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Absorption, Translocation, and Metabolism of Glyphosate and Imazethapyr in Smooth Pigweed with Multiple Resistance

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Abstract: The evolution of herbicide-resistant weeds is the major challenge for chemical management worldwide, increasing production costs, and reducing yield. This work aimed to evaluate the putative resistance of the *Amaranthus hybridus* population from Candido Mota (CMT) to glyphosate and imazethapyr and to investigate the non-target site mechanisms involved. Dose–response studies were conducted under greenhouse conditions and the control and biomass reduction were evaluated 28 days after application (DAA). Absorption, translocation, and metabolization studies were evaluated at 72 h after treatment (HAT) using radiometric techniques. The dose–response results show different responses among populations to glyphosate and imazethapyr. The CMT population was not controlled with labeled herbicide doses. Based on biomass reduction, the resistance factor was 16.4 and 9.4 to glyphosate and imazethapyr, respectively. The CMT absorbed 66% of ^{14}C-glyphosate and 23% of ^{14}C-imazethapyr at 72 HAT. Although the CMT population absorbed more glyphosate than the susceptible population (12.6%), translocation was impaired for both herbicides in the CMT when compared to the SUS population. There was no evidence that herbicide metabolization was involved in CMT resistance to the herbicides studied. Understanding the mechanisms endowing resistance allows better decision-making. This is the first study that describes non-target-site resistance mechanisms in an *Amaranthus hybridus* population from Brazil.

Keywords: weed control; herbicide resistance; *Amaranthus hybridus*; EPSPs inhibitors; ALS inhibitors; non-target site resistance

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Citation: Nalin, D.; Munhoz-Garcia, G.V.; Witter, A.P.W.; Takeshita, V.; Oliveira, C.d.; Adegas, F.S.; Tornisielo, V.L.; Oliveira Junior, R.S.d.; Constantin, J. Absorption, Translocation, and Metabolism of Glyphosate and Imazethapyr in Smooth Pigweed with Multiple Resistance. *Agronomy* **2023**, *13*, 1720. https://doi.org/10.3390/agronomy13071720

Academic Editors: Candelario Palma-Bautista, José Alfredo Domínguez-Valenzuela and Ricardo Alcántara-de la Cruz

Received: 5 May 2023
Revised: 19 June 2023
Accepted: 20 June 2023
Published: 27 June 2023

1. Introduction

The occurrence of herbicide-resistant weeds during the last few decades has been the main problem to implement chemical management by farmers. Selection pressure on weeds caused by herbicides has resulted in the evolution of resistance to at least one herbicide among 267 species worldwide [1]. Biotech crops such as the technology packages for HR (herbicide-resistant) and mainly GR (glyphosate-resistant) crops have been quickly adopted by farmers around the world [2,3]. Since then, an increase in herbicide selection pressure in a wide range of weeds has been evident [4]. Countries such as Brazil, the USA, India, China, and Argentina are among the biggest producers of soybeans and grains in the world [5]. The main GR crop (genetically modified crop) countries in 2018 were the USA

(75 million hectares), Brazil (51.3 million hectares), and Argentina (23.9 million hectares) [6]. Although herbicides are not the only form of selection pressure in modern agriculture, their use results in faster changes, and weed resistance is more easily observed [7]. HR weeds reduce weed control efficacy and increase production costs in crop production. For example, in corn, the presence of GR weeds decreases the control level by 30% and increases the cost of production [8], or in soybean, where the cost of production may increase from 42% to 222% depending on the HR weed species [9].

Several species of the genus *Amaranthus* are known for their importance as weeds within agricultural cropping systems [10]. Smooth pigweed (*Amaranthus hybridus* L.) is native to South America and widespread in agricultural systems in many countries around the world [11,12]. The genus contains broadleaf weed species with several traits which have become major global weeds that are very difficult to control, including the C4 photosynthetic pathway, a high growth rate, and great genetic variability. Besides, traits include tolerance to stress, high fecundity, a high viable seed production rate, and the ability to be selected for herbicide resistance [11,13–15]. The first report of HR *A. hybridus* was in the USA in 1972 to atrazine [1]. Since then, there have been reports including single, cross, and multiple HR cases in several countries such as Switzerland, South Africa, Italy, Israel, France, Canada, Bolivia, Argentina, Uruguay, Paraguay, and Brazil to many mechanisms of action (MOA) like acetolactate synthase inhibitors (ALS), enolpyruvyl shikimate phosphate synthase inhibitors (EPSPS), photosystem II inhibitors (PSII), protoporphyrinogen oxidase inhibitors (PROTOX), and auxin mimics [1].

The basis of *Amaranthus*'s chemical management used to be the use of ALS-inhibiting herbicides applied pre-emergence followed by glyphosate in the post-emergence of weeds after GR crop introduction [3,16]. This strategy was efficient for a decade in South America, but in 2014, an *A. hybridus* population with multiple resistance to glyphosate and imazethapyr was found in Argentina [1]. Some years later, multiple resistant to synthetic auxins and glyphosate populations were related in the same country [1,12]. In Brazil, Paraguay, and Uruguay, there are reports of *A. hybridus* GR populations and multiple-resistant populations to glyphosate and chlorimuron-ethyl [1,17]. These facts point out that herbicide resistance in *Amaranthus* species has become worrying due to the evolution of multiple resistant populations in recent years [1,3], in number and geographical area.

Some mechanisms explain the process of herbicide resistance in weeds. The target-site resistance (TSR) mechanism is usually single-gene inherited resistance [18–20]. The non-target-site resistance mechanism (NTSR) encompasses all mechanisms that minimize herbicide injury, such as limiting toxic herbicide concentrations reaching herbicide target sites (i.e., reduced herbicide leaf penetration, impaired herbicide translocation, and herbicide metabolism) [20,21]. The TSR mechanisms have been the most cited for glyphosate in different species of *Amaranthus* [22–26]. For resistant *Amaranthus*, target site modification to ALS inhibitors is the most common mechanism [27,28], involving imazethapyr [16,29–31]. Conversely, NTSR mechanisms have been published for many weeds [32–34], including the genus *Amaranthus* [35,36]. Compared to TSR mechanisms, very little is known about NTSR mechanisms [37], and there are still no NTSR mechanism studies for the resistant *A. hybridus* population in Brazil. Identifying resistance and understanding which mechanisms are used by weed species that allow for survival following herbicide application may mitigate further resistance in the future and provide insight for the development of new herbicides and HR crops [20].

Recent reports of *A. hybridus* resistant to EPSPS and ALS herbicides in South America including Brazil, Argentina, Paraguay, and Uruguay may indicate the spread of resistance in a region important to crop production and export. It can cause a substantial economic impact on agricultural systems. Early detection and proactive management may help to mitigate economic losses. The aim was to investigate the resistance factor (RF) of the CMT population collected and whether there were NTSR mechanisms responsible for conferring glyphosate and imazethapyr resistance. Our hypotheses were: (1) the CMT *A. hybridus*

population has evolved to glyphosate and imazethapyr resistance and (2) there are NTSR mechanisms involved in the resistance to the herbicides studied.

2. Materials and Methods

2.1. Plant Material for the Dose–Response Test

Plants from an *Amaranthus* population with putative resistance to glyphosate and imazethapyr were collected from a soybean field in Cândido Mota—SP, Brazil (CMT—22°44′02″ S, 50°25′27″ O) and a susceptible (SUS) population was collected from a field that has never received herbicide treatment in Florínea—SP, Brazil (SUS—22°50′40″ S, 50°33′59″ O) (Figure 1). The distance among the populations was about 25 km.

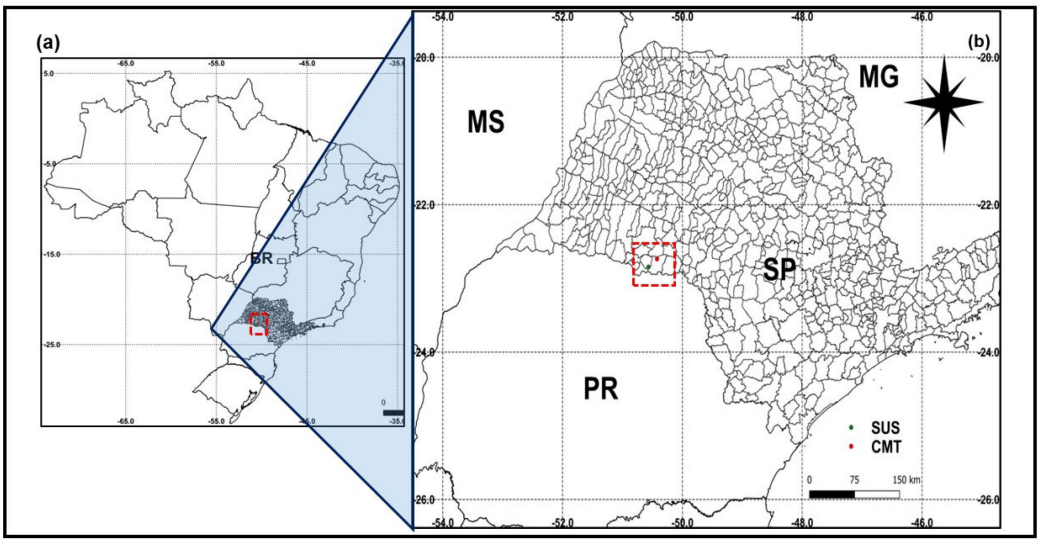

Figure 1. Cities where *Amaranthus hybridus* populations were collected in São Paulo State: (**a**) localization view of the cities at the Brazil level and (**b**) regional localization view of the cities at the São Paulo State level.

The CMT population was collected from a grain-producing area (soybean and corn). This area had a history of glyphosate and imazethapyr herbicide use. Farmers have reported difficulty in controlling these plants in the last few years.

2.2. Resistant Population Confirmation

2.2.1. DNA Extraction

Leaf tissue from the field plants was used for DNA extraction. Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. DNA quantification was performed using a Denovix instrument (Denovix, Wilmington, DE, USA), and DNA quality was verified on a 1% agarose gel. All DNA samples were diluted to 50 ng μL^{-1}, and an aliquot of 2 µL was used for posterior PCR amplification.

2.2.2. *Amaranthus* Species Determination

Species-specific primers available in the literature [38], which are based on intron 1 sequences from the EPSPS gene, were used for the molecular species determination of *Amaranthus* samples. The primer pairs AW473 and AW483 were used in the CMT and SUS populations. PCR reactions were performed in 25 µL volumes with the following reagents: 100 ng DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 200 nM primers, and one-unit GoTaq G2 Hot Start DNA Polymerase (Promega Corporation, Madison, WI, USA). The PCR cycle conditions were the same as those reported by [38]. Amplicons were verified for amplification of the expected band size on 1% agarose gels.

2.3. Dose–Response Assay

Plants from the putative-resistant population were treated using glyphosate (960 g ha^{-1}, Roundup Transorb R, 480 g a.e. kg^{-1}, Monsanto LTDA, São Paulo, SP, Brazil) and imazethapyr (100 g ha^{-1}, Pivot SL® 100 g a.i. L^{-1}, Indaiatuba, SP, Brazil). The surviving plants were kept in a greenhouse for seed production. Seeds from *Amaranthus* populations were germinated in 0.3 L pots filled with a commercial substrate. The plants were potted individually and grown in a greenhouse at 32 °C/25 °C (day/night) under a 15 h photoperiod until they reached the fourth leaf stage.

The dose–response test was performed with the fourth generation of *Amaranthus* (F4). The experiments were conducted separately for each herbicide. The design was completely randomized in a 2 × 9 factorial scheme for glyphosate and imazethapyr, with six replicates. The first factor was two *A. hybridus* populations (CMT and SUS), and the second factor was composed of nine herbicide doses of glyphosate (g a.e. ha^{-1}) or imazethapyr (g a.i. ha^{-1}), separately (Table 1). The same amount of herbicide treatment was maintained for the SUS and CMT populations. However, the doses were different because previous trials showed that the susceptible population was controlled with lower doses, such as 1440 g ae ha^{-1}. The common field doses recommended were 1440 and 100 g ha^{-1} for glyphosate and imazethapyr, respectively. The application was performed in a spray chamber equipped with flat fan nozzles (XR110.02; TeeJet®, Wheaton, IL, USA), calibrated to deliver 200 L ha^{-1} at 200 kPa pressure.

Table 1. Active ingredients, population, field dose (g a.i or e.a ha^{-1}), and doses of the herbicides used in dose-response assays on *Amaranthus hybridus*.

Herbicide	Population	Field Dose	Dose–Response (g a.i. or e.a. ha^{-1})
Glyphosate	CMT	1440 [a]	0, 360, 720, 1440, 2880, 5760, 11,520, 23,040, 46,080
	SUS	1440 [a]	0, 45, 90, 180, 360, 720, 1440, 2880, 5760
Imazethapyr	CMT	100 [b]	0, 25, 50, 100, 200, 400, 800, 1600, 3200
	SUS	100 [b]	0, 6.25, 12.5, 25, 50, 100, 200, 400, 800

[a] Glyphosate in grams of acid equivalent per hectare (g ae ha^{-1}). [b] Imazethapyr in grams of active ingredient per hectare (g ia ha^{-1}).

Plant control and dry mass were evaluated at 28 days after treatment (DAT). The control was assessed using a scale of 0% to 100% [39], where 0% represents no control and 100% represents plant death. The plants were harvested at 28 DAT and oven-dried for 48 h at 60 °C for dry mass determination.

2.4. Absorption, Translocation, and Metabolism Study

2.4.1. Preparation of ^{14}C-Glyphosate and ^{14}C-Imazethapyr Solution

^{14}C-glyphosate (98%) with a specific activity of 55 mCi mmol^{-1} (American Radiolabeled Chemicals, Sant Louis, MO, USA) and ^{14}C-imazethapyr (98%) with a specific activity of 20.66 µCi mg^{-1} (American Cyanamid, Bridgewater Township, NJ, USA) were used. The solution of ^{14}C-glyphosate had 200 µL of radiolabeled solution + conventional formulation (Roundup Transorb R, 480 g a.e. kg^{-1}) containing 66.6 KBq. The ^{14}C-imazethapyr solution was also prepared using a conventional formulation (Pivot SL® 100 g a.i. L^{-1}) mixed with ^{14}C-imazethapyr, resulting in 140 µL of the solution containing 46.6 KBq. In all studies, around 200,000 dpm plant^{-1} (3.3 KBq plant^{-1}) of ^{14}C-glyphosate and ^{14}C-imazethapyr were applied.

2.4.2. Absorption, Translocation, and Metabolism of ^{14}C-Glyphosate and ^{14}C-Imazethapyr

The experimental design was completely randomized in a factorial scheme with two herbicides (glyphosate and imazethapyr) and two *A. hybridus* populations (CMT and SUS), and there were three biological replicates for absorption and translocation. The same design was used for the metabolism experiment. The studies were conducted at

the same time. The fourth youngest leaf was covered with plastic, and non-radiolabeled commercial formulations of glyphosate or imazethapyr were applied to the plants with a CO_2 pressure backpack sprayer calibrated to 200 L ha^{-1} and 250 kPa, equipped with a flat fan spray nozzle (model XR 11002), placed 50 cm from the plants. ^{14}C-glyphosate and ^{14}C-imazethapyr were applied to the fourth fully expanded leaf at a dose of 1440 g a.e. ha^{-1}, and 100 g a.i. ha^{-1} respectively. The plastic bags were taken off to apply the radiolabeled formulations using an automatic microsyringe (Hamilton PB6000 Dispenser, Hamilton, CO, USA). Ten 1-µL droplets were then applied to the upper side of the leaf of each plant.

^{14}C-glyphosate and ^{14}C-imazethapyr absorption and translocation were evaluated 72 h after application (HAT). The treated leaves were subjected to a 3 mL washing procedure using methanol:water solution (50:50, *v*/*v*). Subsequently, two aliquots (500 µL each) of the leaf-washing solution were acquired to enable the quantification of radioactivity via liquid scintillation spectrometry (LSS). This quantification aimed to determine the proportion of herbicide that remained unabsorbed to the total amount applied. The plants were then uprooted, and their roots were washed. Following the washing process, all parts of the plants were dried at a temperature of 45 °C for 24 h using an oven. Subsequently, a qualitative evaluation of the plants was conducted by capturing autoradiography images using a radio scanner (Packard-Cyclone, Perkin Elmer, Shelton, CT, USA). The dried plants were divided into five sections for quantitative analysis, which included the treated leaf, leaves situated above the treated leaf, leaves located below the treated leaf, the stem, and the root. Each section was subjected to combustion in a biological oxidizer (OX 500—R.J. Harvey Instrument Co., Tappan, NY, USA) at a temperature of 950 °C for 3 min. The resultant $^{14}CO_2$ produced during the combustion process in the presence of oxygen was captured in vials containing a scintillation solution. Radioactivity was measured and quantified using LSS. The absorbed percentages of ^{14}C-glyphosate and ^{14}C-imazethapyr were determined by calculating their ratios to the total level of radioactivity initially applied. To assess the translocated herbicide percentage, the radioactivity of each plant section (excluding the treated leaf) was combined and calculated relative to the total absorbed radioactivity.

Metabolism studies were conducted at the same time as absorption and translocation studies. The ^{14}C-imazethapyr was extracted according to Xu et al. (2022) [40] with some changes. After washing, the whole plants applied with ^{14}C-imazethapyr were ground with liquid nitrogen and put into a 50 mL Teflon tube; 4 mL of acetonitrile was added containing 1% acetic acid and 0.5 g NaCl. The tubes were shaken in a tube shaker (AP56-Phoenix. Trammit Medical, Belo Horizonte—MG, Brazil) for two min and centrifuged at 1700× *g* under 4 °C for 5 min. The supernatant obtained from each tube was transferred to a separate tube. Three consecutive cycles of agitation and centrifugation were carried out. After that, the liquid in the second tube was dried in a current of N_2 at 10 kPa and 40 °C (TurboVap® LV, Caliper LifeScience, Hopkinton, MA, USA) and resuspended with 200 µL of acetonitrile for two minutes in an ultrasonic bath (Bransonic®, Branson Ultrasonics Corporation, Danbury, CT, USA). ^{14}C-glyphosate extraction was carried out according to Monquero et al. (2004) [41], with adaptation to the centrifugated conditions and methanol amount used to resuspend the samples. The plants were washed and after, they were grounded with liquid nitrogen and put into a 50 mL Teflon tube. Next, 3 mL of methanol (80%) was added into the tubes and then, they were shaken in a tube shaker (AP56-Phoenix. Trammit Medical, Belo Horizonte—MG, Brazil) for two min, and centrifuged at 7000× *g*, under 4 °C for 10 min. The supernatant from each tube was withdrawn and placed into a second tube. Agitation and centrifugation were performed three times consecutively. After that, the liquid collected in the second tube was dried in a current of N_2 at 10 KPa and 40 °C (TurboVap® LV, Caliper LifeScience, Hopkinton, MA, USA) and resuspended with 200 µL of methanol for two minutes in an ultrasonic bath (Bransonic®, Branson Ultrasonic Corporation, Danbury, CT, USA).

Metabolite separation from imazethapyr and glyphosate was achieved using thin-layer chromatography (TLC) with silica gel plates employed as the stationary phase using 100 µL from ^{14}C-herbicide extracted solution per plant. A different solvent system was used as the mobile phase for each herbicide. For imazethapyr, a mixture of chloroform:methanol (90:10 *v*/*v*) and for glyphosate a mixture of ethanol:water:15 N NH_4 OH:trichloroacetic acid (TCA):17N acetic acid (55:35:2.5:3.5 g:2, *v*/*v*/*v*/*w*/*v* with v in mL) were used, according to Xu et al. (2022) [40] and Monquero et al. (2004), and Sprankle et al. (1978) [41,42], respectively. The TLC plates were placed into a glass container containing the mobile phase of the elution process. The readings of the TLC plates were analyzed using a Cyclone® Plus radio scanner (Packard-Cyclone, Perkin Elmer, Shelton, CT, USA). The chemical identity of ^{14}C-glyphosate and ^{14}C-imazethapyr was determined by comparing their retention factor (Rf) values with those of the respective radiolabeled analytical standards.

2.5. Statistical Analysis

The dose–response curve for the control and dry mass was submitted using a three-parameter nonlinear regression model described by Equation (1) [43]. The model parameters were estimated using the drc package in R software [44].

$$y = \frac{a}{\left[1 + \left(\frac{x}{b}\right)c\right]} \quad (1)$$

where y represents the dependent variable (control or dry mass), x represents the independent variable (herbicide dose), a represents the maximum value, b represents the curve slope around c, and c represents the dose that provides 50% response (LD_{50} or GR_{50}). The RF was calculated as the ratio of the LD_{50} and GD_{50} values among the putative-resistant and sensitive populations.

The data regarding herbicide absorption and translocation were assessed for homogeneity of variance and normality of errors. Under the assumption of meeting these criteria, the data underwent analysis of variance (ANOVA) using the F test ($p < 0.05$). Significantly different means were then compared with the Tukey test ($p < 0.05$) (Supplementary Tables S1 and S2).

3. Results

3.1. Amaranthus Species Determination

Molecular species determination using species-specific primers [38] confirmed that the tested populations are *A. hybridus* consistent with morphological identification. The primers for *A. hybridus* were tested with positive controls to amplify the fragment, thereby confirming the specificity and functionality of these primers.

3.2. Plant Dose–Response Curve

Dose–response analysis showed higher control and biomass reduction as the glyphosate dose increased ($p < 0.05$). There were different responses among the populations (Figure 2a,b). Based on the LD_{50} values, the resistance factor (RF) was 13.1, and based on the GR_{50} value, the RF was 16.4 (Table 2) for the CMT population. The highest dose applied in this study (46,080 g a.e. ha^{-1}) was effective in controlling the CMT population (Supplementary Figure S1). The model estimated 29,910.5 and 11,318.4 g a.e. ha^{-1} for LD_{90} and GR_{90}, respectively, for CMT; those are 20× and 7.8× times higher than the labeled dose (1440 g a.e. ha^{-1}). On the other hand, the model estimated 1909.7 and 428 g a.e. ha^{-1} for LD_{90} and GR_{90}, respectively, for SUS.

A non-significant effect was observed for the control in the CMT population for the imazethapyr doses ($p > 0.05$) (Table 2). However, the biomass reduced when the imazethapyr doses increased ($p < 0.05$). The observed response was different among the populations (Figure 2c,d). Based on the GR_{50} value, the RF was 9.4 (Table 2). The highest dose of imazethapyr (3200 g a.i. ha^{-1}) was not effective in completely killing the CMT population (Supplementary Figure S2). The estimative of the model for 90% effective biomass

reduction is 3494 g a.i. ha^{-1}, which is 34× times higher than the field-recommended dose. For the SUS population, the model estimated 39.9 g a.e. ha^{-1} for GR_{90}.

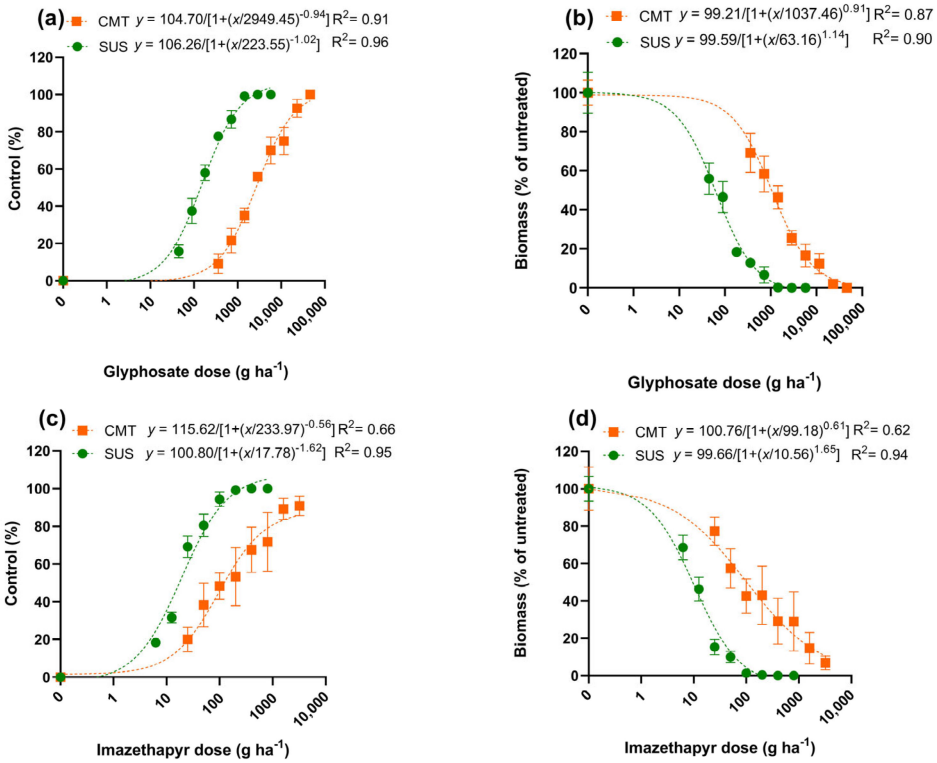

Figure 2. Dose–response curves for the CMT population with multiple resistance to glyphosate and imazethapyr at 28 DAA. (**a**) Control levels and (**b**) biomass reduction under glyphosate doses, and (**c**) control levels and (**d**) biomass reduction under imazethapyr doses. Model fit: $y = a/[1 + (x/c)^b]$. Error bars represent the standard error of the means (n = 6).

Table 2. Glyphosate and imazethapyr dose of 50% and 90% of the control (LD), biomass reduction (GR), and resistant factor (RF) in Smooth pigweed (*Amaranthus hybridus*).

Glyphosate	**Population**	
	SUS	**CMT**
LD_{50}	223.5 ± 40	2949.4 ± 677
RF [1] (LD_{50})	-	13.1
GR_{50}	63.1 ± 13	1037.4 ± 233
RF [1] (GR_{50})	-	16.4
LD_{90}	1909.7 ± 895	29,910.5 ± 19,623
GR_{90}	428 ± 135	11,318.4 ± 3789
Imazethapyr		
LD_{50}	17.7 ± 2.4	233.9 ± 258.1 [ns]
RF [1] (LD_{50})	-	13.2
GR_{50}	10.5 ± 1.8	99.1 ± 36.2
RF [1] (GR_{50})	-	9.4
LD_{90}	68.8 ± 24.0	11,812.1 ± 29,221.3 [ns]
GR_{90}	39.9 ± 12.4	3493.3 ± 2095.7

[1] Calculated by the ratio of LD_{50} and GR_{50} of CMT/SUS. Model fit: $y = a/[1 + (x/c)^b]$. ns: model is not significant ($p \leq 0.05$).

3.3. Absorption and Translocation of ^{14}C-Glyphosate and ^{14}C-Imazethapyr

The absorption and translocation of ^{14}C-glyphosate and ^{14}C-imazethapyr are visually represented in Figure 3 (Tables S1 and S2). Significative effects were found in the interaction

between factors (population × herbicide) for absorption ($p < 0.05$). The CMT absorbed 66.79 ± 2.1% of the ^{14}C-glyphosate total initially applied and SUS absorbed 54.1 ± 6.9% 72 HAA, indicating no differences within the population for glyphosate absorption (Figure 4a). The absorption of ^{14}C-imazethapyr in the resistant population (CMT, 23.8 ± 4.7%) was 2.1-fold smaller than in the susceptible population (51 ± 9.3%). Although the absorption patterns between the herbicides were different, this is not related to resistance. The differences are due to the physical–chemical characteristics of the herbicides.

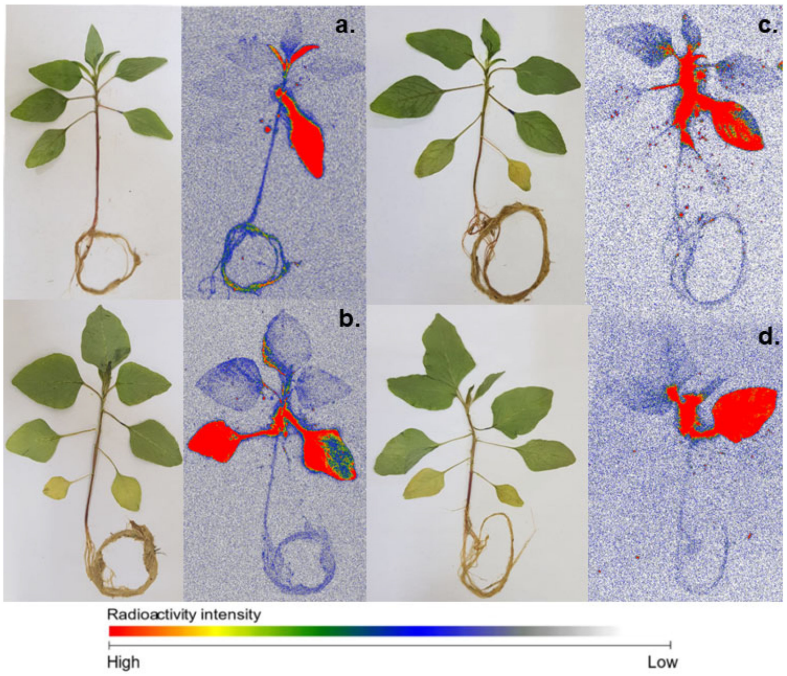

Figure 3. Decreased glyphosate translocation patterns in the *Amaranthus hybridus* CMT population (**a**) compared to the SUS population (**b**), and the translocation patterns of imazethapyr in the CMT (**c**) and SUS (**d**) populations. The red color indicates the major intensity of radioactivity in different parts of the plants.

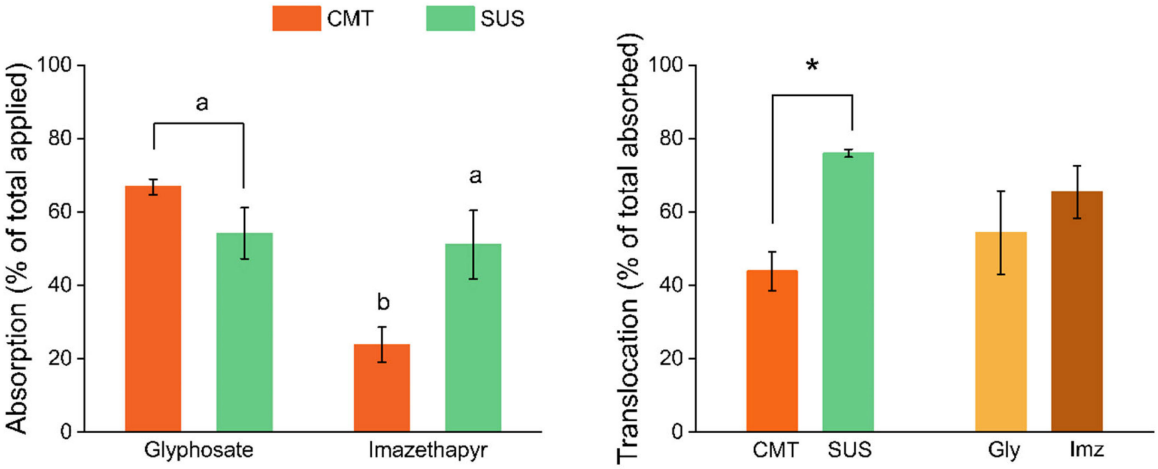

Figure 4. Absorption (a) and translocation (b) of glyphosate and imazethapyr in the CMT and SUS *Amaranthus hybridus* populations at 72 HAT. The populations mean followed by the same lower-case letter do not differ by Tukey test ($p < 0.05$). In translocation data (b), * indicates differences in the populations factor ($p < 0.05$), due to non-significant interaction between both, the population and herbicides application factors.

No significant effects were observed for the interaction between population and herbicides ($p > 0.05$) in ^{14}C-glyphosate and ^{14}C-imazethapyr translocation (Figure 4b). However, the population influenced both herbicide translocations ($p < 0.05$), where CMT plants translocated 43.8 ± 5.2% of the herbicide absorbed (regardless of the herbicide) and SUS plants translocated 75.9 ± 1%. A restricted translocation of herbicides in CMT plants indicates a different pattern of translocation among the tested populations.

3.4. Metabolism of ^{14}C-Glyphosate e ^{14}C-Imazethapyr in Plants

No glyphosate metabolites were found in SUS and CMT populations at 72 HAT (Supplementary Figure S3). The retention factors (Rf) were 0.3 for sample plants and 0.2 for the analytical standard of glyphosate (Supplementary Table S3), indicating high similarity in the RF value. For plants treated with imazethapyr, three metabolites were found in SUS and CMT populations at 72 HAT (Supplementary Figure S4). The analytical standard RF of imazethapyr was 0.4. The retention factors for three metabolites (M) were 0.1 (M1), 0.2 (M2), and 0.33 (M3) for samples of the SUS population, and the CMT population also showed three metabolites with RF values of 0.1, 0.2, and 0.3 for M1, M2, and M3 (Supplementary Table S4), respectively.

4. Discussion

4.1. Plant Dose-Response Curve

The evolution of HR weeds has increased due to the selection pressure caused by the intense use of the same herbicide mechanisms [1,7]. The rapid identification of resistant weeds and the evolutionary processes that drive this phenomenon will aid the proper development of alternative strategies to allow for successful control through integrated weed management programs [45]. Here, we found different responses to glyphosate and imazethapyr among *A. hybridus* populations (SUS and CMT). Resistance to glyphosate was confirmed for the CMT *A. hybridus* population with an RF of 13.1 (based on LD_{50}) and 16.4 (based on GR_{50}). Similar RF (13 and 14) values for glyphosate-resistant *A. hybridus* populations in Brazil were found by [17]. The resistance factor (RF) shows how much more resistant an investigated population is to an herbicide compared to a known susceptible plant of the same species. After glyphosate application, chlorosis followed by necrosis in the leaves was observed in the SUS population (observation data), but the same was not observed in the CMT population. In sensitive plants, shikimate accumulates after inhibition of EPSPS activity (which inhibits the synthesis of tyrosine, phenylalanine, and tryptophan) and leads to eventual plant death [46,47].

In South America, the first report of glyphosate-resistant *A. hybridus* was in Argentina in 2013 with an RF of 314 [1,25]. Another study found high RF values (125) in a different population in the same country [3]. In Brazil the first report was in 2018, the population was considered to have multiple resistance to glyphosate and chlorimuron (EPSPs and ALS inhibitors) [1]. Resistance to imazethapyr was confirmed for the CMT population based on GR_{50} with an RF of 9.4. Acetolactate synthase (ALS) is a critical enzyme that catalyzes the first step of the biosynthesis of essential amino acids, i.e., valine, leucine, and isoleucine [48]. The highest imazethapyr dose (3200 g a.i. ha^{-1}) was not effective in controlling all the plants from the CMT population (Supplementary Figure S2), and the GD_{90} estimated was 3493.3 g a.i. ha^{-1}. Recently, cross-resistance to imazethapyr and chlorimuron-ethyl was confirmed in Argentina to *A. hybridus* [31]. Another study in Brazil found cross-resistance to ALS-inhibiting herbicides in an *A. hybridus* population, with resistance factors of 6.9 and 6.5 for chlorimuron and metsulfuron-ethyl, respectively, but resistance to imazethapyr was not verified [49].

The results have confirmed the CMT population's resistance to glyphosate and imazethapyr. However, it is important to understand the mechanisms responsible for herbicide resistance. Thus, we started to investigate the influence of the NTSR mechanisms of resistance (absorption, translocation, and metabolization) in the CMT population.

4.2. Absorption and Translocation of ^{14}C-Glyphosate and ^{14}C-Imazethapyr

Reduced absorption and impaired translocation are important NTSR mechanisms for weed resistance [37]. When compared with the TSR mechanism, relatively few cases in which the cause of resistance is related to NTSR mechanisms have been described. An experimental approach with radiolabeled herbicides allows one to unequivocally identify lower absorption and reduced movement of the herbicide in plants [50]. In this research, populations (CMT and SUS) showed quantitative and qualitative results that revealed altered patterns of ^{14}C-glyphosate and ^{14}C-imazethapyr absorption and translocation.

Our results show no differences among populations for the absorption of ^{14}C-glyphosate at 72 HAT (Figure 4a). On the other hand, the CMT population showed a decrease in absorption for ^{14}C-imazethapyr at 72 HAT (27.2%). Despite that, there is less translocation in CMT (43%) than in SUS (75%) for both herbicides evaluated (Supplementary Table S2). The impaired translocation may be related to the greater retention of herbicide near the treated area [51,52]. These results are consistent with those previously observed in other *Amaranthus* species for glyphosate [23,35,53], and other weed species [47,51,53–55]. Although not evaluated in this research, many authors have shown that glyphosate sequestration into the vacuole is the main NTSR mechanism responsible for altering the translocation patterns of this herbicide [56–58].

Based on the results of ^{14}C-glyphosate and ^{14}C-imazethapyr translocation, reduced translocation appears to be partly responsible for resistance, thus suggesting that the NTSR mechanisms occur in the CMT population. Reports on the translocation and absorption of herbicides in *A. hybridus* are rare in the literature. Despite that, a decrease in translocation of glyphosate has already been reported as being a resistance mechanism in *Amaranthus palmeri* [35], *Amaranthus tuberculatus* [23], *Lolium perenne* [54], *Digitaria insularis* [51], and *Lolium rigidum* [59]. For the ALS-inhibiting herbicides, three major mechanisms of resistance have been identified: metabolism of their chemical structures, mutation of the active site within ALS, and restriction of cell permeability [60]. Most of the characterization studies of resistance mechanisms support that resistance to ALS inhibitors is mainly due to mutations (TSR) at key positions of the encoding ALS gene [61]. Therefore, more TSR mechanism studies are necessary to investigate whether they are related to CMT resistance to imazethapyr and glyphosate. These results reveal that the non-target site mechanisms based on decreased translocation play an important role as a mechanism involved in the resistance to glyphosate and imazethapyr in the CMT population.

4.3. ^{14}C-Glyphosate and ^{14}C-Imazethapyr Metabolism

In some weed species, at least more than one resistance mechanism has been reported for glyphosate [23,55,62]. The non-target site mechanisms of weed resistance may be due to metabolic alterations. Plants contain numerous enzyme-encoding genes that facilitate biochemical reactions involved in the synthesis of secondary metabolites and for detoxifying xenobiotic compounds (e.g., herbicides) [63]. Some authors have proposed glyphosate metabolism as a mechanism of resistance in some weed species [52,64], a mechanism not detected in SUS and CMT populations (Supplementary Figure S3). The analytical standard of glyphosate shows an RF value of 0.2, and a similar RF value of 0.3 (Supplementary Table S3) was verified in the samples (SUS and CMT). Evaluating the separation of glyphosate in TLC, Sprankle et al. (1978) [42] observed RF values of 0.2–0.3 with the same solvent system. Other studies have found glyphosate RF values of 0.2–0.3 [41] and 0.36 [65]. Our results do not support the involvement of glyphosate metabolism in the resistance of the CMT population.

In the plants treated with ^{14}C-imazethapyr, both populations (SUS and CMT) show three metabolites (Supplementary Figure S4). However, there was no difference among the metabolites formed in the SUS and CMT populations. The data show evidence of metabolization but this does not appear to be responsible for the resistance to imazethapyr in the CMT population. Thus, it will be necessary to study more about imazethapyr

metabolization in *A. hybridus* plants to conclude whether there is an influence of this mechanism on imazethapyr resistance in the CMT population.

5. Conclusions

The results confirmed that the CMT *A. hybridus* population is resistant to glyphosate and imazethapyr with RFs of 16.1 and 9.4, respectively. Impaired translocation in plants treated both with imazethapyr and glyphosate has contributed to resistance in the CMT population. Moreover, the CMT population absorbed less imazethapyr than the SUS population. There was no evidence that herbicide metabolism has influenced the resistance to glyphosate and imazethapyr in the CMT population. TSR mechanism studies are recommended to investigate whether they are related to CMT resistance to imazethapyr and glyphosate.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071720/s1. Figure S1: Evaluation of *A. hybridus* after 28 DAA of glyphosate: (a) SUS and (b) CMT population; Figure S2: Evaluation of *A. hybridus* after 28 DAA of imazethapyr: (a) SUS and (b) CMT population; Figure S3: The metabolization of ^{14}C-glyphosate in *A. hybridus* population at 72 HAT; Figure S4: The metabolization of ^{14}C-imazethapyr in *A. hybridus* population at 72 HAT; Table S1: Absorption of ^{14}C-Glyphosate and ^{14}C-Imazethapyr in the SUS and CMT *Amaranthus hybridus* populations; Table S2: Translocation of ^{14}C-Glyphosate and ^{14}C-Imazethapyr in the SUS and CMT *Amaranthus hybridus* populations; Table S3: The metabolization of ^{14}C-glyphosate in the SUS and CMT *Amaranthus hybridus* populations; Table S4: Retention factor (Rf) to ^{14}C-imazethapyr and metabolites in the SUS and CMT *Amaranthus hybridus* populations.

Author Contributions: Conceptualization, J.C., F.S.A., C.d.O. and R.S.d.O.J.; methodology, R.S.d.O.J., D.N., A.P.W.W., G.V.M.-G., V.T. and V.L.T.; formal analysis, D.N. and G.V.M.-G.; data curation, J.C. and R.S.d.O.J.; writing—original draft preparation, D.N., G.V.M.-G., V.T. and R.S.d.O.J.; writing—review and editing, D.N., G.V.M.-G. and V.T.; supervision, R.S.d.O.J., J.C. and V.L.T.; project administration, D.N., A.P.W.W. and C.d.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are grateful to the State University of Maringá (UEM), Advanced Studies Nucleus in Weed Science (NAPD), the Coordination for the Improvement of Higher Education Personnel (CAPES), and the Center of Nuclear Energy in Agriculture (CENA) from University of São Paulo. Furthermore, we are grateful to Sandra Mathioni (Syngenta) for molecular analyses for species identification and for the collaboration of the laboratory technician Rodrigo Floriano Pimpinato (CENA/USP). The authors thank Fundação de Estudos Agrários Luiz de Queiroz (FEALQ) for the contribution in the payment of APC.

Conflicts of Interest: The authors declare no conflict of interest.

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