ± 0.5	99.6 ± 3.9	31 ± 2.1	53.3 ± 4.9	1,565.7 ± 603.1 (0.01)	28.1 ± 10.6 (0.01)
P2	1.9 ± 0.3	130.6 ± 3.6	56.4 ± 4.7	115.1 ± 12.3	2,863.1 ± 1,107.7 (0.01)	60.7 ± 23.2 (0.01)
P3	0.7 ± 0.2	100.5 ± 4	3.9 ± 2	29.6 ± 5.6	198.3 ± 124.7 (0.12)	15.6 ± 6.4 (0.02)
P4	0.3 ± 0	138.8 ± 5.6	0.2 ± 0	1.9 ± 0.7	—	—
				Fluazifop-P-butyl		
P1	1.2 ± 0.5	125.9 ± 7.6	38.4 ± 9.3	165.3 ± 30.7	330.6 ± 105.8 (<0.001)	15.6 ± 6.6 (0.03)
P2	0.7 ± 0.2	100.9 ± 7.8	230.1 ± 90.7	1,346.8 ± 420.3	1,978.6 ± 883.5 (0.03)	126.7 ± 62.3 (0.04)
P3	0.5 ± 0.2	8.9 ± 8.9	6.1 ± 5.5	100.7 ± 46.3	52.1 ± 48.6 (0.29)	9.5 ± 5.7 (0.14)
P4	0.6 ± 0.4	101.3 ± 9	0.1 ± 0.0	10.6 ± 4.0	—	—
				Pinoxaden		
P1	3.5 ± 1.2	89.1 ± 3.6	39.9 ± 3.8	59.2 ± 9.4	3.6 ± 0.5 (<0.001)	2.5 ± 0.8 (0.05)
P2	2.9 ± 0.7	121.8 ± 4.3	64.6 ± 6.4	103.8 ± 15.3	5.9 ± 0.8 (0.001)	4.4 ± 1.4 (0.01)
P3	2.5 ± 0.6	100.2 ± 4.8	8.1 ± 0.7	14.0 ± 2.2	0.7 ± 0.1 (0.02)	0.6 ± 0.2 (0.03)
P4	1.8 ± 0.7	102.2 ± 6.2	10.9 ± 1.3	23.4 ± 6.3	—	—

[a]Dry matter collected at 28 d after treatment fit to log-logistic model. Log-logistic equation: $Y = d/1 + \exp\{[b(\log x - \log GR_{50/80})]\}$, where d is the upper limit, b is the slope of the curve, and $GR_{50/80}$ are the herbicide rates that reduce the response variable by 50% or 80%. $RI_{50/80}$ are the resistance indices for clethodim, fluazifop-P-butyl, pinoxaden, or glyphosate calculated by dividing the GR_{50} or GR_{80} of the resistant population by that of the susceptible population (P4). Mean estimates are followed by their standard errors. P-values for the RIs are provided in parentheses.

Because of the lack of reference sequences for *D. insularis*, we obtained the *EPSPS* (OM311259.1) sequence from a closely related species, large crabgrass [*Digitaria sanguinalis* (L.) Scop.], for alignment and amino acid annotation and the *ACCase* sequence from Italian ryegrass [*Lolium multiflorum* Lam.; syn.: *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (AY710293.1). The resulting *ACCase* and *EPSPS* sequences were visually inspected for mutations in Geneious Prime (v. 2023.1.2, Biomatters, Auckland, New Zealand).

Results and Discussion

Whole-Plant Dose–Response Assays

Our dose–response analyses revealed that populations P1, P2, and P3 are resistant to glyphosate compared with the known susceptible population P4 (Tables 2 and 3). Population P3 exhibited the highest GR_{50} based on visual injury and dry weight, with RI varying between 4.3 and 9.3, while P2 had an intermediate resistance level (RI = 2.9 to 3.0). Population P1 exhibited

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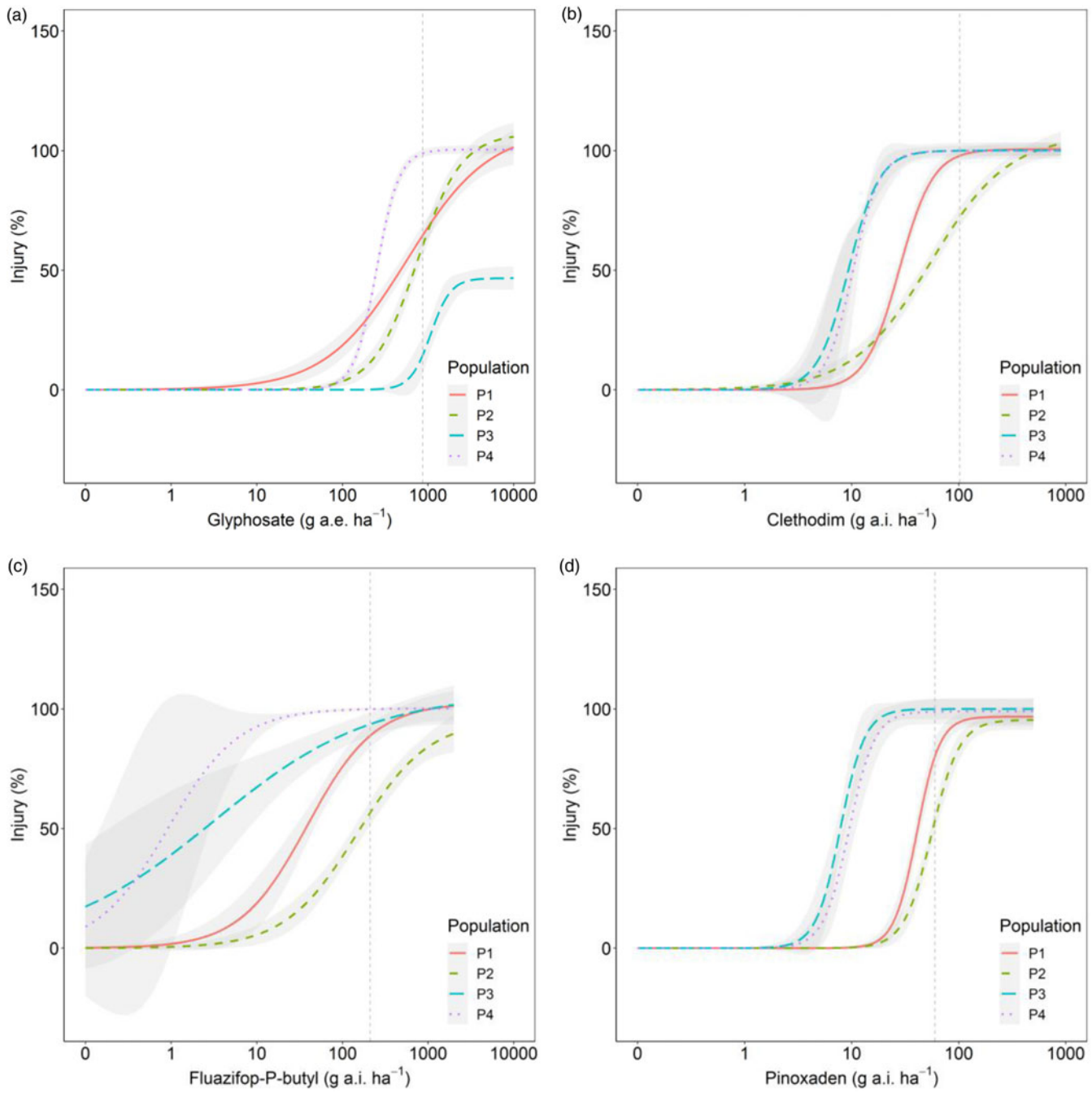

Figure 1. Dose–response curves for glyphosate (A), clethodim (B), fluazifop-P-butyl (C), and pinoxaden (D) based on injury data at 28 d after treatment in *Digitaria insularis* populations. Dotted vertical line represents the labeled rate, and the shaded regions represent the confidence intervals at 95%.

glyphosate resistance based on visual injury, but differences in susceptibility were less clear when the dry weight was analyzed (Figures 1A and 2A), even though we observed greater biomass remaining with the field rates of glyphosate (Figure 1A). However, based on GR_{80}, P1 had RI varying from 2.4 to 6.9 (Figure 2A). These results suggest that glyphosate applications at the recommended field rate (867 g ae ha^{-1}) effectively controlled the susceptible weed population (Figure 1A), but would fail to control P1 to P3. Higher glyphosate rates could provide greater control of resistant populations, particularly P2, that had GR_{80} values of approximately 1,400 and 1,700 g ae ha^{-1} for dry matter and visual injury, respectively. Labeled rates for glyphosate can typically be as high as 1,920 g ae ha^{-1} and, although not commonly utilized by farmers, could still provide *D. insularis* management at acceptable levels (i.e., greater than 80%), with exception for P3, which exhibited high resistance levels.

In other studies, the RI for glyphosate typically exhibits large variation depending on the plant growth stage (Cavalieri et al. 2021). *Digitaria insularis* at the 2- to 4-leaf stage had a lower RI (RI = 8.8) compared with those at the 2- to 4-tiller stage (RI = 13). This suggests that younger plants are more susceptible to glyphosate. The GR_{50} can also vary considerably. Cavalieri et al.

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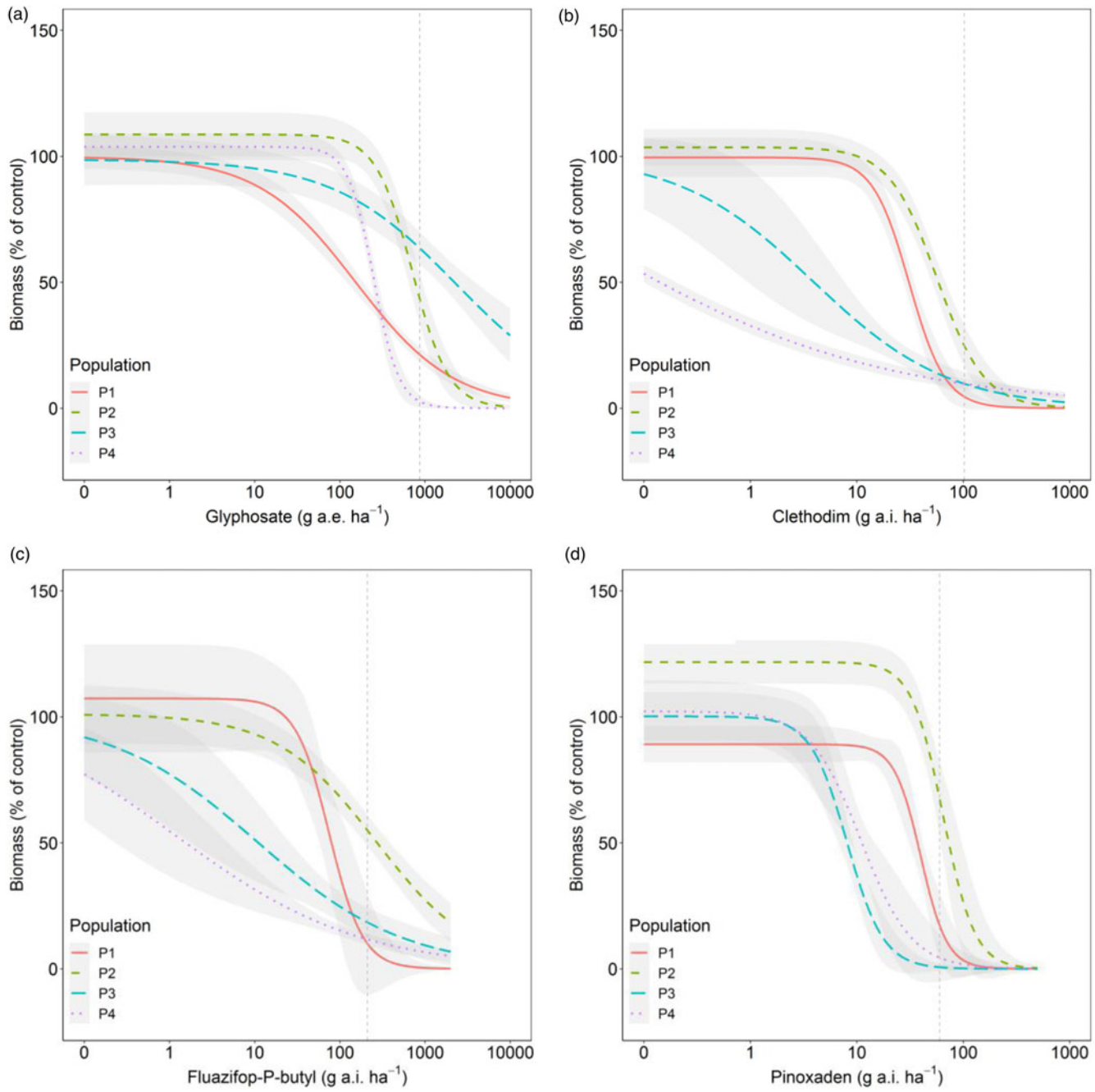

Figure 2. Dose–response curves for glyphosate (A), clethodim (B), fluazifop-P-butyl (C), and pinoxaden (D) based on dry matter data 28 d after treatment in *Digitaria insularis* populations. Dotted vertical line represents the labeled rate, and shaded regions represents the confidence intervals at 95%.

(2021) observed that the GR_{50} of the resistant population ranged from 1,057 to 1,282 g ae ha^{-1} for visual injury and 600 to 1,104 g ae ha^{-1} for dry weight at the 2- to 4-leaf stage. Similarly, Gazola et al. (2019) reported GR_{50} for control and dry weight reduction ranged from 1,115 to 1,405 g ae ha^{-1} and 1,028 to 1,086 g ae ha^{-1}, respectively, while the RI values were between 7.8 and 9.9 for *D. insularis* when treated at the 40- to 60-cm growth heights. The range of GR_{50} values across the previously mentioned studies underscores the significant variation in glyphosate resistance in *D. insularis* populations.

Populations P1 and P2 were cross-resistant to all ACCase inhibitors tested, while P3 and P4 were susceptible (Table 2 and 3; Figure 2B–D). The GR_{50} based on visual injury for population P1 was 28, 39, and 41 g ha^{-1} for clethodim, fluazifop-P-butyl, and pinoxaden, respectively, with RI of 2.7, 87, and 4.3 (Table 2). Comparable values were observed for the dry weight data (Table 3). We observed that the GR_{80} for clethodim in population P1 was lower than the recommended field rate. Population P2 had GR_{50} of 55, 148, and 56 g ai ha^{-1} based on visual injury, and 56, 230 and 65 g ai ha^{-1} based on dry weight for clethodim, fluazifop-P-butyl, and pinoxaden, respectively (Tables 2 and 3). The RIs were as low as 5.3 to >2,800 for clethodim. Overall, population P2 exhibited larger GR_{50}, GR_{80}, and RI values than P1. At the field rate, both P3 and P4 were fully controlled (Figures 1 and 2). The RI_{50} for

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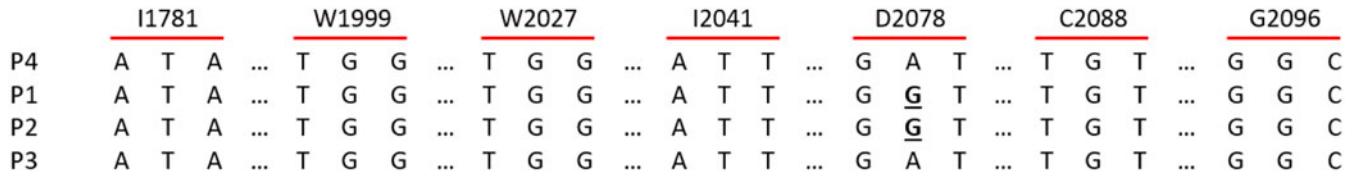

Figure 3. Sequence of the acetyl-CoA carboxylase (*ACCase*) gene from two populations resistant (P1 and P2) and two populations susceptible (P3 and P4) to ACCase-inhibitor herbicide. An amino acid substitution at position 2078 replaced an aspartic acid to a glycine.

Figure 4. Partial sequence of the 5-enolpyrovyl-3-shikimatephosphate synthase (*EPSPS*) gene containing resistance-conferring amino acid substitutions at positions 102 and 106. We observed substitutions at position 102 (a threonine to isoleucine or methionine in population P3) and at position 106 (a proline to alanine or arginine in P1 and P2, and a proline to serine in P3).

clethodim ranged from 2.7 to 5.3 based on visual injury and >1,000 based on dry weight. Fluazifop-P-butyl resistance resulted in large RI_{50} values, ranging from 87.7 to 1,978 depending on assessment type. Finally, resistance to pinoxaden ranged from 3.6 to 5.9. These results collectively indicated that there is wide variation in response to ACCase inhibitors across *D. insularis* populations. Efficacy of ACCase inhibitors has been shown to be reduced at more advanced growth stages (Presoto et al. 2020). Given we treated plants at the BBCH 13-14 (Hess et al. 1997), it is expected that the GR_{80} values would be greater for older plants under field conditions.

Our results indicated that populations P1 and P2 are multiple resistant to glyphosate and ACCase inhibitors based on the dose–response studies. Conversely, P3 is resistant to glyphosate, but remains susceptible to all classes of ACCase inhibitors. Takano et al. (2020) reported on a *D. insularis* population resistant to ACCase inhibitors collected from a soybean–corn–cotton rotation. They observed cross-resistance between haloxyfop and pinoxaden, but not to clethodim. This is not an uncommon cross-resistance pattern, and it has been observed in other species such as *A. myosuroides* (Petit et al. 2010) and *L. multiflorum* (Brunharo and Tranel 2023). This pattern is different from the one we observed in our project, in that cross-resistance across all chemical classes of ACCase inhibitors was detected.

Multiple-herbicide resistance poses a serious challenge for weed management. The cropping systems where our populations were collected rely heavily on herbicides for weed control, with limited alternative chemistries or management practices that effectively control *D. insularis*. Given this weed reproduces via both seed and rhizomes, systemic herbicides are key for effective management. Other complementary management practices, such as the use of preemergence herbicides (Drehmer et al. 2015; Matte et al. 2022) are regaining popularity (Merotto et al. 2022). Nonchemical approaches, such as cover crops and no-till systems, are increasingly popular in South America, now reaching around 50% adoption. However, there are challenges that limit their use to a small percentage of the total agricultural land (Derpsch et al. 2010).

ACCase and EPSPS Gene Sequencing

Sequencing of the CT domain of *ACCase* revealed an SNP that resulted in the substitution of an aspartic acid to a glycine at position 2078 (Figure 3). This mutation was only found in populations P1 and P2, while susceptible populations P3 and P4 exhibited the wild-type aspartic acid. No other amino acid substitutions were observed at the other resistance-endowing positions 1781, 1999, 2027, 2041, 2088, and 2096. We also observed that this mutation was homozygous whenever it occurred, suggesting the advantageous allele is fixed in the resistant populations tested. This mutation has been extensively studied in other weed species; however, this is the first time it has been documented in *D. insularis*. Mutation at position 2078 typically confers resistance to all ACCase-inhibitor chemical groups, while mutations in other positions may exhibit distinct cross-resistance patterns (reviewed by Kaundun 2014). For example, Brunharo and Tranel (2023) observed that all *L. multiflorum* individuals with an amino acid substitution at position 2078 were resistant to clethodim, pinoxaden, and quizalofop. Similar cross-resistance patterns have been observed in other species such as barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] (Fang et al. 2020) and perennial ryegrass (*Lolium perenne* L.) (Yanniccari and Gigón 2020).

It is important to note that other mutations conferring ACCase-inhibitor resistance have been identified in *D. insularis* populations from Brazil, in a region >1,000 km from the location where our populations were identified (Takano et al. 2021). Takano et al. (2020) identified a mutation at position 2027 of *ACCase*. These results strongly suggest, therefore, that the population in our study is the result of an independent evolutionary event due to the local selection pressure from ACCase inhibitors.

EPSPS sequencing identified a diverse array of resistance-endowing polymorphisms. We observed that populations P1 and P2 have synonymous SNPs at coding position 102. We identified nonsynonymous SNPs at position 106 in P1 and P2, where the wild-type proline was substituted by an alanine or arginine, suggesting this is the mechanism of glyphosate resistance in P1 and P2. Interestingly, population P3 had nonsynonymous mutations at both positions 102 and 106. At position 102, we observed the substitution of a threonine by an isoleucine or a methionine, and at position 106, of a proline by a serine (Figure 4). As expected, the susceptible population P4 had the wild-type amino acids at position 102 (threonine) and 106 (proline).

It is unclear whether some of these mutations occur on the same allele, however. Because of the nature of Sanger sequencing, we are unable to parse the alleles, which would require, for instance,

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performing deep amplicon sequencing or cloning. Nonetheless, the sequencing results clearly explain the phenotypic data, in that population P3 exhibited the greatest resistance level of the studied populations, followed by P2 and P1. Double mutations in *EPSPS* have been observed to confer high resistance levels in goosegrass [*Eleusine indica* (L.) Gaertn.] (Han et al. 2017).

There are two possible combinations of evolutionary events. First, each allele has a single mutation at each amino position, which were combined via hybridization. Although single mutations at position 106 are commonly observed, single mutations at position 102 only are uncommon (however, see Li et al. 2018). Alternatively, there is a double mutation at both positions 102 and 106, resulting in a threonine-to-isoleucine or threonine-to-methionine at position 102, as well as a proline-to-serine at position 106. It is possible that we are observing an example of the TIPS mutation (threonine-to-isoleucine at position 102, and proline-to-serine at position 106). This mutation has been characterized in *E. indica* (Han et al. 2017). It is also possible that we observed a novel instance wherein a threonine-to-methionine and proline to serine occurred.

Substitutions at position 106 have been extensively reported in many weed species, including *E. indica* (Ng et al. 2003), *L. multiflorum* (Brunharo and Hanson 2018), annual bluegrass (*Poa annua* L.) (Brunharo et al. 2019), and many others (reviewed by Heap 2024). This mutation alone provides intermediate resistance levels in other species, typically less than 10-fold (Morran et al. 2018; Perez-Jones et al. 2007; Takano et al. 2019). Conversely, populations with double amino acid substitutions, such as the TIPS, result in greater resistance levels (Han et al. 2017; Yu et al. 2015) that typically result in ecological fitness costs (Vila-Aiub et al. 2021).

After over a decade of the first identification of glyphosate resistance in *D. insularis*, resistant populations became widespread throughout Brazil. Studying the population genetics of *D. insularis*, Gonçalves Netto et al. (2021) observed that local gene flow of glyphosate resistance, as well as multiple evolutionary events, shaped the genetic background of various populations. They found evidence of multiple independent events of glyphosate-resistance evolution, supporting the hypothesis that resistance evolution may be dictated by local management practices, rather than the result of a single founder event that spread throughout the country. The authors also observed that some populations had shared genetic background, indicating local movement of alleles plays a role in glyphosate-resistance spread in *D. insularis*.

The fact that we observed homozygosity at the *ACCase* CT domain, but heterozygosity at the *EPSPS* locus, suggests that selection pressure from ACCase inhibitors could be stronger than from glyphosate. Additionally, it is possible that the mutations in *ACCase* evolved before those in the *EPSPS*, because it may take many generations to reach fixation. A more detailed characterization of the genomic landscape surrounding both *ACCase* and *EPSPS* could elucidate the mechanisms of evolution (i.e., new mutations, immigration, standing genetic variation; Lee and Coop 2017).

In summary, our results suggested that multiple-herbicide resistance to glyphosate and ACCase inhibitors evolved in *D. insularis* populations from South America. This is the first time that amino acid substitutions at both 102 and 106 positions in the EPSPS have been found in *D. insularis*, despite glyphosate resistance having been reported in many geographic regions (Gonçalves Netto et al. 2021; Lopez Ovejero et al. 2017). These findings also contribute to the body of literature on multiple mutations within the *EPSPS*, which are more uncommon than single amino acid substitutions. Furthermore, cross-resistance to all ACCase inhibitors is attributed to an amino acid substitution at position 2078 of ACCase. These results suggest an independent evolutionary event compared with previous reports of ACCase-inhibitor resistance in this species (Takano et al. 2020). Although glyphosate resistance has been known in *D. insularis* for over a decade (de Carvalho et al. 2012), this is the first time that a conclusive result on the resistance mechanisms to this herbicide has been observed. Other populations have been shown to have multiple small alterations that resulted in the reduced glyphosate efficacy (de Carvalho et al. 2012), or the resistance mechanisms have remained unknown (Melo et al. 2019; reviewed by Amaral et al. 2023). Given glyphosate resistance in *D. insularis* is widespread in large areas where agronomic crops are grown, farmers relied on ACCase inhibitors as an alternative chemistry for effective control of this weed. It remains unknown whether multiple amino acid substitutions in the EPSPS causes any fitness costs in *D. insularis*, as has been observed in other species (Vila-Aiub et al. 2021). Finally, our results, when contextualized with various previous studies that attempted to elucidate glyphosate resistance in *D. insularis*, underscore the opportunity to utilize *D. insularis* as a model to understand herbicide resistance evolution, not only the underlying physiology, but also evolutionary events that lead to fixations of the herbicide resistance alleles. Our results also highlight that there are a multitude of genetic and/or physiological alterations that could lead to convergent and parallel evolution of the GR phenotype. Most likely, novel glyphosate-resistance mechanisms could be identified in *D. insularis*. Our results underscore the importance of integrated weed management approaches to manage *D. insularis* at the landscape level, with focus on minimizing gene flow and selection pressure from herbicides.

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Competing interests. The authors declare no conflicts of interest.

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