Occurrence of Multiple Glyphosate-Resistant Weeds in Brazilian Citrus Orchards

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Abstract: Glyphosate is the most widely used herbicide for weed control in citrus orchards in Brazil; therefore, it is likely that several species have gained resistance to this herbicide and that more than one resistant species can be found in the same orchard. The objective was to identify weeds resistant to glyphosate in citrus orchards from different regions of the São Paulo State (SP) and determine how many resistant species are present within the same orchard. Seeds of Amaranthus deflexus, A. hybídus, Bidens pilosa, Chloris elata, Conyza bonariensis, Digitaria insularis, Solanum Americanum, and Tridax procumbens, which, as reported by growers, are suspected to be resistant to glyphosate, were collected from plants that survived the last application of this herbicide (≥720 g of acid equivalent [ae] ha−1) in sweet orange and Tahiti acid lime orchards. Based on dose–response and shikimic acid accumulation assays, all populations of A. deflexus, A. hýbídus, B. pilosa, and T. procumbens were sensitive to glyphosate. However, populations of B. pilosa from the Olimpia region (R-NS, R-PT and R-OdA) showed signs of resistance based on plant mortality rates by 50% within a population (LD50 = 355–460 g ae ha−1). All populations of C. bonariensis, C. elata, and D. insularis were resistant to glyphosate, presenting resistance ratios from 1.9 to 27.6 and low shikimate accumulation rates. Solanum americanum also showed resistance, with resistance ratios ranging from 4.3 to 25.4. Most of the citrus orchards sampled presented the occurrence of more than one species resistant to glyphosate: Nossa Senhora—one species; Olhos D’agua and Passatempo—two species; Araras—four species; and Cordeirópolis and Mogi-Mirim—up to five species. The results reported in this paper provide evidence of multiple species in citrus orchards from São Paulo that have exhibited resistance to glyphosate. This underscores the difficulties in managing glyphosate-resistant weeds which are prevalent throughout the country, such as C. bonariensis and D. insularis. The presence of these resistant species further complicates the control of susceptible species that may also develop resistance. In addition, the glyphosate resistance of S. americanum was identified for the first time.

Keywords: black nightshade; flaxleaf fleabane; slender amaranth; sourgrass; tall windmill grass

1. Introduction

By producing 1.1 million tons of orange juice, Brazil will produce three quarters of the world’s orange juice in the 2022/2023 season [1]. Citrus production is mainly concentrated in the Brazilian southeastern region, especially in the state of São Paulo, which is responsible for more than 75% of national production in 366,446 ha (60% of the citrus planted area) [2]. In addition to competing with citrus trees, weeds causing yield reductions of up to 50% can host vector insects and pathogens and hinder cultural practices; thus, their management is essential in order to maintain healthy and highly productive orchards [3,4].
Although some non-chemical weed management practices are being adopted on a small scale by citrus growers [5], weed management in citrus is mainly based on herbicides, directing applications in the intra- (under the canopy of the trees) and inter-row areas of the orchards [6]. However, due to the fact that, in Brazil, there are few authorized herbicides (only 10 active ingredients) for weed management in citrus orchards [7], the most used herbicide is glyphosate, both for the wide spectrum of weeds that it is capable of controlling and for its low cost [5,6,8,9].

Glyphosate is a homolog of phosphoenolpyruvate in the shikimic acid pathway, interrupting 5-enolpyruvylshikimate-3-phosphate (EPSP) biosynthesis by inhibiting the EPSP synthase activity [10]. The interruption of this metabolic pathway causes the accumulation of shikimic acid, affecting the synthesis of three essential aromatic amino acids: phenylalanine, tyrosine, and tryptophan [10]. Therefore, it is possible to determine the susceptibility/resistance to glyphosate through the concentration of shikimic acid present in the plants [11]. This herbicide, in addition to being cheap and effective, has other interesting properties, such as low residual activity in the soil, low toxicity to mammals, minimal environmental impact, among others [12,13], making it the most used agricultural chemical pesticide in the world [10].

Low herbicide rotation and the absence of economically viable integrated weed management strategies have led to an extensive and excessive reliance on glyphosate. As a result, reports of low efficacy with respect to glyphosate in the application of controlling weeds have increased year after year. In addition, most citrus growers commonly increase the rate and frequency of glyphosate application instead of using herbicides with other mechanisms of action [3,6]. Surveys have revealed that 98% of Brazilian citrus growers use glyphosate, with 73% applying doses between 1000 and 2000 g acid equivalent (ae) ha\(^{-1}\) and 11% doses > 2000 g ae ha\(^{-1}\). The frequency of application varies, and only 11% of the producers apply glyphosate once a year; the majority (78%) apply glyphosate two–four times annually, while 11% apply it more than five times annually [9].

In Brazilian citrus orchards, populations of *Conyza bonariensis*, *Conyza canadensis*, and *Digitaria insularis* with resistance to glyphosate have been reported since before 2010 [14,15]. The tropical conditions in which citrus are produced also favor the growth of annual and perennial weeds [16,17]. Species of the genera *Amaranthus*, *Bidens*, *Chloris*, *Conyza*, *Eleusine*, *Lolium*, among others, are weeds that present a high risk of evolving herbicide resistance [18], and some of them have already reportedly become resistant to herbicides in other crops in Brazil [19]. Because species of these genera occur in high densities in citrus orchards [14] and also present high rates of survival in the field after herbicide applications, the possibility that new weed species have developed resistance to glyphosate is likely. In addition, citrus growers have exerted strong selection pressure on weed populations with glyphosate [6,9], so it is likely that more than one resistant species will occur in the same orchard. The occurrence of several herbicide-resistant weed species in the same area considerably increases the costs of resistance management [20].

Since glyphosate is the most used herbicide in Brazil, the aim of this work was to identify glyphosate-resistant weeds in citrus groves from different regions of the São Paulo State and to verify how many resistant weed species can be found within the same orchard. The resistance level was determined by dose–response and shikimic acid accumulation assays.

### 2. Materials and Methods

#### 2.1. Plant Material

Field surveys were carried out in sweet orange [*Citrus sinensis* (L.) Osbeck] and Tahiti acid lime (*C. latifolia* Tan.) orchards in the municipalities of Araras, Cordeirópolis, Mogi-Mirim, and Olimpia, São Paulo State. In these orchards, seeds of at least 20 specimens of each species that survived the last application of this herbicide (>720 g ae ha\(^{-1}\)) were harvested, which, according to growers, showed signs of resistance to glyphosate due to major control failures. Seeds were identified and stored in paper bags. Susceptible seeds
of the same species were collected in an ecologically managed Tahiti acid lime orchard (without the use of herbicides) in the municipality of Mogi-Mirim and on the campus of the Federal University of São Carlos in uncultivated areas. The list of weed species collected at each site are detailed in Table 1.

Table 1. Identification of weed populations with suspected resistance to glyphosate collected in different citrus orchards from the State of São Paulo, Brazil.

<table>
<thead>
<tr>
<th>Population</th>
<th>Species</th>
<th>Botanical Class</th>
<th>Orchard</th>
<th>Municipality</th>
<th>Coordinates</th>
<th>Year(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-lim</td>
<td>Bidens pilosa</td>
<td>Dicotyledonous</td>
<td>Tahiti lime</td>
<td>Mogi-Mirim</td>
<td>22°25’ S, 47°10’ W</td>
<td>2018 and 2019</td>
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<tr>
<td></td>
<td>Chloris clata</td>
<td>Monocotyledonous</td>
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<td></td>
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</tr>
<tr>
<td></td>
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<td>Monocotyledonous</td>
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<td></td>
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<tr>
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<td>No crop</td>
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<td>Tahiti lime</td>
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<td>22°25’ S, 47°09’ W</td>
<td>2018 and 2019</td>
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<tr>
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<td>Dicotyledonous</td>
<td>Pêra sweet orange</td>
<td>Olimpia †—Olhos D’aigua</td>
<td>20° 46’ S, 49° 0’ W</td>
<td>2019</td>
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<td>Pêra sweet orange</td>
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<td>20° 43’ S, 49° 0’ W</td>
<td>2019</td>
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</tbody>
</table>

† The second name of the municipality of Olimpia reflects the names of the farms from which the seed samples were collected.

Seeds from each population were germinated in plastic trays (31 × 15 × 10 cm) containing a mixture of moistened-at-field-capacity commercial substrate and sand (1:1 v/v) and covered with expanded vermiculite. The trays were sealed and kept in a greenhouse until germination (3–6 d). Seedlings of 1–2 true leaves were transplanted individually into 250 mL pots containing substrate, sand, and vermiculite (2:2:1 v/v/v) and fertilized with ~100 mg of 14-14-14 (NPK) (Forth Cote, Osmocote, Froth Jardim Ltd., Cerquilho, Brazil). The plants were kept in a greenhouse until they were required for use in the experiments.
2.2. Dose–Response Assays

Seedlings of different species with 3–4 true leaves (rosette of 6–9 leaves in the case of *C. bonariensis*) were treated with glyphosate (Roundup® Original DI, 370 g ae L⁻¹, Monsanto, Sao Paulo, Brazil). The different doses of glyphosate tested were as follows: 0, 23.12, 46.25, 92.5, 185, 370, 740, 1110, 1480, 2220, and 2960 g ae ha⁻¹. Applications were carried out with a pneumatic knapsack sprayer equipped with a flat fan nozzle (110,015, KGF Spray Nozzles, Vinhedo, Brazil) at 50 cm canopy height. The application pressure was 30 psi, as measured with a glycerin manometer (Model GCN, Cotergavi Instrumentos de Medicação Ltda., São Paulo, Brazil) and adapted to the application bar to deliver 200 L of ha⁻¹ herbicide solution.

Sets of 10 plants from each weed population were treated per herbicide dose. Doses of 23.12 and 46.25 g ha⁻¹ were applied only to dicot weeds, and the highest one (2960 g ha⁻¹) only in monocots.

At 21 d after treatment, fresh weight and plant mortality were recorded, and the values were converted to percentages relative to the untreated control to estimate the GR₅₀ (dose that reduces fresh weight by 50%) and/or LD₅₀ (dose that kills 50% of the individuals in a population) via nonlinear regression analysis and calculate the resistance factor (RF = R/S). The experiments were repeated for populations that showed glyphosate resistance.

2.3. Shikimic Acid Accumulation

Sets of at least 10 plants from each weed population were treated with 370 and 740 g ae ha⁻¹ of glyphosate. A set of untreated plants from each population was reserved as a control group to construct the calibration curve with known concentrations of shikimic acid (0, 0.01, 0.05, 0.1 and 0.2 µg). At 96 h after treatment, 50 mg samples of fresh tissue (from the second and third leaves) of the treated and untreated plants were collected and placed in 1.5 mL tubes containing 1 mL of 0.25 N HCl, frozen in liquid N₂, and stored at −40 °C until required for use. Shikimic acid accumulation was determined following the methodology of Cromartie and Polge [21]. The samples were thawed at room temperature and subsequently incubated for 45 min at 37 °C. Aliquots of 50 µL were transferred to new tubes containing 200 µL of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium m-periodate. After a new incubation at 37 °C for 30 min, 200 µL 0.6 N sodium hydroxide and 0.22 N sodium sulfite was added and homogenized. Volumes of 300 µL were transferred to spectrophotometric cuvettes, which were then supplemented with 600 µL of distilled water.

The absorbance of samples was measured at a wavelength of 380 nm in a diode array spectrophotometer (HP 8425A, Palo Alto, CA, USA). Shikimic acid accumulation was determined from the difference between treated and untreated plants, and the results were expressed in µg of shikimic acid g⁻¹ fresh tissue. Five samples with three technical replicates were analyzed by population in a completely randomized design.

2.4. Statistical Analysis

The GR₅₀ and LD₅₀ values were calculated using the following equation: \[ y = \left( \frac{(d)}{1 + (x/g)^b} \right) \] [22]. In this equation, \( y \) is the response at 50% of the parameter of interest, \( d \) is the upper limit of the curve, \( c \) is the lower limit of the curve, \( b \) is the slope of the curve, \( x \) is the dose(s) of herbicide, and \( g \) is the inflection point of the curve (GR₅₀ or LD₅₀). Regression analyses were performed using SigmaPlot 10.0 (Systat Software Inc. San Jose, CA, USA).

After checking the normality of the shikimic acid accumulation data by using the Shapiro–Wilk test, the data were submitted to ANOVA. When necessary, Tukey’s test at probability \( \alpha = 0.05 \) was performed to compare means. Statistical analyses were performed using Statistics 9.0 software (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Dose–Response Assays

Populations with suspected resistance (*A. deflexus, A. hybridus, B. Pilosa*, and *T. procumbens*) turned out to be sensitive to glyphosate, presenting GR₅₀ and LD₅₀ val-
ues similar to those determined for their susceptible counterparts. In all cases, these values were close to or below 100 g ae ha\(^{-1}\), except the \(B. \) pilosa populations from the Olimpia region (R-NS, R-PT and R-OdA), which presented LD\(_{50}\) values that ranged from 355 to 460 g ae ha\(^{-1}\). All populations of \(C. \) bonariensis, \(C. \) elata, and \(D. \) insularis were resistant to glyphosate. For \(C. \) elata and \(D. \) insularis, the RF’s ranged from 1.9 to 8.3 in relation to their susceptible counterparts. \(Conyza \) bonariensis was the most resistant species, with RF’s of up to 27.6, and in some cases, it was not possible to determine the LD\(_{50}\) since 50% of the plants in a population did not die with the maximum dose evaluated for dicots (2220 g ae ha\(^{-1}\)). \(Solanum \) americanum presented RF’s ranging from 4.3 to 32.5 for GR\(_{50}\) and from 6.8 to 25.4 for LD\(_{50}\) when compared with their respective susceptible populations (Figure 1, Table 2).

![Figure 1](image-url). Dose–response curves of the percentage of plant mortality within a population of weed species with suspected glyphosate resistance collected in citrus orchards from different regions of the São Paulo State, Brazil. Vertical bars ± standard (\(n = 10\)).
Table 2. Effective mean doses of glyphosate to reduce dry weight (GR$_{50}$) and/or plant survival (LD$_{50}$) by 50% in weeds collected in citrus orchards from São Paulo State, Brazil.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>GR$_{50}$ (CI95%)</th>
<th>RF</th>
<th>LD$_{50}$ (CI95%)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. deflexus</td>
<td>S-UFSCar</td>
<td>21.2 (3.5)</td>
<td>-</td>
<td>64.9 (4.1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R-IAC</td>
<td>25.6 (2.8)</td>
<td>1.2</td>
<td>82.3 (6.7)</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>R-lar</td>
<td>26.7 (1.6)</td>
<td>1.3</td>
<td>113.1 (8.6)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

| A. hybridus | S-UFSCar | 40.9 (5.6) | - | 56.9 (0.9) | - |
|            | R-IAC     | 39.6 (2.8) | 1.0 | 46.7 (3.5) | 0.8 |
|            | R-lar     | 41.9 (6.0) | 1.0 | 60.7 (2.1) | 0.5 |
|            | R-OdA     | 45.6 (1.7) | 1.1 | 72.6 (8.7) | 1.3 |
|            | R-PT      | 47.2 (4.6) | 1.2 | 68.2 (6.4) | 1.5 |

| B. pilosa | S-lim     | 46.1 (3.7) | - | 109.6 (12.6) | - |
|           | R-IAC     | 22.1 (3.3) | 0.5 | 79.3 (8.3) | 0.7 |
|           | R-lar     | 32.5 (1.8) | 0.7 | 87.2 (6.7) | 0.8 |
|           | R-NS      | 74.4 (6.7) | 1.6 | 434.2 (32.1) | 4.0 |
|           | R-OdA     | 102.5 (5.9) | 2.2 | 459.4 (27.5) | 4.2 |
|           | R-PT      | 56.3 (4.9) | 1.2 | 355.8 (17.8) | 3.2 |

| C. bonariensis | S-lim | 39.4 (2.2) | - | 157.6 (8.6) | - |
|                | R-IAC  | 793.2 (63.6) | 20.1 | 1643.1 (142.1) | 10.4 |
|                | R-lar  | 201.8 (34.2) | 5.1 | 732.5 (68.2) | 4.6 |
|                | R-PT   | 912.5 (53.4) | 23.2 | 370.7 (36.4) | 3.5 |

| C. elata  | S-lim  | 67.2 (14.6) | - | 105.0 (13.4) | - |
|           | R-Ara  | 193.4 (14.3) | 2.9 | 380.1 (24.3) | 3.6 |
|           | R-IAC  | 174.9 (29.4) | 2.6 | 703.7 (15.6) | 6.7 |
|           | R-lar  | 243.7 (39.4) | 3.6 | 534.4 (55.3) | 5.1 |
|           | R-NS   | 125.3 (16.5) | 1.9 | 702.4 (53.8) | 6.7 |
|           | R-OdA  | 157.2 (11.8) | 2.3 | 429.4 (28.1) | 4.1 |
|           | R-PT   | 148.6 (13.6) | 2.2 | 370.7 (36.4) | 3.5 |

| D. insularis | S-lim | 114.9 (8.3) | - | 130.7 (0.8) | - |
|              | R-Ara  | 265.9 (13.7) | 2.3 | 1052.3 (54.1) | 8.1 |
|              | R-IAC  | 174.9 (65.4) | 5.7 | 917.0 (35.6) | 7.0 |
|              | R-lar  | 406.3 (80.9) | 3.5 | 995.6 (66.7) | 7.6 |
|              | R-OdA  | 732.0 (62.4) | 6.4 | 1081.3 (71.8) | 8.3 |

| S. americanum | S-UFSCar | 22.4 (1.8) | - | 56.2 (1.9) | - |
|               | R-Ara    | 97.4 (40.8) | 4.3 | 387.3 (41.6) | 6.8 |
|               | R-IAC    | 261.5 (25.4) | 11.6 | 757.3 (56.5) | 13.5 |
|               | R-lar    | 728.2 (25.8) | 32.5 | 1430.7 (135.8) | 25.4 |

| T. procumbens | S-UFSCar | 25.2 (2.4) | - | 64.9 (3.8) | - |
|               | R-lar    | 34.3 (2.3) | 1.4 | 80.5 (6.7) | 1.2 |
|               | R-NS     | 28.7 (3.4) | 1.1 | 72.0 (4.6) | 1.1 |

CI95% limits of the confidence intervals at 95% (n = 10). RF = Resistance factors are the R-to-S GR$_{50}$ or LD$_{50}$ ratios.

Most of the citrus orchards sampled with weed management problems with glyphosate presented the occurrence of more than one species resistant to this herbicide. In the orange orchards of the Nossa Senhora ranch, resistance was identified only for C. elata. The citrus orchards from the Olhos D’água (C. elata and D. insularis) and Passatempo (C. bonariensis and C. elata) ranches presented two glyphosate-resistant species. However, all the orchards from the Olimpia region showed signs of resistance to B. pilosa. Four species (C. bonariensis, C. elata, D. insularis, and S. americanum) with different glyphosate resistance levels were identified in the Taiti acid lime orchard in the Araras region and, in the Pêra, the sweet orange orchards of the Cordeiropolis and Mogi-Mirim municipalities exhibited four resistant species (C. bonariensis, C. elata, D. insularis, and S. americanum) (Table 2).
3.2. Shikimic Acid Accumulation

The accumulation of shikimic acid differs between species and populations within species. Similar to the dose–response results, A. deflexus, A. hybridus, and T. procumbens were susceptible to glyphosate, as they accumulated large amounts of shikimic acid both at 370 and 740 g ae ha⁻¹. Plants of B. pilosa from Olimpia and C. elata treated with 370 g ae ha⁻¹ accumulated between 2 and 4 less shikimate than their susceptible counterparts (S-lim); however, when treated with a dose of 740 g ae ha⁻¹, these species accumulated amounts of shikimate similar to S-lim populations. Resistant populations of C. bonariensis, D. insularis, and S. americanum accumulated up to 10 times less shikimate than their susceptible counterparts when treated at 370 g ae ha⁻¹, and even when there was an increase at a dose of 740 g ae ha⁻¹, the accumulation was lower than in the susceptible populations (Figure 2).

Figure 2. Shikimic acid accumulation in weeds collected in citrus orchards from different regions of the São Paulo State, Brazil. Same letter denotes no differences among treatments as determined by Tukey’s test (p < 0.05). ns = non-significant. Vertical bars ± standard (n = 3).

4. Discussion

The recommended dose of glyphosate by manufacturers varies from 370 to 2220 g ae ha⁻¹, depending on the crop and the weed species to be controlled [23]. Due to the need for multiple applications per agricultural year, many growers have adopted a dose of 740 g ae ha⁻¹, which was seen as the standard dose in previous years [8]. However, due to increasing weed management failures, most growers apply doses of at least 1080–1110 g ae ha⁻¹ [9]. Although growers reported glyphosate resistance in A. deflexus, A. hybridus, B. Pilosa, and T. procumbens, the tested populations of these species were susceptible in both the dose–response and shikimate acid accumulation assays. The control
failures observed may be due to the variability involved in the phenological stage of weeds. Although manufacturers recommend application at the early phenological stage of 4–6 true leaves [23] (as in this work), the tropical climate of Brazil [24] and the staggered germination of weeds [25] make it impossible to apply herbicides on uniform populations of weeds. The phenological stage directly influences the efficacy of herbicides. For example, susceptible populations of *C. bonariensis* in the rosette stage presented a GR50 of 15.7 g ae ha\(^{-1}\); however, in bolting and flowering, the values were 87 and 118 g ae ha\(^{-1}\), respectively, i.e., the susceptibility to glyphosate in these phenological stages was 5.4 and 7.3 times less than in the rosette stage [26]. The umbrella (shading) effect imposed by taller plants on smaller plants also affects herbicide efficacy [27], and many growers end up associating resistance with the high plant survival rates of their orchards; however, in many cases, the plants may not have been reached by the herbicide or were reached in non-lethal concentrations.

The populations of *B. pilosa* observed in this study, based on their high weight reduction with low doses of glyphosate, can be classified as susceptible. They require special attention because the populations from the Olimpia region showed signs of glyphosate resistance, with LD50 values ranging between 350 and 450 g ae ha\(^{-1}\). *Bidens pilosa* is a species that is already reportedly highly resistant to glyphosate, according to studies regarding citrus orchards in Mexico [28]. In a parallel study of this research group, resistant populations of this species were identified with LD50 values of up to 880 g ae ha\(^{-1}\) [9]. This suggests that there are already populations of *B. pilosa* with different levels of resistance in citrus orchards in São Paulo. In contrast, all populations of *C. bonariensis*, *C. elata*, and *D. insularis* tested in this study were found to be resistant to glyphosate. *Conyza bonariensis* and *D. insularis* had already been identified as having glyphosate resistance in Brazilian citrus orchards [14,15], and *C. elata* in soybean crops [29]. There are no studies that report the distribution of resistance to glyphosate in species in Brazilian citrus orchards, unlike for other crops [30]. However, based on our results, it can be concluded that resistant populations of these species are widely distributed in the citrus production region of São Paulo. In addition to seed dispersal, resistance may have been produced by independent events [31] since the levels of resistance varied between the populations of each species.

*Solanum americanum* is an unprecedented case of glyphosate resistance in Brazil and in the world. In 2020, *Amaranthus viridis* was also reported to be glyphosate-resistant in some of the citrus orchards evaluated in this study (R-lar and R-IAC) [8], i.e., these two new glyphosate-resistant species join widely distributed resistant species such as *C. bonariensis*, *C. elata*, and *D. insularis* tested in this study were found to be resistant to glyphosate. *Conyza bonariensis* and *D. insularis* had already been identified as having glyphosate resistance in Brazilian citrus orchards [14,15], and *C. elata* in soybean crops [29]. There are no studies that report the distribution of resistance to glyphosate in species in Brazilian citrus orchards, unlike for other crops [30]. However, based on our results, it can be concluded that resistant populations of these species are widely distributed in the citrus production region of São Paulo. In addition to seed dispersal, resistance may have been produced by independent events [31] since the levels of resistance varied between the populations of each species.

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The occurrence of multiple species resistant to glyphosate within the same citrus orchard is worrisome since weed management is more difficult and costly. The average cost of chemical weed management in soybean under Brazilian conditions was estimated to be BRL 120 ha\(^{-1}\) year\(^{-1}\) [20]. That cost increases based on the presence of herbicide resistant weeds. When only one resistant species occurs, the cost increases by BRL 83.5 for *Conyza* sp. species, BRL 98 for *Lolium* sp., and BRL 227 for *D. insularis*; however, when multiple species occur (*Conyza* sp. + *D. insularis*), the average increase is BRL 312 [20], i.e., the cost of resistance management nearly quadruples (BRL 120 + 312) since the application of herbicides with different mechanisms of action is required. Growers are generally reluctant to adopt integrated weed management strategies [33] because these costs often increase, and it is understandable that they would want to strive to reduce production costs. In addition, most of the time, growers do not have a projection of the increase in productivity and profit margins [34]. Research conducted by this research
group shows that, although rising weed management costs regarding citrus orchards can complicate weed management strategies, the productive potential of orchards and the potential economic returns also rise [3]. Mechanical weed control is inefficient and expensive (BRL 696 ha$^{-1}$), but by implementing ecological mowing, economic returns can be doubled compared to unmanaged orchards (BRL 7212 vs. 3344 ha$^{-1}$). Weed management with post-emergence herbicides was three times cheaper than with pre-emergence herbicides (BRL 435 vs. 148 ha$^{-1}$); however, the economic return was similar, and most farmers adopt this method because, in addition to being cheap, it is also easier to execute from a practical point of view. However, the association of pre- and post-emergence herbicides and ecological mowing (BRL 500 ha$^{-1}$) increased yield, improved the quality of fruits, and tripled economic returns (BRL 11,124 ha$^{-1}$). Therefore, researchers have the responsibility of producing and transferring information on herbicide resistance management strategies that are logistically, technically, and economically viable since, most of the time, studies that evaluate management alternatives do not evaluate the costs of each strategy, much less the productive potential.

5. Conclusions

The occurrence of multiple species of glyphosate-resistant weeds was identified in citrus orchards from different regions of São Paulo, Brazil. The incorrect management of widely distributed glyphosate-resistant species such as C. bonariensis and D. insularis make it difficult to control susceptible species, as observed with A. deflexus, A. hybridus, B. pilosa, and T. procumbens. Furthermore, this work identified glyphosate resistance in S. americanum for the first time. Our future research will be focus on characterizing the resistance mechanisms of resistant populations, mainly of S. americanum, aiming to develop resistance management strategies that consider the cost and productive potential of citrus orchards.


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References


8. Alcântara-de la Cruz, R.; Amaral, G.S.; Oliveira, G.M.; Rufino, L.R.; Azevedo, F.A.; Carvalho, L.B.; Silva, M.F.G.F. Glyphosate resistance in *Amaranthus viridis* in Brazilian citrus orchards. *Agriculture* 2020, 10, 304. [CrossRef]

9. Martinelli, R.; Rufino, L.R., Jr.; Alcântara-de la Cruz, R.; da Conceição, P.M.; Monquero, P.A.; de Azevedo, F.A. Glyphosate excessive use affects citrus growth and yield: The vicious (and unsustainable) circle in Brazilian orchards. *Agronomy* 2022, 12, 453. [CrossRef]


34. Livingston, M.; Fernandez-Cornejo, J.; Frisvold, G.B. Economic returns to herbicide resistance management in the short and long run: The role of neighbor effects. *Weed Sci.* 2016, 64, 595–608. [CrossRef]

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