



Research article

Physiological, biochemical and molecular bases of resistance to tribenuron-methyl and glyphosate in *Conyza canadensis* from olive groves in southern Spain

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ABSTRACT

Multiple resistance to acetolactate synthase (ALS, EC 2.2.1.6) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) inhibitor herbicides was studied in two populations of *Conyza canadensis* (RTG and STG) harvested in southern Spain. Dose-response and enzymatic activity studies for the ALS-inhibiting herbicides showed only cross-resistance to sulfonylureas group but not to the other ALS chemical groups in the RTG population. Regarding glyphosate, the dose-response studies showed that the RTG population was 11.8 times more resistant than the STG population, while the inhibition of EPSPS enzyme (I_{50}) was similar for both populations. Altered/reduced absorption and translocation were the main resistance mechanisms for glyphosate but not for tribenuron-methyl. The metabolic studies to find differences in the amounts of metabolites between the two populations were carried out using thin layer chromatography (for tribenuron-methyl) and capillary electrophoresis (for glyphosate). Metabolites were significantly differed among the two populations for tribenuron-methyl but not for glyphosate. The sequencing of the target-site ALS gene from RTG plants revealed a single point mutation, Pro-197-Ala, that causes resistance to sulfonylurea herbicide in *C. canadensis*.

1. Introduction

Horseweed (*Conyza canadensis*) is a dominant weed of annual and perennial crops, occurring mainly in orchards and vineyards, as well as in pastures and fallows, especially under minimum tillage systems (Shrestha et al., 2008; Weaver, 2011). It is native to the more temperate regions of South America, but it is currently widespread in regions of Africa, Asia-Pacific, Europe and Central America (Wu et al., 2007; Sansom et al., 2013). In southern Spain, it is considered as a typical weed of citrus and olive groves (Amaro-Blanco et al., 2018; González-Torralva et al., 2010). The horseweed plants produce many wind-dispersed seeds (≤ 375561 seeds per plant), making them easily spread over long distances (Bhowmik and Bekech, 1993). This trait, combined with the invasive behaviour and competitiveness of horseweed, places this species among the most difficult ones to control worldwide.

The most commonly used methods to control horseweed include the application of different pre-emergent and post-emergent herbicides,

depending on the crop. In Spanish olive groves, the main herbicides employed are acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors (Palma-Bautista et al., 2018; Shrestha et al., 2007). The prevalent use of ALS inhibitors to control horseweed is largely due to the diverse and large number of ALS chemistries for the farmers to choose from based on the price and formulations. The ALS inhibitors are classified in five chemical families, the sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-benzoates (PYB), and sulfonylamino-cabonyl-triazolinones (SCT), and some of their herbicides are tribenuron, imazamox, pennisulam, bispyribac and flucarbazone, respectively. Glyphosate (EPSPS inhibitor) is a highly effective broad-spectrum herbicide that is toxicologically and environmentally safe and so widely used that there are different formulations with different prices (Duke and Powles, 2008).

As noted above, glyphosate is a commonly used herbicide. This herbicide is a nonselective, post-emergent systemic herbicide that is

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used for the control of annual and perennial weeds in agricultural and non-agricultural areas (Caseley and Copping, 2000). It kills plants by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) of the shikimate pathway (Duke and Powles, 2008). As a result, the biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan is stopped (Martinez et al., 2018; Siehl, 1997).

Another herbicide to consider is tribenuron-methyl, which is a selective post-emergent herbicide, used to control broadleaf weeds. This herbicide inhibits the biosynthesis of branched amino acids (valine, leucine and isoleucine) by binding to the enzyme system that catalyses the formation of these amino acids, the acetolactate synthase (ALS, EC 2.2.1.6) (Bhattacharjee and Dureja, 1999). The lack of branched-chain amino acids results in the subsequent plant death (Deng et al., 2015).

However, intensive herbicide application has led to weed species shifts and numerous cases of glyphosate-resistant horseweed with resistance to different groups of herbicides, such as ALS inhibitors, Photosystem I (PSI) Electron Diverter and Photosystem II inhibitors (PSII) (Heap, 2018). In Spain, the first case of glyphosate-resistant *Conyza* spp. was reported in groves located in southern Spain (Urbano et al., 2007). Impaired translocation and high basal EPSPS transcript levels were the two different resistance mechanisms that appeared to be related to glyphosate resistance (Dinelli et al., 2008; Ge et al., 2010). To date, two more cases of resistant horseweed have been reported (Amaro-Blanco et al., 2018).

Similarly, repeated use of tribenuron-methyl has resulted in the evolution of resistant white mustard (*Sinapis alba*) populations in southern Spain during the last decade. The resistance was due to a target-site mutation in the ALS enzyme that reduces the binding affinity of tribenuron-methyl (Cruz-Hipolito et al., 2013). In horseweed, resistance to more than one herbicide has been reported in California and Hungary, where all paraquat- and flazasulfuron-resistant accessions identified were also resistant to glyphosate (Moretti et al., 2016; Palma-Bautista et al., 2018).

In view of the above-stated facts, this study had the following aims: (1) to confirm resistance of horseweed to glyphosate and tribenuron-methyl and (2) to investigate the target-site and non-target site resistance mechanisms involved in the resistant *C. canadensis* populations harvested from olive groves in southern Spain.

2. Material and methods

2.1. Chemicals

¹⁴C-tribenuron-methyl together with its metabolites (metsulfuron-methyl [MM] and hydroxylated metsulfuron-methyl [OH-MM]) were supplied by Dupont de Nemours & Co (Nambesheim, France). The ¹⁴C-glyphosate was from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). The technical grade herbicides used were from

different companies, as shown in Table 1.

2.2. Plant materials

One suspected glyphosate-resistant (RTG) *C. canadensis* population was studied. It was provided by a farmer in Baena (Córdoba, southern Spain), who harvested seeds from 30 plants (approximately) in different points of an olive grove. This site or this field has been treated with glyphosate since 1990 and other herbicides such as tribenuron or flazasulfuron, alone or in combination with glyphosate (Table 1). In addition, one STG population of *C. canadensis* from noncrop areas (characterized as sensitive-glyphosate biotype by University of Cordoba, Spain) was used as a negative control.

Mature seeds were germinated in peat moistened at field capacity (trays 15 × 15 × 8 cm) and 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 75% relative humidity. The plant populations were transplanted into pots (one per pot) containing sand/peat at a 1:2 (v/v) ratio and placed in a greenhouse at 28/18 °C (day/night).

2.3. Herbicide dose-responses in plants

C. canadensis plants from both populations (RTG and STG), in the rosette stage BBCH 16–18 (Hess et al., 1997), were treated with the herbicides, and doses are shown in Table 2. A treatment chamber model SBS-060 (De Vries Manufacturing, Hollandale, MN, USA) equipped with a flat fan nozzle 8002 was used to apply the herbicides to the plants. It was calibrated to spray 250 L ha⁻¹ at 250 kPa. Twenty-one days after treatment (DAT), the survival (LD₅₀) values were obtained, and the plants were cut to obtain the fresh weight reduction (GR₅₀). In each treatment, the RTG population was compared with the STG population. Eight plants per dose were used in each population.

2.4. ¹⁴C-tribenuron-methyl and ¹⁴C-glyphosate absorption and translocation

The ¹⁴C-glyphosate and ¹⁴C-tribenuron-methyl absorption and translocation assays were carried out separately as described by Amaro-Blanco et al. (2018) and Cruz-Hipolito et al. (2013), respectively.

¹⁴C-tribenuron-methyl was mixed with a commercial formulation of tribenuron-methyl. The final concentration of tribenuron-methyl was 15 g ai ha⁻¹ in 250 L ha⁻¹ with a specific activity of 0.834 kBq μL⁻¹. For the ¹⁴C-glyphosate experiment, the final concentration was 360 g ae ha⁻¹ of commercial glyphosate, with the same specific activity. Five plants per time and population were used with each herbicide. Plants in the rosette stage (BBCH 16–18) of the RTG and STG populations were treated with a drop (1 μL plant⁻¹) of radiolabelled solution on the adaxial surface of the second youngest leaf, in a completely randomized design.

Table 1

Main herbicides used to control *C. canadensis* in olive orchards in Spain.

Herbicide	MOA ^a	FYA ^b	AT ^c	Trade name	Field dose (g ai ha ⁻¹)
2,4-D	Auxinic	before 1990	postemergence	U-46D® (Nufarm)	600
Diquat	PSI	before 1990	postemergence	Reglone® (Syngent)	400
Florasulam ^d	ALS	2017	postemergence	Ruedo ^{sd} (Dow AgroSciences)	7.5
Glyphosate	EPSPS	before 1990	postemergence	Roundup Energy® (Monsanto)	1080 ^e
Iodosulfuron	ALS	after 2010	postemergence	Hussar® (Bayer)	10
Flazasulfuron	ALS	after 2000	erly postemergence	Terafit® (Syngenta)	50
Penoxsulam ^d	ALS	2017	postemergence	Ruedo ^{sd} (Dow AgroSciences)	15
Tribenuron	ALS	after 2000	postemergence	Granstar® (DuPont)	20

Where.

^a Indicates mechanism of action.

^b First year of application.

^c Application time.

^d Mix herbicides

^e Doses expressed as g acid equivalent (ae) ha⁻¹ (50.9% potassium salt of glyphosate equals 450 g ae L⁻¹).

Table 2

Herbicide treatments used for the dose-response experiments and parameters estimates from the logistic analysis of growth reduction (GR₅₀) and plant survival (LD₅₀) of resistant (RTG) and susceptible (STG) *Coryza canadensis* populations treated with those main herbicides used in olive orchards (^a g ae ha⁻¹).

Herbicide	Dose-response (g ia ha ⁻¹)	Population	d	b	R ²	GR ₅₀ (g ia ha ⁻¹)	FR (GR ₅₀ RTG/ GR ₅₀ STG)	d	b	R ²	LD ₅₀ (g ia ha ⁻¹)	FR (LD ₅₀ RTG/ LD ₅₀ STG)
2,4-D	0, 22.5, 45, 90, 180, 360, 720	RTG	101.36	0.35	0.99	126 ± 3.57	1.28	100.85	1.07	0.99	202 ± 1.53	1.09
		STG	99.36	1.28	0.99	98 ± 2.07	–	99.28	0.21	0.99	185 ± 1.18	–
Diquat	0, 3.1, 6.2, 12.5, 25, 50, 100	RTG	100.04	0.28	0.99	7.01 ± 0.56	1.16	94.40	0.49	0.99	15.11 ± 2.88	1.07
		STG	99.65	0.20	0.99	6.04 ± 1.50	–	98.16	0.65	0.99	14.06 ± 2.70	–
Florasulam	0, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.92	RTG	100.02	0.49	0.99	0.09 ± 0.02	1.5	100.01	3.1	0.99	1.42 ± 0.32	1.28
		STG	99.92	0.78	0.99	0.06 ± 0.01	–	98.5	2.25	0.98	1.11 ± 0.21	–
Glyphosate ^a	0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000	RTG	97.27	0.01	0.99	1507.82 ± 1.95	34.55	98.08	2.11	0.99	3609.91 ± 1.87	11.80
		STG	100.04	1.60	0.99	43.64 ± 1.28	–	96.23	0.02	0.99	305.70 ± 2.69	–
Iodosulfuron	0, 0.5, 1, 2, 4, 8, 16, 32, 64	RTG	97.78	1.25	0.99	12.63 ± 2.38	16.84	100.39	2.31	0.99	31.24 ± 3.62	12.25
		STG	99.99	2.59	0.99	0.75 ± 0.03	–	101.66	3.98	0.99	2.55 ± 0.13	–
Flazasulfuron	0, 3.1, 6.25, 12.5, 25, 50, 100, 200, 400	RTG	100.40	1.26	0.99	56.46 ± 2.63	12.11	100.18	–0.93	0.99	164.34 ± 2.58	28.68
		STG	99.31	1.46	0.99	4.66 ± 3.70	–	99.79	1.03	0.99	6.94 ± 1.10	–
Penoxsulam	0, 0.5, 1, 2, 4, 8, 16, 32, 64	RTG	99.99	2.38	0.99	2.9 ± 0.11	1.28	100.09	3.64	0.99	9.79 ± 1.26	1.47
		STG	99.98	2.41	0.99	2.26 ± 0.16	–	100.12	3.38	0.99	6.64 ± 0.92	–
Tribenuron	0, 1.25, 2.5, 5, 10, 20, 40, 80, 160	RTG	98.82	1.55	0.99	25.95 ± 2.39	19.66	98.54	0.60	0.99	100.40 ± 1.28	24.13
		STG	100.08	0.57	0.98	1.32 ± 0.6	–	100.01	0.51	0.99	4.16 ± 0.25	–

The plants were harvested at 12, 24, 48, 72, 96 and 120 h after the treatment (HAT). The unabsorbed ¹⁴C-herbicide was removed from the treated leaf with a water-acetone solution (1:1 v/v) by washing the plants three times separately with 1 mL of that solution. The rinse solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard) and was measured by liquid scintillation spectrophotometry (LSS) using a Beckman LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton CA). The whole washed plants were removed from the pot and sectioned into treated leaves, the rest of the plant and roots. The plant sections were individually stored in cellulose cones for combustion, dried at 60 °C for 96 h and burned using a biological oxidant (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA). The CO₂ released from combustion was captured in 18 mL of a mixture of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each sample was measured for 10 min by LSS. The radioactive values of absorption and translocation of ¹⁴C were expressed as a percentage of the total ¹⁴C-herbicide applied and recovered, respectively.

2.5. ¹⁴C-tribenuron-methyl metabolism

Young plants from the two populations (RTG and STG) were treated following the experimental procedure of ¹⁴C-tribenuron-methyl absorption and translocation (0.834 kBq), and at 96 HAT, the non-absorbed herbicide was removed with the same technique and washing solution.

The tribenuron-methyl metabolism was determined according to the methodology described by Cruz-Hipolito et al. (2013). The plants were sectioned in aerial parts (leaf tissue) and roots and stored at –40 °C until analysis. The samples were ground in a mortar with 3 mL of a methanol-water solution (4:1 v/v). The total mix was recovered twice with 3 mL of the aforementioned solution and centrifuged at 20,000 g (Avanti® J-25, Beckman Coulter™) for 10 min at 4 °C. The supernatants were analysed. Aliquots of 100 µL were taken to quantify the total radioactivity by LSS. The supernatants were dried at room temperature under a flow of liquid nitrogen at 0.25 atm. The dried extract was suspended in 200 µL of isopropanol. ¹⁴C-tribenuron-methyl and its metabolites were separated by thin layer chromatography (TLC) on a 400 cm² silica gel plate (silica gel 60, Merck, Darmstadt, Germany) with isopropanol, ethyl acetate, ammonia and distilled water (10:6:3:1 v:v:v:v).

Radio chromatograms of the radioactive areas were obtained with a scanner. The radioactivity of the products was quantified separately with a linear plate analyser (Berthold LB 2821, Wildbald, Germany).

Each product was determined by comparison with known standards (tribenuron-methyl, metsulfuron-methyl and metsulfuron-methylhydroxylate). Three plants from each population (RTG and STG) were used, and the quantification was repeated twice.

2.6. Glyphosate metabolism

Young plants (rosette stage BBCH 16–18) from the RTG and STG populations were treated with glyphosate at 360 g ea ha⁻¹. At 120 HAT, the foliar tissue of the leaf was washed with distilled water and frozen at –40 °C until analysis. The methodology used for the determination of glyphosate and metabolites (aminomethylphosphonic acid –AMPA–, glyoxylate, formaldehyde and sarcosine) was as described by Rojano-Delgado et al. (2010). The analysis was carried out by capillary electrophoresis using an Agilent G1600A 3D electrophoresis instrument equipped with a diode array detector (DAD) (wavelength 190–600 nm). The standards used for glyphosate and its metabolites were provided by Sigma-Aldrich (Spain). The average amount of glyoxylate that was produced naturally by the plants was subtracted from the average glyoxylate produced (glyphosate metabolism) by the treated plants. The two populations were organized in a completely randomized design of six representatives each in two replicates.

2.7. ALS enzyme activity assay

The ALS activity was determined following the methodology described by Palma-Bautista et al. (2018). Three-gram samples of young leaf tissue were taken from each population. The samples were mixed in a mortar with 5 mg of polyvinylpyrrolidone (PVPP). A 1 M K-phosphate buffer solution (pH 7.5) was used as the extraction buffer. This buffer also contained 10 mM sodium pyruvate, 5 mM MgCl₂, 50 mM thiamine pyrophosphate, 100 µM flavin adenine dinucleotide (FAD), 12 mM dithiothreitol and glycerol (1:9 v/v). The mixture was stirred for 10 min at 4 °C. The homogenate was filtered through four layers of gauze and centrifuged (20000 rpm for 20 min). The supernatant containing a crude extract of ALS enzyme was immediately used for enzyme analysis.

To analyse the ALS activity, 90 µL of enzyme extract was added to 110 µL of freshly prepared assay buffer (0.08 M K-phosphate buffer at pH 7.5, 0.5 M sodium pyruvate, 0.1 M MgCl₂, 0.5 mM thiamine pyrophosphate and 1 µM FAD). Increasing concentrations of herbicide inhibitors of ALS (0, 0.1, 1, 5, 10, 50, 100 and 1000 µM) (Table 1) were added.

The mixture was incubated for 60 min at 37 °C. The reaction was

stopped after the addition of 50 μL of H_2SO_4 and incubated at 60 °C for 15 min. Finally, 250 μL of a freshly prepared solution of creatine in water (5 g L^{-1}) and 250 μL of a solution of naphthol in NaOH 5 M (50 g L^{-1}) was added. It was again incubated at 60 °C for 15 min to facilitate decarboxylation of acetolactate to acetoin. Acetoin absorbance was measured with a spectrophotometer (Beckman DU-640, Fullerton, CA, USA) at 520 nm. The total content of protein in crude extract was measured at 595 nm with the Bradford (1976) colorimetric method using the Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain). The experiment was carried out three times with five repetitions per herbicide concentration and per population, following a completely randomized design.

2.8. EPSPS enzyme activity assay

Five-gram samples of young leaf tissue of STG and RTG were powered for extraction of the enzyme following the protocol described by Dayan et al. (2015). The tissue powder was transferred to tubes with 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl and 0.5 mM benzamidine) containing 70 μL of β -mercaptoethanol and 1% polyvinylpyrrolidone (PVPP). The solution was centrifuged for 40 min (18000 g) at 4 °C. The supernatant was decanted into a beaker using a cheesecloth. $(\text{NH}_4)_2\text{SO}_4$ (45% w/v) was added to the solution to precipitate the protein in the extracts. The mixture was centrifuged at 20000 g for 30 min at 4 °C. The previous step was repeated with 80% (w/v) of $(\text{NH}_4)_2\text{SO}_4$. Finally, it was centrifuged at 20000 g for 30 min at 4 °C.

The specific activity of EPSPS was studied in the presence and absence (basal activity) of glyphosate. The EPSPS activity was determined using an EnzChek Phosphate Analysis Kit (Invitrogen, Carlsbad, CA, USA). The substrates for the EPSPS enzyme reaction were phosphoenolpyruvate (1.02 mM) and shikimate-3-phosphate (0.41 mM), supplied by Sigma-Aldrich (Madrid, Spain). The glyphosate concentrations used to determine the inhibition of enzymatic activity (I_{50}) were 0, 0.1, 1, 10, 100 and 1000 μM . The assay buffer was composed of 1 mM MgCl_2 , 10% glycerol, 100 mM MOPS, 2 mM sodium molybdate and 200 mM NaF. EPSPS activity was measured for 10 min at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, USA) to determine the amount of inorganic phosphate (μmol) released, measured in $\mu\text{g}^{-1} \text{ TSP min}^{-1}$. The total content of protein in crude extract was measured at 595 nm with the Bradford colorimetric method using the Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain). Three replicates per population and glyphosate concentration were used, and the experiment was repeated three times.

2.9. ALS gene sequencing

DNA was isolated from six plants per population, which were used for herbicide screening with tribenuron at 20 g ai ha^{-1} . Leaf tissue was collected in liquid nitrogen from RTG and STG plants and stored at -80 °C until further analysis. DNA was isolated from the leaves using the SpeedTools Tissue DNA Extraction Kit (Biotools B & M Labs. S.A, Madrid, Spain). The quantity and quality of DNA were determined with a spectrophotometer (NanoDrop 1000, ThermoFisher Scientific, Waltham, MA).

Gene-specific primers from *C. canadensis* were used to amplify and sequence all of the nucleic acid sites associated with resistance to ALS (Matzrafi et al., 2015; Tranel et al., 2018). All PCR amplifications were performed following the conditions described by Matzrafi et al. (2015). Aliquots of PCR products were loaded on a 1% agarose gel to assess amplification of the correct band. Sequencing of the purified genomic DNA was performed in the Genomic Unit Investigation Central Service of Extremadura University, Spain.

2.10. EPSPS gene sequence

One hundred milligrams of young leaf tissue from RTG and STG populations was used to obtain total RNA with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Purification of RNA and DNA synthesis were performed following the method described by Amaro-Blanco et al. (2018). The primers used for the study of the EPSPS2 gene sequence were F1-EPSPS2 (5'-TGATGTTTCATTACATGCTTGGAG-3') and R1-EPSPS2 (5'-TGAGGTGAGAGAAATGGGTACA-3') (Amaro-Blanco et al., 2018). PCR products were sequenced (STAB VIDA, Caparica, Portugal), searching for the commonly reported mutations at positions 102 and 106 (Alcántara-de la Cruz et al., 2016).

2.11. Statistical analysis

Dose-response and enzyme activity data were subjected to nonlinear regression analysis using a three-parameter log-logistic equation to determine the glyphosate dose causing 50% reduction in growth (GR_{50}), 50% mortality (LD_{50}), or the herbicide 50% inhibition rate of enzyme activity (I_{50}).

$$y = \left(\frac{d}{1 + (x/g)^b} \right)$$

where y is the above-ground fresh weight, the survival, or the enzyme activity expressed as the percentage of 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 rate.

Regression analyses were conducted using the package drc 55 for the statistical environment R56. Resistance factors ($\text{RF} = \text{RTG}/\text{STG}$) were computed as RTG-to-STG GR_{50} , LD_{50} or I_{50} ratios. To test for a common GR_{50} , LD_{50} , or I_{50} for R- and S-populations, i.e., Resistance Index equal to 1, a lack-of-fit test was used to compare the model consisting of curves with population-specific g values with a reduced model with common g 55.

Analysis of variance (ANOVA) was conducted to test for differences between RTG and STG populations in the different assays. When needed, differences between means were separated using the Tukey HSD test at $P < 0.05$. Model assumptions of normal distribution of errors and homogeneous variance were inspected graphically. ANOVAs were conducted using Statistix (version. 9.0) (Analytical Software, USA) software.

3. Results and discussion

3.1. Herbicide dose-response in plants

The results obtained using glyphosate confirmed the resistance of the RTG population to this herbicide. A large reduction of fresh weight in the STG population was observed at low glyphosate doses in comparison to that of the RTG population. The GR_{50} value for the STG population was 43.64 g ae ha^{-1} , while that for the RTG population was 1507.82 g ae ha^{-1} , with an RF value (using the GR_{50} parameter) of 34.55-fold more resistant than the STG population (Table 2, Supplementary Fig. 1). Taking into account the LD_{50} values, the RTG population was 11.8-fold more resistant than the STG population (Table 2, Supplementary Fig. 2). The glyphosate field rate recommended (1080 g ae ha^{-1}) was enough to control the STG population, but not for the RTG population, which needed 3.34 times the field rate recommended to kill 50% of the individuals.

The results for tribenuron methyl also confirmed the resistance of the RTG population to this herbicide. The GR_{50} values were 1.32 and 25.95 g ai ha^{-1} for the STG and RTG populations, respectively, with an RF value in the RTG population of 19.66 (Table 2, Supplementary Fig. 3). Using the LD_{50} values, the RTG population was 24.13-fold more resistant than the STG population (Supplementary Fig. 4). The

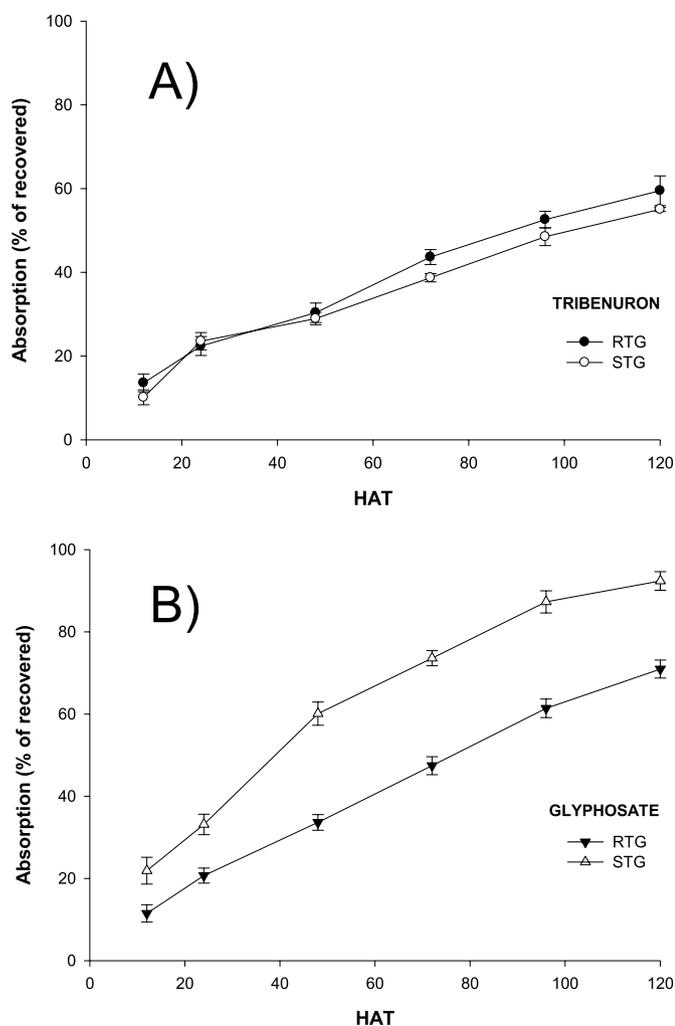


Fig. 1. ^{14}C -tribenuron-methyl (A) and ^{14}C -glyphosate (B) absorption in the *C. canadensis* populations. The vertical bars represent \pm the standard errors of the mean.

tribenuron methyl field rate recommended (20 g ai ha^{-1}) is enough to control the STG population, but not for the RTG population, which needed 5.02 times the field rate recommended to kill 50% of the individuals.

Glyphosate has been the herbicide most used by growers for many years due to its unique properties, such as its broad-spectrum high compatibility with herbicides with different sites of action and relatively low cost (Duke, 2018). Thus, the observed glyphosate resistance of *C. canadensis* populations could have been attributed to the above-described continuous and intensive application. *C. canadensis* has been shown to be resistant to glyphosate, and the control of glyphosate-resistant *C. canadensis* with tribenuron has also resulted in the evolution of resistance (Table 2). Similarly, the use of tribenuron-methyl as an alternative herbicide has led to subsequent tribenuron-methyl resistance. For this reason, the multiple resistance that is indicated in this study should be taken into consideration as a severe threat to crop production systems in Europe.

In addition, other ALS herbicides (such as flazasulfuron and iodosulfuron) have been used by farmers to control this weed species, but unfortunately, this use has led to the concomitant evolution of cross-resistance. However, florasulam (RF = 1.5) and penoxsulam (RF = 1.28) can efficiently control resistant and susceptible *C. canadensis* populations. In parallel, according to the GR_{50} and LD_{50} values of the RTG and STG populations of the treated plants, it seems that the 2,4-D and diquat herbicides can also be applied in rotation with

glyphosate and tribenuron to efficiently control *C. canadensis* (Table 2, Supplementary Figs. 5 and 6).

In 1980, the first population of *C. canadensis* resistant to paraquat was detected in Japan; subsequently, resistance to other herbicides, mainly glyphosate, continued to increase (Heap, 2018). To date, there have been only three cases of multiple resistance to ALS and EPSPS herbicides in North America, and recently, one in Europe (Heap, 2018; Palma-Bautista et al., 2018). The GR_{50} and LD_{50} values found in *C. canadensis* plants treated with glyphosate and flazasulfuron in Spain are similar to those published for the same species in Hungary (Palma-Bautista et al., 2018).

3.2. ^{14}C -tribenuron-methyl and ^{14}C -glyphosate absorption and translocation

The absorption of ^{14}C -tribenuron-methyl were 38.7% and 43.6% at 72 HAT, 48.5% and 52.6% at 96 HAT and finally 55.1% and 59.5% at 120 HAT for the STG and RTG populations, respectively (Fig. 1). However, no significant differences were observed in ^{14}C -tribenuron-methyl absorption and translocation between resistant (RTG) and susceptible (STG) *C. canadensis* populations at 120 HAT (Table 3).

These results suggest that reduced tribenuron-methyl translocation in resistant (RTG) *C. canadensis* plants does not seem to be a resistance mechanism. Similarly, Cruz-Hipolito et al., (2013) found that tribenuron-methyl absorption and translocation from treated leaves were similar between resistant and sensitive *Sinapis alba* populations. Finally, the results of Hatami et al., 's 2016 study indicated that tribenuron-methyl absorption and translocation were not the mechanisms responsible for resistance in turnip weeds (*Rapistrum rugosum*).

Significant differences were observed regarding the absorption and translocation of ^{14}C -glyphosate between resistant (RTG) and susceptible (STG) *C. canadensis* populations. In particular, the maximum glyphosate absorption rate was recorded at 120 HAT, and the value was 21.8% higher in the STG compared to the RTG population (Fig. 1). Moreover, at 120 HAT, the rate of movement of ^{14}C -glyphosate out of the treated leaf was 56.3% higher in the STG compared to the RTG population (Table 3). Similarly, the translocation rates of the herbicide to the rest of the plant and the root system were 28% and 28.3% higher in STG than in the RTG population, respectively (Table 3). Similar results have been reported by Ferreira et al. (2008) and Amaro-Blanco et al. (2018) for glyphosate resistant and susceptible *C. canadensis* populations, which showed consistent differences in their uptake and translocation patterns. In particular, greater glyphosate retention was observed in the resistant population leaves compared to the susceptible population, while susceptible population leaves, stems and roots showed a greater glyphosate concentration, implying that the susceptible population had a higher translocation efficiency. In summary, the results suggest that uptake and translocation likely contribute to glyphosate resistance in these resistant (RTG) *C. canadensis* populations.

3.3. ^{14}C -tribenuron-methyl metabolism

The percentages of ^{14}C -tribenuron-methyl that remained as tribenuron-methyl (TM) in plants of the RTG and STG populations were 35.68% and 90.52% at 96 HAT, respectively (Table 4). The levels of metsulfuron-methyl (MM) were 9.8% and 9.47%, while hydroxylated metsulfuron-methyl (OH-MM) and conjugated -MM levels reached 18.52% and 36.25% in the RTG population, respectively, and were nondetectable in the STG population (Table 4). The detection of two nontoxic metabolites (OH-MM and conjugated-MM) implies that TM metabolism is involved in the *C. canadensis* resistance to tribenuron-methyl. Enhanced herbicide metabolism in weeds has been explained by the presence of detoxification enzymes, such as the cytochrome P450 monooxygenases system, which metabolizes the herbicide to nontoxic metabolites (Cruz-Hipolito et al., 2013; Hatami et al., 2016; Owen et al., 2012; Werk-Reichert and Feyereisen, 2000; Yuan et al.,

Table 3

¹⁴C-tribenuron-methyl and ¹⁴C-glyphosate absorption and translocation in *C. canadensis* RTG and STG populations at 120 h after treatment (HAT). Means with different letter within a column are statistically different at 95% probability determined by the Tukey test \pm Error standard (n = 5).

Herbicides	Populations	Absorption (% from total applied)	Translocation (% from uptake)		
			Treated leaf	Rest of Plant	Root
¹⁴ C-tribenuron Methyl	STG	60.1 \pm 2.02 A	85.3 \pm 2.18 A	14.1 \pm 3.04 A	0.6 \pm 0.21A
	RTG	59.1 \pm 2.48 A	87.6 \pm 2.38 A	11.5 \pm 1.54 A	0.9 \pm 0.25 A
¹⁴ C-glyphosate	STG	93.5 \pm 2.0 A	36.3 \pm 2.62 B	34.4 \pm 3.36 A	29.3 \pm 3.43 A
	RTG	71.7 \pm 2.99 B	92.6 \pm 2.78 A	6.4 \pm 0.87 B	1 \pm 0.54 B

2007). There are few cases in which the metabolism of sulfonylureas (SU) has been described as the dominant resistance mechanism in dicot weeds (Hatami et al., 2016; Osuna and De Prado, 2003; Veldhuis et al., 2000). However, this mechanism has been found and intensely studied in grass weeds such as *Lolium rigidum* (Christopher et al., 1994), *Echinochloa phyllopogon* (Yun et al., 2005) and *Alopecurus myosuroides* (Gardin et al., 2015).

3.4. Glyphosate metabolism

With regard to glyphosate metabolism in the RTG and STG *C. canadensis* populations, 89.4% and 88.7% of the applied herbicide remained as glyphosate in plants of the RTG and STG populations, respectively (Table 4). The levels of AMPA were 6.3% and 7.1%, while glyoxylate levels reached 4.3% and 4.2% in the RTG and STG populations, respectively (Table 4). However, there were no significant differences in these values between the RTG and STG populations.

Some authors have detected the existence of an enzyme called glyphosate oxidoreductase (GOX) in some plants and bacteria present in the soil, with the capacity to degrade glyphosate to two metabolites with low or no toxicity, such as glyoxylate and AMPA, and later sarcosine and formaldehyde (de Carvalho et al., 2012; Duke et al., 2010; Gonzalez-Torralva et al., 2012; Rojano-Delgado et al., 2012). In this study, we did not find sufficient glyphosate metabolism that could explain the resistance to this herbicide, which agrees with the results found by Feng et al. (2004) and Amaro-Blanco et al. (2018).

3.5. ALS enzyme activity assay

In the absence of tribenuron-methyl, ALS-specific activity levels in the *C. canadensis* RTG and STG populations were 405 \pm 24 and 385 \pm 18 nmol h⁻¹ mg⁻¹ of acetoin, respectively.

The quantity of tribenuron needed to inhibit the ALS activity by 50% (I₅₀) in the STG population was 16.7 μ M, while it was 78.6 μ M in the RTG population. As we can see in Table 5 (also in Supplementary Fig. 7), the resistance factors (FRs) for the sulfonylurea herbicides were between 4.7 and 8.8, while for the other herbicides belonging to other structural groups, such as imidazolinones (imazamox), sulfonylamino-carbonylthiazolinone (flucarbazone), triazolopyrimidines (penoxsulam and florasulam) and pyrimidinylthiobenzoates (bispiribac sodium), the FRs were between 0.7 and 1.6. These values allow us to conclude that

Table 4

Herbicides and their respective metabolites, relative percentage in *C. canadensis* resistant (RTG) and susceptible (STG) populations at 96 HAT. Means in the same column followed by the same letter were not significantly different at $\alpha = 0.05$. ^aMean value (n = 7) \pm Error standard.

Population	TM	MM	OH-MM	Conjugated-MM
RTG	35.68 \pm 1.52 B	9.8 \pm 1.01 A	18.52 \pm 2.13 A	36.25 \pm 1.80 A
STG	90.52 \pm 1.51 A	9.47 \pm 1.30 A	nd	nd
Population	Glyphosate	AMPA	Glyoxylate	Sarcosine
RTG	89.4 \pm 1.69 A	6.3 \pm 1.44 A	4.3 \pm 1.75 A	nd
STG	88.7 \pm 2.12 A	7.1 \pm 1.99 A	4.2 \pm 1.12 A	nd

nd: non-detectable. TM: tribenuron-methyl. MM: metsulfuron-methyl. OH-MM: hydroxylatedmetsulfuron-methyl. AMPA: aminomethylphosphonic acid.

Table 5

Herbicides concentrations required to reduce the enzyme activity to 50% (I₅₀) in *C. canadensis* resistant (RTG) and susceptible (STG) populations. Where ^aRF is resistance factor (I₅₀ RTG/I₅₀ STG).

Enzyme	Herbicide	Population	d	b	R ²	I ₅₀ (μ M)	RF ^a
ALS	Imazamox	RTG	97.7	0.6	0.99	352.86	1.2
		STG	97.6	0.4	0.99	295.35	
	Tribenuron	RTG	99.7	0.8	0.99	78.6	4.7
		STG	98.3	0.7	0.99	16.7	
	Flazasulfuron	RTG	100.9	0.5	0.97	0.79	8.8
		STG	100.2	0.6	0.98	0.09	
	Iodosulfuron	RTG	95	0.8	0.99	1.18	6.2
		STG	101	0.7	0.97	0.19	
	Penoxsulam	RTG	100.6	0.4	0.97	0.14	0.9
		STG	101.6	0.6	0.98	0.15	
	Florasulam	RTG	100.1	0.6	0.99	0.29	0.7
		STG	99.8	0.4	0.99	0.41	
Flucarbazone	RTG	100.1	0.8	0.99	10.86	1.6	
	STG	101.3	0.7	0.99	6.92		
Bispiribac	RTG	99.8	0.6	0.98	231.02	1.5	
	STG	101.1	0.3	0.99	149.98		
EPSPS	Glyphosate	RTG	100.08	0.05	0.99	8.18 \pm 0.95	1.0
		STG	100.13	0.31	0.99	8.37 \pm 1.25	

the RTG population only presents cross-resistance to sulfonylureas (Cruz-Hipolito et al., 2013; Hatami et al., 2016).

3.6. EPSPS enzyme activity assay

In the case of glyphosate, basal EPSPS-specific activity levels in the RTG and STG *C. canadensis* populations were 0.12 \pm 0.03 and 0.10 \pm 0.02 μ mol μ g⁻¹ protein min⁻¹, respectively. The amount of glyphosate needed to inhibit the EPSPS activity by 50% (I₅₀) in the STG population was 8.37 μ M, whereas for the RTG population, it was 8.18 μ M (Table 5, Supplementary Fig. 8).

The similarity between basal EPSPS-specific activity values and I₅₀ values between the two populations leads us to consider the lack of amino acid substitutions at 102 or 106 in the *C. canadensis* studies, as other authors have shown in dicot weeds (Amaro-Blanco et al., 2018; Bracamonte et al., 2017; Palma-Bautista et al., 2018).

3.7. ALS gene sequencing

In this study, sequencing of the target-site ALS gene from RTG plants revealed a single point mutation, a proline to alanine mutation (Pro-197-Ala), which is known to be the genetic basis of resistance to sulfonylurea in weeds (Table 5). The target-site mutations in the ALS gene could result in different cross-resistance patterns to ALS inhibitors (Tranel et al., 2018). The level of cross-resistance to ALS inhibitors depends on the specific amino acid substituted at the Pro-197 site of the ALS enzyme (Park et al., 2012). The low-level resistance to the other ALS-inhibiting herbicides tested in this study is probably due to cross-resistance caused by the Pro-197-Ala mutation.

3.8. EPSPS gene sequencing

The EPSPS gene sequence does not show mutations in the studied positions of Pro-106-Ser, Ala-103-Val or Thr-102-Ile in any of the RTG or STG populations of *C. canadensis*. In the *Coryza* genus, only one mutation, Pro-106-Ser, has been referenced in a glyphosate-resistant population of *C. sumatrensis* (Gonzalez-Torralva et al., 2014; Amaro-Blanco et al., 2018).

4. Conclusions

These results documented the first case of multiple-resistant *C. canadensis* in Europe and in the world, demonstrating TSR and NTSR mechanisms that were involved in the resistance to sulfonylureas, while only the NTSR mechanism was involved in the case of glyphosate. For two decades, European restrictions on the use of herbicides and the number of annual applications for weed control have led farmers to apply the same herbicides (that showed more effectiveness) alone or as a mixture with the same site of action. Normally, the chosen herbicides are glyphosate and ALS inhibitors, alone or in combination. These applications with the same herbicide or the same site of action preferentially carried out at the end of winter and then at the beginning of spring in the south of Spain increase the selection pressure on *Coryza* spp. and result in the species' evolution to new biotypes with multiple resistance to EPSPS and ALS inhibitor herbicides, preferentially.

This study confirmed that the emergence of resistance to glyphosate and tribenuron at higher doses than those that were normally used by farmers (1080 g ae ha⁻¹ and 20 g ai ha⁻¹, respectively) means that these chemicals are lost as rotation alternatives, although the cross-resistance found has only been for sulfonylureas. Other chemical groups, such as triazolopyrimidines (penoxsulam and florasulam) or imidazolinone (imazamox), could be used. It is also true that the continued application of these herbicides facilitates the appearance of new cytochrome P450 genes and/or new ALS mutations with a higher level of resistance to this population of *C. canadensis*. Currently, the best solution would be the use of glyphosate in a mixture with 2,4-D or diquat, which would provide wide control of grass-weeds and dicot weeds. In addition, cover crops and conservation tillage are integrated weed management programmes that farmers are adopting effectively.

Declarations of interest

None.

Contributions

David A. Mora, Nikolina Cheimona, Candelario Palma-Bautista, Antonia M. Rojano-Delgado, María Dolores Osuna-Ruiz, Ricardo Alcántara de la Cruz and Rafael De Prado were responsible for design of and conducting the experiments, planning, analysis of data and preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.09.023>.

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