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Pool of Resistance Mechanisms to Glyphosate in *Digitaria insularis*

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ABSTRACT: *Digitaria insularis* biotypes resistant to glyphosate have been detected in Brazil. Studies were carried out in controlled conditions to determine the role of absorption, translocation, metabolism, and gene mutation as mechanisms of glyphosate resistance in *D. insularis*. The susceptible biotype absorbed at least 12% more ^{14}C-glyphosate up to 48 h after treatment (HAT) than resistant biotypes. High differential ^{14}C-glyphosate translocation was observed at 12 HAT, so that >70% of the absorbed herbicide remained in the treated leaf in resistant biotypes, whereas 42% remained in the susceptible biotype at 96 HAT. Glyphosate was degraded to aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine by >90% in resistant biotypes, whereas a small amount of herbicide (up to 11%) was degraded by the susceptible biotype up to 168 HAT. Two amino acid changes were found at positions 182 and 310 in *EPSPS*, consisting of a proline to threonine and a tyrosine to cysteine substitution, respectively, in resistant biotypes. Therefore, absorption, translocation, metabolism, and gene mutation play an important role in the *D. insularis* glyphosate resistance.

KEYWORDS: *N-phosphonomethylglycine, EPSPS, weed resistance, mechanisms of resistance, sourgrass*

INTRODUCTION

Glyphosate herbicide has been used extensively in agriculture worldwide for about 30 years, and it is the most commercialized herbicide in the world.[1] Glyphosate has a unique chemical structure and a molecular target site related to the inhibition of the shikimate pathway. The inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) results in starvation of EPSP and ensuing metabolic products, such as the aromatic acids phenylalanine, tyrosine, and tryptophan that are required for protein synthesis. The inhibition of the shikimate pathway causes its deregulation, resulting in carbon flow from other pathways, leading to disruption of other metabolic pathways.[2]

Frequent glyphosate applications performed during the past 15 years in no-tilled fields and orchard plantations have been selecting *Digitaria insularis* populations in southeastern Brazil. The species is indigenous to tropical and subtropical America. It is an important weed infesting annual and perennial crops in Brazil. This perennial weed reproduces by seeds and rhizomes. In addition, the small hairy seeds with a high percentage of germination allow *D. insularis* to be a high potential infesting weed.[3]

Strong selection by herbicides has resulted in the widespread evolution of herbicide resistance in populations of agricultural weeds. Weed populations evolve resistance in response to repeated treatment with herbicides having the same mechanism of action or metabolic degradation pathway.[1,4] Therefore, the selection pressure caused by the use of glyphosate is causing the selection of *D. insularis* populations resistant to glyphosate in agricultural areas of Brazil.[3]

Herbicide resistance has been one of the most important challenges in agricultural areas in past years.[3] In addition, population shifts toward glyphosate-resistant weeds are becoming a very significant issue in parts of North and South America.[1,4] Today, in the American continent, glyphosate-resistant weed populations have been identified in 15 species and 7 countries, including Brazil.[5] In Brazil, cases of resistance to glyphosate have already been published on biotypes of *Conyza bonariensis*,[6,7] *Conyza canadensis*,[7] *D. insularis*,[3]

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Euphorbia heterophylla,[8] and *Lolium multiflorum*,[9,10] in both annual and perennial crops.

Three relevant factors endowing herbicide resistance in weeds are target enzyme sensitivity, increased herbicide metabolism, and impaired herbicide accumulation at the site of action.[11,12] Glyphosate resistance in weeds has been found to be due to two main mechanisms defined as nontarget site (reduced absorption and/or translocation and vacuolar sequestration) and target site resistance (mutation and gene amplification). Glyphosate resistance due to reduced absorption and/or translocation has been described in resistant biotypes of *Conyza* spp.,[13,14] *C. bonariensis*,[15] *Lolium rigidum*,[16] and *L. multiflorum*.[17,18] Glyphosate resistance due to vacuolar sequestration was described for *C. canadensis*.[19] Different mutations on the *EPSPS* gene have been reported to confer resistance in *Eleusine indica*,[20,21] *L. rigidum*,[16,27] and *L. multiflorum*.[18] *EPSPS* gene amplification was found to confer glyphosate resistance in *Amaranthus palmeri*.[23] However, metabolism of glyphosate has not been found to be a mechanism of resistance.[12,13,24]

The general objective of this research was to identify the resistance mechanisms involved in the glyphosate-resistant *D. insularis* species. The specific objectives were to determine (1) shikimic acid responses, (2) absorption and translocation of ^{14}C-glyphosate, (3) glyphosate metabolism, and (4) mutation(s) in the *EPSPS* gene, in biotypes of *D. insularis* from Brazil.

MATERIALS AND METHODS

Chemicals. Shikimic acid (Sigma-Aldrich), commercial herbicide formulation with 45% of glyphosate SL (Monsanto), [^{14}C]-glyphosate-(phosphonomethyl), specific activity of 52 mc_i $mmol^{-1}$ (American Radiolabel Chemicals), TRIzol reagent (Invitrogen), TURBO DNase (Ambion), Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen), oligo(dT)$_{12-18}$ and random nonamers (Amersham Biosciences), nonproofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BioTools), and ExoSAP-IT (USB Corp.) were used in this study. All other reagents were purchased at analytical grade.

Plant Material. Seeds from suspected glyphosate-resistant *D. insularis* plants (hereafter referred to as resistant biotypes R1 and R2) were harvested from agricultural fields treated for at least 15 continuous years with glyphosate herbicide. In addition, seeds of the species were collected from a natural area never treated with herbicides (hereafter referred to as biotype S). Biotype R1 was collected from an annual crop field, whereas biotype R2 was gathered from an orange orchard, in 2009. In both fields, located within São Paulo State in Brazil, these biotypes were not controlled using glyphosate at 1440 g acid equivalent per hectare (g ae ha^{-1}). All biotypes were previously tested for glyphosate resistance. The glyphosate rate required to inhibit above-ground fresh weight by 50% in the susceptible biotype was 148.8 g ae ha^{-1}, and the resistance factor was 3.90 for both R1 and R2 biotypes.[3]

Growing Conditions. Experiments were conducted in 300 mL plastic pots containing a 1:2 (v/v) mixture of peat and sandy loam, maintaining just one plant per pot. All experiments were performed twice and carried out in a growth chamber model AM0705020 (Eldon) at 28/18 °C (day/night) in a 16 h photoperiod under 850 μmol m^{-2} s^{-1} photosynthetic photon-flux density delivered by a mixture of incandescent and fluorescent lights, with an 80% relative humidity.

Glyphosate Application Conditions. For shikimic acid accumulation and metabolism studies glyphosate was applied at 157.5 g ae ha^{-1}. Glyphosate application was performed on resistant and susceptible plants of all biotypes at the four-leaf growth stage using a laboratory tracking sprayer model SBS-060 (DeVries) equipped with a flat-fan nozzle model 80.02E VS (TeeJet), delivering a spray volume of 200 L ha^{-1} at 200 kPa. For absorption and translocation studies, glyphosate was applied with a microapplicator model PB 600 TA (Hamilton) at the following described concentrations.

Shikimic Acid Extraction. The protocol of Singh and Shaner as modified by Perez-Jones et al.[25] was followed for the extraction of shikimic acid. Plants of all biotypes were harvested for shikimic acid extraction at 24, 48, 72, 96, and 168 hours after treatment (HAT). Leaf tissues (50 mg of fresh weight) were homogenized in a hydrochloric acid solution (treated plants) and some known concentration shikimic acid solutions (untreated plants), and then all samples were frozen in liquid nitrogen. Subsequently, all samples were incubated, mixed with a periodic and metaperiodic acid solution, incubated again, and mixed with a sodium hydroxide and sodium sulfate solution. Shikimic acid accumulation was determined by using a spectrophotometer model DU-640 (Beckman Coulter). The standard curve was determined by using untreated plants and a known concentration of shikimic acid. Shikimic acid accumulation was determined by the difference between shikimic acid concentrations found in treated and untreated plants. The experiments were arranged in a completely randomized design with seven replicates.

Absorption and Translocation Studies. The protocol used by González-Torralva et al.[26] was followed for absorption and translocation studies. ^{14}C-glyphosate was mixed with commercially formulated glyphosate to prepare emulsions with a specific activity of approximately 50000 dpm μL^{-1} (both absorption and translocation studies) and a glyphosate concentration of 3.6 g ae L^{-1} (corresponding to 720 g ae ha^{-1} at 200 L ha^{-1}). The labeled herbicide was applied to the adaxial surface of the second leaf of each plant in one 1.0 μL droplet. At 12, 24, 48, 72, and 96 HAT, unabsorbed ^{14}C-glyphosate was removed from the leaf surface by rinsing the treated area with 3 mL of acetone 50% (v/v). Rinses from batches of five replications of plants were pooled and analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter model LS 6500 (Beckman Counter). Plants of all biotypes were also harvested in each batch at the same times and separated into treated leaf, root, and the rest of the shoot. The plant tissue was dried at 55 °C for 72 h and combusted in a Packard Tri Carb 307 sample oxidizer. The $^{14}CO_2$ evolved was trapped and counted in a 10 mL mixture of Carbo-Sorb E and Permafluor E+ (3:7, v/v) (Perkin-Elmer). The radioactivity was quantified by LSS, and percent herbicide absorbed was expressed as [dpm in combusted tissue/(dpm in combusted tissue + dpm in leaf washes)] × 100. The data of oxidized samples of each plant part were expressed as percentage of the total ^{14}C-glyphosate absorbed (treated leaf + root + rest of shoot) for translocation studies. In addition, treated whole plants were oven-dried (50 °C, 4 days), pressed against a 25 cm ×12.5 cm phosphor storage film during 12 h, and scanned for radiolabel dispersion using a storage phosphor system model Cyclone (Perkin-Elmer). The experiments were arranged in a completely randomized design with five replicates.

Metabolism Study. The protocol described by Rojano-Delgado et al.[27] was followed for the metabolism study. The concentration of glyphosate and its metabolites was determined by reversed-polarity capillarity electrophoresis. Glyphosate, aminomethylphosphonic acid (AMPA), glyoxylate, sarcosine, and formaldehyde were detected in leaf tissues of plants of all biotypes at 48, 96, and 168 HAT. Leaf tissues of treated and untreated plants were cut and frozen. Then, samples were washed, ground, mixed with acetone solution, submitted to ultrasound, centrifuged, dried under nitrogen flow, mixed with potassium phthalate, cetyl trimethylammonium bromide, and acrylonitrile, filtered, and then submitted to electrophoresis on equipment model G1600A (Agilent). Electropherograms were obtained, and the concentrations of glyphosate and its metabolites were determined on the basis of standard equations of Rojano-Delgado et al.[27] The experiments were arranged in a completely randomized design with three replicates.

***EPSPS* Gene Sequencing.** Total RNA was extracted from 0.1 g of leaf tissue. Samples were collected from each plant, immediately frozen by immersion in liquid nitrogen, and stored at −80 °C. Total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions, and treated with TURBO DNase to eliminate any

DNA contamination. The resulting RNA was stored at −80 °C. First-strand cDNA (cDNA) synthesis was carried out using 2 μg of total RNA isolated previously and a M-MLV RT in combination with oligo(dT)$_{12-18}$ and random nonamers, according to the manufacturer's instructions. Primers designed by Perez-Jones et al.[25] (sense, 5′ AGCTGTAGTCGTTGGCTGTG 3′; antisense, 5′ GCCAAGAAA-TAGCTCGCACT 3′) were used to amplify a 564 bp fragment of the *EPSPS* gene from *D. insularis* R1, R2, and S biotypes. Each PCR was carried out in duplicate using the cDNA obtained from 50 ng of total RNA, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1× buffer, and 0.625 unit of a 100:1 enzyme mixture of nonproofreading and proofreading polymerases in a final volume of 25 μL. Cycling conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 30 s, and a final extension cycle of 72 °C 5 min. An aliquot of the PCR product was loaded in a 1% agarose gel and the rest purified using ExoSAP-IT for PCR Product Clean-Up, following the manufacturer's instructions. The purified PCR products were sequenced by the sequencing service (SCAI) in the University of Córdoba. Three biological replicates from each R1, R2, and S biotype were used for RNA extraction and amplification of the *EPSPS* gene.

Statistical Analysis. Shikimic acid, absorption, translocation, and metabolism data were submitted to ANOVA and Tukey's HSD post hoc test at the 5% probability. ANOVA and Tukey's test were performed by using software Statistix version 8.0 (Analytical Software).

RESULTS AND DISCUSSION

Shikimic Acid Extraction. A factorial scheme with two factors, biotypes and evaluation times, was tested by ANOVA. Interaction between the studied factors was highly significant ($P < 0.001$). Shikimic acid did not accumulate in biotypes R1 and R2 after glyphosate application in the range of HAT tested (Table 1). The concentration of shikimic acid in the S biotype was similar to that of the resistant biotypes up to 72 HAT. However, a strong accumulation of shikimic acid was observed in the S biotype after 72 HAT. At 168 HAT, 350.4, 69.9, and 61.0 μg g^{-1} fresh weight of shikimic acid was detected in leaf tissues of biotypes S, R1, and R2, respectivelyl that is, biotype S accumulated 5.0 and 5.7 times more shikimic acid than biotypes R1 and R2, respectively, at 168 HAT.

Table 1. Shikimic Acid Accumulation in Leaves of Susceptible (S) and Resistant (R1 and R2) *D. insularis* Biotypes, at Different Hours after Treatment (HAT) with Glyphosate

biotype	HAT	shikimic acid[a] (μg g^{-1} fresh weight)
S	24	47.5 ± 5.6 A
	48	54.7 ± 6.5 A
	72	56.2 ± 9.3 A
	96	197.3 ± 47.7 B
	168	350.4 ± 46.7 C
R1	24	42.1 ± 2.8 A
	48	36.4 ± 4.7 A
	72	50.5 ± 4.9 A
	96	82.6 ± 8.6 A
	168	69.9 ± 4.8 A
R2	24	45.1 ± 5.6 A
	48	54.5 ± 3.7 A
	72	58.0 ± 2.1 A
	96	59.5 ± 3.9 A
	168	61.0 ± 6.6 A

[a]Means within a column followed by the same letter are not significantly different at the 5% level as determined by the Tukey HSD test. Values ± standard error of the mean.

A high accumulation of shikimic acid in the S biotype indicated susceptibility to glyphosate. On the other hand, shikimic acid was found below the detection limit in biotypes R1 and R2, indicating low susceptibility to glyphosate. Elevated shikimic acid accumulation is used as an early and highly sensitive indicator of glyphosate effects on glyphosate-sensitive plant tissues. Changes in shikimic acid levels in plants are specifically the result of inhibition of EPSPS and have been used as a marker for EPSPS sensitivity in plants to glyphosate.[26] These results confirmed that biotypes R1 and R2 were glyphosate-resistant.

In the susceptible *D. insularis* plants, shikimic acid accumulation was slower as well as its concentration being lower than in other grass weeds studied, such as *L. multiflorum*[18,25,28] and *L. rigidum*.[28] This fact indicates that the glyphosate absorption may be slower in this weed species, as the accumulation of shikimic acid is an indicator of whether glyphosate is reaching the target enzyme.[29]

Absorption Study. A factorial scheme with two factors, biotypes and evaluation times, was tested by ANOVA. Interaction between the studied factors was significant ($P = 0.015$). The patterns of ^{14}C-glyphosate absorption were different between the susceptible biotype and resistant ones (Table 2). At 12 HAT, <20% of glyphosate had absorbed by plants of the resistant biotypes, whereas approximately 36% was found in susceptible plants. However, there was no difference in absorption of ^{14}C-glyphosate among the biotypes after 72 HAT. At 96 HAT, 48.4, 44.6, and 47.7% of the recovered radioactivity had been absorbed by plants of biotypes S, R1, and R2, respectively. The initial difference in herbicide absorption, between S and R1/R2, biotypes could play an important role for *D. insularis* resistant biotypes because differential metabolism was also verified, as discussed under Metabolism Study.

Herbicide absorption by plants was almost 50% in both susceptible and resistant biotypes (Table 2). In general, this result is in agreement with some resistant weeds,[11] but other distinct absorption results were reported for other studied grass weeds. Thus, susceptible and resistant biotypes of *L. multiflorum* from Chile absorbed >90% of ^{14}C-glyphosate,[30] whereas biotypes from the United States had absorbed almost 60% at 48 HAT.[31] At 72 HAT, a susceptible and two resistant biotypes (from Chile and the United States) of *L. multiflorum* absorbed <40% of radioactivity.[25] Thus, herbicide absorption could be dependent on biotype, growing conditions, and environmental plant adaptation.

Differences in glyphosate absorption were observed in *L. multiflorum* biotypes.[30,31] Michitte et al.[32] examined the involvement of the cuticle properties on the resistance and did not find evidence of wax crystallization in either susceptible or resistant biotypes, although certain zones on the surface of resistant biotype had wrinkles and the leaf cuticle was thicker than in the susceptible one. Differences in herbicide absorption between leaves with thicker and thinner cuticles were recently observed in *Abutilon theophrasti*, so that lower absorption of acifluorfen, a moderately polar herbicide, was observed in leaves of thicker epicuticular wax.[33] Thus, because glyphosate is a polar herbicide, lower absorption may occur in plants with thicker cuticles.[34]

Differences in glyphosate absorption may be also related to distinct leaf epicuticular wax composition,[31] as observed between glyphosate-resistant and -susceptible biotypes of *L.*

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Table 2. Absorption (Percentage of Recovered Radioactivity) and Translocation (Percentage of Absorbed Radioactivity) of ^{14}C-Glyphosate in Susceptible (S) and Resistant (R1 and R2) *D. insularis* Biotypes, at Different Hours after Treatment (HAT) with ^{14}C-Glyphosate

			translocation[/a]		
biotype	HAT	absorption[/a]	treated leaf	root	rest of shoot
S	12	35.9 ± 1.4 AB	69.8 ± 3.1 D	15.7 ± 0.8 JKLMNO	14.5 ± 0.3 LMNO
	24	40.3 ± 3.2 AB	59.5 ± 1.0 E	20.6 ± 0.8 HIJKLM	20.9 ± 1.7 HIJKL
	48	41.1 ± 1.7 AB	56.8 ± 1.2 E	20.7 ± 0.9 HI	22.4 ± 1.3 HIJK
	72	47.7 ± 0.8 A	53.2 ± 2.0 E	21.4 ± 1.6 HIJK	25.4 ± 0.6 HIJ
	96	48.4 ± 1.6 A	41.9 ± 1.4 F	32.2 ± 1.4 G	25.8 ± 1.3 GH
R1	12	12.0 ± 0.9 D	82.3 ± 3.2 A	8.7 ± 0.6 O	9.0 ± 0.8 O
	24	16.8 ± 1.4 CD	82.0 ± 1.3 AB	8.3 ± 0.7 O	9.6 ± 0.7 O
	48	19.7 ± 2.9 CD	79.2 ± 1.6 ABC	11.7 ± 0.5 NO	9.1 ± 1.1 O
	72	37.3 ± 1.3 AB	75.9 ± 1.3 ABCD	12.3 ± 0.8 NO	11.8 ± 0.8 NO
	96	44.6 ± 3.0 A	74.5 ± 1.3 BCD	14.5 ± 0.2 LMNO	11.0 ± 1.1 NO
R2	12	19.4 ± 4.2 CD	75.4 ± 2.4 ABCD	11.1 ± 0.5 NO	13.5 ± 0.6 LMNO
	24	27.1 ± 3.9 BC	74.0 ± 0.4 CD	12.9 ± 0.8 MNO	13.1 ± 0.9 MNO
	48	27.9 ± 5.7 BC	71.7 ± 1.8 CD	14.5 ± 0.5 LMNO	13.7 ± 0.3 LMNO
	72	46.1 ± 4.0 A	69.5 ± 1.1 D	16.2 ± 1.0 KLMNO	14.3 ± 1.3 LMNO
	96	47.7 ± 3.8 A	69.0 ± 2.9 D	17.8 ± 1.2 IJKLMN	13.2 ± 0.4 NO

[/a]Means within a column, for absorption studies, and within a column and/or line, for translocation studies, followed by the same letter are not significantly different at the 5% level as determined by the Tukey HSD test. Values ± standard errors of the mean.

multiflorum.[34] Authors found up to 5% more alcohols and aldehydes (polar components) in resistant biotype, increasing slightly the leaf polarity. These results suggest that the nature of cuticle properties may play an important role in the absorption differences between biotypes S and R1 and R2 tested in this work.

Contact angle of droplets and foliar herbicide retention can also play an important role in glyphosate absorption.[30] However, according to previous studies, *D. insularis* biotypes showed similar contact angle and foliar retention,[3] so that these characteristics did not influence glyphosate absorption in either susceptible or resistant biotypes.

Translocation Study. A factorial scheme with three factors, biotypes, plant parts, and evaluation times, was tested by ANOVA. Interaction between the studied factors was highly significant ($P < 0.001$). *D. insularis* biotypes also showed a distinct pattern of translocation of ^{14}C-glyphosate, according to the evaluation time (Table 2). This pattern was a little different even among the resistant biotypes. At 48 HAT, there was a clear difference in percentage of ^{14}C-glyphosate translocated from the treated leaf to the roots and the rest of the shoot in susceptible and resistant biotypes. At this time, 56.8, 79.2, and 71.7% of ^{14}C-glyphosate absorbed remained in the treated leaf for S, R1, and R2 biotypes, respectively. In addition, 20.7, 11.7, and 14.5% had been translocated to roots and 22.4, 9.1, and 13.7% had been translocated to the rest of the shoot in S, R1, and R2 biotypes, respectively.

According to Lorraine-Colwill et al.,[24] Wakelin et al.,[16] Perez-Jones et al.,[25] and Yu et al.,[22] more glyphosate remained in treated leaves and less glyphosate was translocated to young leaves in resistant biotypes compared to susceptible ones; in contrast, in the susceptible biotype, a greater percentage of glyphosate is moved to untreated leaves, roots, and the stem, as observed in this study. This different pattern confirms a distinct behavior of herbicide translocation in susceptible and resistant *D. insularis* biotypes. Differences in ^{14}C-glyphosate translocation were confirmed through phosphorimaging (Figure 1). These results indicate that herbicide translocation was found as a mechanism of resistant to glyphosate.

^{14}C-glyphosate was widely distributed among leaves, root, and the rest of the shoot with appreciable acropetal and/or basipetal herbicide translocation in the S biotypes at 48 HAT (Figure 1). On the other hand, a low translocation of ^{14}C-glyphosate from treated leaf to the rest of the shoot was observed in R1 and R2 biotypes. In addition, an acropetal herbicide translocation was verified in the resistant biotypes. Like in the other species studied so far,[35] glyphosate was ambimobile in *D. insularis* (Figure 1), as seen from radioactivity migrating to the tips of the treated leaves (translocation in the xylem) and out of the treated leaves (translocation in the phloem) as well.[25] This suggests that an altered symplast transport is responsible for a differential glyphosate translocation between susceptible and resistant biotypes.[13,16,24] These authors discussed the existence of an altered cellular transport that would retain glyphosate in the apoplastic space, thus preventing its entry into the phloem tissues.

Altered glyphosate symplast transport has been associated with lack of translocation in weeds resistant to glyphosate, such as *Conyza* spp.,[13−15] *L. multiflorum*,[30] and *L. rigidum*.[24,29] An explanation for differences in glyphosate translocation was provided by Ge et al.,[19] studying *C. canadensis* resistance to glyphosate. Glyphosate enters the cytoplasm of both resistant and susceptible plant variants at the same rate, but begins to occupy the vacuole in the resistant but not the susceptible biotype. Glyphosate in the cytoplasmic pool is available for translocation to sink tissues. However, glyphosate sequestered within the vacuole is effectively removed from the phloem-accessible pool of glyphosate. The resistance mechanism for resistant plants reflects an inherent ability[36] to sequester glyphosate in the vacuole, where, presumably, it stays indefinitely or is released slowly at a sublethal rate.

Metabolism Study. A factorial scheme with three factors, biotypes, compounds, and evaluation times, was tested by ANOVA. Interaction between the studied factors was highly

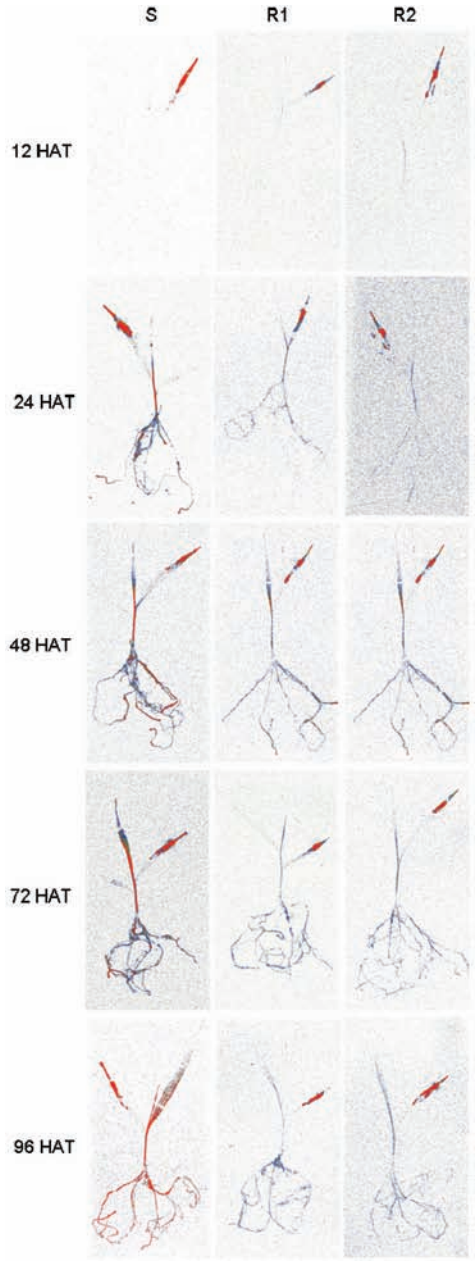

Figure 1. Phosphorimaging visualization of ^{14}C-glyphosate translocation in susceptible (S) and resistant (R1 and R2) *D. insularis* biotypes. HAT indicates hours after treatment. Intensity in red coloration shows greater ^{14}C-glyphosate concentrations.

significant ($P < 0.001$). Glyphosate decreased, whereas its metabolites increased, up to 168 HAT in the leaf tissues of both susceptible and resistant biotypes, but this event was much more intense in the resistant ones. More than 90% of glyphosate, in relation to its metabolites, was detected in the susceptible biotype up to 48 HAT, whereas 25.0 and 59.1% of glyphosate was observed in R1 and R2 biotypes, respectively. At 168 HAT, <10% of glyphosate was detected in the resistant biotypes, whereas about 80% of glyphosate was found in the susceptible biotype, in relation to its metabolites (Table 3).

In the susceptible biotype, AMPA and sarcosine were not detected up to 96 HAT, whereas almost 64 and 37% of AMPA and 15 and 32% of glyoxylate were found in R1 and R2 biotypes, respectively (Figure 2). In addition, sarcosine was also

Table 3. Relative Percentage of Glyphosate and Its Metabolites (AMPA + Glyoxylate + Sarcosine) in Susceptible (S) and Resistant (R1 and R2) *D. insularis* Biotypes, at Different Hours after Treatment (HAT) with Glyphosate

biotype	HAT	glyphosate[a]	metabolites[a]
S	48	92.0 ± 3.0 AB	8.0 ± 2.2 GH
	96	91.1 ± 2.9 AB	8.9 ± 0.5 GH
	168	80.2 ± 6.5 C	10.8 ± 0.5 F
R1	48	25.0 ± 3.3 F	75.0 ± 1.5 C
	96	10.0 ± 3.7 G	90.0 ± 5.9 B
	168	1.6 ± 0.6 GH	98.4 ± 1.2 A
R2	48	59.1 ± 1.1 D	40.9 ± 3.1 E
	96	23.7 ± 1.1 F	76.3 ± 2.1 C
	168	7.3 ± 0.2 GH	92.7 ± 3.9 AB

[a]Means within a column followed by the same letter are not significantly different at the 5% level as determined by the Tukey HSD test. Values ± standard errors of the mean.

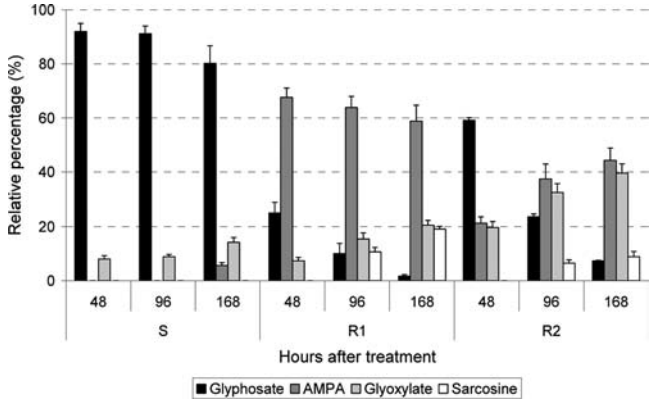

Figure 2. Relative percentage of glyphosate, aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine detected in leaves of susceptible (S) and resistant (R1 and R2) *D. insularis* biotypes, at different times after glyphosate application.

detected in the resistant biotypes. At 168 HAT, AMPA and glyoxylate were detected in the S biotype, indicating degradation of glyphosate, but, in the resistant biotypes, glyphosate was degraded to AMPA, glyoxylate, and sarcosine more rapidly than in the susceptible biotype. These results indicate that more rapid glyphosate degradation occurred in the resistant biotypes.

A difference in glyphosate degradation was also observed between the resistant biotypes. Glyphosate was more rapidly degraded in the R1 than in R2 biotype up to 168 HAT, when less glyphosate and more metabolites were detected in biotype R1 (Table 3 and Figure 2). This indicates that glyphosate metabolism in the R1 biotype was more intense than in biotype R2. The fact of increasing AMPA in the R2 biotype (Figure 2), whereas AMPA was decreased in the R1 biotype with time, supports this conclusion.

Two metabolic pathways were described for glyphosate degradation by soil microorganisms.[12,37] In plants, a first degradation pathway involves the herbicide degradation by glyphosate dehydrogenase enzyme action, originating AMPA and glyoxylate.[37] AMPA is the main metabolite originated from glyphosate degradation, whereas glyoxylate, despite being a metabolite derived of glyphosate degradation, is also a plant

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Figure 3. Partial protein sequence alignment of the EPSPS of S, R1, and R2 *D. insularis* biotypes. The highlighted color shows a proline (P) to threonine (T) and a tyrosine (Y) to cysteine (C) substitution at amino acids 182 and 310, respectively, in the resistant biotypes compared with the susceptible one (amino acid number based on the start codon (ATG) of *A. thaliana* [GenBank: CAA29828.1] EPSP sequence). An asterisk (∗) indicates differences in the protein sequence between *A. thaliana* and *D. insularis* biotypes.

endogenous metabolite involved in different metabolic pathways.[27] AMPA is degraded to methylamine by C−P lyase enzyme action, and by methylamine dehydrogenase enzyme action, methylamine generates formaldehyde. A second pathway is glyphosate degradation to sarcosine by direct C−P lyase enzyme action.[37] Therefore, the appearance of these metabolites indicates that both pathways should be part of the metabolism of glyphosate in plants. Thus, the relative percentage of glyphosate and these metabolites can be used as an indicator of glyphosate metabolism in plants.[27]

Glyphosate metabolism in plants is very limited. Although in some studies metabolism of glyphosate was demonstrated in plants, transformation products did not significantly reduce its phytotoxicity.[38,39] The fact that glyphosate metabolism does not contribute to glyphosate resistance has been reported in *L. rigidum* from Australia,[24,40] in *E. indica* from Malaysia,[41] and in horseweed across the United States.[13] Although evidence on the importance of glyphosate metabolism was not found as a mechanism of weed resistance,[12] according to the results of this study, we can affirm that the metabolism of this herbicide was found to play an important role in the resistance of *D. insularis* to glyphosate.

That differential metabolism associated with the initial difference in herbicide absorption, up to 48 HAT, allows us to affirm that a very low amount of glyphosate could attain its site of action in the resistant biotypes due to rapid herbicide degradation. This is not true for the susceptible biotype, because absorption was faster and degradation was very slower than for the resistant ones. In this case, metabolism also allows absorption to play an important role for glyphosate resistance in *D. insularis* at least up to 48 HAT.

***EPSPS* Gene Sequencing.** A 522 bp fragment of the *EPSPS* gene from susceptible and resistant *D. insularis* biotypes was sequenced. The predicted protein sequences were searched using the BLASTp algorithm. Results showed a high similarity with *E. indica* EPSPS proteins, GenBank accessions AEB80423.2, AEB80422.2, and CAD01096.1, showing *E* values of 8e−122, 1e−121, and 2e−121, respectively. Protein alignment between *A. thaliana* (GenBank: CAA29828.1) and *D. insularis* biotypes (Figure 3) showed a high similarity in the sequences of 83.3%.

The comparison of cDNA sequences of the *EPSPS* between R1, R2, and S biotypes presented many silent mutations. However, in the predicted protein sequences, both resistant biotypes (R1 and R2) showed two amino acid changes in comparison to the S biotype. One nucleotide change (cytosine to adenine, not shown) in the first position of codon 182 (considering position 1 as the starting methionine (ATG codon) of the *A. thaliana* protein) resulted in a proline (S biotype) to threonine (R1 and R2 biotypes) amino acid substitution; in codon 310, one nucleotide change in the second position (adenine to guanine, not shown) resulted in a tyrosine (S biotype) to cysteine (R1 and R2 biotypes) substitution (Figure 3).

Changes in the EPSPS amino acid sequence have been demonstrated to confer glyphosate resistance. Those changes include the proline to serine, proline to threonine, proline to alanine, and proline to leucine substitutions at position 106.[20−22,25,42−44] The proline to threonine amino acid change found at position 182 in our assays is consistent with that reported by Ng et al.[21] and Wakelin and Preston;[42] however, those authors reported this mutation at position 106 of the EPSPS protein instead of position 182 reported in this work. In vitro EPSPS assays will have to be conducted to determine the effect of the tyrosine to cysteine change found at position 310 to determine whether this mutation contributes to glyphosate resistance in *D. insularis* biotypes.

Summarizing, taken together, our results allow us to conclude that (1) shikimic acid does not accumulate in resistant biotypes after glyphosate application, whereas high accumulation occurs in the susceptible biotype, indicating the resistance of *D. insularis* to glyphosate; (2) initial herbicide absorption can play an important role as a mechanism of resistance up to 48 HAT, in association with metabolism, whereas a limited herbicide translocation plays a very important role as a mechanism of glyphosate resistance in *D. insularis*; (3) rapid degradation of glyphosate to AMPA, glyoxylate, and sarcosine indicates that herbicide metabolism plays an important role as a mechanism of glyphosate resistance in *D. insularis*; and (4) two amino acid changes at positions 182 and 310 in *EPSPS*, consisting of a proline to threonine and a tyrosine to cysteine substitution, respectively, in the resistant

biotypes, can contribute as a mechanism of glyphosate resistance in *D. insularis*.

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