

Article

# Target-Site Resistance to Glyphosate in *Chloris Virgata* Biotypes and Alternative Herbicide Options for its Control

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Received: 31 July 2020; Accepted: 25 August 2020; Published: 27 August 2020



**Abstract:** Due to the overdependence on glyphosate to manage weeds in fallow conditions, glyphosate resistance has developed in various biotypes of several grass weeds, including *Chloris virgata* Sw. The first case of glyphosate resistance in *C. virgata* was found in 2015 in Australia, and since then several cases have been confirmed in several biotypes across Australia. Pot studies were conducted with 10 biotypes of *C. virgata* to determine glyphosate resistance levels. The biotypes were identified as either susceptible, moderately resistant or highly resistant based on the glyphosate dose required to kill 50% of plants. Two glyphosate-susceptible (GS) and two glyphosate-resistant (GR) biotypes were identified by the dose-response study and analyzed for the presence of target-site mutation in the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene. Performance of alternative herbicides to glyphosate as well as the double-knock herbicide approach was evaluated on the two GS (Ch and SGM2) and two GR (SGW2 and CP2) biotypes. Three herbicides, clethodim, haloxyfop and paraquat, were found to be effective (100% control) against all four biotypes when applied at the 4–5 leaf stage. All the sequential herbicide treatments, such as glyphosate followed by paraquat and glufosinate-ammonium followed by paraquat, provided 100% control of all four biotypes of *C. virgata*. This study identified effective herbicide options for the control of GR *C. virgata* and showed that target-site mutations were involved in the resistance of two biotypes to glyphosate (SGW2 and CP2). Results could aid farmers in selecting herbicides to manage *C. virgata* in their fields.

**Keywords:** feathertop Rhodes grass; DNA sequencing; sequential herbicide application; missense mutation; silent mutation; *EPSPS*; double knock

## 1. Introduction

*Chloris virgata* Sw. is a summer annual grass weed of the Poaceae family, commonly referred to by several other names such as feathertop Rhodes grass, feathered finger grass, oldland grass, feathered windmill grass, feathered *Chloris* and sweetgrass [1]. This weed species exhibits a C<sub>4</sub> photosynthesis mechanism [2], and has been identified as a host for aphids, barley yellow dwarf and cereal yellow dwarf viruses and some nematode species [1]. *C. virgata* is widely distributed across the mainland of Australia and is considered a major weed of South Australian vineyards and orchards, grain farming systems in Queensland, Western Australia and the Northern Territory, and cotton farming systems across Australia mainly in New South Wales [1]. It has been included in the top 20 weeds of major concern in Australia, infesting an area of 118,000 ha of Australian grain cropping land and contributing to a huge loss in yield (39,300 tons of grain per year) and revenue (AUD 7.7 million per year) [3]. In mungbean, 22–25 plants/m<sup>2</sup> of *C. virgata* reduced grain yield by 50% compared with the

weed-free treatment (Manalil and Chauhan, unpublished data). However, due to high seed retention at crop maturity, *C. virgata* seeds could be captured through harvest weed seed control in sorghum [4].

*Chloris virgata* can achieve a maximum of 1 m height and produce 600 g/m<sup>2</sup> dry matter in summer months at maturity [5]. Additionally, it is a prolific seed bearer (>40,000 seeds per plant) [5]. Seeds of *C. virgata* are light in weight, and due to their aerodynamic shape, they can travel up to 13 m from a mother plant in normal wind speed [1]. The seeds possess two trichomes (hairs) that aid in sticking to agricultural machinery and laborers, which facilitates their transfer from one ecosystem to another. Therefore, considering its high seed production and dispersal ability, *C. virgata* tends to spread rapidly across agroecosystems. Seeds of *C. virgata* follow two kinds of dispersal mechanisms: hydrochory and anemochory (dissemination through water and wind, respectively). Its seeds can germinate under a wide range of temperatures (5 to 35 °C) and tolerate salinities up to 250 mM sodium chloride [6]. Therefore, it is considered a halophyte.

Australian farming systems have shifted from traditional tillage to no-till or minimal tillage systems, and such changes have influenced weed management tactics, causing an over-reliance on glyphosate application in fallow conditions [7]. This has resulted in the development of resistance against glyphosate (inhibitor of enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase) in several weed species, including *C. virgata* [8]. The frequent use of glyphosate and single knockdown herbicide applications may lead to the development of glyphosate-resistant (GR) biotypes. The first case of glyphosate resistance in *C. virgata* was discovered in 2015 in Australia [8], with several more biotypes since being recorded as GR in Australia [9]. Resistance against a herbicide develops due to evolutionary adaptation, and there are two types of herbicide resistance mechanisms, namely target-site resistance (TSR) and non-target site resistance (NTSR) [10]. TSR occurs either due to changes in the structure of the target enzyme, which prevents herbicide binding, or escalating the activity of the target enzyme. NTSR is caused due to changes in any mechanism not involved with the target site, such as reduction of herbicide uptake or translocation in plants, or augmented herbicide detoxification [11].

Biotypes which possess herbicide resistance or are at risk of developing resistance require more strategic control approaches, such as exposure to different groups of herbicides or sequential herbicide applications (double knock), rather than following traditional weed control methods [8]. Sequential herbicide applications, in which different modes of action (MOA) of herbicides are applied in a sequence, has been shown to control several weed species effectively [12]. Applications of different herbicide MOA could lower the risk for development of herbicide resistance in weed biotypes. Therefore, a sequential herbicide application approach and utilizing herbicides other than glyphosate are widely accepted methods in order to control weed biotypes that have a risk of developing glyphosate resistance [8].

A study was conducted to determine glyphosate resistance levels in 10 different biotypes of *C. virgata*. Sequencing of the *EPSPS* gene was also performed to determine whether the resistance in *C. virgata* biotypes was due to target-site or non-target site resistance mechanisms. This study also evaluated the efficacy of alternate herbicide options and double-knock (sequential) herbicide applications on *C. virgata*.

## 2. Materials and Methods

### 2.1. Seed Collection and Storage

Seeds of 10 different biotypes of *C. virgata* were collected in April 2017 from different crop situations (Table 1). Panicles of each plant were detached by utilizing a sickle and immediately placed in paper bags that were labelled for future identification. Seeds were collected from several plants for each biotype. All the seed bags were brought to the Weed Science laboratory, Queensland Alliance for Agriculture and Food Innovation (QAAFI), Gatton, Queensland, Australia. Mature seeds were separated from other material and stored in airtight containers to prevent any unwanted contaminations. Dark conditions and room temperature (20–25 °C) were maintained for the storage of the seed containers [6]. All the

biotypes were sown in 23 (height) × 25 cm (diameter) pots for seed production on 7 January 2019, at the Research Farm of the University of Queensland, Gatton. An automated sprinkler irrigation system was used to facilitate irrigation throughout the growing season. Watering was carried out four times (10 min each time) every day with an interval of 6 h. These seed batches were used in all experiments.

**Table 1.** Biotypes collected from different regions of Queensland, Australia.

Biotype	Location	Situation	Coordinates	Collection Date
D1	Dalby	Sorghum	−27.1844, 151.2668	March 2017
Ch	Chinchilla	Wheat fallow	−26.8264, 150.5802	March 2017
CP1	Cecil Plains	Sorghum	−27.1799, 151.2554	April 2017
D2	Dalby	Mungbean	−27.2866, 151.3228	March 2017
SGM1	St. George	Mungbean boundary	−28.0041, 148.4100	April 2017
SGW1	St. George	Wheat	−28.0775, 148.4100	April 2017
SGM2	St. George	Mungbean	−28.0916, 148.4271	April 2017
SGW2	St. George	Wheat fallow	−28.0454, 148.3158	April 2017
Ga	Gatton	Mungbean	−27.3318, 150.2011	April 2017
CP2	Cecil Plains	Sorghum	−27.2935, 151.1283	April 2017

## 2.2. Experimental Approach and Design

Experiments were performed at the Research Farm of the University of Queensland, Gatton, Australia (−27.5386, 152.3346), in 2019 and 2020. Potting mix (Centenary Landscaping, Mt Ommaney, Queensland) was used as media to conduct trials. Twelve seeds of each biotype were placed on the surface of pots with forceps followed by manual uniform spreading of sieved potting mix over the seeds to achieve a homogeneous depth (0.2 cm). Dark-colored seeds were preferred over ivory-colored seeds because dark-colored seeds are known to have higher germination ability [6]. Trials were established in a randomized complete block design (RCBD) with three replications. Plant were not irrigated for 24 h after herbicide application.

## 2.3. Experiment 1. Glyphosate Dose Response

This experiment was established in pots (12.5 cm diameter) with 3 replications of 10 biotypes (Table 1). Plants were thinned to 4 plants/pot after emergence. Eight different doses of glyphosate (0, 0.143, 0.285, 0.570, 1.140, 2.280, 4.560 and 9.120 kg ae ha<sup>−1</sup>) were applied with the help of a research track sprayer (manufactured by Woodlands Road Engineering, Gatton, Australia), which delivered 114 L ha<sup>−1</sup> spray volume through flat fan nozzles (TeeJet XR 110015). Plants were sprayed at the 4–5 leaf stage.

## 2.4. Experiment 2. EPSPS Gene Sequencing

Two glyphosate-susceptible (GS) (Ch and SGM2) and two GR (SGW2 and CP2) biotypes were selected based on the glyphosate dose-response experiment. The aerial distance between the locations of the four biotypes was measured using Google Maps. The distance between the locations of the SGM2 and SGW2 biotypes is 12 km (Table 1). The aerial distance between the locations of Ch and CP2 is approximately 77 km (Table 1). Chinchilla and Cecil Plains regions are approximately 260 and 285 km from the St George region, respectively. Five plant samples per biotype were analyzed. Fresh plant materials were acquired from the leaves of a young plant, followed by immediate placement on ice, and then taken to the lab for DNA extraction. The cetyl trimethylammonium bromide (CTAB) DNA extraction method with some modifications [13] was followed and, genomic DNA was extracted from leaf tissues. A polymerase chain reaction

(PCR) was carried out to amplify the conserved area of the *EPSPS* gene. Primer sequences were adapted from a previous study [2] (Forward—AACAGTGAGGAYGTYCACTACATGCT; Reverse—CGAACAGGAGGGCMTTCAGTGCCAAG). Each PCR was set up in a total volume of 20  $\mu$ L, containing 4  $\mu$ L 5x MyTaq reaction buffer (Bioline, Australia), 30 ng template DNA, 0.3  $\mu$ M of each primer, 0.2  $\mu$ L MyTaq HS DNA polymerase (Bioline, Australia) and 13.6  $\mu$ L water. A T100 thermal cycler (Bio-Rad, Australia) was used with cycle conditions of 3 min denaturing at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 57 °C, 45 s elongation at 72 °C, and a final extension for 7 min at 72 °C. PCR products were visualized in 1.2% agarose gels stained with GelRed (Biotium, CA, USA) and observed under UV light. The Australian Genome Research Facility, Brisbane, Australia, conducted DNA sequencing using the same primers to acquire forward and reverse sequences. MEGA software was used to check sequencing quality and to perform sequencing alignment.

### 2.5. Experiment 3. Alternative Herbicide Options

An experiment was established to examine the effect of alternative herbicides to glyphosate on the four biotypes studied for sequencing [two GS (Ch and SGM2) and two GR (SGW2 and CP2)]. The experiment was conducted in pots (12.5 cm diameter) with three replications of four seedlings per pot. Six different herbicide treatments, including a control treatment, were applied (Table 2) as mentioned above for the glyphosate dose-response experiment. The experiment was repeated over time (at least one month between the runs).

### 2.6. Experiment 4. Double Knock Approach

This experiment was carried out in pots (12.5 cm diameter) with three replications of four seedlings per pot. Two GS (Ch and SGM2) and two GR (SGW2 and CP2) biotypes were subjected to the sequential herbicide approach, in which different groups of herbicides were applied to each experimental unit maintaining a one-week interval between the spraying of the first and second herbicide. One control, two single and four sequential treatments were applied to each experimental unit (Table 3). Herbicide treatments were applied as described above for the glyphosate dose-response experiment. The experiment was repeated over time (at least one month between the runs).

### 2.7. Response Variables Recorded

The total number of plants per pot were recorded before the application of herbicides. Survival percentages were recorded after the application of herbicides by counting living (green) plants as a percentage of total plants. Dry matter of surviving plants was obtained by cutting them from the base with secateurs and instantly placing them in paper bags before drying all samples in an oven at 70 °C for 72 h. Surviving plants of each biotype and treatment were weighed and then converted into dry matter per plant by dividing the combined dry matter by the total number of surviving plants. Dry matter and surviving plants were recorded 28 days after herbicide application for the glyphosate dose-response and alternative herbicide options experiments. For the double-knock experiment, dry matter and surviving plants were recorded 28 days after the second herbicide treatment.

### 2.8. Statistical Analyses

All experiments were carried out in a randomized complete block design (RCBD) with three replications in each experimental run. Data were pooled across the two runs as no significant difference was observed between the two runs. Nontransformed data were used as data transformation did not improve the homogeneity of variance. Probit analysis was performed using SPSS version 25 software (IBM Corp., Armonk, NY, USA) to evaluate the relationship between survival percentages and herbicide dose-response of glyphosate and to obtain LD<sub>50</sub> (herbicide dose to kill 50% of plants) values for each biotype. The data of survival percentages and dry matter production for alternative herbicide options to glyphosate and double-knock experiments were subjected to analysis of variance (ANOVA) (SPSS software).

**Table 2.** Details of herbicides used as alternatives to glyphosate.

Active Constituent	Chemical Family	Mode of Action	Rates
Clethodim 240 g/L	Cyclohexanediones	Inhibits acetyl co-enzyme A carboxylase	90 g ai ha <sup>-1</sup> + 1% Supercharge
Haloxypop 520 g/L	Aryloxyphenoxypropionates	Inhibits acetyl co-enzyme A carboxylase	78 g ai ha <sup>-1</sup> + 1% Hasten
Imazamox 33 g/L plus Imazapyr 15 g/L	Imidazolinones	Inhibits acetolactate synthesis	25 g ai ha <sup>-1</sup> + 1% Hasten
Paraquat 360 g/L	Bipyridils	Inhibits photo synthesis 1	600 g ai ha <sup>-1</sup> + 1% BS 1000
Pinoxaden 50 g/L	Phenylpyrazoles	Inhibits acetyl co-enzyme A carboxylase	20 g ai ha <sup>-1</sup> + 0.5% Adigor

**Table 3.** Single and sequential herbicide treatments for the double knock approach.

Active Constituent	Application Type	Chemical Family	Mode of Action	Rate
Glyphosate 570 g/L	Single	Glycines	Inhibits EPSP synthase	1.14 kg ae ha <sup>-1</sup>
Glufosinate-ammonium 200 g/L	Single	Phosphinic acids	Inhibits glutamine synthetase	0.75 kg ai ha <sup>-1</sup>
Glyphosate 570 g/L fb Paraquat 360 g/L	Sequential	Glycines fb Bipyridils	Inhibits EPSP synthase fb Inhibits photo synthesis 1	1.14 kg ae ha <sup>-1</sup> fb 0.6 kg ai ha <sup>-1</sup>
Glyphosate 570 g/L fb Paraquat 360 g/L	Sequential	Glycines fb Bipyridils	Inhibits EPSP synthase fb Inhibits photo synthesis 1	1.14 kg ae ha <sup>-1</sup> fb 1.2 kg ai ha <sup>-1</sup>
Glufosinate-ammonium 200 g/L fb Paraquat 360 g/L	Sequential	Phosphinic acids fb Bipyridils	Inhibits glutamine synthetase fb Inhibits photo synthesis 1	0.75 kg ai ha <sup>-1</sup> fb 0.6 kg ai ha <sup>-1</sup>
Glufosinate-ammonium 200 g/L fb Paraquat 360 g/L	Sequential	Phosphinic acids fb Bipyridils	Inhibits glutamine synthetase fb Inhibits photo synthesis 1	0.75 kg ai ha <sup>-1</sup> fb 1.2 kg ai ha <sup>-1</sup>

Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; fb, followed by treatment after one week of the first treatment.

### 3. Results and Discussion

#### 3.1. Experiment 1. Glyphosate Dose-Response

Survival percentages of all the biotypes against different doses of glyphosate were subjected to probit analysis, and LD<sub>50</sub> values were calculated. Biotype Ch was found to be the most susceptible as it showed the lowest LD<sub>50</sub> for glyphosate application (0.21 kg ae ha<sup>-1</sup>) (Table 4). The degree of resistance relative to the most susceptible biotype was then calculated and represented as a resistance/susceptible (R/S) ratio. The ratio was calculated as the LD<sub>50</sub> of each biotype divided by the LD<sub>50</sub> of Ch. The response of each biotype to glyphosate application was grouped into three categories: susceptible (R/S ratio 1 to 11), moderately resistant (R/S ratio 12 to 20) and highly resistant (R/S ratio 21 to 28) (Table 4) [14]. Biotypes Ch and SGM2 possessed the lowest LD<sub>50</sub> values with 0.21 and 0.85 kg ae ha<sup>-1</sup>, respectively; however, SGM2 was 4-fold more resistant to the most susceptible biotype Ch. As such, these two biotypes were chosen for the experiments involving alternative herbicide options and a double-knock approach. The LD<sub>50</sub> of all the biotypes classed as moderately resistant varied from 2.64 to 4.24 kg ae ha<sup>-1</sup>, and the moderately resistant group was on average 16-fold more resistant than the most susceptible biotype Ch. Two biotypes, CP2 and SGW2, were classed as highly resistant and possessed the two highest LD<sub>50</sub> values (5.71 and 5.75 kg ae ha<sup>-1</sup> for CP2 and SGW2, respectively). The highly resistant group was on average 27-fold more resistant compared to the most susceptible biotype Ch. These different levels of glyphosate resistance could mainly be due to either the exposure history to glyphosate or differential herbicide resistance mechanisms within the biotypes of *C. virgata* [2,15].

**Table 4.** Herbicide dose to kill 50% of plants (LD<sub>50</sub>, kg ae ha<sup>-1</sup>) obtained to determine glyphosate resistance levels. Resistance/susceptible (R/S) is a ratio of LD<sub>50</sub> of each biotype compared to the most susceptible biotype (Ch).

Biotypes	LD <sub>50</sub> (kg ae ha <sup>-1</sup> )	R/S Ratio	Resistance Status
Ch	0.21	1	Susceptible
SGM2	0.85	4.04	
D1	2.64	12.53	Moderately Resistant
CP1	2.75	13.03	
SGW1	2.89	13.67	
SGM1	3.88	18.38	
Ga	3.96	18.78	
D2	4.24	20.00	Highly Resistant
CP2	5.72	27.09	
SGW2	5.76	27.28	

These results indicate that higher doses of glyphosate could control *C. virgata*; however, it is not economical and recommended to use higher doses of glyphosate because higher rates may enhance the risk of developing glyphosate resistance [14,16,17]. Differential glyphosate resistance levels between the biotypes suggest the need for area-specific control tactics rather than following species-specific control tactics for *C. virgata*. The occurrence of glyphosate resistance in *C. virgata* has increased since the first case in 2015 in Australia [8], and several other cases have been confirmed in Australia as of 2020 [9]. Some biotypes exhibited a high level of resistance against glyphosate. Therefore, more strategic approaches are required to manage *C. virgata*, such as utilization of alternative options to glyphosate [8], employing a double-knock tactic [18], tank mixture herbicide applications [8] and harvest weed seed control [19].

Dry matter reduction percentage was calculated for each biotype based on the dry matter of the control treatment for each biotype to evaluate the relationship between glyphosate dose and

dry matter per plant of all biotypes. Dry matter per plant decreased with the increasing glyphosate dose (Figure 1B). Due to the susceptibility against glyphosate (Table 4), biotype Ch was completely controlled at a low glyphosate dose ( $0.285 \text{ kg ae ha}^{-1}$ ) and exhibited a 100% reduction in dry matter per plant at this dose. Biotype SGM2 did not survive at  $2.280 \text{ kg ae ha}^{-1}$  (Figure 1A) and hence produced no dry matter (Figure 1A,B). The glyphosate dose required to kill SGM2 ( $2.280 \text{ kg ae ha}^{-1}$ ) was eight times more than the dose required to kill the most susceptible biotype Ch ( $0.285 \text{ kg ae ha}^{-1}$ ). *C. virgata* generally requires a high dose of glyphosate to control it as the species is identified as a glyphosate-tolerant species [2]. Despite that, several biotypes have been identified to be susceptible to glyphosate [2]. Irrespective of resistance levels and survival percentages, all the biotypes exhibited more than 80% reduction in dry matter at  $1.140 \text{ kg ae ha}^{-1}$  (Figure 1B). However, survival percentages of all the biotypes except Ch and SGM2 were more than 80% at  $1.140 \text{ kg ae ha}^{-1}$  (Figure 1A).

These results indicate that the application of glyphosate alone may not control *C. virgata* effectively; however, the ability of glyphosate to suppress dry matter suggests that it could be a good option in a tank mixture and as a first treatment as part of double knock herbicide application. Glyphosate as part of a double knock has been effective in the control of many weed species such as *Conyza bonariensis* (L.) Cronquist [20], *Lolium rigidum* Gaud. [21], *Echinochloa colona* (L.) Link [18] and *Chloris truncata* R. Br. [18]. Glyphosate application along with other herbicides in the tank mixture exhibited considerable control of many weed species such as *Sorghum halepense* (L.) Pers., *Urochloa platyphylla* (Munro ex C. Wright) RD Webster, *Ipomoea lacunosa* L. and *Sesbania herbacea* (Mill.) McVaugh [22]. All the biotypes in this study did not survive and exhibited a 100% reduction in dry matter per plant at the highest glyphosate dose of  $9.120 \text{ kg ae ha}^{-1}$  (Figure 1A,B).

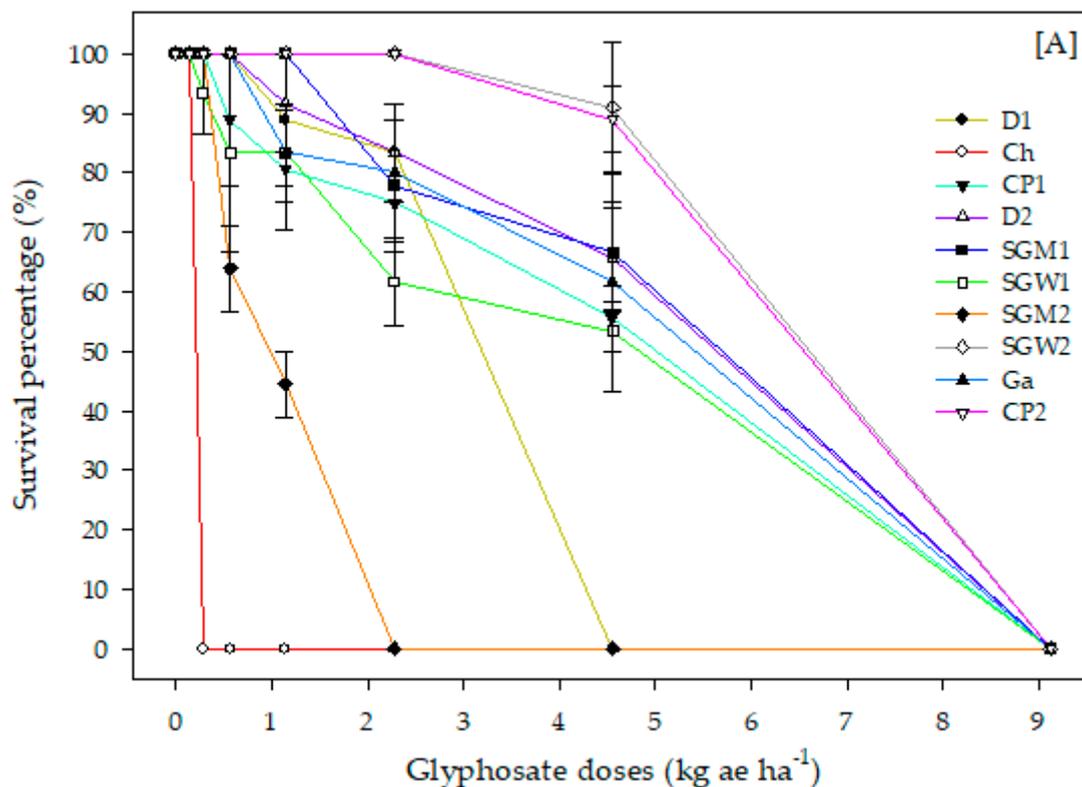
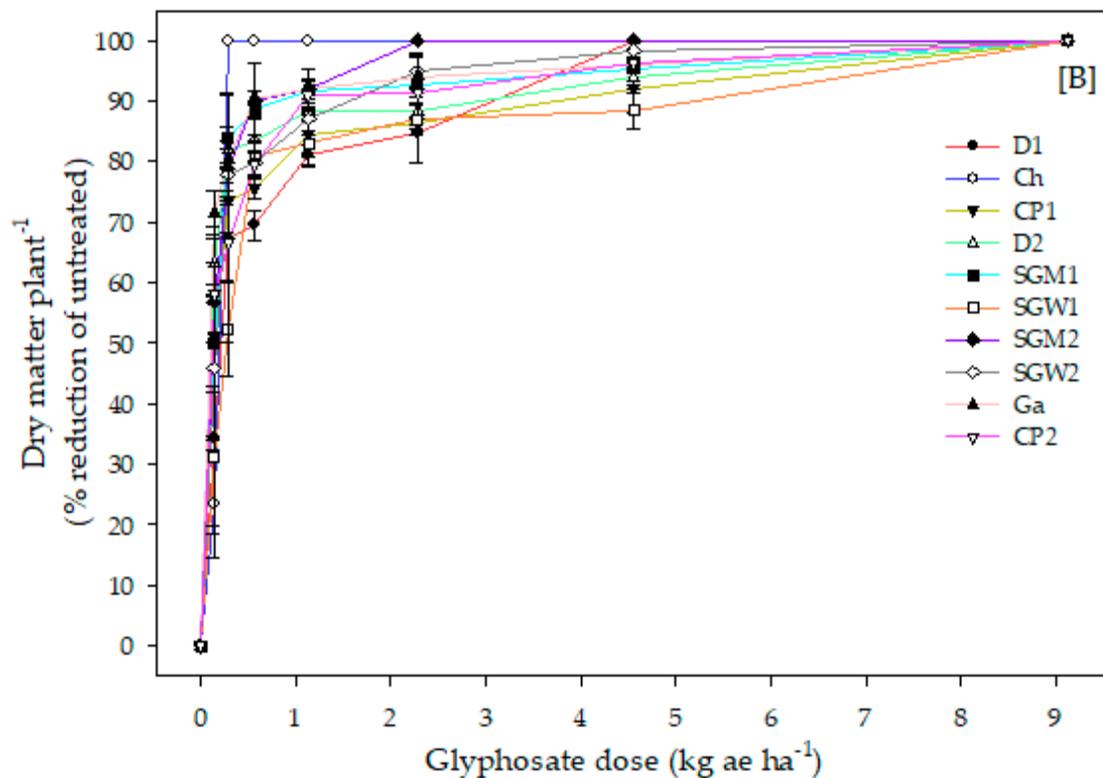


Figure 1. Cont.



**Figure 1.** Effect of glyphosate doses on survival percentage (A) and dry matter plant<sup>-1</sup> (% reduction of untreated) (B) of 10 biotypes of *Chloris virgata*. Error bars represent the standard error of mean. Glyphosate-susceptible biotypes are Ch and SGM2, and glyphosate-resistant biotypes are SGW2 and CP2.

### 3.2. Experiment 2. EPSPS Gene Sequencing

Two GS (Ch and SGM2) and two GR (SGW2 and CP2) biotypes of *C. virgata* were analyzed to identify any mutations within the conserved region of the *EPSPS* gene that could cause target-site resistance to glyphosate. A comparison of sequences revealed that three biotypes (SGM2, SGW2 and CP2) possessed missense mutations (Table 5). Missense mutation was observed in only one sample out of five of SGM2 (GS). This could be because of there are resistant plants in this biotype but at a lower number than for two highly resistant biotypes. Two biotypes (SGM2 and SGW2) exhibited a nucleotide change of CAA to either ACA or ACG in codon 106. This predicts an amino acid change of proline to a threonine. Biotype CP2 possessed a nucleotide change of CCA to TCA or TCG in codon 106, resulting in a change of proline to leucine. Biotype Ch possessed either CCA or CCG in codon 106; however, both of these encode for a proline amino acid and would not cause target-site resistance. Biotype CP2 exhibited silent mutations in some plants at codon 100 (a nucleotide change of GCT to GCG) and another silent mutation at codon 105 (a nucleotide change of CGG to either CCC or CCG).

Of the biotypes which possessed missense mutation (SGM2, SGW2 and CP2), not every plant that was analyzed for each biotype had these mutations. Out of five plants sequenced per biotype, one, two and three samples possessed missense mutations for SGM2, CP2 and SGW2, respectively. Biotype CP2 only had one more plant than SGM2 that had a mutation, yet there was a large difference in the resistance status between these two biotypes (Table 4). Consequently, this suggests that a large sample size would be necessary to truly grasp the prevalence of TSR in these biotypes. These results also suggest that the entire biotype may not have the target-site resistance mechanism. Therefore, non-target-site resistance (NTSR) may be present in these biotypes, and the large gap in resistance levels between the two biotypes may be due to NTSR. As stated previously, the aerial distance between the locations of SGM2 and SGW2 is approximately 12 km. Consequently, the two biotypes from St George both

had the same mutation (Pro > Thr), whereas the more distant biotype CP2 had Pro > Leu. The similar mutation in the St George biotypes could be due to gene flow between biotypes and the different mutation in CP2 is likely because it is more geographically isolated from the other two biotypes and therefore gained the mutation independently. It could also indicate a potential for increasing numbers of resistant plants in biotype SGM2.

Biotypes SGM2 and SGW2 possessed a threonine substitution for proline. This has previously been observed in six different weed species [23–25]. Biotype CP2 exhibited a substitution of a leucine amino acid for proline. This substitution is less common in the literature compared to a threonine substitution, but has been previously observed in *Poa annua* L. [26] and *Lolium rigidum* Gaud. [27].

**Table 5.** Aligned nucleotide sequences of the conserved region of *EPSPS* for biotypes (Ch, SGM2, SGW2 and CP2) of *Chloris virgata* amino acids numbered according to Baerson et al., 2002 [28].

Amino Acid Number:	96	97	98	99	100	101	102	103	104	105	106	107	108
Amino Acids:	Phe	Leu	Gly	Asn	Ala	Gly	Thr	Ala	Met	Arg	Pro	Leu	Thr
Consensus sequence:	TTC	TTG	GGG	AAT	GCT	GGA	ACT	GCA	ATG	CGG	CCA	TTG	ACA
Ch	-	-	-	-	-	-	-	-	-	-	CCX (3)	-	-
SGM2	-	-	-	-	-	-	-	-	-	-	ACX (2)(3)	-	-
SGW2	-	-	-	-	-	-	-	-	-	-	ACX (2)(3)	-	-
CP2	-	-	-	-	GCY (1)(4)	-	-	-	-	CCZ (1)(5)	TCX (2)(3)	-	-

(1) Silent mutation, (2) Missense mutation, (3) X = G or A, (4) Y = G or T, (5) Z = C or G—represents similar sequences to consensus sequence.

### 3.3. Experiment 3. Alternative Options to Glyphosate

Survival percentage differed significantly between herbicide treatments for each biotype ( $P < 0.001$ ) but was not affected by biotypes ( $P = 0.29$ ) (Table 6). All four biotypes reacted similarly to the application of glyphosate-alternative herbicide treatments in terms of survival percentage (Table 6). The interactions between biotype and herbicide treatments were significant ( $P = 0.03$ ) for dry matter per plant, but not for survival percentage ( $P = 0.24$ ).

Applications of clethodim, haloxyfop and paraquat were highly effective for controlling GS and GR biotypes of *C. virgata*, killing 100% of seedlings when sprayed at the 4–5 leaf stage (Table 6). The results suggest that these herbicides are effective at controlling both GS and GR *C. virgata* plants when applied at this early growth stage; however, these herbicides may not provide successful control at later growth stages as the efficacy of herbicides tends to reduce when applied at later leaf stages in *Echinochloa crus-galli* (L.) Beauv., *E. colona* (L.) Link and *Leptochloa chinensis* (L.) [29]. Despite the ability of imazamox+imazapyr to control several annual grass weeds [30], an application of imazamox+imazapyr was not very effective in controlling *C. virgata* as all four biotypes (Ch, SGM2, SGW2 and CP2) possessed high survival percentages (more than 80%) against the application of this commercial mixture (Table 6). However, it was more effective for suppressing the dry matter of all the biotypes, providing more than 70% reduction compared to the untreated control treatment (Table 6). These results indicate that a higher dose of imazamox+imazapyr is required to control *C. virgata* than used in this study (24.8 g ai ha<sup>-1</sup>). Considering the ability of imazamox+imazapyr to reduce dry matter considerably, it could be a good option for a tank mixture herbicide application.

Similarly, an application of pinoxaden did not control *C. virgata* as survival percentages of all the biotypes were 100% (Table 6). Pinoxaden application was also not able to reduce dry matter percentage significantly as reduced dry matter percentage of all the biotypes varied from 2 to 46% (Table 6). Pinoxaden is recommended to control several annual grass weeds [31,32] but did not display effective control on any of the biotypes of *C. virgata* in this study. Pinoxaden, haloxyfop and clethodim inhibit acetyl co-enzyme A carboxylase, however, despite having similar MOA, only haloxyfop and

clethodim were able to control *C. virgata*. Acetyl co-enzyme A carboxylase (ACCase) is an essential enzyme in the synthesis of some fatty acids, and those fatty acids are useful for the construction of cell membranes and waxy cuticles [33,34]. Therefore, in the absence of this enzyme, the plant's growth is prevented. At later growth stages in plants, the production of ACCase declines [35] and so does herbicide efficacy. Therefore, inhibitors of ACCase should be applied at early growth stages in order to achieve optimum control.

Pinoxaden is recommended to control several grass weeds in cereal cropping systems, and it can also be applied as an in-crop herbicide treatment in cereals [36]. However, as suggested by this study, pinoxaden cannot control *C. virgata*. Clethodim and haloxyfop can be effective for targeting *C. virgata*. However, both herbicides can only be used in fallow conditions in cereal cropping because they were developed to control grass weeds in broadleaved cropping systems [37,38]. In our previous research, weed shift phenomenon was observed in *C. virgata* as some biotypes possessed more than 50% germination at 15/5 °C temperature regime despite being a summer annual (unpublished work). Therefore, it suggested that the seeds of *C. virgata* could germinate in winter months and compete with winter cereals. In this situation, farmers would not be able to control *C. virgata* with these glyphosate alternatives.

#### 3.4. Experiment 4. Herbicide Treatments with Double Knock Approach

The interaction between biotype and herbicide treatment was significant for both survival percentages ( $P < 0.001$ ) and dry matter per plant ( $P = 0.002$ ). Single glyphosate treatment (1.14 kg ae ha<sup>-1</sup>) was not effective in controlling the GR biotypes; however, it controlled the most glyphosate susceptible biotype Ch (Table 7). All double knock treatments were highly effective on all four biotypes of *C. virgata* as no plant survived these treatments (Table 7).

The sole application of glyphosate was only effective on Ch (the most glyphosate susceptible biotype), and only a 20% decrease was observed in the survival percentage of the SGM2 biotype. Biotypes (SGW2 and CP2) survived the application of glyphosate due to their high glyphosate resistance levels (Table 4). Glufosinate-ammonium alone, however, completely controlled all four biotypes.

The double knock approach of herbicide application has been found extremely beneficial for controlling several annual grass weeds due to its ability to achieve 100% control even on large weed plants [39]. In a previous study, glyphosate followed by paraquat was highly effective for controlling the summer annual grass weed *E. colona*, but was rarely effective for controlling *C. virgata* [40]. However, in the current study, an application of glyphosate followed by paraquat achieved complete control (Table 7). According to experiment 3, a single application of paraquat completely controlled *C. virgata* when applied at the early vegetative phase. However, a study carried out in Central Queensland in fallow conditions suggested that single herbicide application was less effective as compared to a double knock approach when it comes to controlling large plants of *C. virgata* [1]. Another study suggested that the application of ACCase inhibitors alone can provide satisfactory control of *C. virgata* when applied at early growth stages. However, at the same time, some studies also suggested that the long-term single application of ACCase inhibitors could lead to the development of herbicide resistance. Therefore, an application of paraquat after ACCase inhibitors is recommended to achieve a satisfactory level of control [40]. In this study, an application of glufosinate-ammonium followed by paraquat achieved complete control, which supports the previous findings [40].

The major aim behind the development of the double knock strategy is to prevent the overdependence of glyphosate and reduce the risk of developing glyphosate resistance in weed biotypes [21]. Two glyphosate resistance simulation models predicted that the sequential application of glyphosate and paraquat in the same year could delay the development of glyphosate resistance for approximately 30–50 years, thus making herbicide resistance uncommon [41,42]. Considering these facts and the highly effective nature of the double knock strategy, it can be an effective option for controlling GR weed biotypes.

**Table 6.** Effect of different herbicide treatments on survival percentage and dry matter production of glyphosate-susceptible (Ch and SGM2) and glyphosate-resistant (SGW2 and CP2) biotypes of *Chloris virgata* after 28 days.

Treatments	Survival (%) ± SE				Dry Matter Per Plant (g) ± SE			
	Ch	SGM2	SGW2	CP2	Ch	SGM2	SGW2	CP2
Untreated	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0.964 ± 0.15	0.533 ± 0.10	0.720 ± 0.13	0.930 ± 0.14
Clethodim	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Haloxypfop	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Imazamox + imazapyr	83.3 ± 8.3	100 ± 0	87.5 ± 8.5	94.4 ± 5.6	0.148 ± 0.060 (85)	0.141 ± 0.040 (74)	0.215 ± 0.050 (70)	0.219 ± 0.040 (77)
Paraquat	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Pinoxaden	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0.518 ± 0.020 (46)	0.521 ± 0.100 (2)	0.609 ± 0.100 (15)	0.775 ± 0.080 (17)

Values in parentheses are reduced % of dry matter compared to control (untreated). Values after ± are standard errors (SE) of means.

**Table 7.** Efficacy of double knock approach on glyphosate-susceptible (Ch and SGM2) and glyphosate-resistant (SGW2 and CP2) biotypes after 28 days.

Treatments	Survival % ± SE				Dry Matter Per Plant (g) ± SE			
	Ch	SGM2	SGW2	CP2	Ch	SGM2	SGW2	CP2
Untreated	100 ± 0	100 ± 0	100 ± 0	100 ± 0	1.152 ± 0.1	0.957 ± 0.06	0.870 ± 0.12	1.168 ± 0.25
Glyphosate	0 ± 0	81.66 ± 9.1	100 ± 0	100 ± 0	0 ± 0 (100)	0.479 ± 0.12 (51.83)	0.406 ± 0.05 (53.33)	0.475 ± 0.14 (62.33)
Glufosinate-ammonium	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Glyphosate fb paraquat	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Glyphosate fb paraquat	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Glufosinate-ammonium fb paraquat	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)
Glufosinate-ammonium fb paraquat	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)	0 ± 0 (100)

Parentheses represent reduced dry matter (%) as compared to the control (untreated), and values after ± are standard errors of mean. Acronyms: fb, followed by herbicide treatment after one week of first treatment.

#### 4. Conclusions

Glyphosate resistance levels varied between the biotypes of *C. virgata* analyzed in this study. High resistance to glyphosate in biotypes SGW2 and CP2 may be managed by using very high glyphosate doses, but it is not recommended considering the poor economic viability and risk of developing herbicide resistance to control *C. virgata*. The GS biotype Ch showed no sign of target-site resistance; however, a target-site missense mutation was present in one plant of a biotype of SGM2 (Pro > Thr at codon 106). Therefore, it may mean that resistance could increase in this biotype. Biotypes SGW2 and CP2 exhibited missense mutations in three (Pro > Thr at codon 106) and two (Pro > Leu at codon 106) samples out of five, respectively, thus indicating the presence of target-site resistance. Our results also indicated three herbicides, namely clethodim, haloxyfop and paraquat that could be effective alternative options to glyphosate for controlling glyphosate-resistant *C. virgata* at the early seedling stage. The efficacy of these herbicides, however, should be tested at different growth stages and in field conditions. The double knock approach completely controlled all the plants of *C. virgata* in our study for each treatment, which agreed with previous studies about the highly effective nature of this strategy. Considering the fact that the double-knock approach can delay the development of herbicide resistance in weed biotypes, it could revolutionize chemical weed control in the future.

**Author Contributions:** Writing—Preparation of Original Draft, H.S.D.; Conceptualization, B.S.C.; Methodology, H.S.D., M.T. and B.S.C.; Data Curation & Analysis, H.S.D.; Writing—Review & Editing, B.S.C. and M.T.; Resources, B.S.C.; Project Administration & Fund Acquisition, B.S.C., Supervision, B.S.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research did not receive any specific funding.

**Conflicts of Interest:** The authors declare no conflict of interest.

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