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SPECIAL ISSUE RESEARCH ARTICLE

Amaranthus spp.: a genus of troublesome weeds

Glyphosate-resistant smooth-pigweed (*Amaranthus hybridus*) in Brazil

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Abstract: **Background:** *Amaranthus hybridus*, a common agricultural weed in Brazil, has been associated with complications in the management of major crops. Recently, *A. hybridus* biotypes with suspected resistance to the herbicide glyphosate have been observed in the state of Paraná.

Objective: To characterize glyphosate-resistant *Amaranthus hybridus* biotypes.

Methods: Three independent experiments were conducted to evaluate four biotypes of *A. hybridus*. Twelve doses of glyphosate were applied, including 64D, 32D, 16D, 8D, 4D, 2D, D, 1/2D, 1/4D, 1/8D, 1/16D, and a control with no herbicide. The dose D of glyphosate applied was 1080 g ha^{-1} ae. Four replicates for each treatment and a randomized block design were used. At 28 (DAA), the control (%) and residual dry mass of each plot were analyzed.

Results: The biotypes with suspected resistance (R1, R2, and R3) had a GR$_{50}$ in the range of 3019.13 to 3316.45 g ha^{-1} ae and LD$_{50}$ in the range of 4081.52 to 4310.29 g ha^{-1} ae whereas the susceptible biotype had a GR$_{50}$ of 227.63 g ha^{-1} ae and LD$_{50}$ of 196.59 g ha^{-1} ae.

Conclusions: Biotypes R1, R2, and R3 are resistant to the herbicide glyphosate.

Keywords: Susceptibility; Smooth pigweed; Dose-response; Herbicide resistance

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1. Introduction

The *Amaranthus* genus includes species that are considered as agricultural weeds. *Amaranthus hybridus* L., an annual, dicotyledonous plant, is recognized as a major weed in current agricultural production systems (Larran et al., 2018). This is a competitive species with a high weed capacity owing to its rapid growth and development, long seed viability in the soil, and a high viable seed production rate (Barroso et al., 2012). In addition to these characteristics, the occurrence of its herbicide-resistant biotypes in fields makes it among the most important agronomic weeds.

The first case of herbicide resistance in *A. hybridus*, reported in 1972, was to the herbicide atrazine (Heap, 2021). Since then, a total of 32 cases have been reported worldwide, including from Argentina, Bolivia, Canada, France, Israel, Italy, Africa, Switzerland, and the United States. To date, resistance against five herbicidal mechanisms of action, namely ALS, photosystem II, PPO, EPSPs, and synthetic auxins has been reported (Heap, 2021).

The first report of glyphosate-resistant *A. hybridus* in Argentina was in 2013 in soybean crops (Perotti et al., 2019). Since then, the number of such cases in this species has been increasing steadily. Additionally, multiple resistance to glyphosate and imazethapyr was identified in Argentina in 2014 (Heap, 2021) and four years later, multiple resistance to 2,4-D, dicamba, and glyphosate was reported (Dellaferrera et al., 2018). Recently, a new case of glyphosate-resistant *A. hybridus* was detected in Paraná (Penckowski, Maschietto, 2019) in glyphosate-resistant soybean crops.

Herbicides are commonly used to control weeds in agricultural production systems. In such a scenario, the intense use of glyphosate as an herbicide is notable because of its broad spectrum and simple application (Melo et al., 2019). Glyphosate is a systemic and non-selective herbicide that acts on the shikimate pathway, specifically inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) (Duke, Powles, 2008). However, as a consequence of its continuous and indiscriminate use, chiefly after the introduction of genetically engineered cultivars that are tolerant to this herbicide, there has been an increase in selection pressure on the weed population leading to an increase in glyphosate-resistant weed biotypes (Perotti et al., 2020).

The cases of resistance in the *Amaranthus* genus are concerning as the gene responsible for herbicide resistance may be transferred to other species via interspecific hybridization (Gaines et al., 2012). The potential interspecific transfer of the EPSPs gene and glyphosate resistance within the *Amaranthus* genus has important agronomic significance. Thus, understanding herbicide resistance in *Amaranthus hybridus* is essential for weed management programs in order to slow down the evolution of herbicide resistance.

Since *A. hybridus* is suspected to be resistant to glyphosate, complementary studies to confirm the resistance of this weed biotype are necessary in order to aid future investigations and develop strategies for managing resistance in the field. This study, therefore, aimed to characterize the glyphosate-resistant *A. hybridus* biotypes using dose-response experiments.

2. Material and Methods

The study was carried out in the Department of Plant Production of ESALQ/USP, in Piracicaba city, São Paulo, SP, Brazil (latitude 22° 42' 30" S, longitude 47° 38' 00" W and altitude 546 m) between June 2020 and November 2021. Independent dose-response experiments were carried out in a greenhouse to confirm resistance and determine the resistance level of the suspected biotype.

2.1 Plant Material

Seeds of *Amaranthus hybridus* L. were collected from four locations. Three suspected resistant (R) biotypes were collected from different locations in Ponta Grossa/PR and labeled R1 (25º01'08"S and 50º09'22" W), R2 (25º05'16" S and 50º18'06" W), and R3 (25º15'52" S and 50º08'24" W). The susceptible (S) biotype, which was used as a control, was collected from a location in Piracicaba/SP which has no record of herbicide application in the last 15 years (Table 1).

The seeds of suspected biotypes were systematically collected directly from sites where weed control failures were observed from approximately 40 plants in the each site to compose a bulk sample (Burgos, 2015).

2.2 Dose-response experiments

To verify the suspected resistance of the *Amaranthus* biotypes to glyphosate, three independent experiments were conducted. The experimental design was a randomized block design with four replicates per treatment. The first factor consisted of the biotypes R1 and S for the first experiment, R2 and S for the second experiment, and R3 and S for the third experiment. The second factor consisted of 12 doses (D) of the herbicide glyphosate: 64D, 32D, 16D, 8D, 4D, 2D, D, 1/2D, 1/4D, 1/8D, 1/16D, and a control with no herbicide, the dose D being the amount of glyphosate used in the field (1080 g ha^{-1} ae). All experiments were performed in duplicate at different times for resistance confirmation.

The seeds of each biotype were sown in rectangular plastic boxes filled with the commercial substrate Tropstrato HT Hortaliças®, which is composed of pine bark and vermiculite. At the vegetative development stage of fully expanded cotyledonary leaves (Hess et al., 1997), the seedlings were transplanted into pots with a capacity of 0.2 L filled with the substrate. During the experiment, the pots were fertilized and maintained under sprinkler irrigation for optimum plant growth and development.

The herbicide was sprayed from a height of 0.50 m above the plants at the three to four leaf pair developmental stage using a CO_2 pressurized knapsack sprayer equipped with a TeeJet 110.02 tip, with a volume of 200 L ha^{-1}. The

Table 1 - Parameters for the adjustment of the control and dry mass of the *A. hybridus* to the logistic model.

Variables[1]	Parameters				R^2	LD ou GR		R/S
	P_{min}	a	b	c		50	80	
Biotype R1								
Control[2] (%)	-	103.75	4961.36	-0.37	0.98	4081.52	>69.120	21
Dry mass (%)	-13.59	115.91	4504.67	0.58	0.99	3211.67	21.291.81	14
Biotype R2								
Control (%)	-	106.32	5988.90	-0.36	0.99	4310.29	>69.120	22
Dry mass (%)	-13.91	115.09	5315.32	0.47	0.99	3316.45	33.912.56	15
Biotype R3								
Control (%)	-	95.94	3422.24	-0.41	0.99	4208.53	>69.120	21
Dry mass (%)	-16.05	117.46	5040.08	0.49	0.99	3019.13	26.660.04	13
Biotype S								
Control (%)	-	107.61	241.08	-0.69	0.94	196.59	1116.89	-
Dry mass (%)	-0.76	104.69	215.58	1.11	0.97	227.63	755.53	-

[1] Mathematical model: $y=a/(1+(x/b)^c)$ or $y=P_{min} + a/(1+(x/b)^c)$; [2] 28 DAA= days after application; *significative to 1% of probability.

applications were performed on two dates: 06/15/2020 and 09/23/2020. The climatic conditions on the first and second days were: temperature (27.9 °C and 28.5 °C), relative humidity (67.2% and 71.5%), and wind speed (1.2 $m.s^{-1}$ and 1.5 $m.s^{-1}$) respectively.

Twenty-eight days after the application of treatments (DAA), the control (%) and biomass of the plots were calculated. Control was evaluated using a percentage scale ranging from 0% to 100%, where 0% represents the absence of symptoms and 100% represents plant death (Velini et al., 1995). The plant material remaining in the pots was collected and placed in previously labeled paper bags in a forced circulation drying oven at 70 °C for 72 h. After drying, the dry weight was corrected to percentage values by comparing with the weight obtained in the treatments without herbicide application, which was considered as 100%.

2.3 2.3 Statistical Analysis

Data from two repeated greenhouse dose-response experiments were combined for analysis. Subsequently, analysis of variance (ANOVA) was performed and the significance was checked by the F test ($p<0.0001$) to verify if any differences were present between the resistant and susceptible biotypes. When the glyphosate dose x biotype interaction was significant, the data was adjusted to estimate the parameters and adjust the dose-response curve.

The data was adjusted for logistic-type nonlinear regression models according to Streibig (1988) corresponding to percentage control (1) and to residual dry mass (2) Seefeldt et al. (1995).

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

$$y = Pmin + \frac{a}{\left[1 + \left(\frac{x}{b}\right)^{c}\right]} \tag{2}$$

where y is the percentage control or residual dry mass, x is the dose of herbicide (g ha^{-1}), P*min* is the minimum point of the curve, and *a, b,* and *c* are the estimated parameters of the equation, where *a* is the range between the maximum and minimum points of the variable, *b* is the dose that provides 50% of the response of the variable, and *c* is the slope of the curve around *b*.

Although one of the parameters provides an estimate of the value that provides 50% response or mass reduction (DL_{50} or GR_{50}), a calculation for inversion of the log-logistic model was performed (Carvalho et al., 2005). From these data, the resistance factor was calculated using the formula GR_{50} (resistant)/ GR_{50} (susceptible) (Christoffoleti, 2002). The statistical procedures were performed using the Tablecurve 2D version 5.01 software (Jandel Scientific, 1991) and Sisvar software (Ferreira, 2011).

3. Results and Discussion

We observed a correlation between the glyphosate dose and biotype ($p<0.0001$) and obtained results for control (%) and mass reduction (%) of *A. hybridus*. The use of logistic-type mathematical models provided a good adjustment of the dataset with determination coefficients greater than 94% (Table 1).

Control was observed for the susceptible biotype (S) subjected to the application of glyphosate with similar LD_{50} and GR_{50} values. For this biotype, the value of GR_{50} was 227.63 g ha^{-1} ae, as evaluated by mass reduction and 196.59 g ha^{-1} ae for LD_{50} (Table 1). Carvalho et al. (2015) reported similar results in *A. hybridus* with values of LD_{50} from 168.30 to 252.82 g ha^{-1} ae and GR_{50} from 146.70 to 149.60 g ha^{-1} ae.

Differences in dry mass amongst the biotypes are shown in Figure 1. The dose-response curve analysis highlights mass reduction with increasing doses of glyphosate. For biotypes with suspected resistance (R1, R2 and R3), 3019.13 to 3316.45 g ha^{-1} ae of glyphosate was required to reduce the mass by 50% (Table 1). Biotype S could be controlled with lower doses than biotypes R1, R2, and R3. According to the GR_{50} parameter, the quantity of glyphosate required to reduce the mass of R1, R2, and R3 was 13 to 15 times greater than that required for the S biotype.

There was an increase in the percentage of weed control with the increase in glyphosate dosage. For biotype S, 196.59 g ha^{-1} ae was sufficient to achieve 50% control. In contrast, for biotypes with suspected resistance (R1, R2 and R3), 4080.52 to 4310.29 g ha^{-1} ae of glyphosate was necessary to obtain 50% weed control. The plants of these biotypes that survived, even at the highest dose (64D), continued to grow and produce seeds. Estimation of the dose necessary to obtain DL_{80} for the control variable (R) was not possible as the result was higher than the maximum dose used in the experiment (69.120 g ha^{-1} ae).

The first ever case of glyphosate-resistance in *A. hybridus* occurred in Argentina in 2013 (Heap, 2021). Subsequently, several cases of herbicide resistance involving various mechanisms of action have been described. García et al. (2019), studying populations *of A. hybridus*, found a high level of resistance, GR_{50}= 1395.20 g ha^{-1} ae and LD_{50}= 3503.40 g ha^{-1} ae. These results corroborate those of Dellaferra et al. (2018), who found a value of GR_{50}= 5018.2 g ha^{-1} ae in a population with multiple resistance to auxin and glyphosate herbicides. These results are in agreement with those obtained for the biotypes collected in the state of Paraná.

Target-site resistance (TSR) mechanisms related to EPSPs have been described in several weed species. A single mutation causing P106S substitution in EPSPs was first reported in *Eleusine indica* (Baerson et al., 2002). Another substitution in P106A was later reported in *Lolium rigidum* (Yu et al., 2007) and substitutions P106T and P106L were reported in *Chloris virgata* (Ngo et al., 2018). Another

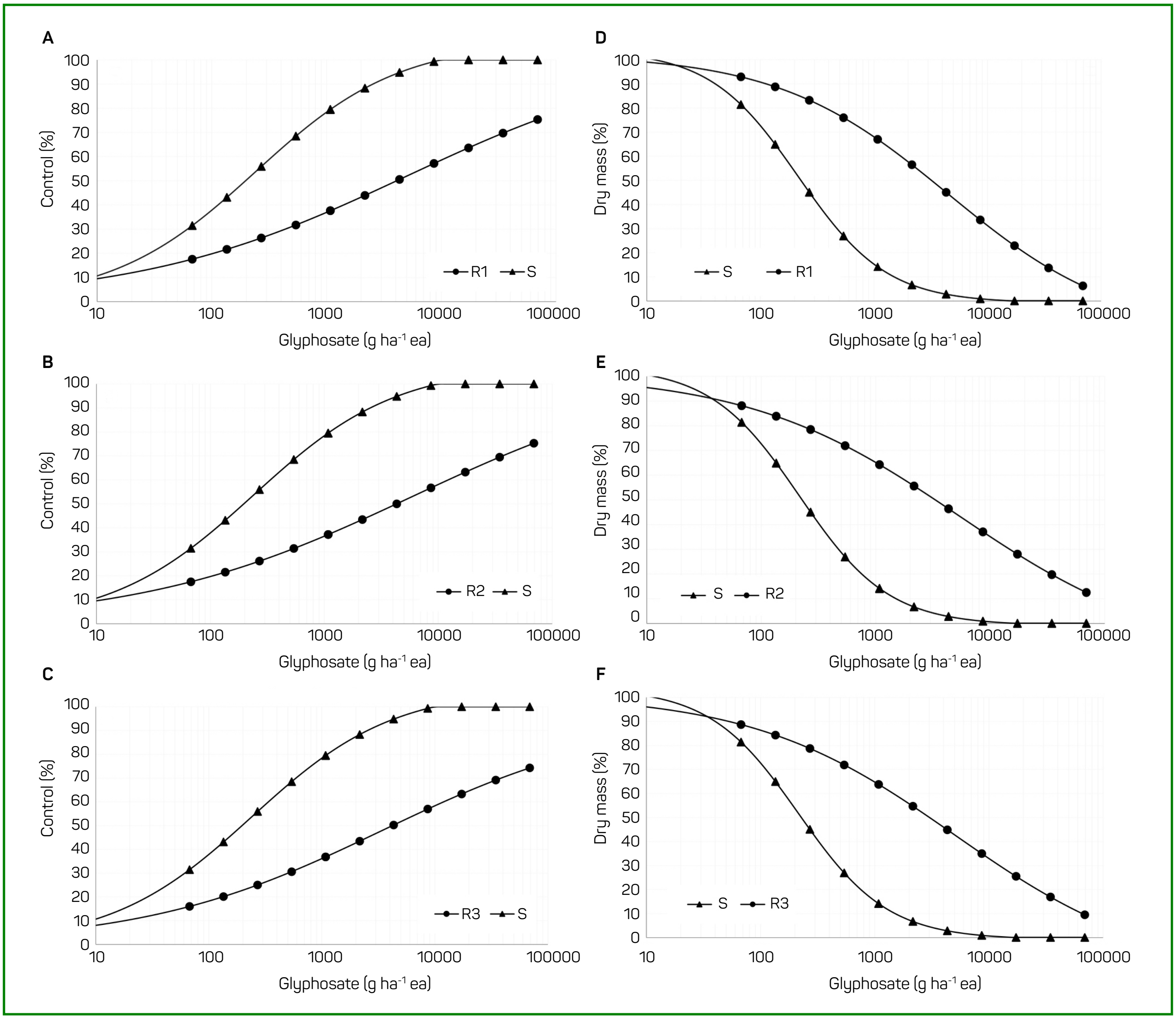

Figure 1 - Control and Dry mass (%) of *Amaranthus hybridus* biotypes submitted to different glyphosate application rates and evaluated at 28 DAA

known mutation, T102S, has been reported in the biotypes of *Tridax procumbens* (Li et al., 2018). Single EPSP mutations generally confer low levels of resistance, but they are sufficient for the biotypes to survive the recommended field doses (Yu et al., 2015). In addition, double mutations have been reported, such as T102I + P106S, also known as TIPS, which confer a high level of resistance in biotypes of *Eleusine indica* (Yu et al., 2015) and *Bidens pilosa* (Alcántara-de la Cruz et al., 2016). Finally, a triple amino acid substitution in the EPSPs was reported, involving TAP-IVS: (T102I, A103V, and P106S) in *Amaranthus hybridus*, revealing a high level of resistance in biotypes from Argentina (Perotti et al., 2019). Another target-site mechanism of resistance refers to the copy number of EPSPs. This mechanism of resistance was reported for the first time in *Amaranthus palmeri* by Gaines et al., (2010).

However, non-target site resistance (NTSR) is known to confer resistance to glyphosate by means of absorption and/or translocation (Fernández-Moreno et al., 2017), changes in glyphosate metabolism (Carvalho et al., 2012) and sequestration of the herbicide into vacuoles (Ge et al., 2012), all of which result in reduced levels of glyphosate interacting with EPSPs. NTSR confers unpredictable levels of resistance. Furthermore, the existence of both TSR and NTSR mechanisms in the same resistant population can result in this population becoming more resistant than if it possessed only one of the two mechanisms (Amaro-Blanco et al., 2018).

The present study has considerable potential for practical application once herbicide resistance becomes a national-level problem. Brazilian farmers often use glyphosate tolerance technology, mainly for soybean and corn crops. Therefore, weed control and management methods need to be better understood, along with the monitoring of infested areas. The glyphosate-resistant *A. hybridus* biotypes identified in this study could influence the choice of herbicides as well as alter the cost of production for future crops, thus increasing complexity in the field of weed management.

The next important step in this research will be to evaluate the mechanisms of resistance of *Amaranthus hybridus* biotypes. Most of the reported cases of glyphosate-resistance in *Amaranthus* suggest altered site of action. Apparently, there is a high probability that this is the mechanism of resistance due to the high level of resistance found in biotypes studied.

4. Conclusions

It can be concluded that the *Amaranthus hybridus* biotypes R1, R2, and R3, collected in the state of Paraná, Brazil, are resistant to glyphosate.

Author's contributions

LSR: conceptualization of the manuscript and development of the methodology. PJC: supervision and project administration. AGN, JCP, and MN: data analysis and data interpretation. EHGM, EFB, and LHP: data collection and curation. All authors read and agreed to the published version of the manuscript.

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