Multiple Herbicide Resistance Evolution: The Case of *Eleusine indica* in Brazil

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ABSTRACT: The occurrence of multiple herbicide resistant weeds has increased considerably in glyphosate-resistant soybean fields in Brazil; however, the mechanisms governing this resistance have not been studied. In its study, the target-site and nontarget-site mechanisms were characterized in an *Eleusine indica* population (R-15) with multiple resistance to the acetyl-CoA carboxylase (ACCase) inhibitors, glyphosate, imazamox, and paraquat. Absorption and translocation rates of ^{14}C-diclofop-methyl ^{14}C-imazamox and ^{14}C-glyphosate of the R-15 population were similar to those of a susceptible (S-15) population; however, the R-15 population translocated ~38% less ^{14}C-paraquat to the rest of plant and roots than the S-15 population. Furthermore, the R-15 plants metabolized (by P450 cytochrome) 55% and 88% more diclofop-methyl (conjugate) and imazamox (imazamox−OH and conjugate), respectively, than the S-15 plants. In addition, the Pro-106-Ser mutation was found in the *EPSPS* gene of this population. This report describes the first characterization of the resistance mechanisms in a multiple herbicide resistant weed from Brazil.

KEYWORDS: cytochrome P450, goosegrass, ^{14}C-herbicide, nontarget-site, target-site

INTRODUCTION

Eleusine indica (L.) Gaertn. is a diploid grass from Asia that is adapted to a wide range of temperatures, and at present it is a common weed in tropical, subtropical, and temperate regions of the world.[1] This species can evolve resistance to a wide range of herbicides. According to *The International Herbicide-Resistant Weed Database*, *E. indica* has evolved resistance to eight sites of action mainly across annual and perennial crop fields in America and Asia.[2,3]

In the last several decades of herbicide use, weeds have developed a vast array of generalist nontarget-site (NTS) and specialist target-site (TS) herbicide resistance mechanisms.[4] TS mechanisms involve key mutations in genes encoding the target site enzymes (limiting the herbicide interaction), and target protein overproduction due to increased gene expression or duplication. NTS mechanisms (reduced absorption, impaired translocation, vacuolar sequestration, enhanced metabolism, and hypersensitivity) are regulated by a large number of genes not related to the target site.[5] NTS-based resistance has become increasingly relevant in recent years, as resistance cases involving these mechanisms are becoming more frequent.[4] Restricted translocation due to vacuolar sequestration is recognized as the NTS mechanism of resistance to paraquat and glyphosate.[6,7] On the other hand, enhanced herbicide metabolism, regulated by cytochrome P450 (Cyp-P450) monooxygenases, glutathione S-transferases, or glycosyl transferases, is by far the main NTS mechanism of resistance to herbicides other than glyphosate and paraquat.[4] Depending on the metabolic enzymes involved, the plant may have broad herbicide resistance, even to action modes never used.

Brazil is the world's largest soybean producer and exporter due to its ability to expand cultivable areas.[8] In the 2019/2020 season, Brazil produced 124 million tons of soybean in 36.8 million ha (~45% of the total planted area).[9] This record production has been made possible by the introduction and rapid adoption of herbicide resistant crops, mainly those resistant to glyphosate (GR), which were officially introduced in 2005.[10] From 2008 to 2018, the area cultivated with GR-soybeans went from 14.1 (65% of the soybean planted soybean) to 33 (95% of the soybean planted area) million hectares.[8] Resistance to acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors in soybean cultivation was already widespread by the mid-2000s in Brazil.[11] GR crops together with glyphosate [5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitor] were a successful means of solving this problem. However, 3−5 years after intensive use of glyphosate in multiple agricultural tasks (chemical fallow, weed management, and desiccation) in the same growing season, this herbicide has no longer been effective in controlling some weed populations that have evolved resistance.[8] This outcome forced farmers to return to ALS and ACCase inhibitors in addition to including herbicides such as 2,4-D, glufosinate, diuron, and paraquat (photosystem I inhibitor, PSI) to improve weed control.[12,13]
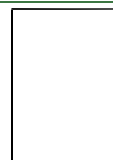
Table 1. Herbicide Treatments Used for Dose Response Curves in *E. indica* Populations

mechanisms of action	herbicides	company	doses used (g ai ha^{-1})	field dose (g ai ha^{-1})
ACCase	haloxyfop-*p*-methyl	52% w/v EC, Gallant, Dow AgroSciences	0, 10, 25, 50, 100, 200, 400, 600, 800, and 1000	50
	diclofop-methyl	36% w/v EC, Firelo, DuPont	0, 25, 50, 100, 200, 250, 500, 1000, 2000, and 3000	1080
	sethoxydim	18.4% w/v EC, Poast, BASF	0, 5, 10, 20, 50, 100, 200, 500, 800, and 1200	368
	tralkoxydim	40% w/v EC, Splendor 40 SC, Nufarm	0, 200, 300, 400, 500, 600, and 1000	400
	pinoxaden	6% w/v EC, Axial Pro, Syngenta	0, 4, 8, 16, 25, 32, 50, 64, 100, 200, and 400	40
ALS	imazamox	4% w/v SL, Pulsar 40, BASF	0, 1, 2, 4, 8, 16, 20, 40, 80, 120 and 240	40
EPSPS	glyphosate[a]	36% w/v SL, Roundup, Monsanto	0, 32.25, 62.5, 125, 250, 500, 1000, 2000 and 4000	720
PS I	paraquat	25% SL, Gramoxone, Syngenta	0, 25, 50, 100, 200, 400, 800, and 3200	500

[a]g ae ha^{-1}.

Soybean cultivation is responsible for 52% of the national consumption of pesticides, 60% of which are herbicides.[8] The almost exclusive use of herbicides for weed management has led to the selection of resistant populations, and of the 17 cases of multiple- or cross-herbicide resistance known in Brazil 10 were reported in soybeans.[2] Currently, resistant populations of *Conyza* spp., *Digitaria insularis*, and *Lolium multiflorum* are a great management challenge in Brazil, since they infest more than 20 million ha of soybeans,[11] but it is believed that *E. indica* will become an even more challenging weed in the coming years. This species was found to be resistant to aryloxyphenoxypropionates (FOP) and cyclohexanediones (DIM), both of which are ACCase inhibitors, in 2003.[14] In 2013, *E. indica* evolved resistance to glyphosate[15] and in 2017, the species showed multiple resistance to ACCase and EPSPS inhibitors.[2] The three cases were found in soybean plantations of the Mato Grosso and Paraná states.

Despite the increasing number of cases of multiple- and cross-resistance to herbicides in soybean plantations of Brazil, there are no studies to characterize resistance mechanisms in these weeds. This work attempts to elucidate the TS and NTS mechanisms in a population of *E. indica* collected in soybeans in 2015, which was confirmed to be resistant to FOP and DIM due to the Asp-2078-Gly mutation in the gene encoding the ACCase in 2006.[16] After 10 years of diversified management with ALS, EPSPS and PSI inhibiting herbicides, multiple resistance has evolved.

■ MATERIALS AND METHODS

Plant Material and Growing Conditions. Seeds of a resistant *E. indica* population were collected in 2015 in a soybean field located in the Lucas do Rio Verde region, Mato Grosso (Brazil), where the first population resistant to ACCase inhibitors was found in 2006 (referred to as R-06).[14] Postemergence FOP and/or DIM herbicides have been applied at least once a year in this field for 20 years prior to 2006. Ten years after GR-soybean varieties adoption, pinoxaden (phenylpyrazolines, DEN) and other herbicides (ALS, EPSPS, and PSI inhibitors) were also used; however, in recent years the control of *E. indica* has not been satisfactory with some of these herbicides. Similar to the resistant population, seeds from a susceptible (S-15) population were collected from the same site (São Paulo) where S seeds (S-06) were collected in 2006. Seeds were stored in paper bags at 4 °C, and in all cases germination tests were performed every year and these seeds were renewed if necessary.

Seeds of the resistant population of *E. indica* collected in 2015 were sown in trays (40 × 60 × 15 cm) containing sand and peat (2:1 v/v) and were placed in a greenhouse at 28/20 °C day/night with a 16 h photoperiod, a 200 mmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. *Eleusine indica* plants with 3–4 true leaves were treated with glyphosate (1080 g ae ha^{-1}) and pinoxaden (40 g ai ha^{-1}) using a laboratory spray chamber equipped with a flat fan nozzle

(TeeJet 8002 EVS) delivering 250 L ha^{-1} at 200 kPa. This laboratory spray chamber was employed in all subsequent experiments. Plants that survived (>60%) the glyphosate and pinoxaden treatments were allowed to produce seeds (F$_1$ progeny), and these new seeds, referred to as R-15, were used for all subsequent trials.

Herbicide Dose Response. Seeds of the S-06, R-06, S-15, and R-15 populations of *E. indica* were germinated in trays as described above. Seedlings were transplanted individually into 8 × 8 × 10 cm pots. *Eleusine indica* plants at the 3–4 leaf stage of the S-06, R-06, S-15, and R-15 were sprayed with different herbicides and doses (Table 1). Because amitrole and malathion are two potent inhibitors of the Cyp-P450, an enzyme complex capable of metabolizing herbicides in nontoxic forms,[5] a set of dose−response curves with plants were pretreated with these compounds for the ACCase and ALS inhibiting herbicides. Amitrole at 13.1 g ha^{-1} (for the ACCase inhibitors curves) and malathion at 2000 g ia ha^{-1} (for the ALS inhibitors curves) were applied 24 and 2 h before herbicide application, respectively. Plants were harvested at ground level at 28 days after treatment (DAT), except for paraquat, and immediately weighed to determine the dose that caused a 50% reduction in fresh weight (GR$_{50}$) and mortality (LD$_{50}$) compared with the untreated control. In the paraquat dose−response curves, the plants were cut and weighed at 7 DAT. The study was arranged in a completely random design with 10 replicates per dose, and all dose−response curves were repeated twice.

^{14}C-Herbicide Absorption and Translocation. The ^{14}C-herbicides (diclofop-methyl, imazamox, paraqua,t and glyphosate) (Table S1)[17−20] were mixed with their respective commercial formulation in order to have the necessary adjuvants for absorption. The concentration of the herbicide solutions corresponded to the half field dose of the herbicides in 250 L ha^{-1} (Table 1), and they had a specific activity of 0.834 kBq μL^{-1}. The radio-labeled herbicides were applied (1 μL drop) on the adaxial surface of the second leaf on 3−4 leaf stage (BBCH 13−14) S-15 and R-15 *E. indica* plants using a microapplicator. In the light, paraquat exhibits restricted movement;[7] therefore, plants treated with this ^{14}C-herbicide were immediately placed in the dark for 12 h and then under 12 h light.

Unabsorbed ^{14}C-herbcides were washed three times with 1 mL of organic solvent solutions (Table S1), depending on the herbicide applied, at 24 (paraquat) or 96 (the other herbicides) h after treatment (HAT). The rinsed solution of each wash was mixed with 2 mL of scintillation fluid (Ready Safe TM, Beckman Coulter). Treated plants were carefully removed from the pot. Subsequently, plants were separated into treated leaf (TL), the remainder of the plant (RP), and roots (R) and dried at 60 °C for 96 h. Samples were combusted in an automatic preparation and oxidation system (Packard Tri Carb 307, PerkinElmer Inc., MA, U.S.A.), and the ^{14}CO$_2$ released was trapped in 18 mL of a mixture of a radioactive dioxide absorber and liquid scintillation cocktail (1:1, v/v) (Carbo-Sorb E and Permafluor, respectively, PerkinElmer, Packard Bioscience BV). The radioactivity of the washes and combustions was quantified by liquid scintillation spectrometry in a LS-650 counter (Beckman Coulter Inc., CA, U.S.A.). Experiments were repeated twice (three plants per replicate) for each herbicide in a completely random design.
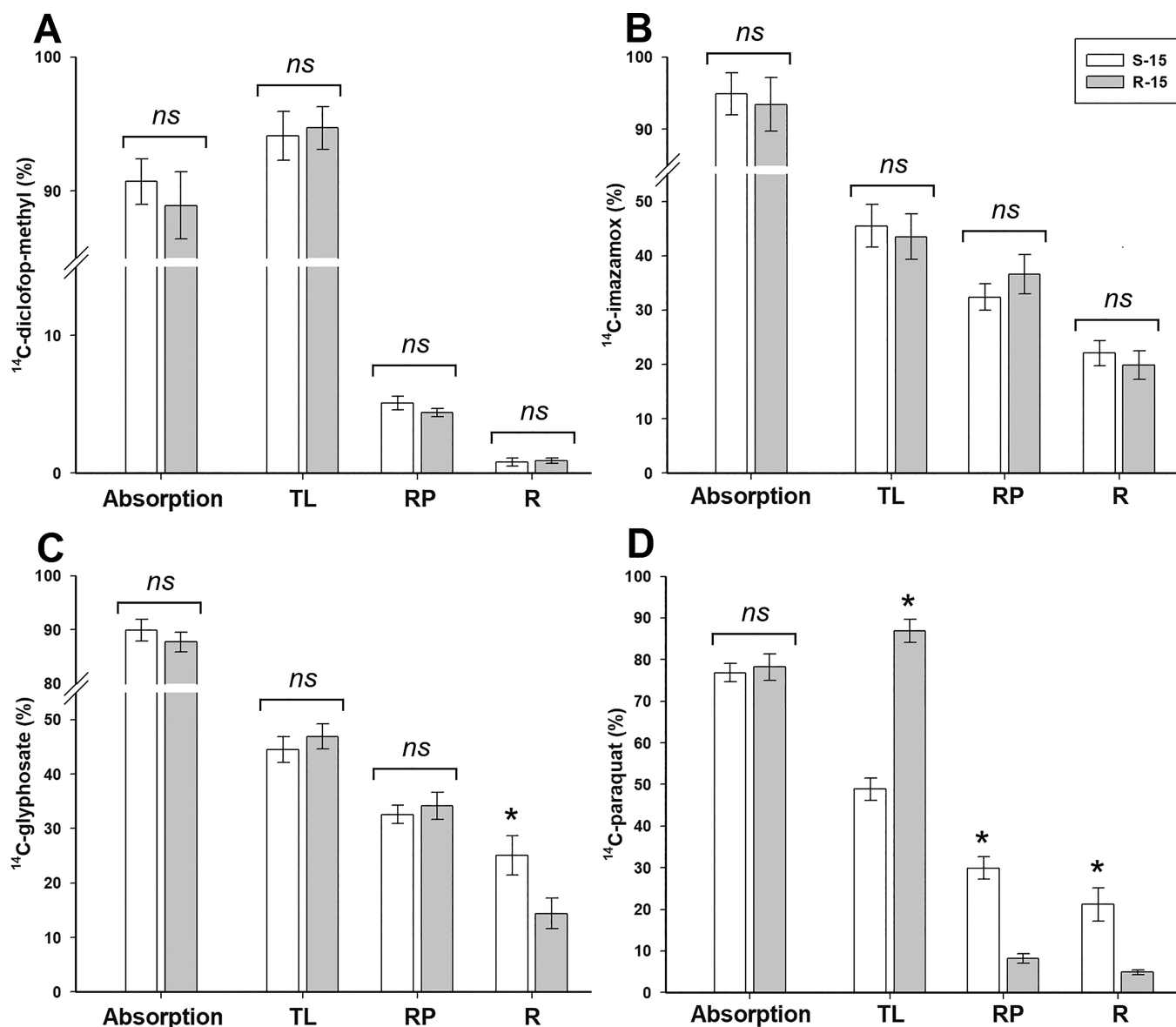
Table 2. Herbicide Dose (g ai ha^{-1}) Required to Reduce the Fresh Weight (GR$_{50}$) or Plant Mortality (LD$_{50}$) by 50% in the R-06, S-16, R-15, and S-15 Populations of *E. indica* and Resistance Factors (RF)[a]

herbicide[b]	population	GR$_{50}$	RF	LD$_{50}$	RF	population PSI inhibitor	GR$_{50}$	RF[e]	LD$_{50}$	RF[e]	GR$_{50}$	RF[e]	LD$_{50}$	RF[e]
ACCase inhibitors														
							− amitrol				+ amitrole			
haloxyfop-*p*-methyl	R-06	184.0	12.5	286.9	13.6	R-15	252.1	16.4	385.6	18.6	146.4	13.9	283.5	14.2
	S-06	14.7		21.1		S-15	15.4		20.7		10.5		20.0	
diclofop-methyl	R-06	282.2	4.9	1569.2	16.4	R-15	497.5	8.6	1856.4	18.6	262.7	5.6	1174.3	11.8
	S-06	57.9		95.7		S-15	57.6		99.8		47.1		99.9	
sethoxydim	R-06	316.6	13.5	660.8	7.8	R-15	561.1	23.5	794.0	9.6	275.8	15.6	663.1	8.5
	S-06	23.5		84.7		S-15	23.9		82.7		17.7		78.2	
tralkoxydim	R-06	636.9	4.7	718.0	3.9	R-15	872.3	6.4	1376.1	7.3	438.9	4.3	903.8	8.1
	S-06	135.5		184.1		S-15	136.3		188.5		133.0		173.8	
pinoxaden	R-06	15.4	1.1	16.6	1.1	R-15	41.6	2.9	73.9	4.9	14.5	1.2	15.9	1.2
	S-06	13.7		14.6		S-15	14.6		15.1		11.9		13.5	
ALS inhibitor														
							− malathion				+ malathion			
imazamox[c]	R-06	2.4	1.1	5.0	0.9	R-15	105.8	37.4	231.4	35.01	2.5	1.2	7.1	1.2
	S-06	2.13		5.7		S-15	2.8		6.6		2.1		5.7	
EPSPS inhibitor														
glyphosate[d]	R-06	138.0	0.9	284.5	1.0	R-15	812.4	5.1	1543.2	5.3	nd		nd	
	S-06	150.2		276.9		S-15	158.4		287.4		nd		nd	
paraquat	R-06	88.4	1.1	223.5	0.9	R-15	668.4	7.3	1834.3	8.5	nd		nd	
	S-06	78.4		234.8		S-15	91.4		215.8		nd		nd	

[a]The R-15 and S-15 populations were treated (+) or not treated (−) with the cytochrome P450 inhibitors amitrole or malathion. [b]ALS, acetolactate synthase; ACCase, acetyl-CoA carboxylase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase, and PSI, photosystem I. [c]Malathion. [d]g ae ha^{-1}; [e]RF = GR$_{50}$ R or LD$_{50}$ R/GR$_{50}$ S or LD$_{50}$ S. nd, nondetermined.

The recovery, absorption, and translocation rates of the ^{14}C-herbicides were calculated for each plant with the following equations: % recovery = [(Bq from washes + Bq in TL + Bq in RP + Bq in R)/Bq total applied)] × 100; % absorption = [(Bq in TL + Bq in RP + Bq in R)/(Bq total applied)] × 100; and % translocation = [(Bq in RP or Bq in R)/(Bq in TL + Bq in RP + Bq in R)] × 100. In these experiments, the radioactivity recovery averaged from 88% (±3.1) to 96% (±2.6) for all tested herbicides. Absorption was expressed as a percentage of recovered radioactivity and translocation relative to absorbed radioactivity.

Herbicide Metabolism Studies. Plants (5- to 6-leaf stage) of the S-15 and R-15 populations were treated with diclofop-methyl, glyphosate, and imazamox at the field dose using the same media as the dose−response assays. In the case of diclofop-methyl metabolism, one leaf was selected to receive a ^{14}C-diclofop-methyl treatment; therefore, this leaf was protected with a paper envelope prior to herbicide application. After the treatment, the ^{14}C-diclofop-methyl solution (1 Kb μL^{-1}) was applied (10 drops of 0.5 μL, that is, 5 kBq plant^{-1}) to the protected leaf as described in the previous section (^{14}C-Herbicide Absorption and Translocation). Fresh tissue samples were taken for herbicide metabolism at 96 HAT, which was performed following the appropriate methodology for each herbicide evaluated (Table S2).[17,21,22] The experiment was repeated twice with three replicates in each repetition.

Target Enzyme Activity Studies. The interaction of the different herbicides with their target enzymes was assayed *in vitro* using younger plants of both S-15 and R-15 *E. indica* populations. To study the enzymatic activity of the ACCase, ALS, and EPSPS, the detailed methodologies by Golmohammadzadeh et al.,[17] Rojano-Delgado et al.,[18] and Dayan et al.,[23] respectively, were followed. Herbicide and the concentrations tested, the main reagents and substrates, as well as the technical characteristic for each enzyme assay are listed in Table S3. In all three cases, methodologies include the following two main steps:

Extraction and preconcentration: In this step, the plant material was ground (with liquid nitrogen). In a series of processes with corresponding extraction buffers, the enzyme extract was obtained. In some cases, this raw extract can be directly used for the next step (as in the ALS) or must be cleaned and preconcentrated, passing through a desalinization column or with a dialysis cassette (as in the EPSPS). In this step, it is important to perform procedures and sample handling in cold conditions (4 °C).

Enzyme assay: Different herbicide concentrations were added to the crude enzyme extract obtained in the previous step together with a reaction buffer and the substrates such that the enzyme would work at the best conditions. Enzymatic activity in many cases is measured through the product originated by the enzyme (either directly or derivatized to colored complexes) or through a product of a coupled reaction (as in EPSPS). The chemical characteristics of the substances monitored were measured with different detection methods (Table S3).

The results were expressed as the concentration of herbicide required to inhibit 50% (I$_{50}$) of enzyme activity. To obtain the basal enzyme activity (without herbicide), the total soluble protein (TSP) of each enzyme was determined by the Bradford method.[24] The experiment was carried out twice with three technical replicates per herbicide concentration and per population, following a completely randomized design.

ACCase and EPSPS Gene Sequencing. Plant tissue samples (100−150 μg) of young leaves were taken from 20 plants (BBCH-14 stage) of the R-06, R-15, and S-15 *E. indica* populations and immediately stored at −20 °C. Then, plants were treated with sethoxidim and glyphosate at the field dose as determined in the dose−response assays. Genomic DNA (gDNA) was extracted from 13 plants that survived the herbicide treatment at 21 DAT. The Qiagen DNA Extraction Kit was used to extract the gDNA of the frozen leaf material following the manufacturer instructions. DNA was quantified with a NanoDrop and then immediately used for PCR analyses or stored at −20 °C until use.

Primers ELEIN_1781F, ELEIN_1781R, ELEIN_2027_f and ELEIN_2027_r were used to amplify two regions in the CT domain of the *ACCase* gene.[15] Primers DEF and DER were used to amplify the conserved region of the *EPSPS* gene. Fragments were amplified separately in a 20 μL volume [50 ng of gDNA, 0.5 μM of each primer,

Figure 1. Absorption (from % recovered) and translocation (from % absorbed) of (A) ^{14}C-diclofop-methyl, (B) ^{14}C-imazamox, (C) ^{14}C-glyphosate (at 96 h after treatment), and (D) ^{14}C-paraquat (24 h after treatment) in R-15 and S-15 *E. indica* populations. TL, treated leaf; RP, remainder of the plant, and R, roots. Vertical lines represent the SE of means ($n = 6$). * Represents significance according to Student's *t* test ($P < 0.05$). ns = nonsignificant.

0.2-mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Spain), 2 mM MgCl2, 1× buffer and 0.625 units of polymerases (100:1 of *Thermus thermophilus* and *Pyrococcus furiosus*; Biotools, Madrid, Spain)] following the PCR conditions described by Osuna et al.[15] for the *ACCase* gene and by Chen et al.[25] for the *EPSPS* gene. Ten microliters of PCR products were used to check the size of fragments in 1% agarose gels, and the other 10 μL were purified using ExoSAP-IT for PCR Product Clean-Up (USB, Ohio, U.S.A.) following the manufacturer's instruction. Fifteen microliters of purified PCR product per sample (three technical replicates per plant and gene) were sequenced by the SCAI (Servicio Central de Apoyo a la Investigación) at the University of Cordoba, Spain.

Statistical Data. Dose–response and enzyme activity data were subjected to nonlinear regression analysis using the following three-parameter log–logistic equation: $Y = ([d/1 + (x/g) \times b]) + c$, where Y is the percentage of above ground fresh weight, plant survival or enzyme inhibition in relation to the nontreated control, d is the upper asymptote, b is the slope of the line, g is the inflection point halfway (GR_{50}, LD_{50}, or I_{50}), and x (independent variable) is the herbicide

rate. Regression analyses were conducted in the statistical freeware program R 3.2.4 using the *drc* package.[26] Resistance indices were computed as R-to-S ratios with the g values, corresponding to the herbicide concentration (g ha^{-1} or μM) that caused the reduction of fresh weight (GR_{50}), plant mortality (LD_{50}), or enzyme inhibition (I_{50}) by 50% in each population of *E. indica* for each herbicide.

The variance stability tests of control rate data showed no difference for both cropping seasons, and data were pooled for further analyses. Inspection of error distributions and scatter plots among variables of absorption, translocation, and metabolic data suggested that assumptions of linearity and normality held reasonably well for all analyses. Data were analyzed using the Student's *t* test to compare pairwise within each point between S and R populations, and $p < 0.05$ values were considered to be significant.

■ **RESULTS**

Herbicide Dose–Response Assays. All tested herbicides controlled the S-06 and S-15 *E. indica* populations in their respective field dose. Regarding to ACCase inhibitors, the R-06

Figure 2. Metabolism rates of diclofop-methyl (A) and imazamox (B) in S-15 and R-15 *E. indica* populations 96 h after treatment. Vertical lines represent the SE of means ($n = 6$). * Represents significance according to Student's *t* test ($P < 0.05$).

population showed cross resistance to the FOP and DIM herbicides tested with RI ranging from 3.9 to 16.4 based on weight reduction and from 6.4 to 23.5 in plant mortality, but cross-resistance to DEN herbicides was not observed. However, the R-15 population in addition to cross-resistance to FOP and DIM herbicides, which almost doubled compared to the R-06 population, evolved resistance to pinoxaden, showing GR_{50} (41.6) and LD_{50} (73.9) values close to or higher than the field dose (40 g ha^{-1}). In R-15 plants pretreated with amitrole, the GR_{50} and LD_{50} values decreased to values close to those observed in the R-06 population (Table 2).

R-06 and S-06 *E. indica* populations were controlled with imazamox ($LD_{50} \leq 10$ g ha^{-1}), that is, they showed no resistance to ALS inhibitors. However, the R-15 population presented higher GR_{50} and LD_{50} values (105.8 and 231.4 g ha^{-1}, respectively). Similar to ACCase inhibitors, malathion pretreatment led the R-15 population to have a GR_{50} and LD_{50} close to those observed in the S-06 and R-06 populations when treated with imazamox. The 2006 populations were susceptible to both glyphosate and paraquat with GR_{50} values less than 150 g ha-1, and LD_{50} values were within 200 and 300 g ha^{-1}. The R-15 *E. indica* population evolved resistance to these herbicides, showing IR values of 5.1 and 5.3 for glyphosate based on weight reduction and plant mortality, respectively, and 7.3 and 8.5 for paraquat compared to the S-15 population (Table 2).

[14]C-herbicide Absorption and Translocation in R-15 and S-15 Plants. Absorption and translocation patterns of ^{14}C-diclofop-methyl and ^{14}C-imazamox were similar between R-15 and S-15 populations at 96 HAT. The absorption of these herbicides was ≤90%, but ^{14}C-diclofop-methyl was retained mainly in the TL (~94%) while high rates of the absorbed ^{14}C-imazamox were translocated to the RP (~34%) and R (~21%) (Figure 1A,B). Glyphosate, which had absorption rates similar (close to 90%) to ACCase and ALS inhibitors, and paraquat, which was absorbed in up to 77%, showed differences in translocation rates. The S-15 population translocated 9% more ^{14}C-glyphosate to R compared to the R-15 population, but there were no differences in relation to the herbicide found in the TL (~45%) and RP (~33%) (Figure 1C). At 24 HAT,

[14]C-paraquat was less translocated to RP and R in the R-15 population (13.1%) compared to the S-15 population (51%). The amount of ^{14}C-paraquat found in RP and R of the S-15 population was 3.6 and 4.3 times, respectively, greater than the R-15 (Figure 1D).

Herbicide Metabolism Studies. Metabolism of diclofop-methyl and imazamox was different between R-15 and S-15 *E. indica* populations at 96 HAT (Figure 2). Approximately 15% and 24% of ^{14}C-diclofop-methyl was not metabolized in S-15 and R-15 populations, respectively. In the S-15 population, the herbicide was primarily bioactivated in diclofop-acid (76%), whereas in the R-15 population, the herbicide was transformed into conjugates (66%) (Figure 2A). In the case of imazamox, the R-15 was able to metabolize up to 88% of the herbicide versus 7.7% for S-15. The R-15 population transformed imazamox to imazamox−OH (34%) and imazamox-conjugate (54.6%) (Figure 2B). Regarding glyphosate, the *E. indica* populations did not show significant metabolism rates and more than 97% of the herbicide was not metabolized (data not shown).

ACCase, ALS, and EPSPS Enzyme Assay. The *in vitro* enzyme activities of the ACCase (R = 3.3 and S = 3.1 pmol ^{14}C-malonyl-CoA μg^{-1} TSP min^{-1}), ALS (R = 3.94 and S = 3.81 pmol of acetoin μg^{-1} TSP min^{-1}) and EPSPS (R = 24.2 and S = 26.1 nmol Pi μg^{-1} TSP min^{-1}) in the absence of herbicide was similar between R-15 and S-15 *E. indica* populations.

The amount of herbicide needed to inhibit the ACCase activity by 50% ranged from 2.1 to 1308.6 μM, depending on the herbicide (FOP, DIM, and DEN) and the resistance status of the *E. indica* populations, but there were no differences in relation to the harvest year (2006 or 2015). The RI ranged from 7.2 to 12.6 for FOP herbicides and from 8.4 to 12.5 for DIM herbicides, whereas for DEN the I_{50} was similar ($I_{50} \approx 34$ μM pinoxaden) between R and S populations. Similar to pinoxaden, the concentration of imazamox needed to inhibit ALS was similar ($I_{50} \approx 3.6$ μM) between R and S populations, regardless of harvest year. In the case of glyphosate, the R-06, S-06, and S-15 populations had similar I_{50} values (~5.4 μM), but the R-15 population required 21.8 times more herbicide in

Table 3. Herbicide Concentration (μM) to Inhibit the Activity,[a]

herbicide	population	I_{50}	RF	population	I_{50}	RF
ACCase						
haloxyfop-*p*-methyl	R-06	39.1	7.2	R-15	40.3	7.9
	S-06	5.4		S-15	5.1	
diclofop-methyl	R-06	55.9	12.2	R-15	62.7	12.6
	S-06	4.6		S-15	5.0	
sethoxydim	R-06	1308.6	9.4	R-15	1244.5	8.4
	S-06	139.8		S-15	147.7	
tralkoxydim	R-06	25.9	12.5	R-15	28.3	11.8
	S-06	2.1		S-15	2.4	
pinoxadem	R-06	32.9	0.9	R-15	35.4	1.0
	S-06	34.9		S-15	35.1	
ALS						
imazamox	R-06	3.7	1.3	R-15	4.3	1.4
	S-06	2.9		S-15	3.0	
EPSPS						
glyphosate	R-06	5.2	1.0	R-15	124.3	21.8
	S-06	5.4		S-15	5.7	

[a]Inhibiting the activity of the acetolactate synthase (ALS), acetyl-CoA carboxylase (ACCase), and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor by 50% in R-06, S-06, R-15, and S-15 populations of *E. indica*.

relation to the S-15 population to inhibit the EPSPS in the same proportion (50%) (Table 3).

ACCase and EPSPS Gene Sequencing. As expected, the R-06 population had a substitution of Asp (wild-codon ATC) by Gly (mutant-codon GTC) in the 2078 position of the *ACCase* gene. The R-15 population also had this amino acid substitution. Regarding the *EPSPS* gene, sequenced only in the S-15 and R-15 populations, the substitution of Pro (wild-codon CCA) by Ser (mutant-codon TCA) at the 106 position was found in the resistant population. No additional mutations were found associated with resistance to ACCase and EPSPS inhibitors.

DISCUSSION

Resistance to ACCase-inhibiting herbicides in grasses occurring in soybean fields in Brazil has been observed since the late 1990s.[2] Dose−response assays revealed an increase in resistance level to FOPs and DIMs, and new resistance to DEN in the R-15 in comparison to the R-06 population. This increase suggests that new resistance mechanism(s) evolved in *E. indica*, because only the single Asp-2078-Gly mutation was found again in the R-15 population. This increase in the level of resistance to ACCase inhibitors cannot be explained by TSR mechanisms, since this mutation has been only reported to confer resistance to FOP and DIM herbicides in *E. indica* from Brazil.[16] This may be because the binding site of the pinoxaden in the ACCase structure is different from the FOP and DIM herbicides, besides the Asp-2078 position is not found in a direct herbicide-binding site.[27] In addition, in homologous *E. indica* populations (R or S) of different harvest years (2006 or 2015), the ACCase was inhibited by each herbicide to a similar extent, corroborating cross-resistance at the target-site level only to FOPs and DIMs but not to DEN herbicides. Therefore, the increase in resistance to ACCase inhibitors was based on NTS mechanisms, as will be addressed later. The Asp-2078-Gly-based dual cross-resistance observed in this study was also reported in other grass weeds such as *Alopecurus aequalis*,[28] *Avena sterilis*,[29] *Phalaris paradoxa*,[30] and in *E. indica* from the U.S.A.[31] However, this mutation has been reported to confer broad resistance to all ACCase inhibitors in *A. myosuroides*,[32]

A. japonicus,[33] and *Bechmannia syzigachne*,[34] among other grass weeds.

The R15 *E. indica* population showed resistance to imazamox, the representative herbicide of ALS inhibitors, in dose−response assays. TS-based resistance was ruled out because enzyme activity assays, both basal and inhibitory, showed no differences between R and S populations, regardless of the collection year. Therefore, resistance to imazamox, as well as increased resistance to ACCase inhibitors, in the R-15 population involved NTS mechanisms.

Because the absorption and translocation patterns of ^{14}C-diclofop-methyl and ^{14}C-imazamox were similar between R and S populations, this resistance was restricted to metabolic process. Both susceptible and resistant weed plants are able to metabolize herbicides into nontoxic secondary metabolites; however, susceptible plants do so to a limited degree.[35] In addition, some herbicides need to be bioactivated to be toxic, as in the case of diclofop-methyl, which is transformed into diclofop-acid by hydrolysis.[36] Both R-15 and S-15 plants metabolized diclofop-methyl and imazamox into nontoxic conjugates. The high percentage of these compounds in the R-15 population confirmed the enhanced herbicide metabolism as the mechanism of resistance to ACCase and ALS inhibitors. Participation of this NTS mechanism is traditionally confirmed by reversing resistance by pretreatment with potent Cyp-P450 inhibitors.[37] Pretreatments with amitrole and malathion reversed resistance to ACCase and ALS inhibitors in R-15 plants to levels similar to those observed in the R-06 population. Metabolic resistance to these herbicides mediated by the Cyp-P450 has been widely documented in mono and dicot weeds around the world.[4] Malathion and/or amitrole also reversed resistance to chlorsulfuron (ALS) and diclofop-methyl (ACCase) in *Lolium rigidim* from Australia,[38] accessions of *Brachypodium* spp. collected in different habitats across Israel,[39,40] and *A. fatua* from U.S.A. and Canada,[41,42] among other examples. Transcriptome surveys have corroborated the upregulation of several Cyp-P450 detoxification genes in *L. rigidum* resistance to ACCase inhibitors.[43] Therefore, the increase in resistance to FOP and DIM herbicides, as well as the new resistance to pinoxaden and

imazamox observed in the R-15 *E. indica* population was due to the enhanced herbicide metabolism regulated by the Cyp-P450.

Regarding glyphosate resistance in the R-15 *E. indica* population, differences in translocation rates to roots were observed, but there was no difference in absorption. However, these differences may not be relevant to resistance, since the amount of glyphosate in the TL was similar between R and S plants. Glyphosate resistant *E. indica* plants from Mexico retained almost twice the ^{14}C-herbicide in the TL than S plants and translocated less than 20% to the RP and R.[44] A R *E. indica* population, also from Brazil (Paraná state), showed similar absorption and translocation patterns to its counterpart S.[45] On the other hand, although glyphosate metabolism was corroborated in *D. insularis* in 2012[46] and also recently in *Echinochloa colona*,[47] our glyphosate metabolism data suggest that this mechanism did not contribute to resistance in the R-15 population, that is, the NTS mechanisms were not involved. This fact is supported by the literature, which documents that *E. indica* has a great adaptive capacity to develop TS mechanisms that confer resistance to glyphosate than NTS mechanisms. Mutations that can affect the interaction of glyphosate with EPSPS and that confer some level of resistance must occur between amino acid positions 95 and 107 of the *EPSPS* gene.[48] The first mutation (Pro-106-Ser) capable of conferring glyphosate resistance in weeds was reported in a resistant population of *E. indica* from Malaysia.[49] In addition, this species was also the first to show a double TIPS mutation (Thr-102-Ile + Pro-106-Ser) in populations from different countries (Malaysia and China).[25,50] *Eleusine indica* was not the first weed to evolve EPSPS overexpression as a resistance mechanism, but it was the first species to show two combined TS mechanisms (Pro-106-Ser + EPSPS overexpression) in populations of Mexico.[44] Enzyme activity assays suggested that the R-15 population had no EPSPS overexpression (basal activity similar to the S-15 population) but the EPSPS gene sequencing revealed the occurrence of the Pro-106-Ser mutation. Glyphosate resistance of the *E. indica* population from Paraná was also conferred by this mutation.[45]

In addition to resistance to ACCAase, ALS, and EPSPS inhibitors, the R-15 population of *E. indica* also showed resistance to paraquat. Because restricted translocation is recognized as the mechanism of resistance to this herbicide,[51] we only evaluated the absorption and translocation rates of ^{14}C-paraquat as a possible mechanism of resistance. Absorption was not the cause of resistance because both R-15 and S-15 populations absorbed similar amounts of ^{14}C-paraquat; however, while the S-15 population moved large amounts of herbicide outside the TL, in the R-15 population, the herbicide was restricted to the TL. Reduced translocation of paraquat in R plants is due to sequestration in metabolically inactive compartments, primarily the vacuoles as demonstrated by Yu et al.,[52] who also isolated this organelle in resistant *L. rigidum* plants that contained 2- to 3-fold more paraquat than vacuoles of susceptible plants. This mechanism has been widely documented in various paraquat resistance species, such as *Conyza bonariensis*, *C. canadensis*,[53] and *L. perenne*.[7] In *E. indica*, there is evidence that polyamine transporters play a key role in paraquat resistance, since paraquat has a similar structure to endogenous polyamines that are carried in vacuoles.[54] In our case, we can affirm that the paraquat resistance of the R-15 population was due to the reduced translocation of the herbicide, although we do not know if the herbicide was sequestered in the vacuoles.

■ CONCLUSIONS

The results of this study indicate that *E. indica*, previously characterized with cross resistance to FOPs and DIMs, evolved broad resistance to ACCase inhibitors and multiple resistance to ALS, EPSPS, and PSI inhibitors. A TS mechanism governed glyphosate resistance (Pro-106-Ser mutation), whereas the NTS mechanisms were responsible for resistance to other herbicides. The increase in resistance to FOP and DIM, as well as the new resistance found to pinoxaden and imazamox, was conferred by an enhanced herbicide metabolism mediated by the Cyp-P450, whereas the paraquat resistance was due to restricted translocation. This study is the first to characterize the mechanisms of a weed with multiple resistances to herbicides from Brazil.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c03999.

Additional tables (PDF)

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Notes

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■ REFERENCES

(1) Nishimoto, R. K.; McCarty, L. B. Fluctuating temperature and light influence seed germination of goosegrass (*Eleusine indica*). *Weed Sci.* **1997**, *45*, 426–429.

(2) Heap, I. International herbicide-resistant weed database. http://www.weedscience.org/Home.aspx (accessed on June 22, 2020).

(3) Jalaludin, A.; Yu, Q.; Powles, S. B. Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population. *Weed Res.* **2015**, *55*, 82–89.

(4) Gaines, T. A.; Duke, S. O.; Morran, S. Mechanisms of evolved herbicide resistance. *J. Biol. Chem.* **2020**, *295*, 10307.

(5) Ghanizadeh, H.; Harrington, K. C. Non-target site mechanisms of resistance to herbicides. *Crit. Rev. Plant Sci.* **2017**, *36*, 24–34.

(6) Ge, X.; d'Avignon, D. A.; Ackerman, J. J. H.; et al. Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (*Lolium* spp.) from Australia, South America, and Europe: A ^{31}P NMR investigation. *J. Agric. Food Chem.* **2012**, *60*, 1243–1250.

(7) Brunharo, C. A. C. G.; Hanson, B. D. Vacuolar sequestration of paraquat is involved in the resistance mechanism in *Lolium perenne* L. spp *multiflorum*. *Front. Plant Sci.* **2017**, *8*, 1485.

(8) Alcántara-de la Cruz, R.; Oliveira, G. M.; Carvalho, L. B.; Silva, M. F. G. F. Herbicide resistance in Brazil: Status, impacts, and future challenges. In *Pests, Weeds and Diseases in Agricultural Crop and Animal Husbandry* Production, 1st ed.; Kontogiannatos, D.; Ferreira, K. M., Ed.; IntechOpen: London, UK, 2020; pp. 1–25.

(9) USDA – United States Department of Agriculture. World agricultural production. https://apps.fas.usda.gov/psdonline/circulars/production.pdf (accessed on June 6, 2020).

(10) Pashaei Kamali, F.; Meuwissen, M. P.M.; de Boer, I. J.M.; van Middelaar, C. E.; Moreira, A.; Oude Lansink, A. G.J.M. Evaluation of environmental, economic, and social performance of soybean farming systems in southern Brazil. *J. Cleaner Prod.* **2017**, *142*, 385–394.

(11) Adegas, F. S.; Vargas, L.; Pisa Gazziero, D. L. Karam. Impacto econômico da resistência de plantas daninhas a herbicidas no Brasil. Embrapa: Circular técnica. **2017**, *132.* 1–11.

(12) Lopes Ovejero, R.F.; Soares, D.J.; Oliveira, W.S.; Fonseca, L.B.; Berger, G.U.; Soteres, J.K.; Christoffoleti, P.J. Residual herbicides in weed management for glyphosate-resistant soybean in Brazil. *Planta Daninha* **2013**, *31*, 947–959.

(13) Gazziero, D. L. P. Mixtures of pesticides in tank, in Brazilian farms. *Planta Daninha* **2015**, *33* (1), 83–92.

(14) Vidal, R. A.; Portes, E. S.; Lamego, F. P.; Trezzi, M. M. *Eleusine indica* resistance to ACCase inhibitors. *Planta Daninha* **2006**, *24*, 163–171.

(15) Takano, H. K.; Oliveira, R. S., Jr.; Constantin, J.; Braz, G. B. P.; Gheno, E. A. Goosegrass resistant to glyphosate in Brazil. *Planta Daninha* **2017**, *35*, 1–9.

(16) Osuna, M. D.; Goulart, I. C. G. R.; Vidal, R. A.; et al. Resistance to ACCase inhibitors in *Eleusine indica* from Brazil involves a target site mutation. *Planta Daninha* **2012**, *30*, 675–681.

(17) Golmohammadzadeh, S.; Rojano-Delgado, A. M.; Vázquez-García, J. G.; et al. Cross-resistance mechanisms to ACCase-inhibiting herbicides in short-spike canarygrass (*Phalaris brachystachys*). *Plant Physiol. Biochem.* **2020**, *151*, 681–688.

(18) Rojano-Delgado, A. M.; Portugal, J. M.; Palma-Bautista, C.; Alcantara-de la Cruz, R.; Torra, J.; Alcantara, E.; De Prado, R. Target site as the main mechanism of resistance to imazamox in a *Euphorbia heterophylla* biotype. *Sci. Rep.* **2019**, *9*, 15423.

(19) Tahmasebi, B. K.; Alcantara-de la Cruz, R.; Alcantara, E.; Torra, J.; Dominguez-Valenzuela, J. A.; Cruz-Hipolito, H. E.; Rojano-Delgado, A. M.; De Prado, R. Multiple resistance evolution in bipyridylium-resistant *Epilobium ciliatum* after recurrent selection. *Front. Plant Sci.* **2018**, *9*, 695.

(20) Alcantara-de la Cruz, R.; Fernandez-Moreno, P. T.; Ozuna, C. V.; Rojano-Delgado, A. M.; Cruz-Hipolito, H. E.; Dominguez-Valenzuela, J. A.; Barro, F.; De Prado, R. Target and non-target site mechanisms developed by glyphosate-resistant hairy beggarticks (*Bidens pilosa* L.). *Front. Plant Sci.* **2016**, *7*, 1492.

(21) Rojano-Delgado, A. M.; Priego-Capote, F.; De Prado, R.; Luque de Castro, M. D. Ultrasound-assisted extraction with LC-TOF/MS identification and LC-UV determination of imazamox and its metabolites in leaves of wheat plants. *Phytochem. Anal.* **2014**, *25*, 357–363.

(22) Rojano-Delgado, A. M.; Ruíz-Jiménez, J.; Luque de Castro, M. D.; De Prado, R. Determination of glyphosate and its metabolites in plant material by reversed-polarity CE with indirect absorptiometric detection. *Electrophoresis* **2010**, *31*, 1423–1430.

(23) Dayan, F. E.; Owens, D. K.; Corniani, N.; et al. Biochemical markers and enzyme assays for herbicide mode of action and resistance studies. *Weed Sci.* **2015**, *63*, 23–63.

(24) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(25) Chen, J.; Huang, C.; Wei, S.; et al. Mutations and amplification of *EPSPS* gene confer resistance to glyphosate in goosegrass (*Eleusine indica*). *Planta* **2015**, *242*, 859–868.

(26) Ritz, C.; Baty, F.; Streibig, J.; Gerhard, D. Dose-response analysis using R. *PLoS One* **2015**, *10*, No. e0146021.

(27) Jang, S.; Marjanovic, J.; Gornicki, P. Resistance to herbicides caused by single amino acid mutations in acetyl-CoA carboxylase in resistant populations of grassy weeds. *New Phytol.* **2013**, *197*, 1110–1116.

(28) Guo, W.; Yuan, G.; Liu, W.; et al. Multiple resistance to ACCase and AHAS-inhibiting herbicides in shortawn foxtail (*Alopecurus aequalis* Sobol.) from China. *Pest. Pestic. Biochem. Physiol.* **2015**, *124*, 66–72.

(29) Liu, W.; Harrison, D. K.; Chalupska, D. Single-site mutations in the carboxyltransferase domain of plastid acetyl-CoA carboxylase confer resistance to grass-specific herbicides. *Proc. Natl. Acad. Sci.* **2007**, *104*, 3627–3632.

(30) Hochberg, O.; Sibony, M.; Rubin, B. The response of ACCase-resistant *Phalaris paradoxa* populations involves two different target site mutations. *Weed Res.* **2009**, *49*, 37–46.

(31) McCullough, P. E.; Yu, J.; Raymer, P. L.; Chen, Z. First report of ACCase-resistant goosegrass (*Eleusine indica*) in the United States. *Weed Sci.* **2016**, *64*, 399–408.

(32) Petit, C.; Bay, G.; Pernin, F.; Délye, C. Prevalence of cross- or multiple resistance to the acetyl-coenzyme A carboxylase inhibitors fenoxaprop, clodinafop and pinoxaden in black-grass (*Alopecurus myosuroides* Huds.) in France. *Pest Manage. Sci.* **2010**, *66*, 168–177.

(33) Chen, G.; Wang, L.; Xu, H.; Wu, X.; Pan, L.; Dong, L. Cross-resistance patterns to acetyl-CoA carboxylase inhibitors associated with different mutations in Japanese foxtail (*Alopecurus japonicus*). *Weed Sci.* **2017**, *65*, 444–451.

(34) Tang, W.; Zhou, F.; Zhang, Y.; Chen, J. Resistance of American sloughgrass (*Bechmannia syzigachne*) populations to ACCase-inhibiting herbicides involves three different target site mutations from China. *Pestic. Biochem. Physiol.* **2015**, *124*, 93–96.

(35) Yu, Q.; Powles, S. Metabolism-based herbicide resistance and cross-resistance in crops weeds: A threat to herbicide sustainability and global crop production. *Plant Physiol.* **2014**, *166*, 1106–1118.

(36) Adkins, A. Degradation of the phenoxy acid herbicide diclofop-methyl by *Sphingomonas paucimobilis* isolated from a Canadian prairie soil. *J. Ind. Microbiol. Biotechnol.* **1999**, *23*, 332–335.

(37) Keith, B. K.; Lehnhoff, E. A.; Burns, E. E.; Menalled, F. D.; Dyer, W. E. Characterisation of *Avena fatua* populations with resistance to multiple herbicides. *Weed Res.* **2015**, *55*, 621–630.

(38) Yu, Q.; Abdallah, I.; Han, H.; Owen, M.; Powles, S. Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. *Planta* **2009**, *230*, 713–723.

(39) Matzrafi, M.; Gadri, Y.; Frenkel, E.; Rubin, B.; Peleg, Z. Evolution of herbicide resistance mechanisms in grass weeds. *Plant Sci.* **2014**, *229*, 43−52.

(40) Matzrafi, M.; Seiwert, B.; Reemtsma, T.; Rubin, B.; Peleg, Z. Climate change increases the risk of herbicide-resistant weeds due to enhanced detoxification. *Planta* **2016**, *244*, 1217−1227.

(41) Beckie, H. J.; Warwick, S.; Sauder, C. A. Basis for herbicide resistance in Canadian populations of wild oat (*Avena fatua*). *Weed Sci.* **2012**, *60*, 10−18.

(42) Keith, B. K.; Lehnhoff, E. A.; Burns, E. E.; Menalled, F. D.; Dyer, W. E. Characterisation of *Avena fatua* populations with resistance to multiple herbicides. *Weed Res.* **2015**, *55*, 621−630.

(43) Gaines, T. A.; Lorentz, L.; Figge, A.; et al. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. *Plant J.* **2014**, *78*, 865−876.

(44) Gherekhloo, J.; Fernandez-Moreno, P. T.; Alcantara-de la Cruz, R.; Sanchez-Gonzalez, E.; Cruz-Hipolito, H. E.; Dominguez-Valenzuela, J. A.; De Prado, R. Pro-106-Ser. mutation and EPSPS overexpression acting together simultaneously in glyphosate-resistant goosegrass (*Eleusine indica*). *Sci. Rep.* **2017**, *7*, 6702.

(45) Takano, H. K.; Mendes, R. R.; Scoz, L. B.; et al. Proline-106 EPSPS mutation imparting glyphosate resistance in goosegrass (*Eleusine indica*) emerges in South America. *Weed Sci.* **2019**, *67*, 48−56.

(46) de Carvalho, L. B.; Alves, P. L. d. C. A.; Gonzalez-Torralva, F.; Cruz-Hipolito, H. E.; Rojano-Delgado, A. M.; De Prado, R.; Gil-Humanes, J.; Barro, F.; Luque de Castro, M. D. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. *J. Agric. Food Chem.* **2012**, *60*, 615−622.

(47) Pan, L.; Yu, Q.; Han, H.; et al. Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochlpoa colona*. *Plant Physiol.* **2019**, *181*, 1519−1534.

(48) Funke, T.; Yang, Y.; Han, H.; et al. Structural basis of glyphosate resistance resulting from the double mutation Thr 97 Ile and Pro 101 Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. *J. Biol. Chem.* **2009**, *284*, 9854−9860.

(49) Baerson, S. R.; Rodriguez, D. J.; Tran, M.; et al. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Plant Physiol.* **2002**, *129*, 1265−1275.

(50) Yu, Q.; Jalaludin, A.; Han, H.; et al. Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate esistance. *Plant Physiol.* **2015**, *167*, 1440−1447.

(51) Hawkes, T. R. Mechanisms of resistance to paraquat in plants. *Pest Manage. Sci.* **2014**, *70*, 1316−1323.

(52) Yu, Q.; Huang, S.; Powles, S. Direct measurement of paraquat in leaf protoplasts indicates vacuolar paraquat sequestration as resistance mechanism in *Lolium rigidum*. *Pestic. Biochem. Physiol.* **2010**, *98*, 104−109.

(53) Moretti, M. L.; Hanson, B. D. Reduced translocation is involved in resistance to glyphosate and paraquat in *Conyza bonariensis* and *Conyza canadensis* from California. *Weed Res.* **2017**, *57*, 25−34.

(54) Luo, Q.; Wei, J.; Dong, Z.; Shen, X.; Chen, Y. Differences of endogenous polyamines and putative genes associated with paraquat resistance in goosegrass (*Eleusine indica* L.). *PLoS One* **2019**, *14*, No. e0216513.