

## First evidence of multiple resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to five- mode-of-action herbicides

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### Abstract

Herbicide resistance is the evolutionary response of weeds to the selection pressure caused by repeated application of the same active ingredient. It can result from two different mechanisms, known as target site resistance (TSR) and non-target site resistance (NTSR). In addition to single-herbicide resistance, multiple resistance can occur due to herbicides selection or accumulation of resistance genes by cross-pollination. The aim of this research was to investigate the suspect of multiple herbicide resistance of Sumatran Fleabane (*Conyza sumatrensis* (Retz.) E.Walker) to herbicides frequently used as a burndown application. Dose-responses in a whole-plant assay were carried out to investigate multiple-resistance of Sumatran fleabane to paraquat, saflufenacil, diuron, 2,4-D and glyphosate. Results indicated that the resistance index (ratio R/S) based on herbicide rate to cause 50% mortality (LD<sub>50</sub>) were 25.51, 1.39, 7.29, 1.84 and 7.55 for paraquat, saflufenacil, diuron, 2,4-D and glyphosate, respectively. Based on herbicide rate required to cause a 50% reduction in plant growth (GR<sub>50</sub>), the resistant index were 51.83, 14.10, 5.05, 3.96 and 32.90 for the same herbicides, respectively. Our results confirmed multiple resistance of *Conyza sumatrensis* from Paraná-Brazil to herbicides from five-mode of-action. This was the first report of *Conyza sumatrensis* resistant to 2,4-D and the first case of *Conyza sumatrensis* presenting multiple resistant to herbicides from five-mode of-action in the world.

**Keywords:** Dose-response; herbicide; mode of action; multiple resistance, Sumatran fleabane.

**Abbreviations:** ae\_acid equivalent; ai\_active ingredient; ALS\_acetolactate synthase; APX\_ascorbate peroxidase; DAA\_days after application; DAT\_days after treatment; EPSPS\_5-enolpyruvyl-shikimate-3-phosphate synthase; GR\_Glutathione reductase; GR<sub>50</sub>\_rate required to cause a 50% reduction in plant growth; ha\_ectare; kPa\_kilopascal; LD<sub>50</sub>\_rate required to cause a 50% mortality; NMR\_nuclear magnetic resonance; PPO\_protoporphyrinogen oxidase; PSI\_photosystem; PSII\_photosystem II; R\_resistant; RI\_resistance index; S\_susceptible; TSR\_target site resistance; non-target site resistance (NTSR); UPCB\_Herbarium of the State University of Paraná.

### Introduction

Brazil is one of the largest grain producers in the world due to its extensive arable land and favorable climate for the production. However, weeds cause serious loss due to competition with the crop of interest for essential resources (Swanton et al., 2015; Jha et al., 2017; Gharde et al., 2018). *Conyza* spp. is currently considered as one of the main weeds in the crop production. Among the *Conyza* genus, there are nearly 50 species (Kissmann and Groth, 1999) and *C. bonariensis*, *C. canadensis* and *Conyza sumatrensis* (Retz.) E.Walker species have been often associated with cases of herbicide resistance in Brazil. It is a cosmopolitan weed, where minimum soil disturbance associated with fallow, perennial

cropping systems, and reduced tillage management practices of annual crops. These factors have provided a favorable niche for the ecological adaptation of *Conyza*s (Murphy and Lemerle, 2006), as one of the main current weed problems in Brazil.

The occurrence of this plant is concentrated in the late autumn and early spring, which coincides with the fallow or winter growing season (Tozzi and Van Acker, 2014). The absence of plants in the fallow favors the establishment of *Conyza* spp. in the areas. These species produce many seeds that can spread long distances by wind (Wu et al., 2007; Savage et al., 2014). These biological characteristics and the selection of herbicide-

resistant biotypes contribute to the wide and increasing dispersion of *Conyza* spp.

Herbicide resistance causes greater short-term cost to manage weed population, including yield loss, reduced commodity prices because of weed-seed contamination, reduced land values, costs of mechanical and cultural controls, additional expense of alternative herbicides or cropping systems or both for managing the resistant weed. Several recent studies have described the added costs associated with the management of herbicide-resistance weeds (Norsworthy et al., 2012). In Brazil, the presence of herbicide-resistant *Conyza* spp. increases control costs by 40%. The scenario is even worse with the occurrence of *Conyza* spp. and *Digitaria insularis* in the same production area, which increase costs by 200% (Adegas et al., 2017).

Treatment with herbicides is the primary method of controlling weed populations in modern agriculture. Furthermore, strong selection by herbicides has resulted in widespread evolution of resistance to herbicides in weed populations (Délye et al., 2013; Powles and Yu, 2010). Currently, there are 100 cases of *Conyza* spp. resistant to herbicides in the world (Heap, 2018). In Brazil, the first report of herbicide-resistant *Conyza* spp. was observed in 2005, when glyphosate no longer controlled these plants (Moreira et al., 2007; Heap, 2018). Because of glyphosate resistance, ALS-inhibitors herbicides started to be used to control weeds in the soybean crop. In 2011, multiple resistance to both mode of action was reported as a result of the high selection pressure exerted by the ALS-inhibitor herbicides (Santos et al., 2014; Heap, 2018). Populations resistant to glyphosate, ALS-inhibitors and other herbicides are of major concerns because these herbicides are the most effective post-emergence chemicals currently available to growers to manage horseweed in soybean. In addition, in 2016 and 2017, single resistance to paraquat and saflufenacil and multiple resistance to chlorimuron, glyphosate and paraquat were reported (Heap, 2018).

Multiple-herbicide resistance can arise by field selection of herbicides or due to accumulation of resistant genes by cross-pollination (Beckie and Tardif, 2012). The multiple resistance to five-herbicides-modes-of-action (5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitors, photosystem I (PSI) diverters, photosystem II (PSII) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors and 2,4-D) was reported by farmers who observed no efficiency to control Sumatran fleabane after different herbicide applications in the last growing season at Western region of Paraná State-Brazil. In no-tillage systems, the non-selective herbicides, such as glyphosate, paraquat or saflufenacil, has been widely used. The presence of *Conyza sumatrensis* requires application of glyphosate + 2,4-D followed by pre-planting spray of paraquat or saflufenacil. This management system is effective for controlling weeds, but when repeated over the years may promote the selection of biotypes with multiple resistance. These escapes in weed control were related to 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitors, acetolactate synthase (ALS) inhibitors, photosystem I (PSI) diverters, photosystem II (PSII) inhibitors and protoporphyrinogen oxidase (PPO) inhibitors and 2,4-D.

The aim of this research was to investigate the first case of multiple resistance of *Conyza sumatrensis* to herbicides from five-mode-of action frequently used as a burndown application prior to soybean planting.

## Results

The dose-response assay showed 100% mortality in the *C. sumatrensis* - S biotype. On the other hand, the R biotype was significantly less affected by herbicides and it was required higher doses than normal. The LD<sub>50</sub> and GR<sub>50</sub> values for R plants were higher for S biotype (Fig. 1 to 5; Table 1).

### Paraquat dose-reponse

Paraquat controlled 100% of S plants. In contrast, R plants were not controlled even at the 16X rate (6,400 g a.i. ha<sup>-1</sup>). Paraquat LD<sub>50</sub> values were 38.24 and 975.59 g a.i. ha<sup>-1</sup> for S and R plants, respectively. Even the highest paraquat dose used in the dose-response assay could not kill the R plants. The maximum control for R plants was 57%. The GR<sub>50</sub> values for paraquat showed that the dose needed to reduce 50% of dry mass was 159.17 and 8,249.65 g a.i. ha<sup>-1</sup>, for S and R biotype, respectively (Fig. 1). The resistance index (R/S) was calculated from LD<sub>50</sub> and GR<sub>50</sub> values and showed that R biotype was 25.51 and 51.83-fold more resistant than S plants (Table 1).

### Saflufenacil dose-response

The S biotype was 100% controlled by saflufenacil. For the R plants more than 8X dose (560 g a.i. ha<sup>-1</sup>) was required to satisfactorily control the weed. The dry mass of S plants was reduced to less than 10% with 2X dose (140 g a.i. ha<sup>-1</sup>), while 8X could reduce about 50% dry mass of R plants. Saflufenacil LD<sub>50</sub> values were 13.19 and 18.36 g a.i. ha<sup>-1</sup> for S and R plants, respectively. The GR<sub>50</sub> values for saflufenacil showed that the dose needed to reduce 50% of dry mass was 45.29 and 638.93 g a.i. ha<sup>-1</sup>, for S and R biotype, respectively (Fig. 2). Based on the LD<sub>50</sub> and GR<sub>50</sub> values, the resistance index revealed that R population was 1.39 and 14.10-fold less responsive to saflufenacil compared to S plants (Table 1).

### Diuron dose-response

Diuron controlled 100% of S plants. On the other hand, R plants were controlled only when 8X (16,000 g a.i. ha<sup>-1</sup>) was applied. The LD<sub>50</sub> values for diuron were 534.88 and 3,900.53 g a.i. ha<sup>-1</sup> for S and R plants, respectively, while the GR<sub>50</sub> values ranged from 1,035.16 to 5,227.16 g a.i. ha<sup>-1</sup> for S and R plants, respectively (Fig. 3). The resistance index (R/S) calculated from LD<sub>50</sub> and GR<sub>50</sub> values showed that R biotype was 7.29 and 5.05-fold more resistant than S plants (Table 1).

### 2,4-D dose response

The herbicide 2,4-D conferred 100% of control to S plants. On the other hand, R plants were controlled only when 8X applied

(8,040 g a.e. ha<sup>-1</sup>). Considering the biomass, 2X dose was enough to reduce the dry mass to zero in the S biotype, while we needed 8X to obtain similar results in R plants. The LD<sub>50</sub> values for 2,4-D were 371.97 and 686.01 g a.e. ha<sup>-1</sup> for S and R plants, respectively, while the GR<sub>50</sub> values were 1,495.67 and 5,918.67 g a.e. ha<sup>-1</sup> for S and R plants, respectively (Fig. 4). The resistance index calculated based on LD<sub>50</sub> and GR<sub>50</sub> showed that R biotype was 1.84 and 3.96 more resistant than S plants (Table 1).

#### **Glyphosate dose-response**

The S plants showed high susceptibility to glyphosate with 95% control at 0.5X dose. The maximum control for R plants was reached only when 16X (11,520 g a.e. ha<sup>-1</sup>) applied, showing high resistance level. The dry mass of S plants was reduced to zero even 0.5X dose applied. For the R plants, 100% of dry mass reduction was observed only under the highest dose. Glyphosate LD<sub>50</sub> values were 52.67 and 397.77 g a.e. ha<sup>-1</sup> for S and R plants, respectively. Based on dry mass reduction, GR<sub>50</sub> estimated for S and R plants was 121.02 and 3,981.13 g a.e. ha<sup>-1</sup>, respectively (Fig. 5). The resistance index (R/S) calculated from LD<sub>50</sub> and GR<sub>50</sub> values showed that R biotype was 7.55 and 32.90-fold more resistant than S plants (Table 1).

#### **Confirmation criteria**

The population of *Conyza sumatrensis* meets all the criteria set to confirm a new case of multiple resistance to paraquat, saflufenacil, diuron, 2,4-D and glyphosate (Heap 2005). Criterion 1: the plants from these populations have survived and reproduced after their exposure to a herbicide dose that was lethal to the susceptible population; Criterion 2: the resistance factors were high and the recommended dose to the species did not provide satisfactory control; Criterion 3: the plants from the F2 generation of these populations were considered resistant; Criterion 4: escaped plants are being observed on the field; Criterion 5: random plants of these population were properly classified as *Conyza sumatrensis*.

#### **Discussion**

The *Conyza* spp. control strategy has used a burndown application of glyphosate associated with 2,4-D, followed a sequential application of a contact herbicide, such as paraquat, paraquat+diuron or saflufenacil (Werth et al., 2010; Peterson et al., 2017). This strategy has been widely used to *Conyza* spp. control, but unfortunately reports of herbicides resistant are now occurring.

A dose-response experiment not only determines the discriminating dose for large-scale resistance testing as mentioned in the previous section, but also evaluates the highest dose that a population can tolerate and to obtain clues for the potential resistance mechanism(s). High resistance level may not only indicate targeted genetic point mutation, but also could be indicative of nontarget site resistance mechanism (Burgos et al., 2015). The R/S ratios of LD<sub>xx</sub> or GR<sub>xx</sub> values are used to compare the magnitude of resistance of different populations (Burgos et al., 2013).

Evolved resistance to paraquat has been reported in 68 biotypes of 32 weed species in 18 countries. Nineteen of these

resistant biotypes are from *Conyza* species (horseweeds and fleabanes) (Heap, 2018). The S biotype, at all rates, and R plants sprayed with 8 and 16X displayed rapid desiccation and necrosis, but the R plants recovered two weeks after treatment, while the S plants were died. Some reports of *Conyza* spp. exhibit the level of paraquat resistance values ranging from 9 to 352.5-fold, when compared with S populations (Van Gessel et al., 2006; Eubank et al., 2012; Moretti et al., 2017). In the present study, the R biotype showed resistant index of 25.51 and 51.83-fold based on LD<sub>50</sub> and GR<sub>50</sub>, respectively.

The ratio of paraquat resistance between R and S biotypes ranged from 10 and 30-fold at vegetative rosette stage to 100-fold or more after 10 weeks, when plants were entered their reproductive cycle (Ye and Gressel, 2000). It makes the control of weeds more difficult at the advanced stages. Current understanding of the molecular mechanism of paraquat resistance in higher plants includes sequestration of paraquat to the vacuoles and/or enhanced activity of antioxidative enzymes (Szigeti, 2005). Although sequestration into the vacuole remains the best hypothesis to account high-level paraquat resistance that is inherited as a semi-dominant single gene trait, multiple mechanisms governed by more complex inheritance might combine to confer tolerance at a lower level (Hawkes, 2014).

Saflufenacil is effective against plants that are difficult to control, such as *Conyza* spp. It has been widely used as a strategy to control *Conyza* spp. resistant to glyphosate and paraquat plants (Mellendorf et al., 2013; Waggoner et al., 2013; Moretti et al., 2015). However, as observed in this study, weed populations from Brazil showed resistance indexes of 1.39 and 14.10 to saflufenacil, based on LD<sub>50</sub> and GR<sub>50</sub>, respectively. A common approach to manage herbicide-resistant weeds is to shift to alternative herbicides or herbicide mixtures that still effectively control the resistant populations, or as part of program that integrates PRE herbicides along with POST-applied (Beckie, 2006).

There are a few cases of PPO-inhibitor resistance already reported. The first case was the multiple resistance of *Amaranthus tuberculatus* (Tall waterhemp) to ALS and PPO-inhibitors in the United States recorded in 2001. So far, thirteen species were identified worldwide. Only three biotypes were reported as resistant to saflufenacil, all of them in Brazil, including *Euphorbia heterophylla* in 2004 and *Conyza sumatrensis* in 2017 (Heap, 2018). The resistance mechanism to saflufenacil remains unknown, but resistance to PPO-herbicides in *Amaranthus* spp. was attributed to a target-site mutation in the PPX2 gene only, characterized by a Gly-210 deletion (Patzoldt et al., 2006; Salas et al., 2016; Salas-Perez et al., 2017).

PSII inhibitors block electron transfer to the secondary QB plastoquinone (QB-site) acceptor of the D1 subunit protein, resulting in interruption of electron transfer from the primary plastoquinone site (QA-site) to QB (Powles and Yu, 2010). The interrupted electron transfer causes oxidative stress by production of reactive oxygen species, which cause rapid cellular degradation (Menne, 2005). There are currently nine biotypes resistant to diuron herbicide. The first case was reported in 1987, when *Chloris barbata* was identified in the United States.

**Table 1.** The LD<sub>50</sub>, GR<sub>50</sub> and resistance index (RI) values for the dose–response assay.

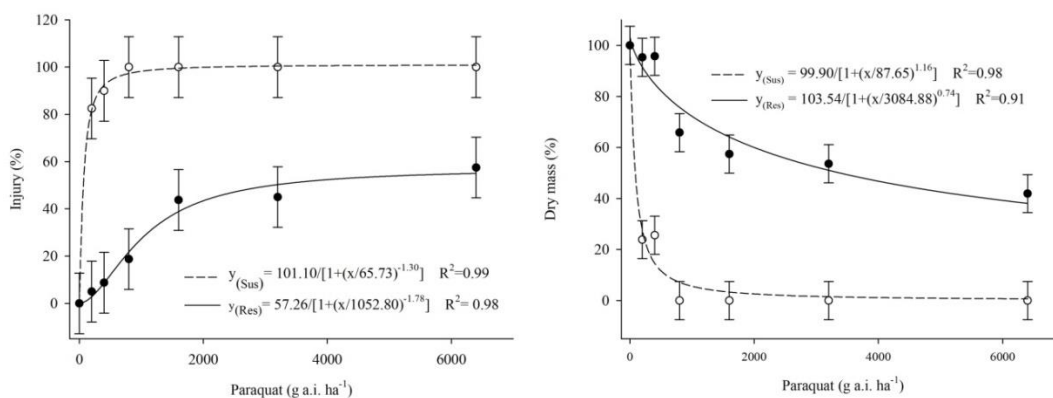
Biotype <sup>†</sup>	Estimated Parameters			
	LD <sub>50</sub> <sup>‡</sup>	GR <sub>50</sub> <sup>§</sup>	RI <sub>LD50</sub> <sup>¶</sup>	RI <sub>GR50</sub>
	<b>Paraquat (g a.i. ha<sup>-1</sup>)</b>			
S	38.24	159.17		
R	975.59	8,249.65	25.51	51.83
	<b>Saflufenacil (g a.i. ha<sup>-1</sup>)</b>			
S	13.19	45.29		
R	18.36	638.93	1.39	14.10
	<b>Diuron (g a.i. ha<sup>-1</sup>)</b>			
S	534.88	1,035.16		
R	3,900.53	5,227.16	7.29	5.05
	<b>2,4-D (g a.e. ha<sup>-1</sup>)</b>			
S	371.97	1,495.67		
R	686.01	5,918.67	1.84	3.96
	<b>Glyphosate (g a.e. ha<sup>-1</sup>)</b>			
S	52.67	121.02		
R	397.77	3,981.13	7.55	32.90

<sup>†</sup> Susceptible – S and suspected of resistance – R.

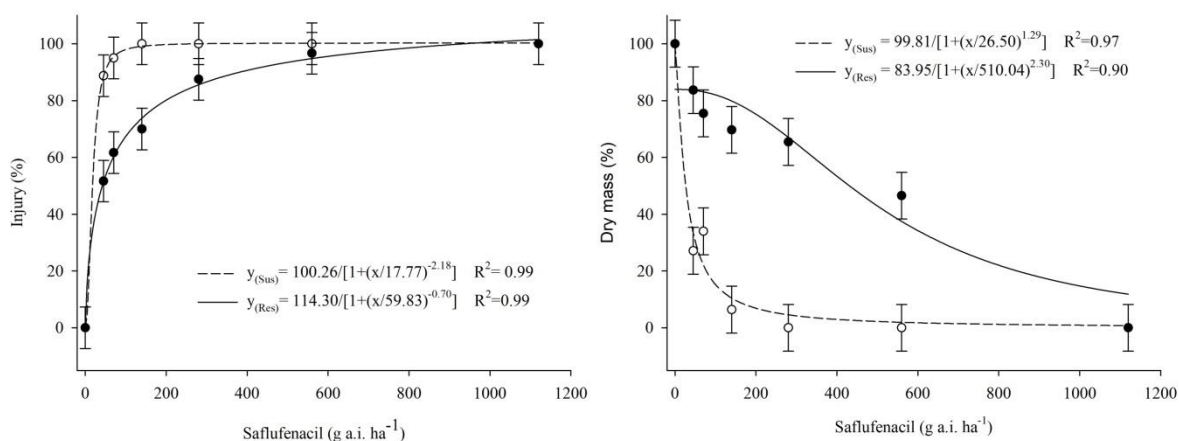
<sup>‡</sup> Rate required to cause a 50% mortality.

<sup>§</sup> Rate required to cause a 50% reduction in plant growth.

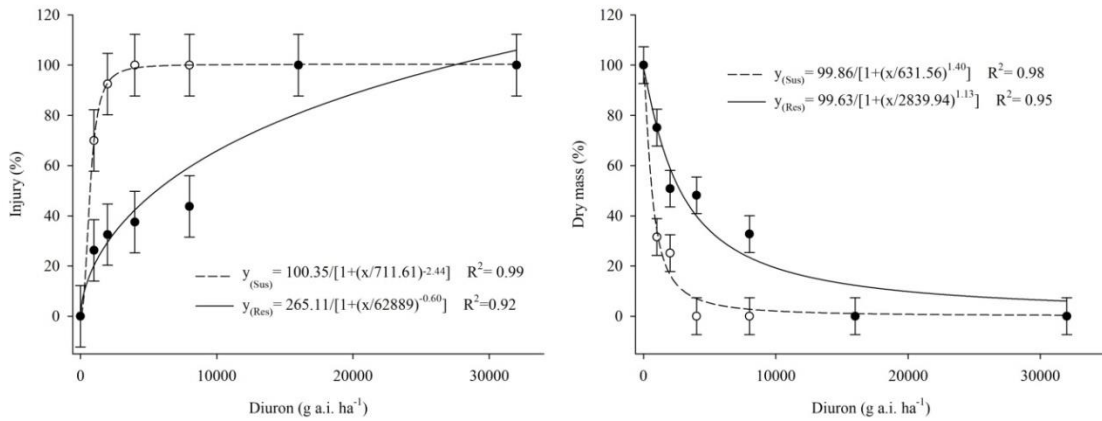
<sup>¶</sup> Resistance index.



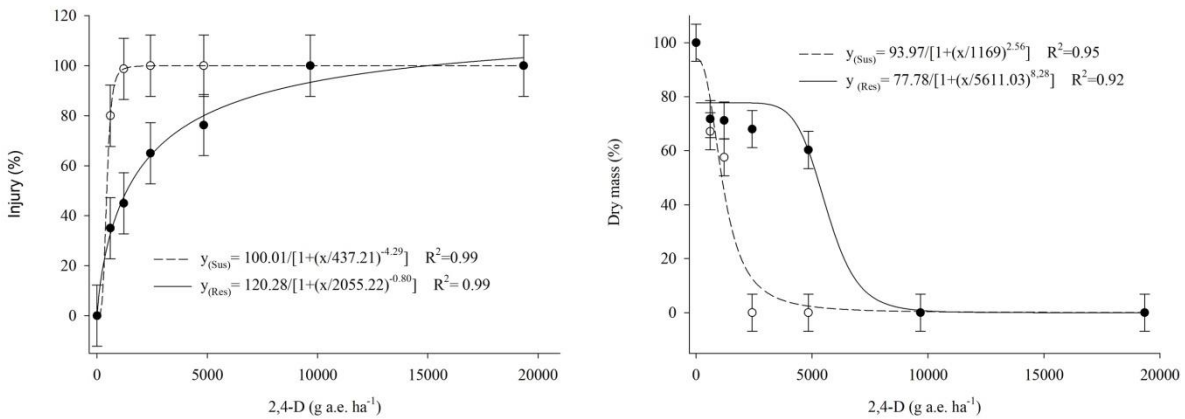
**Fig 1.** Injury and dry mass observed by dose–response assay of herbicide resistant (R) and susceptible (S) *Conyza sumatrensis* plants treated with paraquat at 28 days after herbicide application.



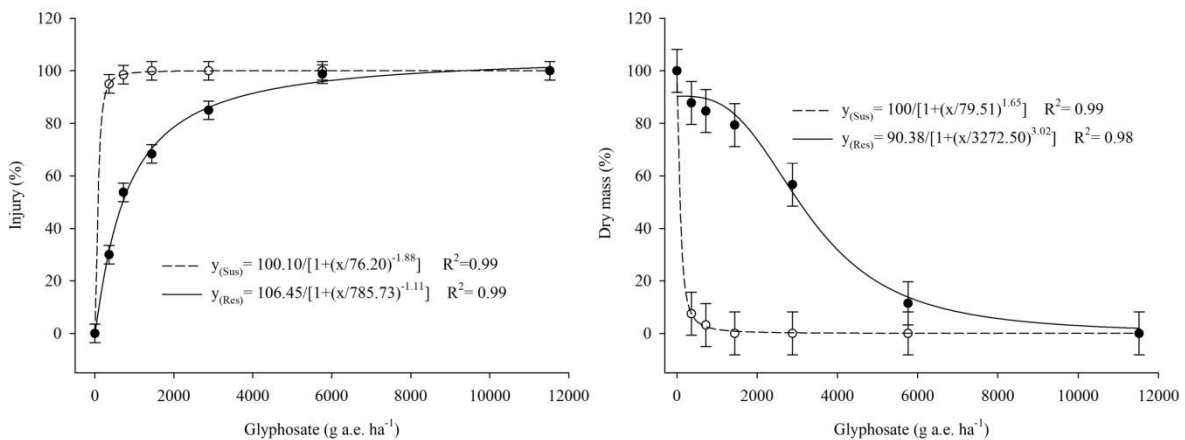
**Fig 2.** Injury and dry mass observed by dose–response assay of herbicide resistant (R) and susceptible (S) *Conyza sumatrensis* plants treated with saflufenacil at 28 days after herbicide application.



**Fig 3.** Injury and dry mass observed by dose–response assay of herbicide resistant (R) and susceptible (S) *Conyza sumatrensis* plants treated with diuron at 28 days after herbicide application.



**Fig 4.** Injury and dry mass observed by dose–response assay of herbicide resistant (R) and susceptible (S) *Conyza sumatrensis* plants treated with 2,4-D at 28 days after herbicide application.



**Fig 5.** Injury and dry mass observed by dose–response assay of herbicide resistant (R) and susceptible (S) *Conyza sumatrensis* plants treated with glyphosate at 28 days after herbicide application.

In 2002, cross-resistance to PSII-inhibitors including atrazine, simazine and diuron was reported in *Conyza canadensis* present in blueberry fields (Heap, 2018). Our study is the first case of *Conyza sumatrensis* resistant to diuron worldwide with resistance index of 7.29 and 5.05, based on LD<sub>50</sub> and GR<sub>50</sub>, respectively.

So far, sequencing of the *psbA* gene has indicated two mutations point: Ser<sub>264</sub>Gly and Val<sub>219</sub>Ile, responsible for resistance to PSII inhibitors (Mengistu et al., 2000; Perry et al., 2012). Ser<sub>264</sub>Gly mutation confers cross-resistance to other PSII-inhibiting herbicides, such as triazolinone family (Dayan et al., 2009; Perry et al., 2012; Yu et al., 2013), while Val<sub>219</sub>Ile substitution have also been correlated to resistant to diuron and metribuzin (Mengistu et al., 2000). The non-target site resistance (NTSR) to PSII-inhibiting herbicides has been related to increased levels of herbicide detoxification in velvetleaf (*Abutilon theophrasti* Medik.) (Yuan et al., 2007), and Cytochrome P450 monooxygenases can provide resistance to multiple herbicides, including PSII inhibitors simazine and metribuzin in rigid ryegrass (Preston et al., 1996; De Prado et al., 1997; Preston, 2003).

The first cases of evolved herbicide resistance were to one of the first commercialized synthetic herbicides, 2,4-D. Wild carrot (*Daucus carota* L.) (Switzer, 1957) and spreading dayflower (*Commelina diffusa* Burm.) (Hilton, 1957) were both reported to have evolved resistance to 2,4-D in 1957, around 10 years after the use of 2,4-D had become widespread. Currently, there are 38 species resistant to synthetic auxins and 22 are resistant to 2,4-D (Heap, 2018). In this study, we identified the first case of *Conyza sumatrensis* resistant to 2,4-D in the world, with resistance index of 1.84 and 3.96, respectively, based on LD<sub>50</sub> and GR<sub>50</sub>. The resistant biotype shows rapid desiccation after herbicide spray. In about 6 hours, the old leaves of resistant biotype showed necrosis while the susceptible did not show any symptom. Later, the susceptible plants developed typical symptoms of auxin herbicides while the young leaves of resistance plants remained intact. This response could be related to low herbicide translocation, allowing the plants to survive and ensuring resistance to this herbicide. This phenomenon was already reported, but it was associated with glyphosate-resistance in *Ambrosia trifida* (Van Horn et al., 2017; Moretti et al., 2018) and called as rapid-necrosis symptom.

Glyphosate is one of the most widely used herbicides. The intensive use of glyphosate (no-till, fallow and/or glyphosate tolerant crops) has resulted in the evolution of glyphosate-resistant worldwide (Powles, 2008; Beckie and Harker, 2017). Glyphosate-resistant biotypes of *C. bonariensis*, *C. canadensis* and *C. sumatrensis* have been reported in many countries, including Brazil. There are currently 300 unique cases of glyphosate-resistant weeds in the world, including 62 *Conyzas*. The first case of *Conyza* resistant to glyphosate was observed in the United States, in 2000. In Brazil, both *C. bonariensis* and *C. canadensis* were reported as glyphosate-resistant in 2005. Later, in 2010, *C. sumatrensis* was also reported as resistance to glyphosate (Heap, 2018). In this study, a glyphosate-resistant biotype was identified with higher resistance level, about 7.55 and 32.90-fold, based on LD<sub>50</sub> and GR<sub>50</sub> respectively. The level of glyphosate-resistance *Conyza* spp. for

biotype R was 2.5-12-fold, when compared with S (Eubank et al., 2012; Kumar et al., 2017). In Brazil, the glyphosate-resistance level ranged from 1.96 to 4.08 (Santos et al., 2014). Also, the multiple resistance levels ranging from 1.66 to 6.74 and from 18.93 to 47.02 for glyphosate and chlorimuron-ethyl, respectively were observed (Santos et al., 2014b).

Glyphosate-resistance mechanisms includes target-site mutation, target-site gene duplication, active vacuole sequestration, limited cellular uptake and a rapid necrosis response (Sammons and Gaines, 2014). Currently, the resistance mechanisms to glyphosate in *Conyza* spp. was identified as the substitution of proline to serine in the codon 106 (Amaro-Blanco et al., 2018; Page et al., 2018), overexpression of *epsps* gene and ABC transporters (Ge et al., 2011; Tani et al., 2015) and reduced herbicide translocation (Nol et al., 2012; Gonzalez-Torralva et al., 2014, 2017; Kleinman and Rubin, 2017). Calvin cycle enzymes and proteins of unknown function were also identified as possible candidates involved to glyphosate-resistance *Conyza canadensis* (Gonzalez-Torralva et al., 2017).

Target-site mutations for glyphosate resistance endow lower levels of resistance than target-site mutations for some other mechanisms of action (Preston et al., 2009). A double mutation found recently in some weeds, Pro<sub>106</sub>Ser and Thr<sub>102</sub>Ile, greatly increases resistance levels (Yu et al., 2015). Pro106 target-site mutations normally act together with reduced translocation to provide higher-level of resistance (Kaundun et al., 2011; Bostamam et al., 2012; Nandula et al., 2013; Gonzalez-Torralva et al., 2014). Experiments using nuclear magnetic resonance (NMR) to observe glyphosate *in vivo* entering cells and cellular compartments have identified a new resistance mechanism that result in restricted translocation correlated with the glyphosate resistance. The difference in glyphosate vacuole sequestration rates between resistant and susceptible *C. canadensis* was about 10X (Ge et al., 2014). A whole-plant assay in the cold (8°C) demonstrated that vacuole sequestration was inhibited. In this situation, resistant plants of *C. canadensis* were become susceptible to glyphosate (Ge et al., 2011). This demonstrated that the resistance was due to the vacuolar sequestration and that physiological properties of the resistance mechanism could be utilized as a mechanism to reverse the resistance.

#### **Impact on weed management**

The large difference in the R/S LD<sub>50</sub> and GR<sub>50</sub> ratio obtained for paraquat, saflufenacil and glyphosate response in this *Conyza sumatrensis* population indicates that the potential resistance mechanism(s) may incur fitness cost in the presence of herbicide. In view of the confirmation of resistance, proactive resistance management strategies should be proposed, predicting the behavior of other herbicides to control these species. Herbicides should be used wisely (e.g. in rotation or mixture) and in combination with other nonchemical control options.

Integrated weed management practices should be adopted in regions of existence or potential existence of multiple-resistant *Conyza* spp. including the use of cover crops, management of weed seed bank, usage of herbicides with different

mechanisms of action in the same spray tank, and the appropriate and accurate application of herbicides (Norsworthy et al., 2012). Herbicide resistance screening and monitoring should be adopted by farmers since multiple resistance is a challenge and presents additional risks.

## Materials and Methods

### Plant material

Samples originated from failures in weed control were collected from the field with history of herbicide application including several applications of glyphosate, 2,4-D, paraquat+diuron and saflufenacil to confirm the resistance to paraquat. The survived plants were self-pollinated and the F<sub>1</sub> seeds of *C. sumatrensis* from plants were identified as resistant to paraquat and also to saflufenacil (Heap, 2018). These plants were selected and grown in greenhouse for the production of the F<sub>2</sub> generation seeds, with the objective to complete a dose-response assay with herbicides that included different modes of action. The original collection site was in Assis Chateaubriand-Paraná, Brazil (24°16'53.8"S 53°30'47.5"W). Another population suspected to be susceptible to all modes of actions were collected from Rio de Janeiro.

The seeds were sown in 2.5 dm<sup>3</sup> pots filled with potting mix soil and seedlings were thinned after emergence to one seedling per pot. Plants were grown out until reproductive stage, which were properly identified as Sumatran Fleabane at Herbarium of the State University of Paraná (UPCB) and at Institute of Biology – Herbarium Uberlandense at Federal University of Uberlândia.

### Greenhouse trials: Dose-response assays

The *C. sumatrensis* plants were tested for the possible multiple resistance. A dose-response assay was carried out in a randomized block design with four replications, using herbicides of five different modes of action: photosystem-I-electron diversion (PSI), protoporphyrinogen oxidase (PPO) inhibitors, photosystem II inhibitors (PSII), synthetic auxin and 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitors, represented by paraquat, saflufenacil, diuron, 2,4-D and glyphosate, respectively.

The experimental units were kept under greenhouse conditions and were irrigated daily. The recommended rate (1X) was defined to be 400 g ai ha<sup>-1</sup> for paraquat, 70 g ai ha<sup>-1</sup> for saflufenacil, 2,000 g ai ha<sup>-1</sup> for diuron, 1,005 g ae ha<sup>-1</sup> for 2,4-D and 720 g ae ha<sup>-1</sup> for glyphosate. A dose range of 0.5, 1, 2, 4, 8 and 16 times (X) the recommended dose were used. The non-treated plants were considered as control. Paraquat and saflufenacil were applied with non-ionic surfactant Agral 1% and 0.5% (v/v) (Agral®, Syngenta Crop Protection, Greensboro, NC), respectively. The treatments were sprayed to both biotypes (susceptible – S and suspected of resistance – R).

### Herbicide spray

The herbicide application was performed when the plants reached 12-15 cm height measured from the ground to the last

leaf insertion. Herbicides treatments were applied using a CO<sub>2</sub> pressurized backpack sprayer with four XR-110015 flat fan nozzles (TeeJet Technologies, Wheaton, IL), delivering 200 L ha<sup>-1</sup> at 240 kPa.

### Injury and biomass analysis

Visible injury and plant mortality was evaluated 28 days after treatment (DAT) compared to the non-treated control using a 0 to 100 % scale, in which 0 was no effect and 100 as complete control (Velini et al., 1995). Plant growth reduction and dry mass evaluation of shoots was performed at 28 days after application (DAA). The plants were collected and dried in oven at 70°C for 72 hours and then weighed on scale. Biomass data were expressed as percentages of the non-treated mean control to standardize the comparisons among populations.

### Data analysis

Plant mortality (LD) and reduction in plant growth (GR) were measured 28 DAT. After estimating LD<sub>50</sub> (herbicide rate required to cause a 50% control) and GR<sub>50</sub> (herbicide rate required to cause a 50% reduction in plant growth relative to nontreated plants) values using log-logistic models proposed by Streibig (1988) and Seefeldt et al. (1995):

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

Where;  $y$  is the response based on visible injury estimate or dry weight,  $a$  is the amplitude between the maximum and minimum point of the variable,  $x$  is the dose of the herbicide (g a.i. ha<sup>-1</sup>),  $b$  is the herbicide dose giving a 50% response (plant mortality [LD<sub>50</sub>] or growth reduction [GR<sub>50</sub>]) and  $c$  is the inflection point around  $b$ .

The log-logistic model provided an estimate of the parameter LD<sub>50</sub> or GR<sub>50</sub>. It was decided to use the mathematical calculation through the inverse equation making it possible to calculate the LD<sub>50</sub> and GR<sub>50</sub>:

$$x = b \left( \left( \frac{a}{y} - 1 \right) \right)^{\frac{1}{c}}$$

The resistance index (RI = R/S) were computed as R-to-S LD<sub>50</sub> or GR<sub>50</sub> ratios. The graphics were plotted using Sigma Plot 12.5.

### Conclusion

Based on our results, we can confirm multiple resistance of *C. sumatrensis* to five-modes-of-action to herbicides such as paraquat, saflufenacil, diuron, 2,4-D and glyphosate in Paraná-Brazil. This is the first report of *C. sumatrensis* resistant to diuron and 2,4-D and the first case of *C. sumatrensis* with multiple resistance to five modes of action in the world. Research is underway to elucidate the mechanisms of resistance. In this moment, actions are being taken to monitor the areas, where the resistant biotypes were collected as well as other areas with biotypes suspected resistance.


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## Disclosure Statement

The authors declare no conflicts of interest.™® = Trademark of Dow AgrosSciences LLC, DuPont or Pioneer and their respective owners and their affiliated companies.

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