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Article

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 agricultural areas throughout the world, mainly to its ability to evolve herbicide resistance. Specifically, in Hungary, extensive areas have been infested by this species due to the difficulty in controlling it with glyphosate. To corroborate this fact as resistance and not as an incorrect herbicide application, eight suspicious glyphosate-resistant *C. canadensis* populations from different Hungarian regions were studied. In dose-response assays with glyphosate, the LD_{50} and GR_{50} values indicated that populations 1 to 5 were resistant to this herbicide (H-5 population the most resistant). Besides, the shikimic acid accumulation tests corroborated the results observed in the dose-response assays. 11 alternative herbicides from 6 different mode 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 was 63.3 fold 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 this two MOA. For that reason, the other herbicides with different MOA have to be tested here.

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 an herbicide to sustain growth with little or no symptomology [1]. Factors that are important in the development of herbicide-resistant weed species include a strict dependence on one herbicidal mode of action (MOA) and its continuous use [2].

One of the most used herbicides over four decades has been glyphosate, which has a demonstrated high efficiency in weed control [3]. Its excessive (doses higher than recommended by the manufacturer) and continued use, has resulted in the evolution glyphosate-resistance in a large number of weed species [4]. Some of which 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 *C. canadensis* with resistance to glyphosate was found in North America in 2000 [8]. Since then, there have been many cases of resistance in these dicotyledonous species around the world [4]. *Conyza* species are one of the most prone to evolve resistance to glyphosate; afterwards, the incidence of glyphosate resistance in Europe for the studied species could be reported here.

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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 by reduced absorption and/or translocation, increased vacuolar sequestration [9], and/or metabolism into non-toxic compounds [10,11]. In 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 and potentially greater problem is when multiple resistance or coexisting resistance mechanisms for different modes of action (MOA) herbicides in the population appear. Given its importance in agriculture, the most serious multiple herbicide resistance cases are those involving glyphosate. Half of the glyphosate-resistance cases include cases of multiple resistance in the world [4].

The continued use of herbicides with different MOA (ie, acetolactate synthase–ALS–inhibitor) to control glyphosate-resistant weeds under non-herbicide-rotation regimes have resulted in decreased weed control efficiencies [14], leading to the appearance of multiple resistance.

In addition, Hungary is a country with great agricultural activity, due in part to its favorable climatic conditions [15]. In recent years, this country 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 current study were: to evaluate the level of glyphosate resistance in eight suspicious populations of *C. canadensis* from two different vineyard regions of Hungary; to evaluate chemical control alternatives in two glyphosate resistant populations; and to determinate the level of multiple resistance if it was found.

2. Materials and Methods

2.1. Plant material

Eight suspicious glyphosate-resistant (GR) *C. canadensis* populations from two different regions of Hungary were studied. They were provided by Monsanto Europe and denominated as H-1 to H-8 (Table 1). 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 ten mature plants in vineyard crops and non crops areas.

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Table 1. *Conyza canadensis* populations harvested in different Hungarian and Spanish areas.

C. canadensis	Country/Location	Crops	Herbicide application	Dose/year	Coordinate
GR	Spain/Córdoba	Olive grove	Glyphosate	1440[a] /20	37.999139, -4.448414
GS	Spain/Córdoba	Railway	Mechanical control	------	37.915686, -4.717149
H-1	Hungary/Badacsony	Vineyard	Glyphosate +2,4-D	1440[a] /10+600[b]/5	46.786291, 17.382222
H-2	Hungary/Badacsony	Vineyard	Glyphosate + flazasulfuron	1440[a] /10 +750[c]/4	46.789740, 17.427881
H-3	Hungary/Badacsony	Vineyard	Glyphosate	1800[a] /20	46.786496, 17.448939
H-4	Hungary/Balaton	Vineyard	Glyphosate	1800[a] /20	46.786663, 17.715590
H-5	Hungary/Balaton	Vineyard	Glyphosate + flazasulfuron	1800[a] /20+ 750[c]/7	46.788120, 17.769552
H-6	Hungary/Balaton	Vineyard	Organiccrop	---/20	46.810988, 17.830547
H-7	Hungary/Balaton	No crop	No herbicide	------	46.871464, 17.944467
H-8	Hungary/Badacsony	Vineyard	Organic crop	---/10	46.787210, 17.486736

[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, and they 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. The plants populations were transplanted into pots [one per pot (0.448 cm^3)] containing sand/peat at a 1:2 (v/v) ratio and were then placed in a greenhouse at 28/18 °C (day/night).

2.2. Dose-response assays with glyphosate

Glyphosate treatments were applied at the rosette stage (BBCH 16-18 stage, described in Hess et al. [16] of *C. canadensis* plants. Applications were applied with a laboratory chamber sprayer (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with a 8002 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. Plants were cut at the soil surface 28 days after treatment (DAT), and the fresh weight reduction (GR_{50}) and survival (LD_{50}) at 50%, in respect to untreated control plants were measured. All populations were compared to the GS population.

Treatments were replicated three times in a completely randomized design, using 5 plants per dose and population.

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Table 2. Herbicide treatments applied at the 3-4 leaf growth stage of *C. canadensis* populations from Spain (GR and GS) and Hungary (H1–H8).

Herbicide	HRAC group	Formulation	Company	Doses (g ai ha^{-1})	Recommended field dose (g ai ha^{-1})
Glyphosate*	G	Roundup Energy®. (Glyphosate 45 % p/v. SL)	Monsanto	0/31.25/62.5/125/250/500/ 1000/2000/4000/6000	1080
Flazasulfuron	B	Terafit®. (Flazasulfuron 25% WG).	Syngenta	0/5/10/20/40/50/100/200	80
2,4-D	O	U 46 D Complet®. (2,4-D 60% p/v. SL).	Nufarm	0/45/90/180/360/720/1200	600
Carfentrazone	E	Affinity 240 CE®. (Carfentrazone ethyl 22.3% CE).	FMC	0/3.75/7.5/15/30/60/100	100
Flumioxazin	E	Pledge®. (Flumioxazin 50% WP)	Kenogard	0/25/50/100/300/600	400
Fluroxypyr	O	Praxis®. (Fluroxypyr 20% p/v. EC).	Nufarm	0/25/50/100/200/400	200
Diflufenican	F1	Mohican 50 SC® (diflufenican 50% p/v. SC)	Sapec	0/125/250/500/1000/2000	375
Fomesafen	E	Flex 25 SL®. (Fomesafen 25% p/v. EC).	Syngenta	0/50/100/200/300/600	400
MCPA	O	U 46 SP Fluid®. (MCPA 40% p/v. SL).	Nufarm	0/250/500/750/1000/2000	1000
Pyraflufen-ethyl	E	Gozai®. (Pyraflufenethyl 2.65% p/v. EC).	Belchin	0/1/2/3/6/8	6.62
Glufosinate	H	Finale®, (glufosinate15 % p/v. SL)	Bayer CropScience	0/31.25/62.5/125/250/500/1000/2000/4000	750
Diquat	D	Reglone®, (diquat 20 % p/v. SL)	Syngenta	0/5/25/50/100/200/400/600/800	400

gai ha^{-1} = grams of active ingredient per hectare.

* g ae ha^{-1} = grams of acid equivalent per hectare.

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

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 (aprox. 0.05 grams) 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 conditions of temperature, humidity and light. After 24 h, the tubes were stored at -20 ºC until further analysis. For analysis, tubes were thawed at 60 ºC 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 ºC for 15 min and then again shaken for the same time. 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, 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 create an integrated weed management (IWM) program, in which herbicide treatments were applied at the same conditions and spraying volume as the previous assay, H-5 and H-6 populations (from the Balaton region) were used in this assay. The different herbicides and doses used are shown in Table 2. H-5 was compared to the H-6 population, which was considered susceptible. Plants were cut at 28 DAT, and GR_{50} and LD_{50} values were determinate. Treatments also were replicated three times in a completely randomized design, using 5 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 powdered 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 (Spain). The homogenate was filtered through four layers of cheesecloth and centrifuged in an Avanti J-25 Beckman Coulter centrifuge (Fullerton, USA) at 20000 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 10000 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 1 hour. The reaction was stopped with 50 µL of H_2SO_4. (1:50 v/v) and heated at 60°C for 15 min.

To decarboxylate acetolactate to acetoin 0.25 mL of 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 an incubation at 60°C for 15 min. Acetoin 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 5 repetitions per herbicide concentration and population following a randomized design.

2.6. Statistical analysis

To determine the dose of glyphosate and alternatives herbicides causing reduction in growth (GR_{50}), mortality (LD_{50}), or inhibition of 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 (Equation 1):

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

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* packagein R (version 3.2.5) were used to conduct the regression analyses [21]. Plots were generated with SigmaPlot 11.0 (Systat Software, Inc. 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, USA) was used to conduct the Analysis of variance (ANOVA) to test for differences between populations in terms of the shikimic acid accumulation. Differences between means were separated using the Tukey HSD test.

3. Results

3.1. Dose-response assays with glyphosate

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, Table 3).

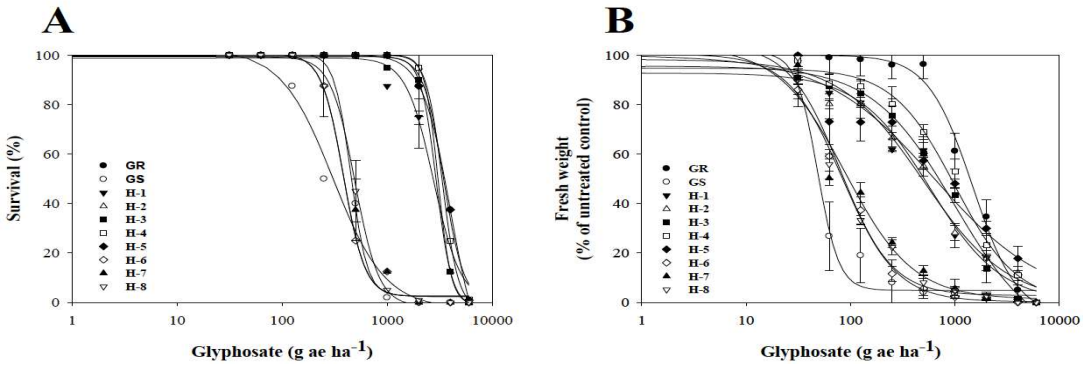

Figure 1. Glyphosate dose-response on (A) survival and (B) fresh weight reduction expressed as a percentage of the mean untreated control of the populations of *C. canadensis* from Spain (GR and GS) and Hungary (H1–H8). Symbols denote the mean (n=15) ± standard errors.

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Table 3. Parameters of the log-logistic equations used to calculate the glyphosate rates 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}	R^2	RF*	*P*	d	b	GR_{50}	R^2	RF*	*P*
GR	100.31	4.53	3453.61 ± 91.35	0.99	11.29	0.0001	99.99	2.03	1473.98 ± 106.41	0.99	30.49	0.0001
GS	102.12	1.81	305.69 ± 33.14	0.99			101.25	4.23	48.33 ± 4.27	0.98		
H-1	98.91	3.33	2761.84 ± 62.5	0.99	9.03	0.0001	98.74	1.26	574.52 ± 38.60	0.99	11.88	0.0001
H-2	100.24	6.96	3055.76 ± 87.24	0.98	9.99	0.0001	98.27	1.08	500.12 ± 29.81	0.98	10.34	0.0001
H-3	99.33	5.88	2937.71 ± 83.81	0.99	9.61	0.0001	99.55	1.10	990.91 ± 102.36	0.97	20.50	0.0001
H-4	99.57	5.72	3358.57 ± 102.94	0.99	10.98	0.0001	99.69	1.46	995.53 ± 74.36	0.98	20.59	0.0001
H-5	100.04	3.18	4029.40 ± 115.37	0.98	13.18	0.0001	99.93	0.82	638.42 ± 21.45	0.99	13.20	0.0001
H-6	100.42	4.20	382.99 ± 21.87	0.99	1.50	0.2671	100.45	1.67	83.33 ± 5.38	0.99	1.72	0.1227
H-7	100.91	5.09	436.37 ± 38.94	0.97	1.42	0.1089	102.41	1.33	89.41 ± 9.61	0.98	1.84	0.1098
H-8	99.27	3.78	493.24 ± 17.49	0.99	1.61	0.3516	100.48	1.76	79.69 ± 12.01	0.99	1.64	0.2539

*RF= each population was compared with respect to GS.

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 (Table 3).

These five populations also survived at the recommended field dose and had values similar to the GR *C. canadensis* used as references (Table 3).

In contrast, the H-6, H-7, and H-8 populations demonstrated glyphosate susceptibility. Differences between these three populations, compared to the GS population used as reference, were not found (Table 3). 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).

3.2. Shikimic acid accumulation

From 10 to 500 μM of glyphosate, the accumulation of shikimic acid increased slightly in each population. However, after 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 4-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 at different concentrations of glyphosate in *C. canadensis* populations from Spain (GR and GS) and Hungary (H1–H8).

Populations	Glyphosate (μM)[a,b]				
	10	50	100	500	1000
GR	4.25 ± 0.69 G	23.52 ± 3.91 DE	54.91 ± 6.37 C	63.05 ± 7.32 B	65.81 ± 6.09 B
GS	10.19 ± 1.78 EF	78.65 ± 7.58 B	160.38 ± 15.26 B	273.65 ± 23.14 A	289.51 ± 24.30 A
H-1	6.21 ± 1.27 G	20.13 ± 4.59 E	60.11 ± 8.07 C	66.72 ± 5.48 B	71.53 ± 7.47 B
H-2	13.92 ± 2.06 CDE	21.98 ± 2.57 DE	56.64 ± 6.93 C	62.49 ± 7.01 B	69.40 ± 5.38 B
H-3	10.54 ± 3.29 DEF	25.47 ± 3.03 CD	61.75 ± 5.41 C	70.39 ± 6.11 B	76.02 ± 8.25 B
H-4	7.66 ± 2.47 FG	29.12 ± 4.42 C	52.26 ± 7.11 C	64.50 ± 5.77 B	71.53 ± 7.11 B
H-5	14.31 ± 3.76 BCD	25.06 ± 3.67 CDE	61.33 ± 6.45 C	68.25 ± 7.34 B	74.95 ± 6.40 B
H-6	18.23 ± 3.05 AB	83.90 ± 6.12 A	176.51 ± 20.09 AB	250.93 ± 24.26 A	276.88 ± 29.15 A
H-7	16.87 ± 2.44 ABC	75.61 ± 7.36 B	171.93 ± 17.62 AB	268.75 ± 27.31 A	283.20 ± 25.07 A
H-8	20.09 ± 5.38 A	80.44 ± 6.03 AB	182.68 ± 18.34 AB	276.33 ± 30.55 A	288.90 ± 27.35 A

[a] Mean values (n=15) ± standard error of the mean; Means with different letter within a column are statistically different at 95% probability determined by the Tukey's test.

[b] Results expressed as μg of shikimic acid g^{-1}fresh weight.

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 GR_{50} and LD_{50} values of the alternative herbicides with different MOAs had no significant differences between them and displayed excellent effectiveness even at lower rates than the recommended field dose (Table 1 and 5), except H-5 with flazasulfuron.

The LD_{50} value of the H-5 population with flazasulfuron was two-fold higher than the recommended field dose and was 27.8-fold more resistant than for the H-6 population (Figure 2).

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Table 5. Parameters of the log-logistic equations used to calculate the herbicide rates 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).

	Population	d	b	LD_{50}	R^2	RF	*P*	d	b	GR_{50}	R^2	RF	*P*
Flazasulfuron	**H-5**	100.56	2.41	161.15 ± 24.35	0.99	27.88	0.0001	98.51	1.15	52.57 ± 5.67	0.99	16.53	0.0001
	H-6	99.98	3.55	5.78 ± 0.41	0.98			102.54	2.18	3.18 ± 0.33	0.99		
2,4-D	**H-5**	102.73	2.11	184.43 ± 20.99	0.99	1.11	0.3902	99.34	0.82	124.76 ± 25.39	0.98	1.57	0.0968
	H-6	101.55	1.72	164.75 ± 22.65	0.99			102.29	0.87	79.46 ± 18.59	0.99		
Carfentrazone	**H-5**	100.89	2.64	30.89 ± 0.79	0.97	1.30	0.2571	99.02	1.13	19.09 ± 1.71	0.99	1.26	0.2861
	H-6	100.37	1.76	23.62 ± 1.64	0.98			100.64	1.14	15.14 ± 2.38	0.99		
Flumioxazin	**H-5**	100.31	2.92	200.58 ± 25.87	0.99	1.41	0.0984	101.36	0.97	75.72 ± 7.91	0.99	1.59	0.3875
	H-6	103.31	2.01	141.48 ± 20.55	0.97			99.85	0.79	47.37 ± 10.83	0.98		
Fluroxypyr	**H-5**	101.32	3.75	114.79 ± 4.61	0.99	1.13	0.0996	100.03	3.31	28.66 ± 1.22	0.97	1.16	0.4392
	H-6	100.53	2.69	101.17 ± 2.31	0.99			100.27	2.90	24.53 ± 3.96	0.99		
Diflufenican	**H-5**	100.96	3.01	257.96 ± 32.17	0.99	1.11	0.1583	101.25	2.99	208.72 ± 13.81	0.97	1.13	0.2447
	H-6	101.25	5.86	231.37 ± 9.26	0.98			100.49	2.78	183.25 ± 10.81	0.98		
Fomesafen	**H-5**	102.12	3.53	206.81 ± 15.49	0.98	1.04	0.1034	98.53	1.66	189.18 ± 17.66	0.97	1.44	0.2816
	H-6	99.12	3.58	198.55 ± 10.94	0.99			97.11	1.65	131.19 ± 21.82	0.98		
MCPA	**H-5**	100.42	6.03	545.19 ± 12.33	0.97	1.07	0.2190	99.88	0.97	172.27 ± 21.07	0.99	1.33	0.1648
	H-6	96.75	4.13	506.09 ± 27.73	0.99			99.95	0.93	130.33 ± 18.39	0.98		
Pyraflufen-ethyl	**H-5**	96.28	6.03	2.22 ± 0.11	0.97	1.11	0.1693	99.91	1.02	1.31 ± 0.32	0.99	1.06	0.1739
	H-6	97.40	3.88	1.99 ± 0.09	0.98			100.04	1.39	1.23 ± 0.17	0.99		
Glufosinate	**H-5**	100.64	2.47	77.71 ± 6.04	0.99	1.23	0.3591	101.49	2.05	46.14 ± 4.37	0.99	1.21	0.1520
	H-6	100.11	3.66	62.83 ± 3.51	0.98			102.82	3.23	38.13 ± 5.46	0.99		
Diquat	**H-5**	100.28	1.77	14.12 ± 1.91	0.98	1.27	0.2745	102.04	0.98	7.27 ± 0.57	0.99	1.27	0.3435
	H-6	99.85	2.42	11.08 ± 1.39	0.99			101.05	1.11	5.72 ± 0.33	0.99		

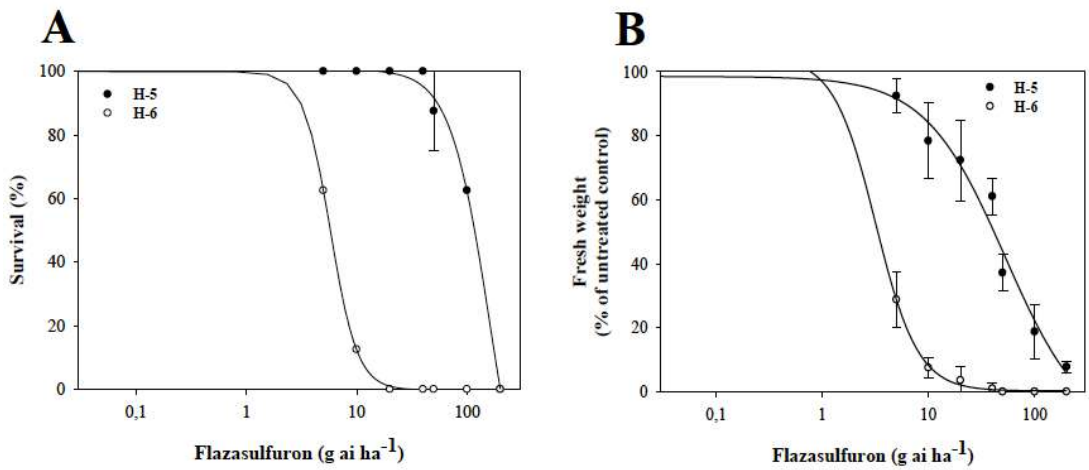

Figure 2. Flazasulfuron dose-response on (A) survival and (B) fresh weight reduction expressed as a percentage of the mean untreated control of the populations of *C. canadensis* from Spain (GR and GS) and Hungary (H1–H8). Symbols denote the mean (n=15) ± standard errors.

3.4. ALS enzyme activity

The test for a lack of fit comparing a reduced model with a common g parameter for the H-5 and H-6 populations with a full model with population-specific parameter values was significant (P<0.0001). The I_{50} results were 603.71 ± 17.63 and 9.54 ± 1.64 µM for the H-5 and H-6 populations, respectively. The results indicated that resistance was 63.3-fold higher in the H-5 population than in the H-6 population (Figure 3).

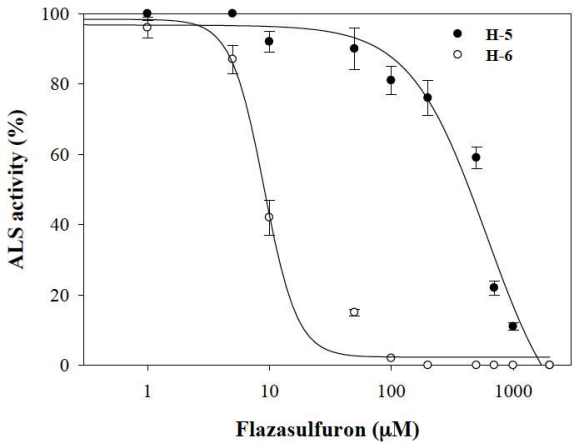

Figure 3. ALS enzyme activity was determined using flazasulfuron in the H-5 and -6 populations from Hungary. The equations of log–logistic curves to estimates the I_{50} values are: H-5: $Y = \{(99.15)/[1 + (dose/I_{50})^{1.38}]\}$ and H-6: $Y = \{(100.39)/[1 + (dose/I_{50})^{3.21}]\}$. Symbols denoted mean ($n$=15) ± standard errors.

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4. Discussion

3.1. Dose-response assays with glyphosate

The observed resistance in H-1–H-5 populations may be due to numerous herbicide applications in 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.

The RFs variability may be attributed to different species, the greater or lesser susceptibility of the S-populations used for comparison to the R-populations, the different mechanisms, and/or the existence of multiple or cross-resistance [23–25].

3.2. Shikimic acid accumulation

Differential accumulation of shikimic acid occurs when glyphosate does not reach the target-site in sufficient amounts to completely inhibit the EPSPS enzyme [13,26,27]. For glyphosate to be effective in plants with these low shikimic acid accumulations, larger amounts of herbicide are required, which indicates an increase in herbicide doses. The different shikimic acid accumulation between the *C. canadensis* populations were in agreement with those observed in the dose-response assays, confirming the resistance to glyphosate of the H-1 to H-5 populations.

3.3. Dose-response assays with alternative herbicides

The positive results found in the H-6 population treated with flazasulfuron evidenced that it is an efficient herbicide, but only if an IWM plan is followed with other MOA herbicides. Although not yet reported, the multiple resistance (flazasulfuron-glyphosate), as a consecuence of the same herbicide alternatives, is beginning to be observed in the European Mediterranean area. Some farmers are now reporting low effectiveness due to the continuous use of flazasulfuron over a 5 year period without alternative MOA herbicides. This effect may be soon found in Hungary.

3.4. ALS enzyme activity

In consideration of the results reported here, a goal for further studies will be to identify the resistance mechanisms that are involved in both herbicides, glyphosate and flazasulfuron. Studying the NTSR and TSR mechanisms may help us to understand how the resistance is reached and also to find both mechanisms in the same populations, as reported recently in many other weed species [14,28,29]. In glyphosate studies, reduced glyphosate absorption/translocation and/or amino acid substitution(s) are commonly observed [26]. However, in ALS studies, 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]. 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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