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Multiple Resistance to Glyphosate and 2,4-D in *Carduus acanthoides* L. from Argentina and Alternative Control Solutions

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Abstract: *Carduus acanthoides* L. is an invasive species native to Europe and distributed in other parts of the world, including North and South America. In Cordoba, Argentina, control failures of this species have been reported in Roundup Ready (RR) soybean crops where glyphosate and 2,4-D have frequently been applied, although there are no confirmed reports worldwide of resistance to glyphosate and 2,4-D in this species. Dose–response tests showed multiple-resistance to both active principles. The resistant population (R) had LD_{50} values of 1854.27 and 1577.18 g ae ha^{-1} (grams of acid equivalent per hectare), while the susceptible (S) population had LD_{50} values of 195.56 and 111.78 g ae ha^{-1} for glyphosate and 2,4-D, respectively. Low accumulations of shikimic acid (glyphosate) and ethylene (2,4-D) at different doses in the R population compared to the S population support the results observed in the dose–response curves. No significant differences in leaf retention were observed for glyphosate and 2,4-D in the R and S populations. However, the use of adjuvants increased the retention capacity of herbicides in both populations. Ten alternative herbicides with seven different action mechanisms (MOAs) were evaluated and the most effective active principles were dicamba, bromoxynil, atrazine, tembotrione, flazasulfuron, glufosinate, and paraquat. These findings are the first evidence of glyphosate and 2,4 D resistance in *C. acanthoides*.

Keywords: dose–response; shikimate accumulation; ethylene accumulation; adjuvants; efficacy of herbicides; alternative chemical control

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1. Introduction

Glyphosate [n-(phosphonomethyl) glycine], has been the most widely used herbicide in the world due to its physicochemical characteristics [1–3]. Poor implementation of intensively cultivated glyphosate-resistant crops and poor management of herbicide application programs have generated significant dependence on glyphosate, resulting in the evolution of weed resistance to this herbicide [4–6]. Glyphosate was introduced in 1974 and weed resistance was not reported until 1995, when a population of resistant *Lolium rigidum* was detected in Australia [7]. Currently in Argentina, more than 90% of the soybean fields are planted with glyphosate-resistant soybeans. The intense use of glyphosate has contributed to the spread of weeds with resistance to this herbicide in Argentina, including species such as *Sorghum halepense*, *Lolium multiflorum*, *Lolium perenne*, *Cynodon hirsutus*, *Echinochloa colona*, *Eleusine indica*, *Conyza bonariensis*, *Brassica rapa*, *Amaranthus quitensis*, *Amaranthus palmeri*, *Bromus catharticus*, *Urochloa panicoides*, *Echinochloa crus-galli*, and recently, *Carduus acanthoides* [8,9]. The greatest problem with resistant weeds are estimated to be in the province of Córdoba [10]. Globally, 48 weed species, thus far, have been confirmed to be resistant to glyphosate [8].

Since its introduction in 1946, 2,4-D (2,4-dichlorophenoxyacetic acid), known as a synthetic auxin, has been used as a selective herbicide to control broadleaf weeds and it remains one of the most competitive herbicides due to its broad spectrum control and efficiency [11,12]. In Argentina, 2,4-D is widely used from May to September, up to a month before planting Roundup Ready (RR) soybeans. Resistance to 2,4-D requires more generations to evolve than in herbicides with other mechanisms of action, although resistance associated with cereals or monoculture systems have been reported [12–14]. The first cases of 2,4-D resistance were reported in *Daucus carota* and *Commelina diffusa* in 1957 [15,16]. There are currently 45 reported cases of weed resistance to synthetic auxins in the world and most of these cases include 2,4-D [8]. In Argentina, four cases of 2,4-D resistance have been reported in different dicotyledonous species such as *Brasica rapa*, *Hirschfeldia incana*, *Amaranthus hybridus*, and *C. acanthoides* [8].

Herbicide retention plays a fundamental role in the efficacy of the herbicide and is closely related to penetration, which is very important for the uptake of concentrations of the herbicide sufficient to inhibit vital processes and cause plant death [17,18]. Glyphosate controls weeds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), disrupting the shikimate pathway, which is important for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Different levels of shikimic acid concentrations have been accepted as a quick and easy indicator to determine the level of resistance to glyphosate [19,20]. While 2,4-D mainly kills plants in three ways: by altering the plasticity of the cell walls, influencing the amount of protein production, or increasing ethylene production. Therefore, ethylene can be used as an indicator to determine whether a population is susceptible or resistant [11,21,22]. The use of herbicides with a different mechanism of action (MOA), alone or in a mixture, are the main tool to combat the emergence of resistance or resistance in a specific group of weeds [23]. However, inadequate implementation of control strategy can lead to the selection of general resistance mechanisms of target (TSR) or non-target (NTSR) sites, inducing the evolution of biotypes of weeds resistant to multiple MOA [24,25]. In some cases, weed species have evolved multiple herbicide resistances, posing a great challenge to herbicide sustainability in world agriculture [26]. In that sense, the most problematic cases involve resistance to multiple sites of action; however, only a handful of weed species have repeatedly evolved multiple resistances and, consequently, anticipating which species will become a troublesome multiple herbicide-resistant weed is crucial [27].

Carduus acanthoides is an invasive species in the Asteraceae family native to Europe and distributed in other parts of the world, such as North and South America. This species is annual or biennial and reproduced by seeds. The plant is 20–150 cm tall. The flowers are about 20 mm in diameter. Seeds are oblong, striate, and slightly curved. A typical plant produces 35–60 capitula. Mean seed set averages 56–83 seeds per seed head. Germination occurs mainly in the spring and fall, with resulting plants acting either as winter annuals or as spring or fall biennials [28]. In Canada, it has been reported that *Carduus* can cause reduced productivity of pastures and rangeland by suppressing growth of desirable vegetation, preventing livestock from eating plants growing in the vicinity of thistle stands [28]. In Argentina, competition from *C. acanthoides* with the crops destined for harvesting, occurs both in the initial and intermediate stages of the crops, preventing their normal implantation and hindering their normal development. *C. acanthoides* is a common weed in fields intended for the production of wheat and soybean. In the Pampas region, it can also cause disturbances at harvest, due to the introduction of green material in the harvesting machines [29]. Recently, glyphosate and 2,4-D control failures were reported in *C. acanthoides* in soybean fields in the province of Córdoba (Argentina). In this region, glyphosate and 2,4-D have been widely used as the main weed control tools for several years. The objectives of this work were (1) to confirm resistance to glyphosate and 2,4-D in a *C. acanthoides* population from Córdoba, Argentina through dose–response assay in greenhouse and biochemical such as the accumulation of shikimic acid and ethylene, (2) quantify the foliar retention and the efficacy of glyphosate and 2,4-D with the use of adjuvants, and (3) seek alternative herbicides for the control of this species.

2. Materials and Methods

2.1. Plant Materials

Mature seeds of a *C. acanthoides* population suspected of having multiple resistance (R) to glyphosate and 2,4-D used in this research were collected from 25 plants in a soybean field (RR). These plants were treated with both herbicides alone or in combination at the field dose (720 g ae (grams of acid equivalent per hectare) ha^{-1} and 400 g ae ha^{-1} of glyphosate and 2,4-D, respectively) for over 20 years in an agricultural area (33°18′59.7″ S 62°26′00.5″ W) of Marcos Juárez in the Province of Córdoba (Argentina). Seeds from susceptible populations (S) were also collected from 25 plants from a nearby area in 2019 (1000 m between the R and S plants) in which no herbicides had ever been applied.

2.2. Glyphosate and 2,4-D Dose–Response Assay

Seeds were germinated in Petri dishes containing filter paper moistened with distilled water. The Petri dishes were placed in a growth chamber at 28/18 °C (day/night) with a photoperiod of 16 h, 850 µmol m^{-2} s^{-1} of photosynthetic photon flux, and 60% relative humidity. All of the seedlings were transplanted in pots (one plant per pot) containing sand/peat in a ratio of 1:1 (*v*/*v*) and placed in a greenhouse with a photoperiod of 16 h and a temperature of 26/18 °C (day/night). Plants from S and R populations of *C. acanthoides* were treated at the four-leaf growth stage using a laboratory system (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 200 L ha^{-1} at 250 kPa at a height of 50 cm from the plant level.

Glyphosate (Roundup Energy® SL, 480 g ae L^{-1} (grams of acid equivalent per liter) as isopropylamine salt, Monsanto) was applied in eight doses (10 plants dose-1) including 0, 31.25, 62.5, 125, 250, 500, 1000, and 2000 g ae ha^{-1}; 2,4-D (Esteron 60%, 600 g ae L^{-1} as 2,4-dichlorophenoxyacetic acid butyl ester, Dow AgroSciences) doses used in this experiment on both S and R plants were 0, 40, 80, 160, 320, 640, 1280, and 2560 g ae ha^{-1}. Non-treated plants were used as a control. The experiment was organized in a completely randomized design and was repeated twice at different times.

The dose resulting in plant survival (LD_{50}) and fresh/dry (60 °C for 4 days for dry weight) weight reduction on the soil per plant by 50% (GR_{50}) were determined at 28 days after treatment (DAT). The data were expressed as percentages in relation to the untreated control. Resistance index (RI) were computed as R-to-S GR_{50} or LD_{50}.

2.3. Shikimic Acid Accumulation

Young leaf discs (4 mm in diameter) were sampled for a total of 50 mg of plant tissue from the R and S populations of *C. acanthoides*. Shikimic acid accumulation was determined according to the methodology described by Shaner et al. [19]. The glyphosate concentrations used were 0, 250, 500, and 1000 µM. The sample absorbance was measured in a Beckman DU-640 spectrophotometer at 380 nm. The test was performed in triplicate on five treated and five non-treated plants of each population in a completely random design and repeated twice. The results were expressed in micrograms of shikimate per milliliter of HCl solution (µg/mL).

2.4. Ethylene Accumulation

Plants at the 3–4 leaf stage were applied with 2,4-D solutions (0, 50, 100, 200, 300, 400, 600, 800, and 1000 g ae ha^{-1}) as in the dose–response curves. Twenty-four hours after treatment (HAT) the seedlings were cut at ground level and 400 g shoot fresh weight were placed in a 10 mL syringe with 1 mL of distilled water and sealed [30]. The syringes were placed in a dark incubator at 27 °C for 4 h and 1 mL of the headspace gas was analyzed for ethylene (C_2H_4) content by gas chromatography [31]. C_2H_4

was expressed as nanoliters per gram of fresh weight per hour (nL g^{-1} fresh weight h^{-1}). There were five replicates per treatment and the experiment was repeated twice.

2.5. Foliar Retention and Efficacy of Herbicides

Foliar retention and herbicide efficacy with and without adjuvants were carried out following the methodology used by Gauvrit [32]. Young *C. acanthoides* plants in the 3–6 true leaf stage were sprayed with glyphosate and 2,4-D separately with and without adjuvants (2 mL L^{-1} Retenol and 1 mL L^{-1} Trend 90) using the sprayer described in the dose–response assay. The dose of glyphosate applied was 360 g ae ha^{-1} in a volume of 200 L of water and 100 g of Na-fluorescein, and the 2,4-D dose was 400 g ae ha^{-1} at the same volume of application and concentration of fluorescein as glyphosate. The plants were cut at ground level and submerged and washed in test tubes with 50 mL of a 5 mM NaOH solution for 30 s. Absorbance readings were measured using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) at 490/510 nm. A calibration standard curve was constructed with serial dilutions of Na-fluorescein [33]. The aerial part of the plant was dried in an oven at 80 °C for 48 h and the dry matter weight was recorded. Six repetitions were used for each treatment in a completely randomized design. The experiment was repeated twice and the results were combined for analysis.

For the herbicide efficacy, 10 plants were sprayed in each trial with glyphosate doses of 400 g ae ha^{-1} for the R population and 200 g ae ha^{-1} for the S population, with and without adjuvants (adjuvants: 2 mL L^{-1} Retenol and 1 mL L^{-1} Trend 90). On the other hand, the doses of 2,4-D sprayed were 200 and 40 g ae ha^{-1} for the R and S populations, respectively, with and without adjuvants. The selected doses were less than the estimated GR_{50}. Applications were performed with the sprayer used in the dose–response assay at an application volume of 200 L ha^{-1} at 250 kPa (2.2 Glyphosate and 2,4-D Dose–response Assay). At 28 DAT, the dry weights (dw) of the aerial parts of the plants were recorded for each experiment. The experiment was repeated twice and the data were combined.

2.6. Alternative Chemical Control

The aim of this trial was to contribute an important part within an integrated weed management (IWM) programme in which herbicide treatments were applied under the same conditions and spraying volumes used as in the previous dose–response assay (Section 2.2). The different herbicides were applied in field doses, described in Table 1, on young plants with four true leaves of the putative population S and the putative population R of *C. acanthoides*. The application of each herbicide was carried out in 10 plants of each population. The experiments were replicated twice at different times in a completely randomized design. At 28 DAT, visual evaluation and plant survival were carried out and plants were cut to obtain the weights and determine the values of fresh weight reduction. For visual evaluation, a linear scale was used to evaluate weed control described by Vanhala et al. [34]. Ten untreated plants were used as a control for all herbicides.

2.7. Statistical Analysis

The dose–response was subjected to nonlinear regression analysis adjusted to a three-parameter log-logistic model (1) using the R package drc (R Core Team) [35], to determine the glyphosate and 2,4-D dose resulting in reduction in growth (GR_{50}) and plant survival (LD_{50}) by 50% in each *C. acanthoides* population.

$$Y = c + \{(d - c)/[1 + {}^{b}(\log(x) - \log(e))]\}, \quad (1)$$

where Y is the reduction in dry/fresh weight or plant mortality by 50% of the harvested plants expressed as a percentage of the untreated control, d is the coefficient corresponding to the upper asymptote, c is the lower limit (fixed at 0), the coefficient b is the slope at the inflection point, e is the herbicide concentration required to inhibit shoot growth or plant survival by 50% (i.e., GR_{50} or LD_{50}, respectively), and x is the herbicide dose. The data were plotted using SigmaPlot 12.0 (Systat Software Inc, San Jose, CA, USA).

Table 1. Main characteristics of the herbicides used in the investigation of alternative chemical control in the greenhouse.

Herbicide	HRAC Group	Trade Name	Application Time	Recommended Field Dose (g ai ha^{-1})
Dicamba	O	Banvel® (Dicamba 57.8% SL)	postemergence	150
Fluroxypyr	O	Starane® (Fluroxypyr 20% p/v EC)	postemergence	150
Bromoxynil	C	Buctril® (Bromoxynil 21.8% w/w)	postemergence	400
Atrazine	C	Gesaprim® (Atrazine 90% WG)	postemergence	2000
Diflufenican	F1	Mamut® (Diflufenican 50% p/v SC)	pre- and postemergence	150
Fomesafen	E	Flex 25 SL® (Fomesafen 25% p/v EC)	postemergence	76
Tembotrione	F2	Laudis® (Tembotrione 42% SC)	postemergence	120
Flazasulfuron	B	Terafit® (Flazasulfuron 25% WG)	pre- and postemergence	50
Glufosinate	H	Finale® (Glufosinate 15% p/v SL)	postemergence	500
Paraquat	D	Gramoxone® (Paraquat 27.6% SL)	postemergence	400

g ai ha^{-1} = grams of active ingredient ha^{-1}. HRAC: Herbicide Resistance Action Committee; B: acetolactate synthase (ALS) inhibitor; C: Photosystem II (PSII) inhibitors; O: Synthetic auxins; E: Protoporphyrinogen oxidase (PPO) inhibitor; F2: 4-hydroxyphenyl-pyruvatedioxygenase (HPPD) inhibitor; H: Glutamine synthase inhibitor; D: Photosystem I (PSI) electron diverter. Formulations: soluble concentrate (SL); emulsifiable concentrate (EC); water dispersible granule (WG); suspension concentrate (SC).

Analysis of variance (ANOVA) was performed using Statistix 10.0 (Analytical Software, Tallahassee, FL, USA) to verify differences between the R and S populations in the accumulation of shikimate and ethylene at the different concentrations used for glyphosate and 2,4-D, respectively, in the leaf retention test for each herbicide (with and without adjuvants) and for the alternative chemical control only for the percentage of fresh weight reduction. For efficacy data, R and S populations were analyzed separately with and without adjuvants for glyphosate and 2,4-D. Percentage data were previously transformed (arcsine of the square root) to meet model assumptions of normality of the error distribution and variance homogeneity. Model assumptions were graphically inspected. When needed, differences between means were separated using a Tukey's honestly significant difference (HSD) test. Replicates of the experiments were pooled due to a lack of statistical difference between them.

3. Results

3.1. Dose–Response Assays

The dose–response assays showed differences in the GR_{50} and LD_{50} values of the R and S populations of *C. acanthoides*. The data of free and dry weight were analyzed. It was found that there were no significant differences ($p = 0.1614$) between these values for the two herbicides as shown in Figure 1A,C. The S population was well controlled without surviving plants at doses lower than those recommended in the field of glyphosate and 2,4-D in this area (720 and 160 g ae ha^{-1}, respectively). The GR50 estimated values for glyphosate in the R population were 534.5/594.1 (fresh/dry), while for 2.4-D they were 266.5/246.0 (fresh/dry) (Table 2). Based on these values, the RI calculated in dry weight for glyphosate showed that the R population was 4.7 times more than the S population and for 2,4-D it was 5.5 times more (Table 2). The LD_{50} in the R population for glyphosate was 1854.2 g ae ha^{-1} and

1577.1 g ae ha^{-1} for 2.4-D, values much higher than the recommended rates (glyphosate 720 and 2,4-D 400 g ae ha^{-1}). Therefore, the data showed the existence of multiple resistance to glyphosate and 2,4-D for this population of *C. acanthoides*.

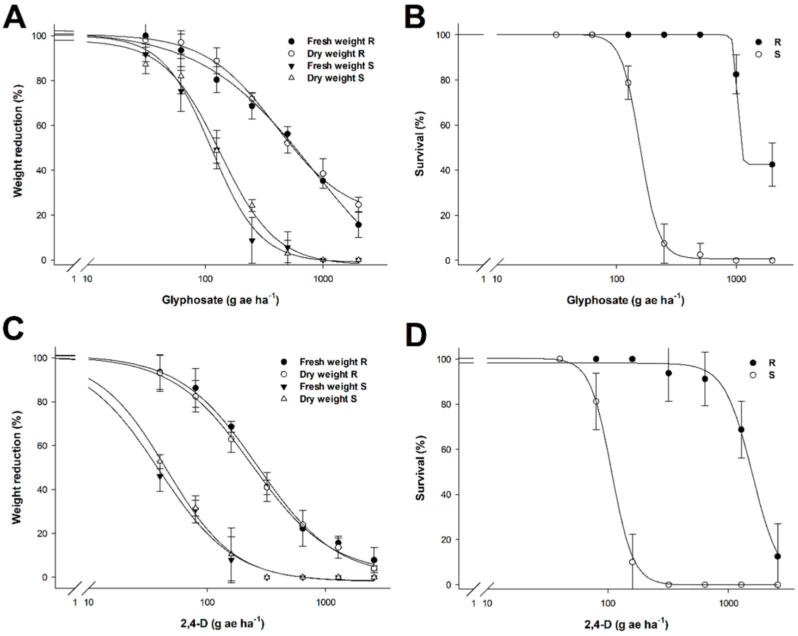

Figure 1. Dose–response curves in % reduction in fresh and dry weight (**A**,**C**) and % survival (**B**,**D**) treated with different doses of glyphosate and 2,4-D, respectively, expressed as a percentage of the mean of the untreated control of the R and S populations of *C. acanthoides*. Vertical bars represent the standard error of the mean (n = 10).

Table 2. Parameters of the log-logistic equation used to estimate the doses of glyphosate and 2,4-D (g ae ha $^{-1}$) required to decrease 50% of the fresh and dry/fresh weight (GR$_{50}$) or survival (LD$_{50}$) of the R and S populations of *C. acanthoides*.

Herbicide	Population	b	d	GR$_{50}$ (*g ae ha*$^{-1}$)	*p*-Value	RI
			Fresh weight			
Glyphosate	R	1.10	101.15	534.59 ± 89.82	<0.001	4.60
	S	2.26	97.79	115.37 ± 17.26	<0.001	-
2,4-D	R	1.32	101.28	266.56 ± 19.87	<0.001	6.88
	S	1.49	99.71	38.71 ± 8.29	<0.001	-
			Dry weight			
Glyphosate	R	1.06	102.54	594.12 ± 98.48	<0.001	4.67
	S	2.06	97.28	127.13 ± 18.56	<0.001	-
2,4-D	R	1.25	101.16	246.01 ± 45.48	<0.001	5.48
	S	1.62	99.72	44.85 ± 9.84	<0.001	-
			Plant survival			
Herbicide		**b**	**d**	**LD$_{50}$ (*g ae ha*$^{-1}$)**	***p*-value**	**RI**
Glyphosate	R	3.05	99.45	1854.27 ± 141.05	<0.001	9.48
	S	3.11	100.89	195.56 ± 87.01	<0.001	-
2,4-D	R	3.86	98.50	1577.18 ± 117.08	<0.001	13.39
	S	3.31	101.28	111.78 ± 12.12	<0.001	-

RI = Resistance index, calculated as GR$_{50}$ or LD$_{50}$(R)/GR$_{50}$ or LD$_{50}$(S) ± standard error of the mean (n = 10).

3.2. Shikimic Acid Accumulation

The accumulation of shikimic acid was greater in the S population with the three doses of glyphosate used compared to the R population. The greatest difference in the accumulation of shikimic acid was observed in the 500 µM and 1000 µM concentrations of glyphosate (Figure 2). The accumulations in the S population were 6.1, 11.5, and 19 µg shikimate g^{-1} of fresh weight at concentrations of 250, 500, and 1000 µM, respectively. The accumulation of shikimic acid in the R population was 4.5 µg shikimate g^{-1} of fresh weight at the dose of 250 µM; it then remained constant at approximately 7.6 µg at concentrations of 500 µM and 1000 µM (Figure 2).

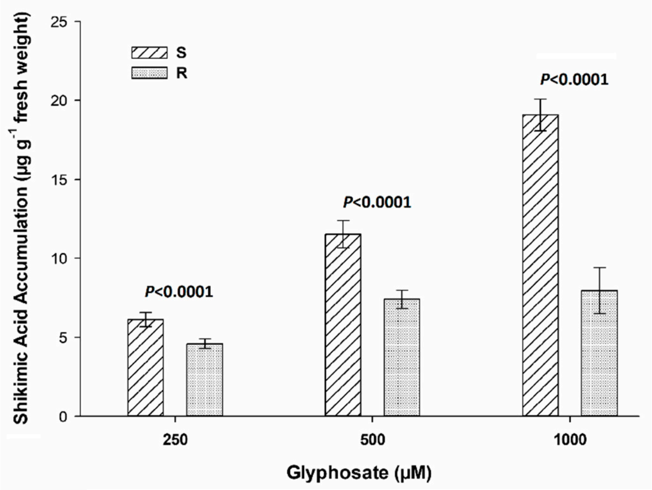

Figure 2. Shikimic acid accumulation of susceptible (S) and resistant (R) *C. acanthoides* plants at different glyphosate concentrations. Vertical bars represent the standard error of the mean ($n = 3$ per glyphosate concentration).

3.3. Ethylene Accumulation

The S population of *C. acanthoides* accumulated more ethylene than the R population at increasing doses of 2,4-D. The differences in ethylene accumulation between the R and S populations were observed from 50 to 1000 g ae ha^{-1} (Figure 3). In population R, the accumulation increased slowly and then remained constant from 200 to 1000 g ae ha^{-1}, since there were no significant differences in the accumulation between them (Figure 3). The accumulation of ethylene at 1000 g ae ha^{-1} was 2.7 times greater in the S population compared to the R population.

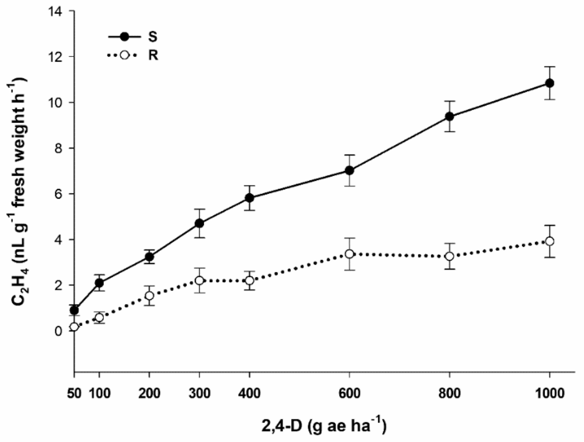

Figure 3. Accumulation of ethylene (C_2H_4) following the application of the herbicide 2,4-D in populations of *C. acanthoides*. Vertical bars represent the standard error of the mean ($n = 4$).

3.4. Foliar Retention and the Efficacy of Herbicides

The ANOVA analysis of spray retention for glyphosate and 2,4-D with and without adjuvants did not show any significant differences (p = 0.2141) between the R and S populations of *C. acanthoides* (Table 3). Therefore, the spray retention did not influence resistance to glyphosate and 2,4-D, likewise the adjuvants Retenol and Trend 90 did not show any improvements in the retention of the spray when compared to the corresponding control treated only with glyphosate and 2,4-D, respectively.

Table 3. Foliar retention of glyphosate and 2,4-D with and without adjuvants and increased effectiveness

Treatment	Herbicide Dose	Foliar Retention (µL g^{-1} Dry Weight)	
		R	**S**
Gly [a]	360 g ae ha^{-1}	689.78 a	684.57 a
Gly + Retenol		826.35 a	779.26 a
Gly + Trend 90		853.83 a	796.74 a
2,4-D	400 g ae ha^{-1}	348.52 a	349.95 a
2,4-D + Retenol		396.93 a	394.38 a
2,4-D + Trend 90		400.97 a	396.26 a

[a] Glyphosate. 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 HSD test; n = 6.

Efficacy is a fundamental parameter for evaluating weed control. Sublethal doses of glyphosate (400 and 200 g ae ha^{-1} for R and S, respectively) and 2,4-D (200 and 40 g ae ha^{-1} for R and S, respectively) were applied to determine if there was an increase in yield when it was mixed with adjuvants. The highest efficacy in glyphosate occurred when Trend 90 was added (Table 4). The efficacy of glyphosate in the R population improved by 19.8% when Trend 90 was added. While for the S population an improvement of 24.01% was observed. Glyphosate applications with Retenol showed no improvement when compared with the glyphosate-only treated control. The application of adjuvants did not improve the efficacy and activity of 2,4-D for the putative R and S populations of *C. acantohides* (Table 4).

Table 4. Efficacy of glyphosate and 2,4-D with and without adjuvants in the R and S populations of *C. acanthoides*.

Treatment	% Dry Weight Reduction	
	R (400 g ae ha^{-1})	**S (200 g ae ha^{-1})**
Gly [a]	51.82 b	47.04 b
Gly + Retenol	58.12 a	54.08 ab
Gly + Trend 90	61.72 a	61.91 a
	R (200 g ae ha^{-1})	**S (40 g ae ha^{-1})**
2,4-D	37.22 a	37.02 a
2,4-D + Retenol	44.10 a	45.50 a
2,4-D + Trend 90	47.75 a	47.20 a

[a] Glyphosate. The means that different letters within a column and for each herbicide are significantly different with a 95% probability determined by Tukey HSD test; n = 10.

3.5. Alternative Chemical Control

Herbicidal treatments within integrated weed management (IWM) programmes must meet a minimum control standard (80% for direct field treatments) to be accepted by farmers [36,37]. The response of most of the herbicides applied to the R and S populations of *C. acanthoides* indicated good a performance in the visual evaluation, very low levels of survival, and significant reductions in the fresh weight (Table 5). The control levels were separated into two groups: those that showed high levels of control (<15% survival) and those levels that demonstrated intermediate control (15–50% survival).

Table 5. Percentage of visual evaluation, % survival and % fresh weight (Fw) reduction compared to the untreated controls in resistant (R) and susceptible (S) populations of *C. acanthoides* with alternative control herbicides

Herbicides	Field Doses (g ai ha^{-1})	Visual Evaluation [a]		% Survival [b]		% Fw Reduction	
		R	S	R	S	R	S
Control	-	0	0	100	100	0	0
Dicamba	150	100	90	0	10	100 a	98.31 a
Fluroxypyr	150	100	70	10	10	96.14 a	97.47 a
Bromoxynil	400	100	100	0	0	100 a	100 a
Atrazine	2000	100	100	0	0	100 a	100 a
Diflufenican	150	50	50	50	50	63.12 b	59.34 b
Fomesafen	76	50	50	50	50	80.97 b	77.68 b
Tembotrione	120	100	100	0	0	100 a	100 a
Flazasulfuron	50	100	100	0	0	100 a	100 a
Glufosinate	500	100	100	0	0	100 a	100 a
Paraquat	400	100	100	0	0	100 a	100 a

[a] Visual evaluation considered the vigor and chlorosis of the plant compared to the control; 0% was attributed when there was no damage, and 100% signified total control of the plants by the herbicides [34]. [b] Control was considered unsatisfactory when the survival of the plants was ≥15%. The same letter indicates that there is no significant difference with a 95% probability determined by the Tukey HSD test.

The herbicides that provided high levels of control for the R and S populations were dicamba, bromoxynil, atrazine, tembotrione, flazasulfuron, glufosinate, and paraquat. Since these herbicides resulted in 100% control upon visual evaluation, they consequently also showed 0% survival and close to a 100% reduction in the fresh weight (Table 5).

The herbicides that performed poorly in the control of both the R and S populations were diflufenican and fomesafen. The visual evaluation and survival findings of these two herbicides barely reached 50% and the reduction in fresh weight supported these results. Fluroxypyr was also included in this category because it only showed good control in the R population, while it demonstrated control deficiencies in the S population (Table 5).

4. Discussion

The inclusion of glyphosate in soybean RR cultivation systems plays an important role in weed control [38]. RR crops were quickly adopted by farmers in some regions of the world and Argentine farmers soon embraced these technological advances, mainly in soybeans and corn [5,39].

The emergence of new resistant species, such as *C. acanthoides*, demonstrates the difficulty farmers face due to the lack of knowledge and tools that are as effective as glyphosate in combating the serious problem of resistance in Argentina. The studies performed by Faccini et al. [40] and Montoya et al. [29] used populations of *C. acanthoides* that had been selected from glyphosate-treated areas, showing the great difficulty of controlling *Carduus* with glyphosate, even with pre-emergent herbicides.

The resistance of *C. acanthoides*, as determined by the RI (GR_{50} R/GR_{50} S), is evident when the factor is greater than 4, following the definition of resistance [8]. Furthermore, the LD_{50} parameter is used to define the herbicide dose necessary to reduce the number of individuals in a population to 50%. The field dose is sufficient to completely control a susceptible population but not the population of *C. acanthoides* with R modifications, increasing plant survival. From an agronomic perspective, referring to a resistant population by the LD_{50} value is subjective, since the dose selected in the field is modified according to the environmental conditions of each country [41]. Furthermore, the sensitivity of weed species to herbicide modification differs between species.

Shikimic acid accumulation was found to be significantly higher in S plants than in R plants (Figure 3). In our study, S population *C. acanthoides* plants showed shikimate accumulations more than 4 times higher than those found in R plants, which is consistent with the results obtained in previous experiments using whole plants. These results showed resistance to glyphosate, as has been shown in different dicotyledonous weeds. This difference between the R and S populations could indicate that glyphosate reached its target site (EPSPS) differently [42,43].

For our study, population S accumulated 2.7 times more ethylene than the resistant population. The differences in the accumulation of ethylene by the susceptible population fluctuated exponentially with respect to the increase in the rate, while resistant population accumulation was constant. These results could indicate the existence of non-target site resistance mechanisms (NTSR), such as reduced translocation and recently shown 2,4-D metabolism, in different dicotyledonous species [11,44,45].

Glyphosate efficacy was shown to improve with the addition of adjuvants in our study, suggesting that a lack of penetration could be involved in the resistance of *C. acanthoides* to glyphosate. Similar results have been found in other studies, such as one by Nalewaja et al. [46] in *Kochia scoparia* and one by Sharma et al. [47] that observed better control of *Lantana camara* and *Baccharis halimifolia* when non-ionic adjuvants and organosilicones were added.

Once resistance is confirmed, it is of great importance to propose alternatives for the control of resistant species, so that farmers can use other tools or herbicides for field management. The best way to manage a resistant species is by rotating crops, adding diversity to the agricultural system [48].

Dicamba (3,6-dichloro-2-methoxybenzoic acid) and fluroxypyr [(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy acetic acid] are synthetic auxins that belong to different chemical families and are used to control post-emergence dicots in soybean rotation crops, such as corn and wheat, in Argentina and other regions of the world [49,50]. In our study, the control of *C. acanthoides* was adequate after the application of dicamba and fluroxypyr with these herbicides, where 10% of the plants survived, which should not be a problem due to the high reduction of fresh weight in both populations. These populations will not be able to compete with the cultivation of corn or wheat. However, the option of applying synthetic auxins should be used with caution due to the selection pressure exerted by these herbicides and the appearance of cross-resistance [11,51]. In our study, some herbicides have been proposed for use as a desiccant (glufosinate, paraquat, and diflufenican), while some are selective for soybean (bromoxynil and fomesafen) and others can be used if they are rotated with corn (atrazine and tembotrione). The herbicides with the highest efficacy were paraquat and glufosinate; Eubank et al. [52] obtained similar results, indicating that effective management against resistant *Conyza* spp. includes paraquat and glufosinate in management programs prior to planting. Another study by Kaur et al. [53] concluded that the application of herbicides prior to planting, such as paraquat and glufosinate, are viable options for the control of glyphosate-resistant *Ambrosia trifida* in soybeans. Of the proposed alternative herbicides, fomesafen is the only one that could be used post-emergence after soybeans. However, the control offered against *C. acanthoides* is poor (50%), so it is not recommended for the management of this weed. Another alternative is crop rotation that includes corn, allowing the use of herbicides that offer satisfactory control (100%). In studies carried out by several authors, the advantages of the soybean–corn rotation compared to monoculture were found. In several experiments, increases in the range of 4–25% in soybean yield have been found with systems that include corn as a crop in the rotation, and these results have been confirmed in more than two campaigns [54–57]. Best management practices include using burning herbicides (paraquat, glufosinate, and diflufenican) to begin cleaning and then removing weeds before they produce seeds to significantly decrease the seed bank.

5. Conclusions

The results of this study confirmed the first report of multiple resistance to glyphosate and 2,4-D through dose–response studies and the accumulation of ethylene and shikimic acid in *C. acanthoides*. Glyphosate efficacy tests showed that the addition of the Trend 90 adjuvant improves control.

Alternative herbicide options are available for early season control of *C. acanthoides* using burndown herbicides. Most of the alternative herbicides that can be used in corn cultivation tested in this study provided effective control (100%) of *C. acanthoides*, suggesting that for proper management, they should be included in corn cultivation. This research provides valuable insights into crop rotation options and the management of *C. acanthoides* in soybean cultivation.

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