BIOSCIENCE JOURNAL

GLYPHOSATE-RESISTANT HAIRY FLEABANE (*Conyza bonariensis*) EXHIBITS A LARGER NUMBER OF TRICHOMES AND ALTERED STOMATAL DENSITY RELATIVE TO THE SUSCEPTIBLE COUNTERPART

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How to cite: BACCIN, L.C., et al. Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) exhibits a larger number of trichomes and altered stomatal density relative to the susceptible counterpart. *Bioscience Journal*. 2023, **39**, e39047. https://doi.org/10.14393/BJ-v39n0a2023-64343

Abstract

Following the adoption of Roundup Ready crops, glyphosate spraying frequency increased, while the use of other herbicide modes of action was neglected. Herbicide-resistant biotypes were reported in three major *Conyza* species in Brazil, including *Conyza bonariensis*, increasing growers' bottom line. Considering that leaf surface structures affect proper herbicide deposition, uptake, and performance, this study aimed to characterize epicuticular surface components in glyphosate-resistant (R) and -susceptible (S) *C. bonariensis*. *Conyza* spp. seeds were collected in 36 locations in Brazil, and plants were subjected to resistance screening tests by spraying glyphosate at 720 and 1440 g ae ha^{-1} (0.5X and 1X the label recommended rate, respectively). For resistance level characterization, *C. bonariensis* biotypes with contrasting responses were selected for glyphosate dose-response assays. Leaf tissues for epicuticular surface analysis were harvested from newly-obtained R and S biotypes at two growth stages. Histological cuts were made on a leaf area of 25 mm^2 with a blade. Samples were fixed in Karnowsky solution, gradually changed to 100% ethanol, critical-point dried with CO^2, and coated with gold, followed by stomatal and trichome density quantification using scanning electron microscopy. Results indicated a poor control with glyphosate in 33 of 36 *Conyza* spp. biotypes, and a high (31.5) resistance factor was calculated after dose-response trials. Leaf surface analysis indicated that *C. bonariensis* leaves are amphistomatic and exhibit tectorial trichomes. A higher number of trichomes and altered stomatal density (number.mm^2) were quantified in R compared to the S counterpart, potentially reducing glyphosate uptake and effectiveness.

Keywords: Herbicides. Scanning Electron Microscopy. Stoma. Leaf surface.

1. Introduction

Weed resistance to herbicides occurs naturally and stems from high genetic variability within a plant species, allowing for a specific set of plants to survive and reproduce following exposure to a lethal herbicide rate to the wild biotype. Herbicide resistance is a widely-known and acknowledged phenomenon, with first reports dating back to the late 1950s and 1960s (Switzer 1957; Whitehead and Switzer 1963; Ryan 1970).

Studying resistance mechanisms is vital because it allows for a clearer understanding of resistance evolution and ensures the discovery of valid management options. Resistance mechanisms can be traced

down to target-site (TSR) and non-target-site (NTSR) changes, which ultimately impact herbicide efficacy (Gaines et al. 2020). TSR mechanisms include mutations at genes encoding proteins or enzymes inhibited by a given herbicide and gene amplification. NTSR mechanisms include a broader category of enhanced herbicide metabolism, altered herbicide uptake or translocation, and herbicide compartmentalization in organelles.

Weed species of the *Conyza* genus (syn. Erigeron; commonly named marestail, fleabane, or horseweed) show high morphological similarities and can hybridize. However, these characteristics impair their proper identification in the field, mainly because three common *Conyza* species can simultaneously occur in Brazil: *Conyza bonariensis* (L.) Cronq., *C. sumatrensis* (Retz.) E.H. Walker, and *C. canadensis* (L.) Cronq. (Marochio et al. 2017; Pedroso et al. 2021). Worryingly, there are, to date, nine confirmed cases of herbicide-resistant *Conyza* populations in Brazil, three of which relate to multiple herbicide resistance (Heap 2022) - when two or more herbicide modes of action are affected. One such case regards resistance to five different action modes in C. sumatrensis, including an unprecedented resistance to 2,4-D conferred by rapid necrosis (Queiroz et al. 2020). Other cases have been correlated to herbicide compartmentalization (Ge et al. 2010, 2011), enhanced activity of detoxifying enzymes (Piasecki et al. 2019), and decreased glyphosate movement out of the treated leaf, impairing translocation (Cardinali et al. 2008; Ferreira et al. 2008).

Post-emergent herbicides are valuable options for controlling herbicide-resistant *Conyza* spp. (Cantu et al. 2021; Pedroso et al. 2021). Even though post-emergent herbicide absorption can occur upon contact of molecules with leaves, stems, flowers, and even fruits, the former represents the primary route for herbicide uptake in weeds (Silva 2000). Crucially, leaf morphology directly affects the amount of herbicide intercepted and retained by the foliage of weeds, hence playing a crucial role in overall herbicide absorption rates.

Besides leaf anatomy and architecture, the growth stage might also affect chemical weed control efficacy due to changes in photosynthate partitioning, which, in turn, alter herbicide translocation. Growers are usually advised to spray herbicides onto young (4-8 leaf stage) *Conyza* plants to optimize control efficacy (Johnson and Hoverstad 2002; Silva 2002; Marques et al. 2012) because the overall control of well-established and mature Conyza plants drops substantially.

Anatomical leaf structures have affected the uptake of post-emergent herbicides such as glyphosate in several weed species. Therefore, the present study aimed to (i) select glyphosate-resistant (R) and -susceptible (S) *Conyza* spp. biotypes using dose-response assays and (ii) characterize and compare the epicuticular surface of newly obtained glyphosate-R and -S *C. bonariensis* biotypes to uncover potential differences affecting glyphosate efficacy among biotypes.

2. Material and Methods

Selection of herbicide-R and -S biotypes

Conyza spp. seed samples were collected in August 2018 in 36 locations across five municipalities in southeast Brazil (Table 1). Seeds were sown into plastic flats filled with potting soil, and *Conyza* spp. seedlings were later transplanted into 0.8L pots filled with a 1:1 mix of potting and field soils. The pots remained in a greenhouse at an average temperature of 26°C and average relative humidity of 60% and were irrigated with 5 mm of water daily to prevent moisture stress. To characterize the response to glyphosate of 36 biotypes, plants at the 4-6 leaf stage were treated with glyphosate (Zapp Qi™) at 720 (0.5X) and 1440 (1X) g acid equivalent ha^{-1} using a CO^2-pressurized backpack sprayer equipped with Magnojet 110.02 AD flat-fan nozzle tips, previously calibrated to deliver 200 L ha^{-1} at 200 kPa, and placed 0.5 m above the target plants.

Control efficacy was scored weekly from 7 to 28 days after spraying (DAS) using a percent scale by ALAM (1974) (Table 2). Then, biotype responses were compared to the untreated control. Biotypes whose control after glyphosate spraying was regarded as poor (0-40%) were considered resistant, while those showing excellent control levels (near or equal to 100%) were regarded as susceptible accessions. Two

biotypes with contrasting responses to glyphosate were then selected and subjected to dose-response assays. Both biotypes were identified as *C. bonariensis*, according to Lazarotto et al. (2008).

Table 1. Sample ID, city and state, and geographical coordinates depicting harvest locations for 36 *Conyza* spp. biotypes.

Sample	City/State	Latitude (S)	Longitude (W)	Sample	City/State	Latitude (S)	Longitude (W)	Sample	City/State	Latitude (S)	Longitude (W)
1	Piracicaba/SP	22°42'31.8"	47°37'42.8"	13	Piracicaba/SP	22°42'00.4"	47°37'57.1"	25	Matão/SP	21°35'16.4"	48°18'45.2"
2	Piracicaba/SP	22°42'30.5"	47°37'42.5"	14	Piracicaba/SP	22°41'58.5"	47°37'49.9"	26	Matão/SP	21°35'31.4"	48°19'44.7"
3	Piracicaba/SP	22°42'27.3"	47°37'40.5"	15	Jaboticabal/SP	21°11'03.3"	48°22'19.3"	27	Matão/SP	21°39'54.3"	48°21'25.9"
4	Piracicaba/SP	22°42'28.6"	47°37'38.3"	16	Jaboticabal/SP	21°11'10.7"	48°22'47.9"	28	Matão/SP	21°55'06.9"	48°36'96.9"
5	Piracicaba/SP	22°42'30.9"	47°37'40.9"	17	Jaboticabal/SP	21°11'15.5"	48°23'01.9"	29	Tabatinga/SP	21°44'31.3"	48°38'46.7"
6	Piracicaba/SP	22°42'53.1"	47°37'18.5"	18	Jaboticabal/SP	21°11'11.8"	48°23'10.3"	30	Tabatinga/SP	21°44'33.3"	48°39'05.6"
7	Piracicaba/SP	22°42'54.9"	47°37'07.2"	19	Jaboticabal/SP	21°10'33.1"	48°23'00.7"	31	Tabatinga/SP	21°44'25.8"	48°40'04.3"
8	Piracicaba/SP	22°42'49.4"	47°37'12.7"	20	Jaboticabal/SP	21°10'28.7"	48°22'40.7"	32	Dois Córregos/SP	22°18'24.7"	48°22'48.5"
9	Piracicaba/SP	22°42'59.0"	47°37'04.1"	21	Jaboticabal/SP	21°10'39.5"	48°22'14.8"	33	Dois Córregos/SP	22°18'28.4"	48°22'52.6"
10	Piracicaba/SP	22°43'04.4"	47°36'58.9"	22	Jaboticabal/SP	21°12'07.9"	48°24'48.7"	34	Dois Córregos/SP	22°18'24.4"	48°22'58.8"
11	Piracicaba/SP	22°42'10.6"	47°37'54.1"	23	Jaboticabal/SP	21°12'41.4"	48°24'34.7"	35	Dois Córregos/SP	22°18'16.1"	48°22'43.3"
12	Piracicaba/SP	22°42'04.5"	47°37'52.7"	24	Jaboticabal/SP	21°11'14.2"	48°22'47.1"	36	Dois Córregos/SP	22°18'17.3"	48°22'09.6"

Table 2. Rating scale developed by ALAM (1974) used for visual control assessments.

Index	% Control	Control level
1	91-100	Excellent
2	81-90	Very good
3	71-80	Good
4	61-70	Sufficient
5	41-60	Fair
6	0-40	None or very poor

Glyphosate dose-response assays

The previously selected glyphosate-R and -S *C. bonariensis* biotypes were subjected to dose-response assays at the University of São Paulo, Piracicaba campus, SP/Brazil (22˚42'35.20"S, 47˚37'47.01"W; 610 m elevation). Mature seeds were harvested from untreated control plants at the end of the initial glyphosate response screening trial and sown onto plastic flats filled with potting soil. Next, seedlings were transplanted onto 0.8L pots filled with a 1:1 mix of potting and field soils. The pots remained in a greenhouse under similar conditions to those described previously.

Biotype responses to glyphosate were determined by treating plants at the recommended growth stage (4-6 leaf growth stage) with glyphosate (Zapp Qi™) at 0, 180,360, 720, 1440, 2880, 5760, 11520, and 23040 g ae ha^{-1}, corresponding to rates from 1/8 to 16 times the label recommended use rate, and replicated four times. Treatments were applied with a bench-type sprayer equipped with a Magnojet 110.02 AD flat-fan nozzle calibrated to deliver 200 L ha^{-1} at 200 kPa, placed 0.5 m above target plants.

Visual control was evaluated up to 28 DAS, according to ALAM (1974), and aboveground biomass was harvested afterward by cutting plants at the soil level. Samples were then stored in a dry chamber at 65°C until constant weight, obtaining dry aboveground biomass values. Visual control and dry aboveground biomass data were subjected to analysis of variance (ANOVA) and adjusted to the following non-linear regression model by Streibi (1988), when suitable:

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

where *y* is the response variable (percent of weed control or dry aboveground biomass); *x* is the herbicide rate (g ae ha^{-1}); and *a*, *b*, and *c* are equation parameters indicating the amplitude between the highest and lowest curve values, the dose that decreases the variable by 50%, and the slope of the curve around *b*, respectively. Following visual assessment, the required herbicide rates for reducing plant growth by 50% and 80% (ED_{50} and ED_{80}, respectively) were obtained with the RStudio 2021.09.1 build 372 (RStudio Team 2022) and compared.

Characterization of the epicuticular surface

To characterize leaf morphology, newly-obtained glyphosate-R and -S *C. bonariensis* biotypes were seeded directly onto plastic pots filled with a 1:1 mix of potting and field soils. Then, the pots remained in a greenhouse under similar conditions to those described previously. Experimental units were randomized following a completely randomized design and rearranged weekly.

The R and S biotypes were histologically analyzed with scanning electron microscopy at two growth stages, namely the 4- and 8-10 leaf stage. Upon harvest, histological cuts were made on a leaf area of 25 mm^2 with a blade. Leaf tissues were then prepared according to Kitajima and Leite (1998) by first allowing for tissue fixation for 24 hours in a Karnowsky solution, followed by rinsing in a 0.5-M cacodylate buffer solution. All samples for electro-scanning were then gradually dehydrated with increasing concentrations of ethanol (30, 50, 70, 90, and 100%) and critical-point dried using liquid CO_2.

Upon drying, stubs were mounted with histological leaf cuts and coated with gold. Analyses were performed on an LEO 435 VP Scanning Electron Microscope (LEO Electron Microscopy Ltd., Cambridge, England) at 20 kV, and the resulting images were micrographed. Three replicates for each biotype and growth stage combination were used, and 9-11 micrograph images were taken per replicate (sub-samples) for stomatal and trichome density assessments. Samples were prepared in duplicates. One half was used for evaluating both the adaxial and abaxial (upper and lower) sides of the leaf, and the other half for lateral view micrographs of the leaf profile. Stomatal and trichome densities were quantified on both leaf surfaces (1 mm^2), and mean values were obtained and expressed in number.mm^2.

3. Results

The initial glyphosate resistance screening test (Table 3) indicated that just three of 36 *Conyza* spp. biotypes collected were considered susceptible (S) to glyphosate, as indicated by 100% control (i.e., death of plants) recorded 28 days after spraying (DAS) glyphosate at 1X (1440 g ae ha^{-1}). Conversely, poor (≤40%) control levels 28 DAS were scored for the remaining 33 *Conyza* spp. biotypes (Table 3). Twelve of such biotypes received a control score of zero (e.g., 0% control) 28 DAS, indicating an absolute absence of glyphosate-related damage to treated plants, which matched the growth levels of the untreated control counterparts.

These findings allowed selecting biotypes (samples) P9 and P5 for further studies because they originated from nearby regions and are accordingly referred to as R and S biotypes for displaying either poor (0%) or excellent (100%) control levels after glyphosate spraying at 1440 g ae ha^{-1}, respectively (Table 3). Therefore, seeds of untreated plants were hand-harvested by gently shaking into paper bags and stored. These newly obtained biotypes were identified as *Conyza bonariensis*, according to Lazarotto et al. (2008).

Table 4 presents non-linear regression parameters obtained after glyphosate dose-response assays. The high resistance level of the R (P9) biotype to glyphosate is evidenced by its calculated ED_{50} value (6115.2 g ae ha^{-1}), which is 8.5 times higher than the labeled glyphosate rate. In contrast, an ED_{50} of 194.1 g ae ha^{-1} was calculated for the S biotype (P5). These differences are more evident when comparing the resulting dose-response curves prepared with the data from 28 DAS (Figure 1). An overall resistance factor of 31.49 was calculated based on the ED_{50} values of biotypes. This indicates that glyphosate would have to be sprayed at a rate nearly 31.5 times higher to allow for a 50% control level in R compared to the S counterpart, which is unfeasible from a management standpoint due to high costs, safety concerns, and

environmental issues. Overall, these biotypes were suitable for further characterization of the epicuticular leaf surface because they almost matched the results of the initial glyphosate resistance screening tests.

Table 3. Glyphosate resistance screening test results. Values represent mean visual control values of 36 *Conyza* spp. biotypes collected in Southeast Brazil at 28 days after spraying (DAS) of glyphosate at 1X (1440 g ae ha^{-1}). Only data from the last assessment (28 DAS) are presented as these better reflect control efficacy relative to assessments performed right after spraying.

Sample	City/State	% Control	Sample	City/State	% Control
1	Piracicaba/SP	0	19	Jaboticabal/SP	0
2	Piracicaba/SP	10	20	Jaboticabal/SP	0
3	Piracicaba/SP	15	21	Jaboticabal/SP	0
4	Piracicaba/SP	20	22	Jaboticabal/SP	10
5	Piracicaba/SP	100 *	23	Jaboticabal/SP	15
6	Piracicaba/SP	100	24	Jaboticabal/SP	10
7	Piracicaba/SP	10	25	Matão/SP	30
8	Piracicaba/SP	100	26	Matão/SP	40
9	Piracicaba/SP	0 *	27	Matão/SP	30
10	Piracicaba/SP	10	28	Matão/SP	30
11	Piracicaba/SP	15	29	Tabatinga/SP	40
12	Piracicaba/SP	20	30	Tabatinga/SP	40
13	Piracicaba/SP	20	31	Tabatinga/SP	30
14	Piracicaba/SP	15	32	Dois Córregos/SP	0
15	Jaboticabal/SP	5	33	Dois Córregos/SP	30
16	Jaboticabal/SP	0	34	Dois Córregos/SP	0
17	Jaboticabal/SP	0	35	Dois Córregos/SP	0
18	Jaboticabal/SP	0	36	Dois Córregos/SP	0

*Biotypes selected for further investigation (i.e. dose-response assays and leaf epicuticular surface characterization).

Table 4. Non-linear regression model parameters calculated from dose-response control data obtained 28 days after glyphosate spraying onto newly-obtained glyphosate-resistant (P9) and -susceptible (P5) biotypes.

Biotype	a [a]	b (ED_{50})	c	RF [b]	ED_{80}	RF [c]
Resistant (P9)	142.74	6115.21	-0.53	31.49	84109.06	211.31
Susceptible (P5)	101.01	194.15	-1.93	-	398.03	-

[a] Non-linear regression parameters obtained from the equation $Y= a/ [1 + (x/b)^c]$, where *y* represents the response variable (percent weed control); *x* is the herbicide rate (g ae ha^{-1}); and *a*, *b*, and *c* are equation parameters indicating the amplitude between highest and lowest curve values, the dose which decreases the variable by 50%, and the slope of the curve around *b*, respectively; [b] resistance factor calculated as ED_{50} (R)/ED_{50} (S);[c] resistance factor calculated as ED_{80} (R)/ED_{80} (S).

The leaf surface analysis of the present study indicated that *C. bonariensis* leaves are amphistomatic because they show stomata on both leaf surfaces (Figures 2 and 3). Furthermore, stomata are anomocytic (i.e., irregular-celled) and present on upper and lower leaf surfaces. The stomatal density of each biotype changes over time (Table 5). Compared to younger stage-1 plants (E1 in Table 5), the stomatal density of more developed R plants (E2) increased more at the abaxial (lower) surface and slightly decreased at the adaxial (upper) surface. Unlike R, stomatal density in the S biotype decreased with plant growth at both abaxial and adaxial surfaces.

C. bonariensis also showed long and thin-pointed trichomes of unicellular and multicellular tectorial types (Figures 2D and 3A), which presented high densities (number.mm^2) at the adaxial and abaxial sides of the leaf (Figures 2D and 3B). Trichome density highly increased over time (i.e., from stage-1 to stage-2 plants) on both leaf surfaces in the R biotype (P9), which density increased to 54% and 33% at the abaxial and adaxial leaf surfaces, respectively. Conversely, trichome density decreased on both leaf surfaces by at least 29% when S plants were at the 8-10 leaf stage (e.g., stage 2) compared to younger stage-1 plants. Lastly, even though the technique did not allow the differentiation of other mesophyll structures, positioning the leaf cuts on their side allowed better visualization of trichomes and leaf surface cells (Figure 4).

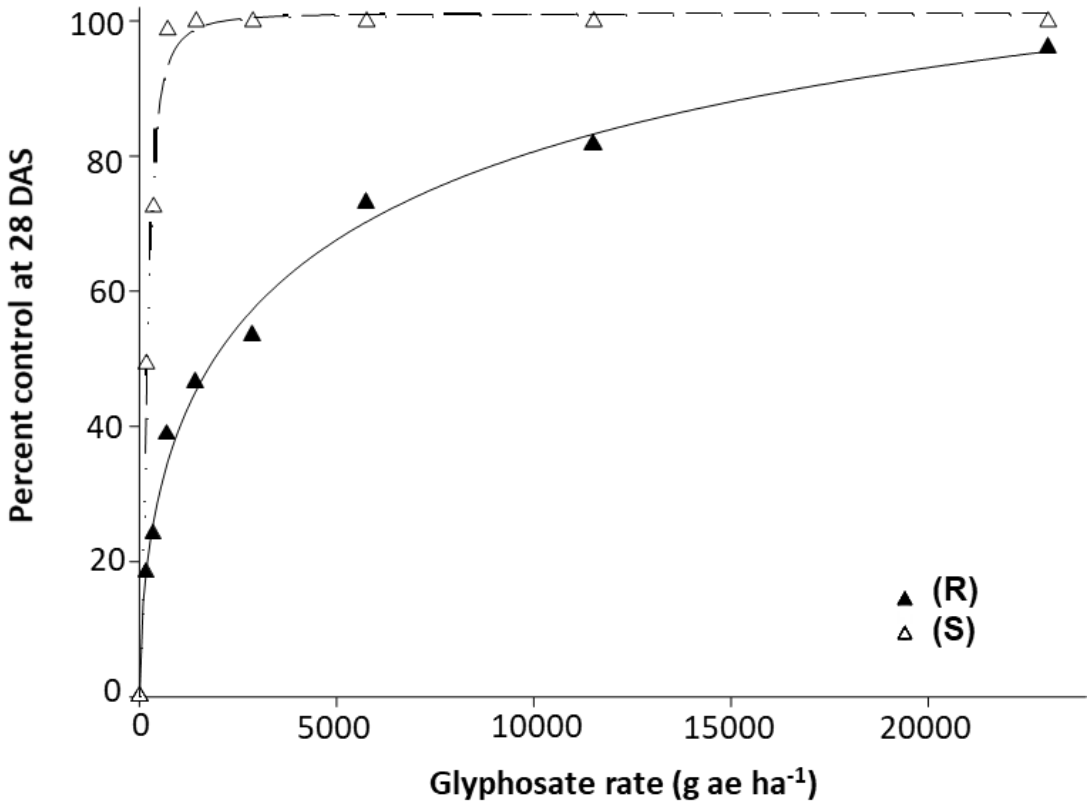

Figure 1. Dose-response curves prepared from control data of newly-obtained glyphosate-R and -S *Conyza bonariensis* biotypes. Symbols represent average control values for each biotype and rate, obtained 28 days after glyphosate spraying.

Table 5. Stomata and trichome density (number mm^2) at the abaxial (lower) and adaxial (upper) leaf surfaces in newly-obtained glyphosate-resistant (P9) and -susceptible (P5) biotypes. Numbers represent mean density values obtained at two growth stages, namely E1 (4-leaf) and E2 (8-10 leaf stage) from 9-11 micrograph images taken per replicate.

Biotype	Growth stage [a]	Trichome density		Stomata density	
		Abaxial	Adaxial	Abaxial	Adaxial
		number mm^2			
Resistant (P9)	E1	15.0	18.2	116.0	73.3
	E2	23.1	24.2	154.6	61.0
Susceptible (P5)	E1	13.2	13.8	156.6	120.0
	E2	7.7	9.3	90.5	103.7

[a]Growth stage during epicuticular surface characterization; E1 represents leaf cuts obtained from plants the 4- leaf stage whereas E2 is the 8-10 leaf stage.

4. Discussion

The present study collected 36 *Conyza* spp. biotypes in various cropping systems in southeast Brazil and found a complete absence of control upon glyphosate spraying at 1440 g ae ha^{-1} (1X) on a third of these biotypes (i.e., 12 of 36) (Table 3). In turn, only three biotypes were considered susceptible (S) to glyphosate, indicated by 100% control 28 days after spraying (DAS). Herbicide resistance is the ability of a given population or set of plants within a species to survive and reproduce after herbicide treatment at a lethal rate to the wild biotype (Pedroso et al. 2021), which was observed in this study. These results were also a troubling confirmation of the extent of glyphosate-resistant (R) populations in the sampled regions. The S biotypes are from plants collected in secluded fields at the University of São Paulo, Piracicaba campus, SP/Brazil, where glyphosate is not often used. However, the ability of *Conyza* spp. seeds to disperse over large distances is well known (Shields et al. 2006; Moreira et al. 2007).

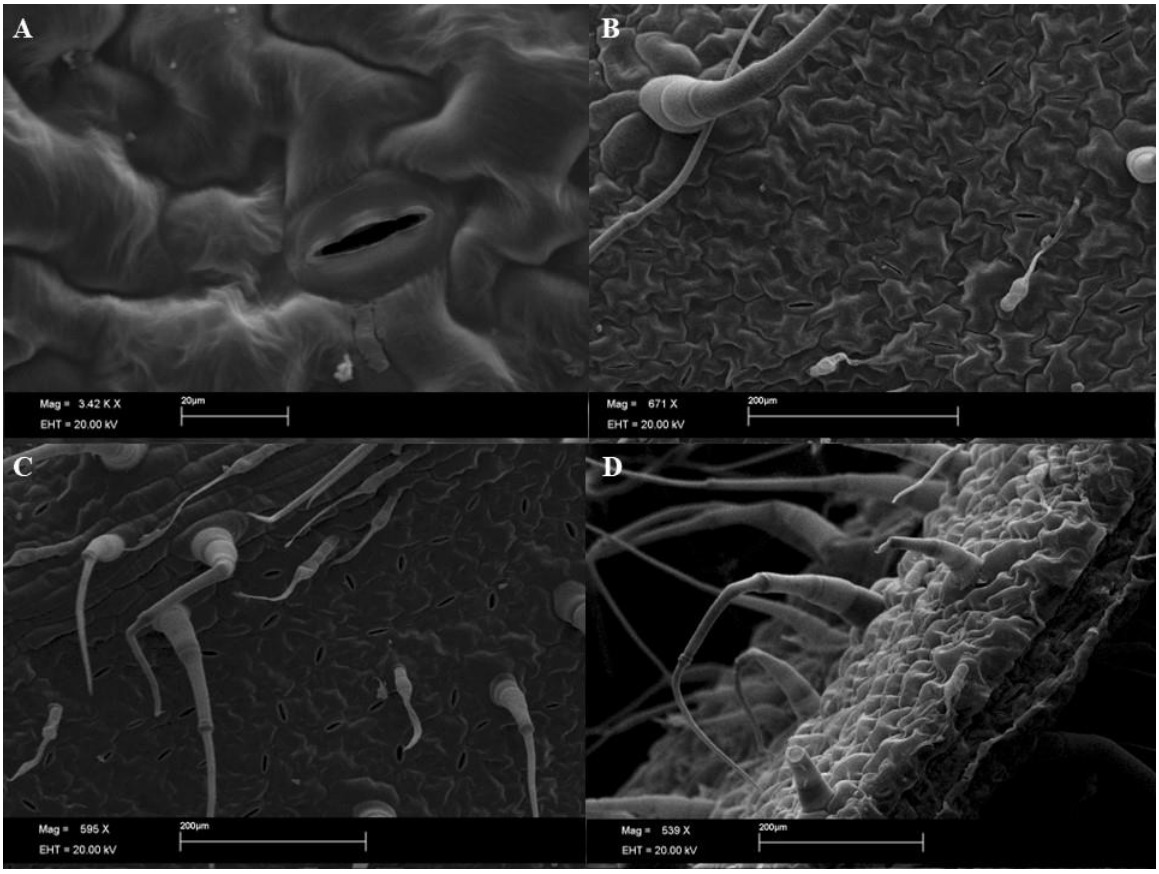

Figure 2. Glyphosate-resistant *Conyza bonariensis* leaf electron micrograph images. A - open stoma (400x magnification); B - adaxial side of the leaf (600x mag); C - tectorial trichomes on the abaxial side (595x mag); D - leaf side view exhibiting tectorial trichomes and mesophyll cells.

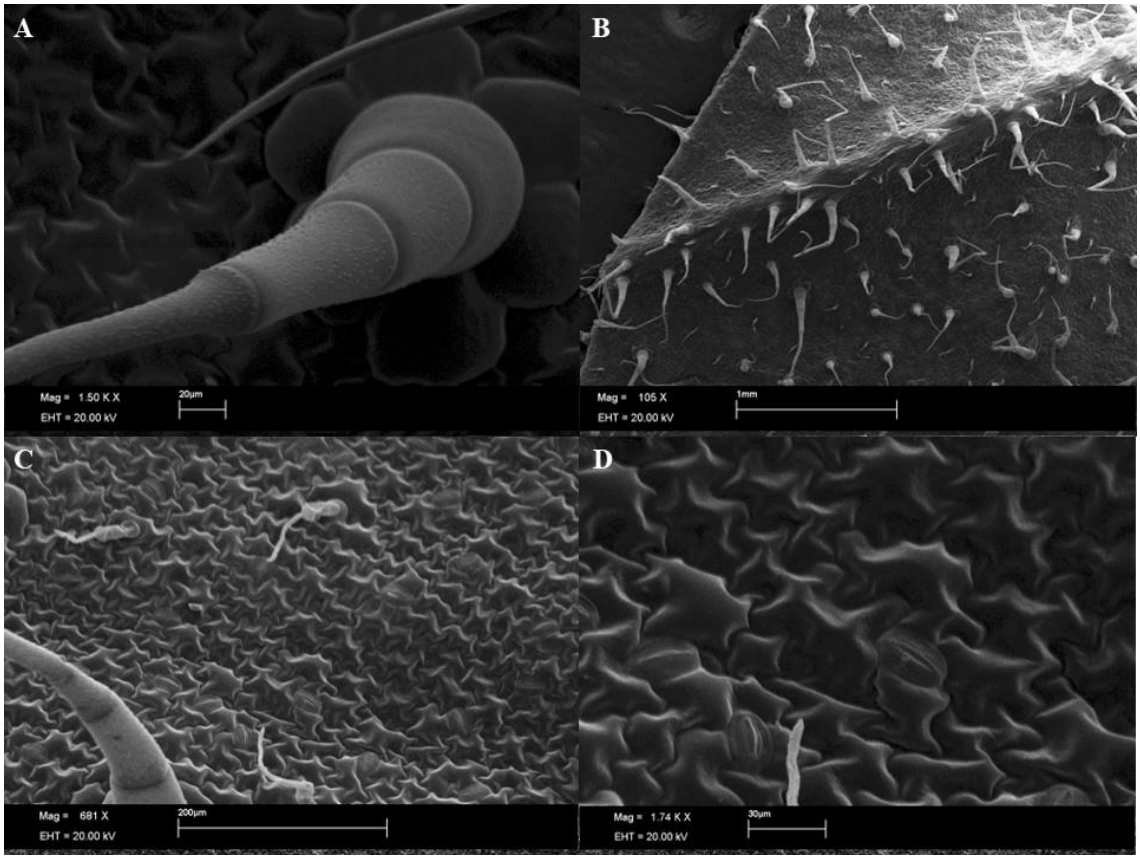

Figure 3. Glyphosate-susceptible *Conyza bonariensis* leaf electron micrograph images. A - multicellular tectorial trichomes (1,500x mag); B - abaxial (lower) surface at (100x mag); C - tectorial trichomes on the adaxial (upper) surface (600x mag); D - stomata and cells on the adaxial surface (1,700x mag).

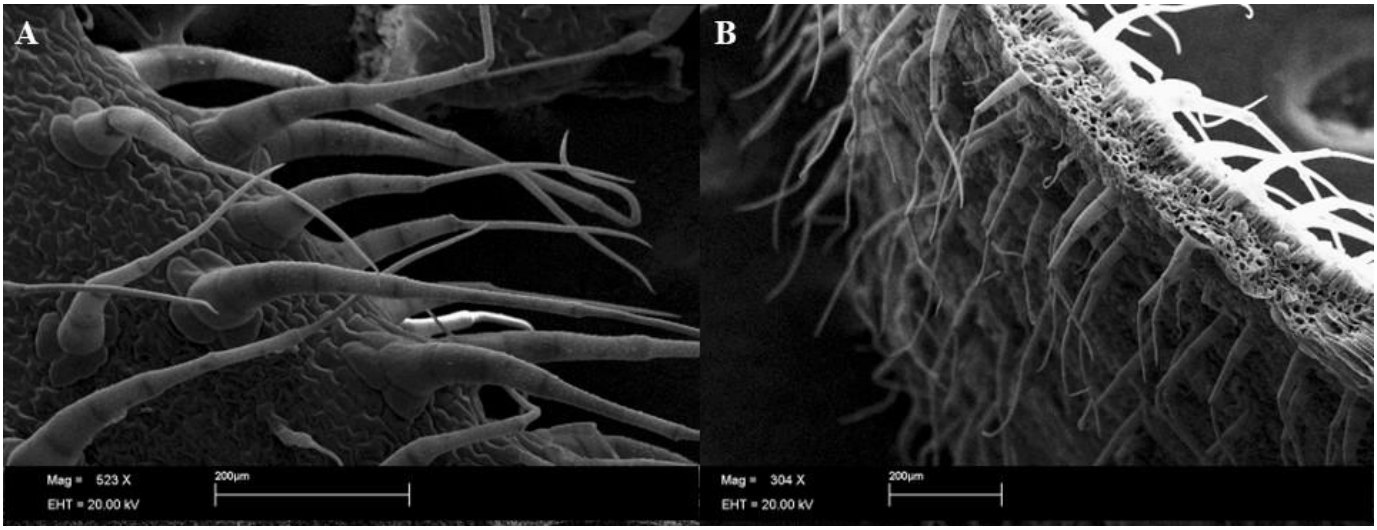

Figure 4. Glyphosate-resistant *Conyza bonariensis* leaf electron micrograph images. A - tectorial trichomes (500x mag); B - leaf cut positioned on its side to allow for better visualization mesophyll and trichome structures (300x mag).

Worryingly, a third of the *Conyza* spp. biotypes collected (i.e., 12 of 36 biotypes) did not show damage from glyphosate spraying at 1X at the end of the trials, an indication of high glyphosate resistance levels. These biotypes originated from grain production fields of the university campus and roadsides and sugarcane/peanut rotation fields in the municipalities of Jaboticabal, Matão, and Tabatinga. The control of biotypes collected in macadamia production fields in Dois Corregos was also poor. Overall, poor *Conyza* spp. control might relate to an intense resistance selection pressure imposed by frequent glyphosate applications (López-Ovejero et al. 2008), which occur in these areas or their vicinities.

Environmental variables such as geographical landscape, temperature and precipitation regimes, and other abiotic stress sources can deliberately alter a given species' genome over time compared to individuals growing in different locations or biomes. That, in turn, can lead to significant genetic differences and heterogeneity among organisms of the same species and affect chemical weed control efficacy (Vila-Aiub et al. 2011). Such allelic variation can be minimized or accounted for with weed ecotypes, such as biotypes that evolved in the same ecoregion as a form of genetic background control so that other genetic differences in these ecotypes might not impact herbicide efficacy, especially when assessing fitness costs associated with resistance. Accordingly, the present study selected glyphosate-R and -S accessions for glyphosate dose-response assays (Table 1) due to their sampling proximity, as they come from areas roughly 2 km apart at the University of São Paulo campus, thus evolving in a similar environment.

Dose-response curves from dry aboveground biomass data (Figure 5) closely resemble those from control data and confirmed the high resistance levels to glyphosate initially hypothesized when analyzing the initial glyphosate resistance screening trials (Table 3). Both biotypes decreased almost 80% in biomass accumulation upon spraying at the highest glyphosate rate (23040 g ae ha^{-1}). However, the S biotype had experienced this level of biomass loss at a rate as low as 720 g ae ha^{-1} (0.5X), and biomass loss of the R biotype was still close to 50% at the 2X rate (2880 g ae ha^{-1}), further indicating its high resistance level. These results corroborate Moreira et al. (2007), who also found that much higher glyphosate rates were required to decrease biomass accumulation of R *Conyza bonariensis* and *C. canadensis* populations compared to their S counterparts.

The high resistance factor presented suggests an intense selection pressure through successive and frequent glyphosate spraying, allowing the survival and selection of *C. bonariensis* individuals with decreased susceptibility to glyphosate. This, in turn, has been associated with naturally-occurring physiological and/or morphological adaptations, which can be transferred to the progeny (Moreira et al. 2007).

The first confirmed case of *C. bonariensis* resistance to glyphosate occurred in Africa in 2003, followed by similar reports in Spain in 2004 and Brazil in the following year (Heap 2022) when Vargas et al.

(2007) reported a lack of control on several biotypes after glyphosate spraying in the field. Upon further tests in the greenhouse, these authors reported that the R biotype plants showed up to 50% damage even when applying 5760 g ae ha^{-1}, and S biotypes were controlled by glyphosate spraying at 360 g ae ha^{-1}, which closely resembles the findings of the present study.

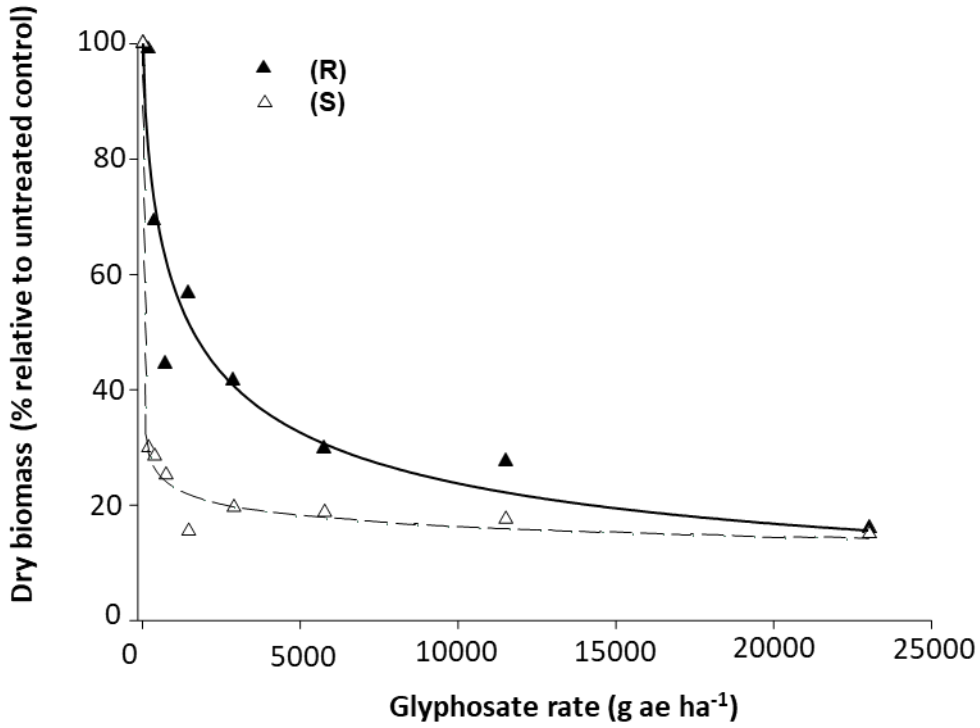

Figure 5. Dose-response curves prepared from dry aboveground biomass data of newly-obtained glyphosate-R and -S *Conyza bonariensis* biotypes. Biomass was harvested 28 days after glyphosate spraying and are expressed as % values relative to accumulation measured at the untreated control.

Leaf epicuticular surface characterization assays revealed that glyphosate-R *C. bonariensis* plants at the 8-10 leaf stage (stage 2 in Table 5) had 70% higher stomatal density at the abaxial (lower) leaf surface compared to the S counterpart. However, there was a 42% decrease in stomatal density at the adaxial (upper) surface in R compared to S plants. Despite a common misconception, higher stomatal densities do not enhance herbicide uptake or efficacy. Accordingly, the literature indicates that uptake occurs primarily through the leaf cuticle and intercellular spaces rather than at stomata openings (Greene and Bulkovac 1974; Schonherr 2006; Barroso et al. 2015). Nonetheless, higher stomatal densities incur more stomata guard cells. The latter usually shows fewer waxes, which, in turn, might facilitate herbicide uptake (Schonherr 2006).

In the present study, a glyphosate-R *C. bonariensis* biotype (P9) with high resistance levels to this EPSPs-inhibiting herbicide consistently showed higher trichome density (number.mm^2) than the glyphosate-S biotype (P5), regardless of the plant growth stage and leaf surface. For instance, older stage-2 R plants presented increased trichome density of 200% and 160% at the abaxial and adaxial leaf surfaces, respectively, compared to S (Table 5). Furthermore, trichome density in the R biotype increased with the plant growth stage, while the opposite holds for the S biotype. Trichomes are tiny outgrowths from the plant epidermis that help maintain a water-vapor saturated atmosphere around the leaves, reducing the gradient for transpiration losses. They also indirectly help regulate leaf temperature by reflecting incident solar radiation. From a weed control standpoint, these structures might intercept spray droplets, decreasing chemical control efficacy by preventing herbicide deposition and uptake through the cuticle (Larcher 2000; Procópio et al. 2003).

Variations in leaf protective structures between glyphosate-R and -S *C. bonariensis* might impact deposition, retention, uptake, and translocation of foliar-applied herbicides, such as glyphosate, working as a barrier and decreasing the number of molecules reaching the target site. Such alteration at the adaxial

leaf side might constitute a significant obstacle for herbicide uptake in R because spray droplets are largely distributed onto this surface due to its position and angle relative to the ground (Procópio et al. 2003). This condition would ultimately cause higher or lower susceptibility to means of chemical control. Accordingly, modifications in leaf morphology among glyphosate-R and -S populations have been reported in *Lolium multiflorum* Lam. (Italian ryegrass), *Digitaria insularis* (L.) Fedde (sourgrass), *Chloris elata* Desv. (tall windmill grass), and paraquat-resistant *C. sumatrensis* (Galvani et al. 2012; Barroso et al. 2015; Placido 2018; Pereira 2019). Increased trichome density and epicuticular waxes have also been associated with decreased glyphosate uptake through the cuticle and lower overall control efficacy in the troublesome glyphosate-tolerant weed species *Commelina benghalensis* L. (Bengal dayflower or tropical spiderwort) (Monquero et al. 2005).

It is also worth noting that mechanisms of evolved herbicide resistance might be correlated to one or more factors, and more than one mechanism might be implicated in the ability of a biotype to survive a lethal herbicide rate to the wild biotype. Feng et al. (2004) indicated lower glyphosate translocation associated with compartmentalization as likely mechanisms of resistance to glyphosate in *C. canadensis*, which Ge et al. (2010) later confirmed as rapid vacuolar sequestration. Using glyphosate formulations labeled with ^{14}C-glyphosate, Ferreira et al. (2008) provided evidence of lower translocation to sink tissues in R *C. bonariensis* biotypes, which showed higher glyphosate retention in treated leaves than the S counterpart.

5. Conclusions

There was a high incidence of *Conyza bonariensis* biotypes whose control after glyphosate spraying was considered poor or insufficient in the sampled region. Comparisons between newly obtained R and S biotypes indicated a high resistance factor. Trichome and stomatal densities significantly decreased in older and more developed plants in the S biotype. More developed R plants showed higher trichome and stomatal densities on the abaxial surface but lower stomatal density at the adaxial leaf surface compared to the S counterpart. These modifications combined might be correlated to higher glyphosate retention at the leaf surface and lower glyphosate control efficacy in the R biotype.

Authors' Contributions: BACCIN, L.C..: conception and design, acquisition of data, analysis and interpretation of data, and drafting the article; ALBRECHT, A.J.P.: conception and design and critical review of important intellectual content; PEDROSO, R.M.: drafting the article and critical review of important intellectual content; ARAÚJO, L.S.: acquisition of data and analysis and interpretation of data; DOTTA, M.A.: acquisition of data and analysis and interpretation of data; VICTORIA FILHO, R.: conception and design and critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

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

Ethics Approval: Not applicable.

Acknowledgments: The authors would like to thank the University of Sao Paulo (USP-ESALQ), for space and equipment's provided for the development of the experiments, Prof. Dr. E.W. Kitajima (USP-ESALQ) for his guidance and for kindly allowing us to use his Electron Microscopy lab. We also thank graduate students Luis Rodolfo Rodrigues and Beatriz Ribeiro da Cunha for their help and support. This research project was partly funded by a CAPES scholarship.

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Received: 22 January 2022 | **Accepted:** 13 October 2022 | **Published:** 31 March 2023