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First Case of *Conyza canadensis* from Hungary with Multiple Resistance to Glyphosate and Flazasulfuron

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Abstract: *Conyza canadensis* is a species invading large areas throughout the world, mainly due to its ability to evolve herbicide resistance. In Hungary, extensive areas have been infested by this species due to the difficulty in controlling it with glyphosate. To determine whether poor control was a result of misapplication or glyphosate resistance, eight suspected glyphosate-resistant *C. canadensis* populations from different Hungarian regions were studied. In whole-plant dose-response assays with glyphosate, the LD_{50} and GR_{50} values (survival and fresh weight reduction at 50% relative to the untreated control, respectively) indicated that resistance was confirmed in five of the eight populations (H-5 population being the most resistant). Additionally, the shikimic acid accumulation tests corroborated the results observed in the dose–response assays. 11 alternative herbicides from six different modes of action (MOA) were applied at field doses as control alternatives on populations H-5 and H-6 (both in the same regions). The H-5 population showed an unexpected resistance to flazasulfuron (ALS-inhibitor). The ALS enzyme activity studies indicated that the I_{50} for H-5 with flazasulfuron was 63.3 times higher compared to its correspondent susceptible population (H-6). Therefore, the H-5 population exhibited multiple-resistance to flazasulfuron and glyphosate, being the first case reported in Europe for these two MOA.

Keywords: ALS-inhibitors; horseweed; multiple-resistance; alternative chemical control

1. Introduction

Herbicide resistance is an evolutionary phenomenon that allows weeds that are exposed to the recommended field dose of a herbicide to maintain growth with little or no symptomology [1]. Factors that are important for the selection of herbicide-resistant weed populations include a strict dependence on herbicides with the same mode of action (MOA) and its continuous use [2].

One of the most widely used herbicides over the last four decades has been glyphosate, which has a demonstrated high efficiency in weed control [3]. Its continued use however, together with the resistance evolution, have resulted in a large number of weed species resistant to glyphosate [4]. Amongst these are the three common species of *Conyza* genus (*Conyza bonariensis* (L.) Cronquist., *C. canadensis* (L.) Cronquist., and *C. sumatrensis* (Retz.) E. Walker), found in many countries [4–7].

The first case of glyphosate resistance in *C. canadensis* was confirmed in North America in 2000 [8]. Since then, there have been many cases of resistance observed in this genus around the world [4].

Conyza species are one of the most prone to evolve resistance to glyphosate. This incidence has been corroborated in Europe in a large number of populations.

The survival of resistant weeds after herbicide applications can occur because of two distinct resistance mechanisms: target-site resistance (TSR) and non-target site resistance (NTSR) [2]. The NTSR mechanisms are caused, for example, by reduced absorption and/or translocation, increased vacuolar sequestration [9], and/or metabolism into non-toxic compounds [10,11]. By contrast, the TSR mechanisms are caused by the increased expression of the target protein or structural changes in the herbicide-binding site [12,13].

Another important problem is when multiple resistances or coexisting resistance mechanisms for different modes of action (MOA) herbicides in the population occur. Given its importance in agriculture, the most serious multiple herbicide resistance cases are those involving glyphosate. Half of the glyphosate-resistance cases around the world include cases of multiple resistance [4]. The continued use of herbicides with different MOA (i.e., acetolactate synthase–ALS–inhibitor) to control glyphosate-resistant weeds under non-herbicide-rotation regimes have resulted in decreased weed control efficiency [14], leading to the appearance of multiple resistance and reducing the alternatives for growers when acting against it.

Hungary is a country with significant agricultural activity, due in part to its favorable climatic conditions [15]. In recent years, Hungary has observed infestations of its crop fields (pastures, vineyards, and corn crops) by weeds—such as *Cirsium arvense*, *Conyza canadensis*, and *Sorghum halepense*—that are herbicide resistant (synthetic auxins, EPSPS inhibitors and ALS inhibitors, respectively). However, no studies have reported the resistance level in these *C. canadensis* which present multiple resistance. According to Heap [4] this would be the first case of multiple resistance to group G and B in Europe in this species.

The objectives of the present study were: to evaluate the level of glyphosate resistance in eight suspected *C. canadensis* populations from two different vineyard regions of Hungary; to evaluate chemical control alternatives in two glyphosate resistant populations; and to determine the level of multiple resistance if it was found.

2. Materials and Methods

2.1. Plant Material

Eight suspected glyphosate-resistant (GR) *C. canadensis* populations from two different regions of Hungary were studied. Populations were provided by Monsanto Europe and denominated as H-1 to H-8. Additionally, two populations, one GR and one GS (glyphosate-susceptible) of *C. canadensis*, (characterized as the R and S-glyphosate populations by University of Cordoba, Spain, respectively) were compared to the Hungarian populations (Table 1). In all cases, seeds were taken from 10 mature plants in vineyard crop and non-crop areas.

Table 1. *Conyza canadensis* populations harvested in different Hungarian (HUN) and Spanish (ESP) areas.

Population	Location	Crops	Herbicide Application	Dose/Year	Coordinate
GR	Córdoba/ESP	Olive grove	Glyphosate	1440 [a]/20	37.999, −4.448
GS	Córdoba/ESP	Railway	Mechanical control	——	37.916, −4.717
H-1	Badacsony/HUN	Vineyard	Glyphosate + 2,4-D	1440 [a]/10 + 600 [b]/5	46.786, 17.382
H-2	Badacsony/HUN	Vineyard	Glyphosate + flazasulfuron	1440 [a]/10 + 750 [c]/4	46.790, 17.428
H-3	Badacsony/HUN	Vineyard	Glyphosate	1800 [a]/20	46.785, 17.449
H-4	Balaton/HUN	Vineyard	Glyphosate	1800 [a]/20	46.787, 17.716
H-5	Balaton/HUN	Vineyard	Glyphosate + flazasulfuron	1800 [a]/20 + 750 [c]/7	46.788, 17.770
H-6	Balaton/HUN	Vineyard	Organic crop	—/20	46.811, 17.830
H-7	Balaton/HUN	No crop	No herbicide	——	46.871, 17.944
H-8	Badacsony/HUN	Vineyard	Organic crop	—/10	46.787, 17.487

[a] glyphosate g ae ha^{-1}, [b] 2,4-D mL ha^{-1}, [c] flazasulfuron g ai ha^{-1}.

Mature seeds were germinated in Petri dishes with filter paper moistened with distilled water. 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} photosynthetic photon flux, and 80% relative humidity. Seedlings from each population were transplanted individually into plastic pots (448 cm^3) containing sand/peat at a 1:2 (*v*/*v*) ratio, and they were then placed in a greenhouse at 28/18 °C (day/night).

2.2. Dose–Response Assays with Glyphosate

Conyza canadensis plants were treated with glyphosate at the rosette stage (BBCH 16–18 stage) [16]. Herbicide treatments were carried out in a laboratory chamber sprayer (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with an 8002 E flat fan nozzle delivering 200 L ha^{-1} at 250 kPa at a height of 50 cm. The different glyphosate doses are shown in Table 2. Plant survival, i.e., plants that survived the herbicide treatment, was recorded at 28 days after treatment (DAT). The plants were then harvested at the ground level and weighed to determine their fresh weight. Fresh weight and survival data were converted to percentages in comparison with the untreated control plants. All populations were compared to the GS population.

The experiment was repeated three times with five replicates per treatment and population combination.

Table 2. Herbicides, formulation (type and concentration) [a]/manufacturer, HRAC group, doses used in the curve dose–response in g ai ha^{-1} (Dose–response), and recommended field doses in g ai ha^{-1} (Dose) applied on *C. canadensis* populations from Spain (GR and GS) and Hungary (H1–H8) at the rosette stage (BBCH 16–18).

Herbicide	HRAC [b]	Formulation/Manufacturer	Dose–Response	Dose
Glyphosate [c]	G	Roundup Energy® (SL 50.9% *w*/*v*)/Monsanto	0/31.25/62.5/125/250/500/1000/2000/4000/6000	1080
Flazasulfuron	B	Terafit® (WG 25% *w*/*w*)/Syngenta	0/5/10/20/40/50/100/200	80
2,4-D	O	U46 D Complet® (SL, 60% *w*/*v*)/Nufarm	0/45/90/180/360/720/1200	600
Carfentrazone	E	Affinity 240 CE® (CE 22.3% *w*/*v*)/FMC	0/3.75/7.5/15/30/60/100	100
Flumioxazin	E	Pledge® (WP 50% *w*/*w*)/Kenogard	0/25/50/100/300/600	400
Fluroxypyr	O	Praxis® (EC 20% *p*/*v*)/Nufarm	0/25/50/100/200/400	200
Diflufenican	F1	Mohican 50 SC® (SC 50% *w*/*v*)/Sapec	0/125/250/500/1000/2000	375
Fomesafen	E	Flex 25 SL® (25% *w*/*v*)/Syngenta	0/50/100/200/300/600	400
MCPA	O	U 46 SP Fluid® (SL 40% *p*/*v*)/Nufarm	0/250/500/750/1000/2000	1000
Pyraflufen-ethyl	E	Gozai® CE, 2.65% *w*/*v*)/Belchim	0/1/2/3/6/8	6.62
Glufosinate	H	Finale® (SL, 20% *w*/*v*)/BayerCropScience	0/31.25/62.5/125/250/500/1000/2000/4000	750
Diquat	D	Reglone® (SL, 17% *w*/*w*)/Syngenta	0/5/25/50/100/200/400/600/800	400

[a] Formulation type: SL, soluble (liquid) concentrate; SC, suspension concentrate; WG, water dispersible granules; EC, emulsifiable concentrate; WP, wettable powder. Concentration in percentage: *w*/*w* = weight/weight or *w*/*v* = weight/volume. Mention of trade names in this publication is solely for providing specific information and does not imply their recommendation. [b] HRAC: Herbicide-Resistance Action Committee; G: EPSPS inhibitors; B: ALS inhibitors; O: Synthetic auxins; E: PPO inhibitors; F1: PDS inhibitors; H: Glutamine synthase inhibitors; D: PSI electron diverter. [c] Doses expressed as g acid equivalent (ae) ha^{-1} (50.9% potassium salt of glyphosate equals 450 g ae L^{-1}).

2.3. Shikimic Acid Accumulation

Leaf disks of 4-mm diameter were harvested from the youngest fully expanded leaf at the BBCH 16–18 stage from each *C. canadensis* population. Shikimate accumulation was determined according to Dayan et al. [17] and Hanson et al. [18]. The disks of fresh tissue (~50 mg) from each population were transferred to 2 mL Eppendorf tubes containing 1 mL of 1 mM $NH_4H_2PO_4$ (pH 4.4). At this point, 1 µL of glyphosate at different concentrations was added to each tube resulting in the following concentrations: 0 (blank), 10, 50, 100, 500, and 1000 µM. The Eppendorfs were incubated in a growth chamber for 24 h under the above temperature, humidity, and light conditions. After 24 h, the tubes were stored at −20 °C for further analysis. For analysis, tubes were thawed at 60 °C for 30 min. Thereafter, 250 µL of 1.25 N HCl were added to each Eppendorf tube and shaken with the mechanical

stirrer Selecta (Barcelona, Spain) for 5 min. The tubes were incubated at 60 °C for 15 min and then shaken again for the same period. A 125 µL aliquot from each Eppendorf tube was pipetted into a new 2 mL Eppendorf tube, and 500 µL of periodic acid and sodium metaperiodate (0.25% (*w*/*v*) each) were added. After incubation at room temperature for 90 min, 500 µL of 0.6 N sodium hydroxide and 0.22 M sodium sulfite were added. Finally, the liquid in the tubes was transferred to glass vials. Within 30 min, the light absorption at 380 nm was measured in a spectrophotometer mod. DU-640 from Beckman Coulter (Fullerton, CA, USA). This experiment was replicated three times with five repetitions for glyphosate concentration and population in a randomized design.

2.4. Dose–Response Assays with Alternative Herbicides

To evaluate the potential efficacy of an integrated weed management (IWM) program and screening for potential multiple herbicide resistances, alternative herbicides were applied at the same conditions and spraying volume as the previous assay on the H-5 and H-6 *C. canadensis* populations, which presented the highest and lowest LD_{50} values for glyphosate, respectively. The different herbicides and doses used are shown in Table 2. H-5 was compared to the H-6 population, which was considered susceptible and from the same region (Balaton, Hungary). Plants were cut at 28 DAT, and GR_{50} and LD_{50} values were determinate. Treatments were replicated three times in a completely randomized design using five plants per dose and population.

2.5. ALS Enzyme Activity

Three grams of young leaf tissues were harvested from the H-5 and H-6 populations according to Hatami et al. [19]. They were ground with liquid N_2 and mixed with an extraction buffer in a proportion of 1:2 (tissue: buffer). This buffer was composed of 0.5 g in polyvinylpyrrolidone (PVP), 1 M K-phosphate (at pH 7.5), 10 mM sodium pyruvate, 5 mM $MgCl_2$, 50 mM thiamine pyrophosphate, 100 µM flavint adenine dinucleotide (FAD), 12 mM dithiothreitol, and glycerol (1:9 *v*/*v*). The mix was agitated for 10 min at 4 °C in a magnetic stirrer from Bunsen (Humanes de Madrid, Spain). The homogenate was filtered through four layers of cheesecloth and centrifuged in an Avanti J-25 Beckman Coulter centrifuge (Fullerton, CA, USA) at 20,000 rpm for 20 min. The supernatant contained a crude ALS enzyme extract, which was immediately used for the enzyme assays.

The ALS activity was assayed by adding 0.09 mL of enzyme extract to 0.11 mL of freshly prepared assay buffer (0.08 M K-phosphate buffer solution at pH 7.5, 0.5 M sodium pyruvate, 0.1 M $MgCl_2$, 0.5 mM thiamine pyrophosphate, and 1 µM FAD) containing increasing concentrations of flazasulfuron (Sulfonylureas): 0, 1, 5, 10, 50, 100, 500, 1000, 5000, and 10,000 µM. A solution of 0.04 M K_2HPO_4 (pH 7.0) was added to complete a final volume of 0.25 mL. This mixture was incubated at 37 °C for one hour. The reaction was stopped with 50 µL of H_2SO_4. (1:50 *v*/*v*) and heated at 60 °C for 15 min. An aliquot of 0.25 mL creatine (5 g L^{-1} freshly prepared in water) and 0.25 mL of 1-naphthol (50 g L^{-1} freshly prepared in 5 N NaOH) were added followed by incubation at 60 °C for 15 min. The acetoin from decarboxylate acetolactate was detected as a colored complex (A_{520} nm) in the spectrophotometer. The background was subtracted using control tubes in which the reaction was stopped prior to incubation.

The protein was determined using the Bradford method [20] in which an acidic solution of Coomassie Brilliant Blue G-250 was used for protein binding. The absorbance used for measurement was 595 nm. The maximum ALS-specific activity (nmol acetoin mg^{-1} STP h^{-1}) was measured without herbicide.

The experiment was performed three times with five repetitions per herbicide concentration and population following a randomized design.

2.6. Statistical Analysis

To determine the dose of glyphosate and alternatives herbicides needed to reduce the fresh weight (GR_{50}), cause mortality (LD_{50}), or inhibit the ALS activity (I_{50}) by 50%, the data of dose–response and

ALS enzyme activity assays were subjected to non-linear regression analysis using a three-parameter log-logistic Equation (1)

$$y = ([(d)/1 + (x/g)^b]) \quad (1)$$

where y is the fresh weight, survival, or enzyme activity expressed as the percentage in relation to the non-treated control; d is the coefficient corresponding to the upper asymptote; b is the slope of the line; g is the GR_{50}, LD_{50}, or I_{50}; and x (independent variable) is the herbicide dose/concentration.

The *drc* package in R (version 3.2.5) was used to conduct the regression analyses [21]. Plots were generated with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). Resistance factors (RF) were obtained as R-to-S GR_{50}, LD_{50}, or I_{50} ratios. A lack-of-fit test was used to compare the model that consisted of curves with population-specific g values to a reduced model with a common g [21].

Statistix 9.0 (Analytical Software, Tallahassee, FL, USA) was used to conduct the analysis of variance (ANOVA) to test for differences between populations in terms of the shikimic acid accumulation. Differences of $p < 0.05$ between means were considered significant and separated using the Tukey HSD test.

3. Results

3.1. Dose–Response Assays with Glyphosate

Numerical differences were observed in the GR_{50} and LD_{50} values of the Hungarian populations in comparison to the GR and GS populations from Spain (used as references) (Figure 1). Of the eight populations studied, the H-4 (RF ≈ 11) and H-5 (RF ≈ 13) populations showed the largest resistance factors (RF) based on the LD_{50} values, followed by the H-2 (RF ≈ 10), H-3 (RF ≈ 9), and H-1 (RF ≈ 9) populations. These five populations survived at the recommended field dose and had values similar to the GR *C. canadensis* used as a references. By contrast, the H-6, H-7, and H-8 populations were susceptible to glyphosate. Differences between these three populations, compared to the GS population used as a reference, were not found. However, a difference of almost 200 g ae ha^{-1} based on the LD_{50} values between the H-8 and GS populations was observed (Table 3).

Table 3. Parameters of the log-logistic equations [a] used to calculate the glyphosate rates (g ea ha^{-1}) required for 50% survival (LD_{50}), or reduction fresh weight (GR_{50}) of *C. canadensis* populations from Spain (GR and GS) and Hungary (H1–H8).

Population	*d*	*b*	LD_{50}	RF *	*P*	*d*	*b*	GR_{50}	RF *	*P*
GR	100.3	4.53	3453.6 ± 91.4	11.3	0.0001	100.0	2.03	1474.0 ± 106.4	30.5	0.0001
GS	102.1	1.81	305.7 ± 33.1			101.3	4.23	48.3 ± 4.3		
H-1	98.9	3.33	2761.8 ± 62.5	9.0	0.0001	98.7	1.26	574.5 ± 38.6	11.9	0.0001
H-2	100.2	6.96	3055.8 ± 87.2	10.	0.0001	98.3	1.08	500.1 ± 29.8	10.3	0.0001
H-3	99.3	5.88	2937.7 ± 83.8	9.6	0.0001	99.6	1.10	990.9 ± 102.4	20.5	0.0001
H-4	99.6	5.72	3358.6 ± 102.9	11.0	0.0001	99.7	1.46	995.5 ± 74.4	20.6	0.0001
H-5	100.0	3.18	4029.4 ± 115.4	13.2	0.0001	99.9	0.82	638.4 ± 21.5	13.2	0.0001
H-6	100.4	4.20	383.0 ± 21.9	1.5	0.2671	100.5	1.67	83.3 ± 5.4	1.7	0.1227
H-7	100.9	5.09	436.4 ± 38.9	1.4	0.1089	102.4	1.33	89.4 ± 9.6	1.8	0.1098
H-8	99.3	3.78	493.2 ± 17.5	1.6	0.3516	100.5	1.76	79.7 ± 12.0	1.6	0.2539

[a] $Y = d/1 + (x/g)^b$: where Y = percentage of survival or fresh weight with respect to the control, d = upper limit, b = slope of the curve, g = herbicide dose at the inflection point (i.e., LD_{50} or GR_{50}), and x = herbicide dose. Resistance factor (RF = LD_{50} or GR_{50} of a resistant population/LD_{50} or GR_{50} of GS).

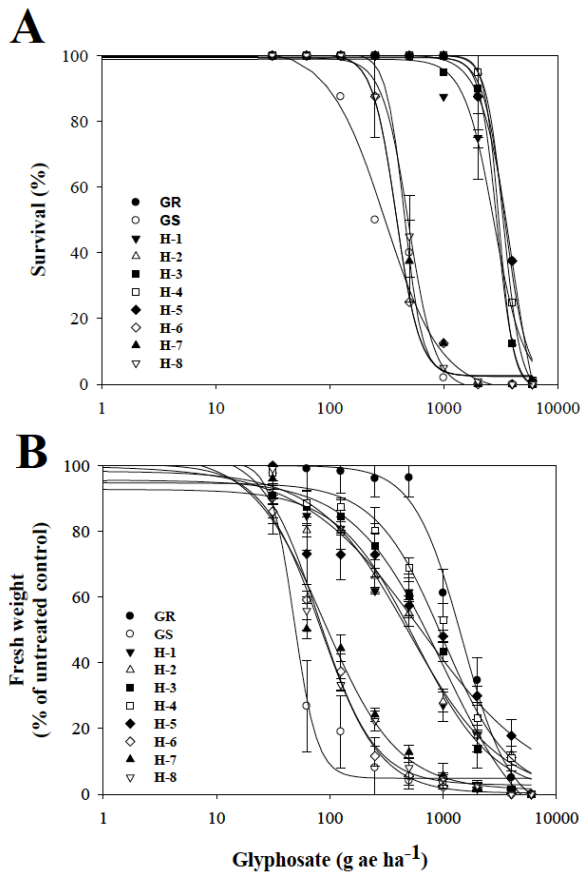

Figure 1. Glyphosate dose–response on (**A**) survival and (**B**) fresh weight reduction expressed as a percentage of the mean untreated control of the populations of *C. canadensis* from Spain (GR and GS) and Hungary (H1–H8). Symbols denote the mean (n = 15) ± standard errors.

3.2. Shikimic Acid Accumulation

From 10 to 500 µM of glyphosate, the accumulation of shikimic acid increased slightly in each population. At 500 µM, the accumulation increased strongly, with the largest amount occurring at 1000 µM. The R-populations (H-1, H-2, H-3, H-4, H-5, and GR) accumulated approximately four-fold less shikimic acid at 1000 µM compared to the H-6, H-7, H-8, and GS populations (Table 4).

Table 4. Shikimic acid accumulation (µg of shikimic acid g^{-1} fresh weight) at different glyphosate concentrations (µM) in *C. canadensis* populations from Spain (GR and GS) and Hungary (H1–H8).

Populations	Glyphosate Concentration				
	10	50	100	500	1000
GR	4.3 ± 0.7 G	23.5 ± 3.9 DE	54.9 ± 6.4 C	63.1 ± 7.3 B	65.8 ± 6.1 B
GS	10.2 ± 1.8 EF	78.7 ± 7.6 B	160.4 ± 15.3 B	273.7 ± 23.1 A	289.5 ± 24.3 A
H-1	6.2 ± 1.3 G	20.1 ± 4.6 E	60.1 ± 8.1 C	66.7 ± 5.5 B	71.5 ± 7.5 B
H-2	13.9 ± 2.1 CDE	22.0 ± 2.6 DE	56.6 ± 6.9 C	62.5 ± 7.0 B	69.4 ± 5.4 B
H-3	10.5 ± 3.3 DEF	25.5 ± 3.0 CD	61.8 ± 5.4 C	70.4 ± 6.1 B	76.0 ± 8.3 B
H-4	7.7 ± 2.5 FG	29.1 ± 4.4 C	52.3 ± 7.1 C	64.5 ± 5.8 B	71.5 ± 7.1 B
H-5	14.3 ± 3.8 BCD	25.1 ± 3.7 CDE	61.3 ± 6.5 C	68.3 ± 7.3 B	75.0 ± 6.4 B
H-6	18.2 ± 3.1 AB	83.9 ± 6.1 A	176.5 ± 20.1 AB	250.9 ± 24.3 A	276.8 ± 29.2 A
H-7	16.9 ± 2.4 ABC	75.6 ± 7.4 B	171.9 ± 17.6 AB	268.8 ± 27.3 A	283.2 ± 25.1 A
H-8	20.1 ± 5.4 A	80.4 ± 6.0 AB	182.7 ± 18.3 AB	276.3 ± 30.6 A	288.9 ± 27.4 A

Means with different letter within are statistically different at 95% probability determined by the Tukey's test. ± Standard error of the mean (n = 15).

3.3. Dose–Response Assays with Alternative Herbicides

The H-6 population from Hungary, that was susceptible to glyphosate, was compared with the H-5 population (the most resistant to glyphosate) in order to avoid variant factors. The resistant factors (RF) obtained for GR_{50} and LD_{50} values of the alternative herbicides with different MOAs were close to unity, except for H-5 with flazasulfuron (Table 5). The LD_{50} value of the H-5 population with flazasulfuron was two times higher than the recommended field dose and was 27.8 times more resistant than for the H-6 population (Figure 2).

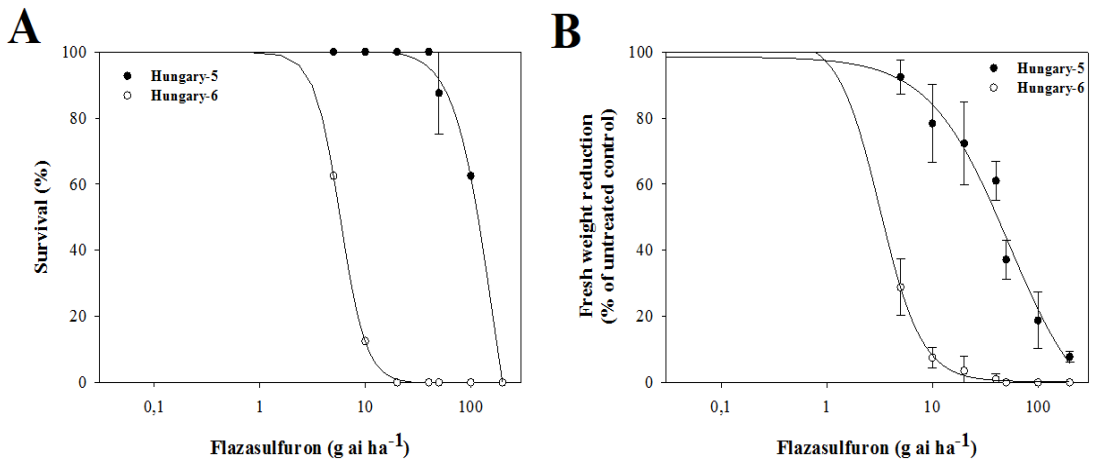

Figure 2. Flazasulfuron dose–response on (**A**) survival and (**B**) fresh weight reduction expressed as a percentage of the mean untreated control of the populations of *C. canadensis* from Spain (GR and GS) and Hungary (H1–H8). Symbols denote the mean (n = 15) ± standard errors.

Table 5. Parameters of the log-logistic equations [a] used to calculate the herbicide rates (g ea ha^{-1}) required for 50% survival (LD_{50}), or reduction fresh weight (GR_{50}) of *C. canadensis* populations H-5 and H-6 from Hungary (n = 15).

Herbicide/Population		*d*	*b*	LD_{50}	RF *	*P*	*d*	*b*	GR_{50}	RF *	*P*
Flazasulfuron	**H-5**	100.6	2.41	161.2 ± 24.4	27.9	0.0001	98.5	1.15	52.6 ± 5.7	16.5	0.0001
	H-6	100.0	3.55	5.8 ± 0.4			102.5	2.18	3.2 ± 0.3		
2,4-D	**H-5**	102.7	2.11	184.4 ± 21.0	1.1	0.3902	99.3	0.82	124.8 ± 25.4	1.6	0.0968
	H-6	101.6	1.72	164.8 ± 22.7			102.3	0.87	79.5 ± 18.6		
Carfentrazone	**H-5**	100.9	2.64	30.9 ± 0.8	1.3	0.2571	99.0	1.13	19.1 ± 1.7	1.3	0.2861
	H-6	100.4	1.76	23.6 ± 1.6			100.6	1.14	15.1 ± 2.4		
Flumioxazin	**H-5**	100.3	2.92	200.6 ± 25.9	1.4	0.0984	101.4	0.97	75.7 ± 7.9	1.6	0.3875
	H-6	103.3	2.01	141.5 ± 20.6			99.9	0.79	47.4 ± 10.8		
Fluroxypyr	**H-5**	101.3	3.75	114.8 ± 4.6	1.1	0.0996	100.0	3.31	28.7 ± 1.2	1.2	0.4392
	H-6	100.5	2.69	101.2 ± 2.3			100.3	2.90	24.5 ± 4.0		
Diflufenican	**H-5**	101.0	3.01	258.0 ± 32.2	1.1	0.1583	101.3	2.99	208.7 ± 13.8	1.1	0.2447
	H-6	101.3	5.86	231.4 ± 9.3			100.5	2.78	183.3 ± 10.8		
Fomesafen	**H-5**	102.1	3.53	206.8 ± 15.5	1.0	0.1034	98.5	1.66	189.2 ± 17.7	1.4	0.2816
	H-6	99.1	3.58	198.6 ± 10.9			97.1	1.65	131.2 ± 21.8		
MCPA	**H-5**	100.4	6.03	545.2 ± 12.3	1.0	0.2190	99.9	0.97	172.3 ± 21.1	1.3	0.1648
	H-6	96.8	4.13	506.01 ± 27.7			100.0	0.93	130.3 ± 18.4		
Pyraflufen-ethyl	**H-5**	96.3	6.03	2.2 ± 0.1	1.1	0.1693	99.9	1.02	1.3 ± 0.3	1.1	0.1739
	H-6	97.4	3.88	2.0 ± 0.1			100.0	1.39	1.2 ± 0.2		
Glufosinate	**H-5**	100.6	2.47	77.7 ± 6.0	1.2	0.3591	101.5	2.05	46.1 ± 4.4	1.2	0.1520
	H-6	100.1	3.66	62.8 ± 3.5			102.8	3.23	38.1 ± 5.5		
Diquat	**H-5**	100.3	1.77	14.1 ± 1.9	1.3	0.2745	102.0	0.98	7.3 ± 0.6	1.3	0.3435
	H-6	99.9	2.42	11.1 ± 1.4			101.1	1.11	5.7 ± 0.3		

[a] $Y = d/1 + (x/g)^b$: where Y = percentage of survival or fresh weight with respect to the control, d = upper limit, b = slope of the curve, g = herbicide dose at the inflection point (i.e., LD_{50} or GR_{50}), and x = herbicide dose. Resistance factor (RF = LD_{50} or GR_{50} of a resistant population/LD_{50} or GR_{50} of GS).

3.4. ALS Enzyme Activity

The I_{50} values determined for the H-5 and H-6 populations were 603.7 ± 17.6 and 9.5 ± 1.6 µM, flazazulfuron respectively. These results indicated that resistance to this herbicide was 63.3 times higher in the H-5 population than in the H-6 population (Figure 3).

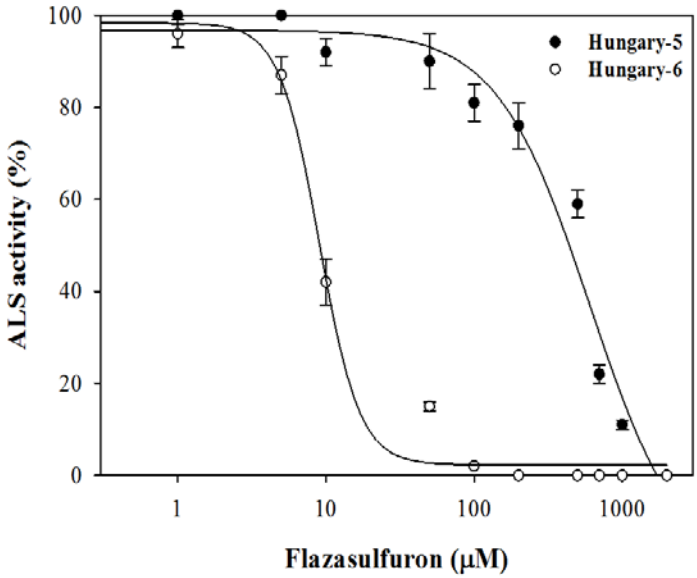

Figure 3. ALS enzyme activity was determined using flazasulfuron in the H-5 and -6 populations from Hungary. The equations of log–logistic curves to estimates the I_{50} values are: H-5: $Y = \{(99.2)/[1 + (dose/I_{50})^{1.38}]\}$ and H-6: $Y = \{(100.4)/[1 + (dose/I_{50})^{3.21}]\}$. Symbols denoted mean ($n$ = 15) ± standard errors.

4. Discussion

4.1. Dose–Response Assays with Glyphosate

Growers use glyphosate because it is a highly versatile, broad-spectrum herbicide for weed control. This herbicide has been an important tool to control perennial and annual weeds in crop areas, highlighting the control of perennial weeds in perennial crops, as well as prior to planting or after harvesting in annual crops. Another important aspect is that glyphosate is a herbicide that has high compatibility in mixture with other herbicides with different MOA, increasing its action spectrum. For these reasons, glyphosate has been used excessively. The appearance of resistance to glyphosate in weeds is a problem for growers who are deprived of this effective molecule for weed control.

The observed resistance in H-1–H-5 populations may be due to numerous herbicide applications over successive years, increases in the recommended field dose, or because the MOA was not changed [14].

Control failures are often due to applications at a later growth stage or because environmental factors during the use of herbicides were ignored [22]. These situations may result in resistance after several years, or a false resistance signal (as a consequence of a bad application and not due to resistance mechanisms). RFs variability between *C. canadensis* populations may be attributed to different resistance mechanisms, and/or the existence of multiple or cross-resistance [23–25].

4.2. Shikimic Acid Accumulation

Differential accumulation of shikimic acid between R and S plants may occurs when glyphosate does not inhibit the EPSPS enzyme mechanisms [13,26,27], due to either target site or non-target site resistance mechanisms [28]. Glyphosate resistant *C. canadensis* plants could accumulate more shikimic

acid; however, larger amounts of herbicide would be needed, i.e., an increase in glyphosate doses. The variable accumulation of shikimic acid between the *C. canadensis* populations was in agreement with that observed in the dose–response assays, and other glyphosate resistant *Conyza* spp. populations from Hungary [29], confirming the resistance to glyphosate of the H-1 to H-5 populations.

4.3. Dose–Response Assays with Alternative Herbicides

The results found in the H-6 population treated with flazasulfuron showed that it is an efficient herbicide, but only if an IWM plan is followed with other MOA herbicides. Although not yet reported, multiple resistance flazasulfuron × glyphosate as a consequence of applying the same herbicide alternatives, it is beginning to be observed in the European Mediterranean region. Some farmers are now reporting low effectiveness due to the continuous use of flazasulfuron over a five year period without alternative MOA herbicides. This effect may soon de observed in Hungary.

4.4. ALS Enzyme Activity

In consideration of the results reported here, a goal for further research will be to identify the resistance mechanisms that are involved in both herbicides, glyphosate, and flazasulfuron. For glyphosate, reduced absorption/translocation and/or amino acid substitution(s) are commonly observed [26]; however, for ALS-inhiting herbicides it is not common to see absorption/translocation as the resistance mechanisms [19,30], although it is common to find metabolism and/or amino acid substitution(s) [11,31]. Studying the NTSR and TSR mechanisms endowing resistance to glyphosate and fazasulfuron in the H-8 *C. canadensis* population may help us to understand how resistance has been selected, as reported recently in many other weed species [14,32,33]. We plan to study these mechanisms in the future; meanwhile, taking into account these results, we have determined multiple-resistance to flazasulfuron (ALS-inhibitors) and glyphosate in *C. canadensis* from Hungary.

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