Confirmation of Multiple Resistant *Chloris radiata* Population, Harvested in Colombian Rice Fields

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Abstract: This paper reports the first *C. radiata* population with resistance to glyphosate and multiple resistance to the acetalactate synthase (ALS) inhibitor, imazamox. Two populations, one putative resistant (R) and one susceptible (S), were used in the studies. Dose–response experiments were performed to evaluate the resistance factor (RF). Shikimic acid accumulation, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and ALS enzyme activities were studied together with chemical integrated weed management (adjuvants and alternative herbicides). The resistance to glyphosate and imazamox was confirmed based on the dry weight reduction, visual evaluation and survival. The results of dose–response curve assays showed for the R population intermediate RF for glyphosate (5.1 and 9.7 for amount of herbicide needed to reduce the dry weight by 50% GR50 and lethal dose of 50% LD50, respectively) and high RF for imazamox (34.9 and 37.4, respectively). The low shikimic acid accumulation in R population confirmed the glyphosate resistance. The glyphosate concentration which inhibited the EPSPS enzyme in 50% (I50) was approximately 20 times higher for R population than the S population, while the imazamox I50 in ALS enzyme for the R plants was 89 times greater than the S plants. In the chemical integrated weed management, the foliar retention and effectivity assays showed that the use of adjuvants improves the retention of glyphosate and imazamox, and the reduction in dry weight of weeds. The alternative herbicides study showed that the acetyl-CoA carboxylase (ACCase) inhibitors, paraquat and glufosinate, had better results for control in this species. However, poor control was observed with bispyribac-sodium, metsulfuron-methyl and quinclorac, indicating possible cross-resistance for ALS-inhibitors and also multiple resistance for auxinic herbicides (quinclorac). Nevertheless dose–response experiments are required to confirm this assumption.

Keywords: glyphosate; ALS-inhibiting herbicides; imazamox; radiate fingergrass; chemical weed control

1. Introduction

Cultivated rice is among the most important cereal crops in the world, and plays an essential role in global food security and reduction in poverty [1,2]. For more than half of the world’s population, rice is a staple food, contributing 21% of their daily calorie intake [3]. For that reason, rice production has been described as the world’s single most important economic activity [4]. In 2018, world rice production was highly localised. Of the total paddy rice harvested area (167.1 million hectares), India was the primary producer with 27.6%, followed by China (18.2%). In America, Brazil ranks first as the highest producer (30.4%), followed by the United States (19.3%), Colombia (10.4%) and Peru (7.1%) [5]. Rice is the fourth most important crop in Colombia after sugarcane, palm oil and banana [6].
In any rice production system, weeds are one of the main biological limitations, which cause yield reductions and affect the quality of paddy rice. Average losses worldwide are around 13%; however, rice cultivation in Colombia has reported losses from 30 to 73% due to weeds [7]. These losses vary depending on the floristic composition, community structure, sowing and tillage systems, water management, continuous planting of rice on the same piece of land, among other factors [8,9]. The most common weeds in rice crops around the world in the last 30 years are species of the Echinochloa genus (E. crus-galli and E. colona mainly) and weedy rice [2]. However, the most common weeds in rice crops in Colombia are Echinochloa colona (barnyardgrass), Oryza sativa (weedy rice), Cyperus iria, Ischaemum rugosum, Eleusine indica and Digitaria bicornis [10–12].

The genus Chloris as a weed is hardly known and includes numerous species distributed in tropical and warm temperate regions [13]. Chloris radiata (L.) Sw. (basionym: Agrostis radiata L.), commonly known as radiate fingergrass, included in the Poaceae family, is a C4, annual and native species in Colombia, growing between 500 and 2000 m above sea level [14]. It has been reported as a weed in rice crops since 2011, specifically in the central zone of the country, with no reports of yield losses in rice cultivation for this species. On the other hand, Chloris polydactyla reduces soybean growth by up to 70% due to competition [15], Chloris truncata decreases wheat biomass and yield by 25% [16] and Chloris virgata causes yield losses in sorghum of about 37 thousand tons [17], showing the importance of the species of this genus and their impact on crops.

Herbicides have become the most used weed control method worldwide. Currently, resistance to herbicides decreases the effectiveness and use of these products due to a lower level of control and the use of a single control method. [13]. Herbicides exert high selection pressure on weeds, and repeated and intensive use of them with the same mechanisms of action can complicate weed control and may hasten herbicide resistance evolution [18]. In Colombian rice crops, chemical control is frequently used and the applications are commonly carried out at different moments: pre-sowing, pre-emergence and postemergence (early–middle–late).

In pre-sowing applications (before the establishment of the crop), an activity known as chemical-burning, non-selective active ingredients are usually used, and the objective is to reduce the density of weeds that could compete with the crop in its early stages of development [19]. The most commonly used herbicide is glyphosate with a frequency of use of 94% [20]. This active ingredient inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), involved in the biosynthesis of the aromatic amino acids, phenylalanine, tyrosine and tryptophan in plants [21], which are essential as precursors for the production of hormones, cell wall formation and defence against pests [22].

In postemergence control, 100% of the farmers perform these applications using 23 different active ingredients [20]. The following active ingredients stand out for postemergence application in Colombian rice producing areas: propanil, bentazon, pyrazosulfuron, metsulfuron-methyl, carfentrazzone, bispyribac-sodium, imazapic, imazamox, imazapyr, cyhalofop, fenoxaprop, profoxydim, 2,4-D, picloram and quinclorac [19]. The most frequent mechanism of action in this stage of application is acetolactate synthase (ALS) inhibitors, and about 50% of the farmers use them [20]. The primary mechanism of action of these herbicides is inhibition of branched chain amino acid synthesis [21].

In recent years, an increase in the distribution and density of C. radiata has been observed, attributable to possible failures of chemical control in rice crops, such as overuse of the same active ingredient or mode of action, overdose, monoculture, among others; suspecting that this weed has evolved resistance to herbicides. Therefore, the objectives of this research were: (1) to confirm glyphosate (EPSPS inhibitor) and imazamox (ALS inhibitor) resistance in one population; (2) to evaluate the resistance level of this population to both herbicides; and (3) study new chemical alternatives to control this resistant C. radiata population.
2. Materials and Methods

2.1. Chemicals

Putative resistant (R) and known susceptible (S) *Chloris radiata* populations to glyphosate and imazamox, were sprayed with commercially formulated glyphosate and alternative herbicides (Table 1). For the foliar retention and effectiveness experiments, we used the following adjuvants: Retenol (66.5% terpenic alcohols w/v, DAYMSA, Zaragoza, Spain) and Trend® 90 (90% isodecyl ethoxylated alcohol w/v, FMC, Valencia, Spain). Retenol is a non-ionic adjuvant used to reduce the surface tension of droplets and thus, is intended to increase wettability, foliar retention and persistence of the active substances. Trend® 90 is a non-ionic wetting agent, which is used to improve the persistence and adherence of plant protection chemicals.

| Herbicide          | HRAC/WSSA Code | MOA        | Commercial Product    | Recommended Field Dose (g ea·ai ha⁻¹) b |
|-------------------|----------------|------------|-----------------------|----------------------------------------|
| Glyphosate a      | 9              | EPSPS      | Roundup Energy® (450 g/L SC) | 960                                    |
| Imazamox          | 2              | ALS        | Pulsar® 40 (4% SC)    | 40                                     |
| Bispyribac-sodium | 2              | ALS        | Bispirel® 100 SC      | 50                                     |
| Metsulfuron-methyl| 2              | ALS        | Ally® 60% WG          | 9                                      |
| Quinclorac        | 4              | ACCase     | Facet (25 SC)         | 375                                    |
| Atrazine          | 5              | PSII       | Gesaprim® 90% WDG     | 2000                                   |
| Glufosinate-ammonium | 10           | GS         | Finale® 15% p/v SL    | 500                                    |
| Oxyfluorfen       | 14             | PPO        | Goal Supreme® 48%     | 480                                    |
| Paraquat          | 22             | FSI        | Gramaxone® 27.6% SL   | 400                                    |
| Tembotrione       | 27             | HPPD       | Laudis® 42% SC        | 120                                    |

* a g ea ha⁻¹ = grams of equivalent acid per hectare. b g ai ha⁻¹ = grams of active ingredient per hectare. HRAC: Herbicide-Resistance Action Committee; WSSA: Weed Science Society of America. MOA: Mode of action.

2.2. Plant Materials

Seeds of *Chloris radiata* were collected in commercial rice fields from the Meseta zone of Ibagué (4.376603°, −75.149832°), Central Zone in Colombia. This population was considered to be resistant as the technicians and farmers of the area reported difficulty in chemical control with glyphosate and imazamox. The seeds of the susceptible population were collected in an area with no previous exposure to any herbicides (5.150360°, −74.154028°). Each population was defined as the mixture of seeds (bulk) collected in a single field.

The seeds were sown in plastic pots (10 × 10 cm × 6.3 cm), filled with peat at field capacity and covered with parafilm. These pots were kept in a growth chamber (28/18 °C day/night), 16-h photoperiod, 850 µmol m⁻² s⁻¹ light density and 60% relative humidity) until emergence (the plumule protruded). Seedlings were transplanted into 250 cm³ pots (1 plant per pot), filled with sand/peat (1:1 v/v). During the experiments, pots were placed in a greenhouse with controlled conditions (under 16-h day length with an average temperature of 28/20 °C day/night, relative humidity of 70–80%, typical conditions for rice growth in Colombia [23]) and were watered daily.

2.3. Fast Screening and Dose–Response Curve Assays

Under controlled conditions, the herbicides, glyphosate and imazamox, were applied at the recommended rate (1X) to confirm the resistance occurrence in the collected *C. radiata* population (Table 1). Herbicides were sprayed when plants reached the 3–4 leaf stage, using a laboratory chamber (De Vries Manufacturing, Hollandale, MN, USA), equipped with a TeeJet 8002 EVS flat fan nozzle calibrated to deliver 200 L ha⁻¹ at 200 kPa at a height of 50 cm. The experiments were organised in a completely randomised design using ten plants (replicates) of each population per herbicide and dose (field dose and non-treated). This experiment was repeated twice. Twenty-eight days after treatment (DAT), visual evaluation of the percentage of control, survival and the reduction in dry
weight were analysed [24,25]. Visual evaluation (%) was based on the modified scale of Frans et al. [26], observing plant vigour and chlorosis compared with the untreated plants: 0% corresponded to no weed reduction or injury, 50% weed injury more lasting, recovery doubtful, and 100% when the herbicide had a total effect on the plants.

For the dose–response curve experiments, the herbicides were applied on the same populations at the same leaf stage (3–4 fully extended leaves). For glyphosate, treatments were performed using the following doses: 0, 31.25, 62.50, 125, 250, 500, 1000, 2000, 3000 and 4000 g ae ha\(^{-1}\). For imazamox, the following doses were used: 0, 0.65, 1.25, 2.5, 5, 10, 20, 30, 40, 80, 160, 320, 640 and 1080 g ai ha\(^{-1}\). The treatments were applied in the laboratory chamber under the same application conditions mentioned above. The experiment was arranged in a completely randomised design using ten plants of each population per herbicide and dose. This experiment was repeated twice.

2.4. Shikimic Accumulation Assay

For this experiment, the methodology described by Fernández-Moreno et al. [27] was followed. Samples (50 mg) of leaf segments were harvested from the youngest fully expanded leaf from a pool of 20 plants per population at the 3–4 leaf stage. The glyphosate concentrations used were: 0, 100, 250, 500 and 1000 \(\mu\)M. The samples were measured in a spectrophotometer at 380 nm within 30 min. The assay was repeated twice with three replicates per glyphosate concentration for each population. The results were expressed as mg shikimic acid g\(^{-1}\) fresh weight.

2.5. EPSPS Enzyme Activity

For this assay the methodology described by Vázquez-García et al. [13] was followed. Approximately 5 g of leaf tissue were powdered using liquid nitrogen. The EPSPS activity was determined using EnzChek Phosphate Analysis Kit (Invitrogen, Carlsbad, CA, USA). The substrates for the EPSPS enzyme reaction were phosphoenolpyruvate (1.02 mM) and shikimate-3-phosphate (0.41 mM), supplied by Sigma-Aldrich (Madrid, Spain). Different glyphosate concentrations (0, 0.1, 1, 10, 100, and 1000 \(\mu\)M) were used to determine the inhibition of enzymatic activity (I\(_{50}\)). The used assay buffer was composed of 1 mM MgCl\(_2\), 10% glycerol, 100 mM MOPS, 2 mM sodium molybdate and 200 mM NaF. EPSPS activity was measured at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, CA, USA) to determine the amount of inorganic phosphate (\(\mu\)mol) released, measured in \(\mu\)g TSP min\(^{-1}\). The total content of protein in crude extract was measured at 595 nm with the Bradford colorimetric method [28]. Three replicates per population and glyphosate concentration were used, and the experiment was repeated twice.

2.6. ALS Enzyme Activity

ALS enzyme activity was measured as described Hatami et al. [29]. For this, 3 g of young foliar tissue (from the 4–5 leaves stage) was cut, frozen in liquid nitrogen and powdered with the addition of PVPP. The supernatant obtained in the extraction step was immediately used for ALS enzyme activity assays.

ALS activity was assayed by adding 0.05 mL of enzyme extract to 0.1 mL of freshly prepared assay buffer (0.08 M potassium phosphate, pH 7.5, 0.15 M sodium pyruvate, 1.5 mM MgCl\(_2\), 1000 \(\mu\)M FAD) and increasing concentrations of technical-grade imazamox. After mixture incubation (37 °C for 1 h), the reaction was stopped by the addition of 0.05 mL of H\(_2\)SO\(_4\) (3 M). The reaction tubes were then heated (15 min at 60 °C) to facilitate decarboxylation of acetolactate to acetoin. Acetoin was detected as a coloured complex (520 nm) formed after the addition of 0.25 mL of creatine (5 g L\(^{-1}\), freshly prepared in water) and 0.25 mL of \(\alpha\)-naphthol (50 g L\(^{-1}\), freshly prepared in 5 M NaOH) and incubated (60 °C for 15 min). The background was determined using control vials, in which the reaction was stopped before incubation and measured. Maximum ALS specific activity (nmol of acetoin mg\(^{-1}\) of protein h\(^{-1}\)) was determined in the absence of herbicide and expressed as 100%. Total protein content was measured using the Bradford method [28]. Three
replicates per population and imazamox concentration were used, and the experiment was repeated twice.

2.7. Chemical Integrated Management

2.7.1. Effectivity with and without Adjuvants

Under greenhouse conditions, an experiment was carried out to evaluate the dry weight reduction (%) of the weed at 28 DAT. For this experiment the dose used for the herbicides was selected according to the dose of the herbicide that causes a growth reduction equivalent to 50% (GR50); in the R population the dose of glyphosate was 485 g ae h$^{-1}$, and 150 g ai h$^{-1}$ for imazamox; in the S population the doses used were 95 g ae h$^{-1}$ and 4.5 g ia h$^{-1}$, respectively. The plants were sprayed with and without adjuvants (Trend® 90 at 2 mL L$^{-1}$ and Retenol® at 4 mL L$^{-1}$). Herbicide applications were made using the same equipment described above at the 3–6 leaf stage. The experiment was repeated twice with 10 replicates per dose of adjuvants and herbicide.

2.7.2. Foliar Retention with and without Adjuvants

The methodology described by Domínguez-Mendez et al. [30] was followed for this experiment. In the foliar retention assays, a solution of glyphosate (360 g ae ha$^{-1}$) and of imazamox (40 g ai ha$^{-1}$) with and without adjuvant (same doses as in effectivity assay) were applied to six plants (replicates) with four leaves, adding a visual indicator (100 mg of fluorescein per litre of 5 mM NaOH). The treatments were applied in the laboratory chamber mentioned in fast screening and dose–response curve assays under the same application conditions. After one hour, once the leaves were dried, the plants were cut at soil level and placed individually in test tubes covered with paraffin paper, containing 50 mL of 5 mM NaOH each. Subsequently, this was vigorously shaken for 30 s to eliminate possible residues of herbicide and dye that could remain on the leaf tissue. In a spectrofluorimeter (F-2500, Hitachi, Tokyo, Japan), the readings of the wash solutions were made, at a wavelength of 490 nm for excitation and 510 nm for emission. Finally, the cut tissues were packed into paper bags and dried in an oven at 80 °C for 72 h for later weighing. The retention was expressed in μL of herbicide per g of dry matter. This experiment was repeated twice.

2.7.3. Alternative Herbicide

Greenhouse experiments were performed in R and S populations of C. radiata, testing ten herbicides with eight different mechanisms of action at the recommended field dose (Table 1). At visual evaluation higher than 80% the control was considered satisfactory, at between 50 and 75% it was considered intermediate and unsatisfactory at less than 50% [26]. Using the same laboratory chamber and calibration mentioned in the dose–response assay, each herbicide was sprayed on ten (replicates) young (3–4 true leaf stage) plants in a completely randomised design, and the experiment was repeated twice. Ten plants were used as a control for all treatments. At 28 DAT, visual evaluation (%), survival (%) and dry weight reduction were measured for each treatment.

2.8. Statistical Analysis

Non-linear regression analysis was conducted to determine the amount of herbicide needed to reduce the dry weight by 50% (GR50), lethal dose of 50% (LD50) and the herbicide concentration causing 50% inhibition of enzyme activity (I50) of each C. radiata population. The log-logistic model with three parameters to inhibit shoot growth, lethal dose and enzyme activity was conducted, under the following equation (Equation (1)):

$$Y = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(c)))}$$

(1)

Model Y represents the growth response (dry weight, survival or enzyme activity) to dose x of the herbicide; d is the upper limit of the curve; c is the lower limit (fixed at 0);
b is the slope at the inflection point (i.e., GR50, LD50 or I50); x is the herbicide dose; e the herbicide concentration required to inhibit shoot growth, lethal dose and enzyme activity by 50% [31,32]. The resistance factors (RF = R/S). were computed as R-to-S GR50, LD50, or I50 ratios.

Statistical analysis was carried out with R version 4.0.4 [33] (Vienna, Austria) and its dose–response curves extension package drc and lmtest [34].

An analysis of variance (ANOVA) was conducted on data for shikimic acid, foliar retention, effectivity using adjuvants and alternative herbicides. For the statistical analysis, model assumptions of normal distribution of errors and homogeneous variance were graphically inspected, data were tested for Shapiro–Wilk normality and Hartley homoscedasticity. Significant differences were compared using Tukey’s test at the 95% probability level. The ANOVAs were performed using Statistix software (version 10.0) from Analytical Software (Tallahassee, FL, USA). To jointly analyse the data of experiments repeated twice, an analysis of residual homogeneity between runs was performed, dividing the largest sum of squares by the smallest, all the results were lesser than seven thus it was considered homogeneity to mix both runs.

3. Results

3.1. Fast Screening and Dose–Response Curve Assays

The fast-screening assay at commercial doses showed that the S population was controlled by glyphosate and imazamox (Table 2). For the R population, both herbicides showed dry weight reduction compared to untreated plants higher than 60%, visual evaluation less than 70% and a survival of 100% (Table 2), confirming resistance to glyphosate and imazamox in this species.

Table 2. Effect of glyphosate and imazamox, and the alternatives herbicides used in “Integrated chemical management assay” in Chloris radiata. Percentage of visual evaluation, survival and dry weight (DW) reduction compared to the untreated controls in the resistant (R) and susceptible (S) populations.

| Herbicide               | Visual Evaluation (%) a | Survival (%) b | DW Reduction (%) |
|-------------------------|-------------------------|----------------|-----------------|
|                         | S  R                    | S  R           | S  R            |
| Glyphosate              | 100 ± 0                 | 50.1 ± 9.2    | 0 100 ± 0       | 96.0 ± 3.3 A | 70.8 ± 8.1 B |
| Imazamox               | 92 ± 7                  | 66.0 ± 9.4    | 10.3 ± 5 100 ± 0 | 94.6 ± 3.8 A | 69.0 ± 5.9 B |
| Bispyribac-sodium      | 100 ± 0                 | 30.3 ± 8.6    | 0 100 ± 0       | 100 ± 0 A 18.5 ± 8.1 C |
| Metsulfuron-methyl     | 100 ± 0                 | 30.0 ± 5.6    | 0 100 ± 0       | 100 ± 0 A 13.2 ± 8.5 C |
| Quinclorac             | 100 ± 0                 | 19.9 ± 7.3    | 0 100 ± 0       | 100 ± 0 A 16.1 ± 8.4 C |
| Atrazine               | 100 ± 0                 | 100 ± 0       | 20.0 ± 6.5 100 ± 0 | 81.9 ± 8.4 B 83.2 ± 9.1 B |
| Glufosinate-ammonium   | 100 ± 0                 | 100 ± 0       | 0 100 ± 0       | 100 ± 0 A 100 ± 0 A |
| Oxyfluorfen            | 100 ± 0                 | 100 ± 0       | 10.0 ± 4 10.1 ± 3 | 85.2 ± 6.8 B 84.6 ± 9.4 B |
| Paraquat               | 100 ± 0                 | 100 ± 0       | 0 100 ± 0       | 100 ± 0 A 100 ± 0 A |
| Tembotrione            | 100 ± 0                 | 100 ± 0       | 0 100 ± 0       | 100 ± 0 A 100 ± 0 A |

a The visual evaluation was based in the modified scale of Frans et al. (1986). b Survival was evaluated by the ability of the plant to produce new leaves and/or have active growth. The means with different letters within a column and for each herbicide are significantly different with a 95% probability determined by Tukey’s test: n = 10.

The glyphosate dose to reduce the dry weight (GR50) and to permit a survival (LD50) by 50% of the R biotype were 481.53 ± 64.06 and 4242.39 ± 561.72 g ae ha⁻¹, respectively, with a RF of 5.1 and 9.68 for these parameters. For imazamox the resistant population needed 34.89-fold the dose of the S to decrease the dry weight by 50% (GR50 = 155.45 ± 7.9 g ai ha⁻¹) and based on LD50 values it was 37-fold more resistant (Figure 1, Table 3).
Figure 1. Effects of the glyphosate and imazamox dose on the dry weight reduction (A,B) and plant survival (C,D) of the untreated (control) Chloris radiata S (○) and R (●) populations, expressed as a percentage of the mean (n = 10) ± SE.

Table 3. Parameters of the log-logistic equation used to calculate the herbicide dose required for a 50% reduction in dry weight (GR50) and lethal dose (LD50).

| Herbicide   | C. radiata | d    | b    | p-Value | GR50/LD50 | RF  |
|-------------|------------|------|------|---------|-----------|-----|
| Glyphosate a | S         | 99.13| 1.45 | <2.2 × 10^{-16} | 95.15 ± 13.59 | 5.06 (5.01–5.09) |
|             | R         | 100.25| 1.48 | <2.2 × 10^{-16} | 481.53 ± 64.06  |
| Imazamox b  | S         | 102.26| 2.26 | 0.9998  | 4.45 ± 0.3   | 34.90 (34.40–35.60) |
|             | R         | 100.64| 3.83 | 0.9999  | 155.45 ± 7.9 |     |

3.2. Shikimate Accumulation

As the dose of glyphosate was increased, the accumulation of shikimic acid gradually increased in both populations (R and S) of C. radiata, but the susceptible populations showed higher accumulation compared with resistant ones. For the S population, 43.5% more shikimic acid accumulates at the highest glyphosate concentration evaluated compared to the lowest concentration. For the R population the difference was 13%. At the lowest
concentration of glyphosate (250 µM), S plants accumulated 2.5 times more shikimic acid than R plants, and at the highest concentration (2000 µM) the difference was 3.1 times greater (~2.1 ± 0.16 mg shikimic acid g⁻¹ fresh weight) than the R population (Figure 2).

**Figure 2.** Shikimic acid accumulation of glyphosate-susceptible and -resistant *Chloris radiata* plants at different glyphosate concentrations. Vertical bars represent the standard error of the mean (n = 3 technical replicates). Means with different letters within are statistically different at 95% probability determined by the Tukey test.

### 3.3. EPSPS Enzyme Activity

The $I_{50}$ (glyphosate concentration to reduce the enzyme activity by 50%) in the S population was 0.22 µM, while for the R population was 4.36 µM. These results showed the activity of the enzyme in the R population was approximately 20 times higher compared to the S population (Figure 3A).

**Figure 3.** (A) EPSPS and (B) ALS enzyme activity expressed as a percentage of the untreated control in leaf extracts of glyphosate/imazamox-susceptible and -resistant *Chloris radiata* plants.

### 3.4. ALS Enzyme Activity

The imazamox concentration needed to inhibit the ALS activity by 50% ($I_{50}$) in the S population was 5.47 µM, while that in the R population was 487 µM, which means the R population was approximately 89-fold more resistant to imazamox than the S population (Figure 3B).
3.5. Integrated Chemical Management

3.5.1. Effectivity with and without Adjuvants

The results of the effectivity experiment were similar to those of foliar retention, showing the effect of the addition of adjuvants in better control of susceptible and resistant populations of *C. radiata*. For glyphosate and imazamox resistant populations, the use of Trend® 90 adjuvant showed the best results, increasing the dry weight reduction by 50% for glyphosate and 32% for imazamox (Table 4).

### Table 4. Dry weight reduction (%) and increased effectiveness (%) with glyphosate and imazamox in the presence and absence of adjuvants from the R and S populations of *Chloris radiata*.

| Treatment                  | Glyphosate | Imazamox |
|----------------------------|------------|----------|
|                            | S (95 g ae ha\(^{-1}\)) | R (485 g ae ha\(^{-1}\)) | S (4.5 g ai ha\(^{-1}\)) | R (150 g ai ha\(^{-1}\)) |
| Only Herbicide             | 51.30 ± 3.82 B | 54.82 ± 3.83 C | 52.06 ± 2.70 B | 48.72 ± 2.42 B |
| Herbicide + Retenol        | 65.68 ± 3.64 A | 71.36 ± 3.98 B | 60.22 ± 4.18 A | 58.06 ± 3.85 A |
| Herbicide + Trend 90       | 70.82 ± 2.11 A | 81.98 ± 2.11 A | 63.20 ± 3.58 A | 64.62 ± 4.08 A |

*a* IE = Increase effectiveness in the control (%); (numbers in parentheses are the confidence). The means with different letters within a column and for each herbicide are significantly different from the 95% probability determined by Tukey’s test: n = 10.

3.5.2. Foliar Retention with and without Adjuvants

The use of adjuvants increased herbicide retention for both R and S populations of *C. radiata*. The increase in foliar retention was higher for glyphosate than imazamox, regardless of the population. For glyphosate, the addition of Trend® 90 showed the best results with increases in retained herbicide higher than 250% in the R and S populations (Table 5). Moreover, this adjuvant also presented the best result for imazamox; nevertheless, the R plants had greater retained herbicide (205%) than the S plants (154%) (Table 5).

### Table 5. Foliar retention (expressed as g\(^{-1}\) dry matter) of glyphosate (360 g ae ha\(^{-1}\)) and imazamox (40 g ai ha\(^{-1}\)) with and without adjuvants and increased retained herbicide in R and S populations of *Chloris radiata*.

| Treatment                  | Glyphosate | Imazamox |
|----------------------------|------------|----------|
|                            | S (µL g\(^{-1}\) dry Matter) | R (µL g\(^{-1}\) dry Matter) | S (µL g\(^{-1}\) dry Matter) | R (µL g\(^{-1}\) dry Matter) |
| Only Herbicide             | 377.68 ± 10.11 C | 335.18 ± 17.37 C | 153.91 ± 17.22 C | 121.07 ± 12.46 C |
| Herbicide + Retenol        | 1146.69 ± 49.95 B | 1034.69 ± 24.30 B | 321.60 ± 4.18 A | 291.71 ± 19.74 B |
| Herbicide + Trend 90       | 1390.17 ± 31.94 A | 1186.96 ± 46.23 A | 390.67 ± 16.91 A | 370.39 ± 12.50 A |

*a* IRH = Increased retained herbicide (%); (numbers in parentheses are the confidence). The means with different letters within the R and S columns and for each herbicide are significantly different from the 95% probability determined by Tukey’s test: n = 6.

3.5.3. Alternative Herbicides

Almost all herbicides used in the postemergence weed treatments were effective for control of resistant population, with values of 100% on visual evaluation, with the exception of bispyribac-sodium, metsulfuron-methyl and quinclorac. For these herbicides, the control was unsatisfactory and there was no decrease in plant growth compared with...
the susceptible population (Table 2). The survival percentage and the dry weight reduction showed the same.

4. Discussion

The *Chloris* genus presents a certain level of natural tolerance to glyphosate, evidenced in the differential response of some species [36–38]. Nonetheless, the results of the present study confirm the first case of *Chloris radiata* with glyphosate and imazamox resistance. Glyphosate resistance has been studied in other species of the same genus, such as *C. polydactyla*, *C. elata*, *C. virgata*, *C. truncata*, *C. barbata* and more recently in *C. distichophylla* [13,36–39]. The resistance factor based on GR$_{50}$ obtained in this study (RF = 5.1) is similar to Brazilian research for *C. distichophylla* (RF = 5.1) [13] and *C. elata* (RF = 5.4) [39], and in Mexico for *C. barbata* (RF = 4.8) [38]. However, the glyphosate dose required to control a plant population by 50% (LD$_{50}$) is higher than that reported in the studies mentioned above but similar to studies conducted in Australia [40,41]. This active ingredient is used in rice crops in Colombia mainly in pre-sowing and can also be used in pre-emergent crop applications, to control weedy rice and other weeds considered difficult to control [19].

The low accumulation of shikimic acid in resistant populations (3.1 times less) compared to susceptible populations evidences the loss of susceptibility to glyphosate (Figure 2). Similar results were reported in *C. elata* from Cuba and Brazil (4.9 and 5.4 times, respectively) [37,39], *C. truncata* (2.4–8.7) and *C. virgata* (2.0–9.7) from Australia [40,41]. Accumulation differences between susceptible and resistant populations are a rapid and reliable indicator of glyphosate resistance [42]. Despite that, this parameter is only an indicator of resistance and indicates a limited interaction between the herbicide and the EPSPS protein [38].

For the genus *Chloris*, the current study is the first report of imazamox resistance. *C. radiata* showed high resistance factors based on GR$_{50}$ and LD$_{50}$ (34.89 and 37.43, respectively). These results differ from those obtained in studies in *Echinochloa colona* (2.5), *Lolium perenne* ssp. *multiflorum* (5.3 and 17.48), and *Bromus tectorum* (110) [43–45]. Imazamox is used in Clearfield production systems [19], which started to be used in Colombia in 2003 [46]. Currently, there is only one report of resistance to imidazolinones in Colombia; weedy rice resistant to imazapyr and imazamox [47].

The confirmation of imazamox resistance is demonstrated by I$_{50}$ for R plants, which was 89 times greater than S plants. This result shows the important role that the enzyme plays in resistance to imazamox [48]. Broadly and based on ALS enzyme inhibition by ALS inhibitors, a high level of resistance indicates the presence of simple or multiple mutations in the ALS enzyme of resistant plants [49]. It is not only mutations that can be the mechanisms in the target site, it also can be due to an increase in the ALS gene copy number and amplification [50].

Based on chemical integrated management of resistant population of *C. radiata* to glyphosate and imazamox, it was evident that the use of adjuvants improved the control of the species for both herbicides. One of the factors to improve the efficacy of herbicides is increasing the absorption into plant foliage, and the use of adjuvants is an important tool for this [51]. Laboratory experiments (foliar retention) showed increases greater than 200% in the effectiveness of the active ingredients, where the use of Trend® 90 presented the best results. Palma-Bautista et al. [35] found similar results when they added adjuvants to glyphosate applications for control of resistant *L. rigidum* and *Conyza canadensis* populations. In greenhouse experiments, the effectiveness test did not show increases as significant as the previous test; nevertheless, it increased the effectiveness with the use of adjuvants by 40% for glyphosate and 25% for imazamox; again Trend® 90 presented the best results. Trend® 90 is a non-ionic surfactant, this kind of adjuvant is the most commonly used in agriculture and its main characteristic is breaking water surface tension which allows a better covering and penetration [51].

In the alternative herbicide test for control of resistant *C. radiata* populations, poor weed control was achieved using both bispyribac-sodium and metsulfuron-methyl, indicat-
ing a possible cross-resistance for other ALS inhibitors. Similarly, low control percentages (<20%) were recorded using auxinic herbicides, showing possible multiple resistance with quinclorac; both cases still await verification. In Colombia, there are some reports of resistance to these active ingredients in Murdannia nudiflora, Ischaemum rugosum and E. colona, also in rice fields [25,52,53]. Initially, when bispyribac-sodium was introduced, the control of different weeds was satisfactory, and farmers preferred using this herbicide than other alternatives due to its crop selectivity, low dosage, high efficiency and versatility, which led to more than 90% of the area being treated with this active ingredient [53]. Quinclorac, a quinolinecarboxylic acid, has 11 reports of resistance, of which ten are associated with rice cultivation [47], due to its selectivity to cultivation and good control of species of the Echinochloa complex [54]. For example, in the central rice zone in Colombia, 54% of the area routinely used quinclorac mainly in late postemergence for control of E. colona [25]. Weed management implemented in Colombian rice showed high dependence on chemical control, including the use of quinclorac, propanil, fenoxaprop-ethyl, profoxydim, sulfonyleurea and bispyribac-sodium [53].

The alternative herbicides evaluated are viable tools to prevent the spread of this species, especially ACCase-inhibiting graminicide herbicides in postemergence applications, and paraquat and glufosinate in pre-sowing applications. Similar results were reported in C. distichophylla from Brazil, where herbicides such as clethodim, quizalofop, paraquat and glufosinate showed total control of the plants [13]; meanwhile paraquat, glufosinate and atrazine have high control in the early stages of the weed [55], and setoxydim and haloxyfop-p-methyl have control higher than 90% [56]. Recent studies on glyphosate-resistant C. virgata and C. truncata with sequential herbicide applications, showed that the best control was haloxyfop-paraquat treatment with a few days (1–4) between applications, and glyphosate-paraquat only for C. truncata [57]. Other research has shown that isoxaflutole, haloxyfop and different photosystem II inhibitors (atrazine, simazine, terbutylazine) in a tank mixture with paraquat were effective in controlling C. virgata [58].

The best strategies for managing herbicide-resistant weeds are in diversification, seeking to reduce selection pressure and to control resistant populations. Our results demonstrate that including different mechanisms of action in integrated weed management is a good alternative. However, farmers should diversify all weed control practices, including rotation in cropping systems [59].

5. Conclusions

Chloris radiata is a species that has become a problem in recent years, attributable to possible failures in chemical control. Our research confirms the first case of glyphosate resistance and multiple resistance to ALS inhibitor (imazamox), with intermedium resistant factor for glyphosate and high for imazamox. The results of alternative herbicide experiments evidenced a possible cross-resistance (bispyribac-sodium and metsulfuron-methyl) and another multiple resistance (quinlorac) resulting in poor control of the species. New studies to confirm these resistances and elucidate resistance mechanisms are being conducted. Based on integrated chemical management, we concluded that the use of adjuvants improved foliar retention and effectiveness of glyphosate and imazamox. On the other hand, the ACCase-inhibitor herbicides, paraquat and glufosinate, showed better results for the control of resistant C. radiata.

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Abbreviations
ALS: acetolactate synthase; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; ACCase: acetyl-CoA carboxylase; R: resistant population; SF: susceptible population; RF: resistance factor; GR50: dose of herbicide needed to reduce the fresh weight by 50%; LD50: lethal dose of 50%. Is50: herbicide concentration causing 50% inhibition of enzyme activity; g a.i. ha−1 = grams of equivalent acid per hectare; g ai ha−1 = grams of active ingredient per hectare; HRAC: Herbicide-Resistance Action Committee; WSSA: Weed Science Society of America; MOA: Mode of action; DAT: days after treatment; DW: dry weight; IE = Increase effectiveness in the control; IRH = Increased retained herbicide.

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