



Determination of glyphosate-resistant *Conyza* spp. in orchards and vineyards in Turkey

Mehmet Nedim Doğan · Emine Kaya-Altop ·
Süleyman Gürdal Türkseven ·
Ahmet Tansel Serim

Received: 13 May 2021 / Accepted: 3 November 2021 / Published online: 24 March 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract *Conyza* spp. are among the most problematic broadleaf weeds in citrus orchards and grape fields in Turkey. Glyphosate is used to control weeds, but *Conyza* spp. can escape the treatment and grow as a monoculture in these fields. To investigate whether glyphosate-resistant biotypes of *Conyza* spp. exist in Turkey, seeds of *Conyza* spp. were collected from 131 citrus fields and 121 vineyards with heavy *Conyza* infestations in the Mediterranean and Aegean regions of the country. Seeds were classified by species, and initial susceptibility screenings were conducted by applying glyphosate potassium salt at 1.323 kg a.i. ha⁻¹ to seedlings at the five- to six-leaf stage. Forty-five biotypes showed less than 80% susceptibility in the screenings and were subsequently used in dose–response experiments. Assays were also conducted to

measure shikimic acid accumulation in resistant and susceptible biotypes after glyphosate treatment, and molecular studies were undertaken to investigate the mechanism of resistance. Among 252 populations collected from fields, 32 biotypes showed resistance to glyphosate. Molecular studies showed that target site mechanisms including mutations or expression of the *EPSPS* gene did not contribute to the mechanism of glyphosate resistance in *Conyza* biotypes from Turkey.

Keywords Glyphosate · Horseweed · Hairy fleabane · Bioassay · Shikimic acid · Resistance

Introduction

Turkey is not top ranked in terms of citrus production; however, based on the quantity of citrus crops such as orange, tangerine, grapefruit, lemon, and lime that it exports, the country is one of the major global exporters (CWMT, 2019). In contrast to citrus production, grape production in Turkey is not located in a specific region, and the Mediterranean, Middle Anatolia, and South East regions have large viticulture fields. The Aegean region also has a considerable number of vineyards (TUIK, 2021). Viticulture fields of Turkey cover nearly 448,000 ha, representing 7% of the world's vineyards (OIV, 2018). Despite being ranked third among major grape-producing countries

M. N. Doğan
Department of Plant Protection, Faculty of Agriculture,
Adnan Menderes University, Aydın, Turkey

E. Kaya-Altop
Department of Plant Protection, Faculty of Agriculture,
Ondokuz Mayıs University, Samsun, Turkey

S. G. Türkseven
Department of Plant Protection, Faculty of Agriculture,
Ege University, İzmir, Turkey

A. T. Serim (✉)
Department of Plant Protection, Faculty of Agriculture
and Natural Sciences, Bilecik Şeyh Edebali University,
Bilecik, Turkey
e-mail: a_serim@hotmail.com

worldwide, Turkey is the largest dried grape producer in the world (Statista, 2019).

Conyza spp. belonging to Asteraceae are among the most problematic broadleaf weeds in citrus orchards and grape fields in Turkey. Representative species include horseweed (*C. canadensis*), hairy fleabane (*C. bonariensis*), and tall fleabane (*C. sumatrensis*). These herbaceous weeds are annual and produce a huge number of light seeds that are wind dispersed (CABI, 2018; Loux et al., 2006). *Conyza* spp. may compete with grapevines and citrus trees for water, nutrients, and sunlight and delay their growth. Weeds can reduce the total dry mass of 1-year-old grape vines by up to 80% (Bordelon & Weller, 1997), and yield losses due to full-season weed competition in 6-year-old grapevines var. Baco Noir were reported to reach 37% (Byrne & Howell, 1978). *Conyza* spp. do not directly affect the yield of grape yield var. Thompson seedless; however, in citrus orchards the weed may cause negative effects on agronomic practices, provide a favourable environment for pests and diseases, and create difficulties during quarantine inspections (Shrestha et al., 2010). Sanguaneko et al. (2009) reported that vines can tolerate competition with weeds to a certain degree, but to reach desired yields, weed control practices such as herbicide application or soil cultivation should be employed.

As with other weed species, control strategies such as tillage, cover crops, and use of selective and non-selective herbicides can easily control or suppress *Conyza* spp. at early growth stages if herbicide resistance is not present in the application area. Some plant genera belonging to Asteraceae, Poaceae, and Amaranthaceae are more prone to developing resistance to herbicides than genera of other families (Heap, 2021; Travlos & Chachalis, 2010). *Conyza* spp. began showing herbicide resistance nearly two decades ago in relation to paraquat, and the number of documented cases of resistance has gradually increased through the years. To date, 65 cases of resistance have been found in *Conyza* spp. collected from areas including pastures and orchard fields, with nearly 20 herbicides being involved (Heap, 2021). Each herbicide treatment may change the balance between resistant and susceptible biotypes in favour of resistant biotypes in terms of the relative crowding coefficient (Travlos & Chachalis, 2013).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is responsible for the synthesis of tryptophan, tyrosine, and phenylalanine, eventually leading to the death in the higher plant (HH, 2014). It is a total herbicide applied post emergence to control grass and broadleaf weed species in orchards and non-agricultural areas such as gardens, parks, pavements, railroads, roadsides, and airports (Fogliatto et al., 2020; Işık et al., 2015; Kudsk & Mathiassen, 2020). Repeated use of the herbicide without rotation in orchards may considerably increase the frequency of glyphosate-resistant (GR) biotypes owing to selection pressure. The first GR horseweed biotype was identified in soybean fields in the United States in 2000, and similar cases were subsequently reported in cotton, soybean, maize, corn, and orchards worldwide (Heap, 2021; VanGesnel, 2001). Increases in the growing areas of genetically modified (GM) crops, especially GR soybean, have accelerated the evolution of glyphosate resistance among *Conyza* spp. (Cerqueira et al., 2011). Since some non-selective herbicides such as paraquat have been banned for use in orchards, glyphosate use has intensified per growing season, without rotation with other herbicides with different modes of action, in Turkey and Greece (PPPT, 2019; Travlos & Chachalis, 2010).

Citrus orchards cover a significant part of the Mediterranean region and a small portion of the Aegean region in Turkey (TUIK, 2021). *Conyza* spp. are frequently found in citrus orchards in the Mediterranean and Aegean regions, and their abundance has gradually increased from year to year. The producers of these crops have reported that glyphosate is insufficient for horseweed control, which has led them to use it repeatedly at higher rates than recommended in a growing season. Since the lack of efficacy of glyphosate or any other herbicide can be related to inappropriate use such as wrong timing or application technique, as well as climatic conditions, long-term repeated use could lead to the selection of resistant biotypes.

Therefore, the objective of the present study was to characterize resistance through whole-plant dose–response, shikimic acid (SA) accumulation assays, and EPSPS gene expression.

Materials and methods

Seed collection

Seeds of *Conyza* spp. were collected from 131 citrus fields and 121 vineyards in the Mediterranean and Aegean regions of Turkey during September and October 2015. About 90% of the total citrus and half of the total vineyard growing areas in Turkey were represented by the collection sites. The location data and some important agricultural features of the sampling points were also recorded. Seed collection sites were selected according to horseweed presence and no known glyphosate application. The distance between sampling locations was at least 2–3 km. Horseweed flower heads were carefully cut with secateurs and placed in paper bags. Seeds were dried in the paper bags under shade conditions for 1–2 weeks, followed by manual cleaning. The seeds were then stored at ambient room temperature until use in a screening test and dose–response assay.

Screening test

Discrimination experiments were conducted in the screen houses at Ege University Agricultural Faculty, Department of Plant Protection (EUAF) and Aydın Adnan Menderes University Agricultural Faculty, Department of Plant Protection (ADUAF) during September and October 2015 to distinguish susceptible and resistant populations. Each of the collected 252 populations was used in both facilities, with a total of 203 populations being successfully tested in both. The seeds were planted separately on the surface of a soil mixture consisting of peat, soil, and perlite (1:1:1) in 0.76-L pots, and the pots were irrigated with tap water. The day-time and night-time screen house temperatures were 22 and 18 °C, respectively. All experiments were conducted with five replicates, and seedlings in each replicate were thinned to one plant in each pot at 7–10 days after emergence. Horseweed seedlings were grown until the rosette stage and irrigated regularly as needed.

The seedlings were treated with glyphosate potassium salt at 1.323 kg a.i. ha⁻¹ when they were at the five- to six-leaf stage. Herbicide was applied in a spray chamber calibrated to deliver 200 L ha⁻¹ at 200 kPa from two flat fan spray nozzle (Lechler 11,002) at AMUAF. At EUAF, herbicide was applied

using a knapsack sprayer to deliver 200 L ha⁻¹ at 200 kPa using a portable boom-mounted four flat fan nozzle (Lechler 11,002). After herbicide application, the seedlings were returned to the screen house. Each population was represented with five treated and five non-treated pots.

At 28 d after treatment (DAT), all seedlings were visually evaluated based on a 0–100 scale on which 0 indicated no damage and 100 indicated a dead seedling. The horseweed biotypes with $\geq 80\%$ proportional survival rate were considered as having suspected resistance to glyphosate, and these biotypes were subsequently used in [dose–response assays](#). The biotypes with $\geq 80\%$ proportional mortality rate were considered to be susceptible to glyphosate (Moss et al., 1999).

Dose–response assays

Dose–response assays were conducted in the screen houses at EUAF and AMUAF during June–October 2016 to characterize the glyphosate resistance levels of the GR and glyphosate-susceptible (GS) *Conyza* accessions. GS biotypes were also added to the experiments to determine resistance factors. The seedlings were grown until the rosette stage (five to six leaves), and herbicide was applied at seven logarithmic rates of glyphosate from 0.25 to 16 times the recommended rate for resistant biotypes and from 0.0625 to 4 times the recommended rate for susceptible biotypes. Non-treated seedlings of both biotypes were used as controls. Afterwards, the seedlings were returned to the screen house. The positions of herbicide-treated seedlings were changed weekly to avoid environmental effects on the uniform growth of the seedlings, which were irrigated with tap water as needed. The seedlings were cut at ground level at 21 DAT, placed in paper bags inside a drying cabinet, and then dried at 65 °C for 3 d. Next, the dry biomass of seedlings of each population was determined and expressed as a percentage based on the dry biomass of non-treated seedlings. GR horseweed biotypes whose resistance index (RI) values were higher than 2 were consecutively grown in greenhouse conditions until the seed production stage, and their seeds (F₂) were then used in further assays to confirm inheritance of herbicide resistance.

The experimental design was a randomized complete block design with five replicates for each

experiment, and it was repeated in both facilities. The data obtained from dose–response experiments were analysed by non-linear regression using the Weibull model described by Ritz and Streibig (2005) Eq. (1). The regression was carried out with R statistical software (R Core Team, 2020).

$$Y = D \times e^{-e^{b \times (\log(x) - \log(ED_{50}))}} \quad (1)$$

where Y refers to the dry biomass of each seedling, D is the upper limit of the dose–response curve, b is the slope of each curve determined by ED_{50} , ED_{50} is the glyphosate dose required to cause 50% response, and x is the glyphosate dose.

The resistance index was used to evaluate the degree of resistance of the GR accessions compared with the GS accessions based on the following formula (Eq. 2):

$$RI = \frac{ED_{50}(GR)}{ED_{50}(GS)} \quad (2)$$

where RI refers to the resistance index (R/S ratio for each population), $ED_{50}(GR)$ is the glyphosate dose required to cause a 50% response for the GR accession, and $ED_{50}(GS)$ is the glyphosate dose required to cause a 50% response for GS accession.

Shikimic acid assays

SA levels of seedlings were determined following Henry et al. (2005) with small modifications to evaluate the relation between glyphosate resistance and SA in horseweed biotypes. Glyphosate was applied at seven logarithmic rates from 0.25 to 16 times the recommended rate for resistant biotypes and from 0.0625 to 4 times the recommended rate for susceptible biotypes. Non-treated seedlings of both biotypes were used as controls. Twelve discs of ~4-mm diameter were taken from the youngest leaves of each seedling 2 DAT with a cork borer and transferred to the laboratory in cooler bags. Each sample was put into a plastic vial to which 1 mL of 0.25 N HCl was added and placed in a freezer (–20 °C). For the assay, samples were thawed at 60 °C in an incubator for 30 min, and a 50-μL aliquot was taken from each. Next, 50 μL of 0.25% periodic acid + 0.25% metaperiodic acid was added to the sample in the vial, and the mixture was incubated for 90 min at 25 °C. A 100-μL mixture consisting of 0.6 N sodium hydroxide and

0.22 M sodium sulphide was then added to each vial. The optical density of the samples was measured in a spectrophotometer at 380 nm, and the background light was quantified based on the untreated control. A calibration curve for shikimate was created using 0.01–1 μg of shikimate in 1 mL of HCl.

Molecular analysis

Amplification and sequencing of the EPSPS gene fragment

Seeds from the putative GR and GS mature plants (F2) were germinated and grown to the four- to six-leaf stage under greenhouse conditions. At 0 and 8 h after treatment, all plants, approximately 100-mg samples were taken from the leaves of both glyphosate-treated and untreated biotypes (CANR1/S1 and BONR2/S2) for RNA and DNA extraction. The study was carried out in three replicates. Total genomic DNA and RNA were isolated from the leaf samples of each S and R *Conyza* biotypes following the application directions of the DNeasy/RNeasy Plant Mini Kits (Qiagen, Qiagen GmbH, Hilden, Germany).

EPSPS cDNA sequencing

Complementary DNA (cDNA) was synthesized from 1 μg total RNA extracted from GR and GS plants using SuperScript II-RT (Invitrogen) according to the manufacturer's instructions. The primers were synthesized by Alpha DNA (Montreal, Canada), and the sequence was determined using the software Primer 3. The following primer sets were used: 5' and 3' RACE (Patzoldt et al., 2006) and EPSPS1F (5'-ATGGCAGCTACTCAC ATTAACAC-3'), EPSPS1R (5'-GCAAACCACTAC CCATAATCAC-3') EPSPS1 (GenBank Accession No. AY545668) (Peng et al., 2010). EPSPS1 x EPSPSR primers included an EPSPS gene fragment possibly containing the P106 mutation from R and S biotypes. PCR was done with Platinum Taq DNA Polymerase High Fidelity kit (Thermo Fisher Scientific) according to the instructions provided. The PCR mix consisted of 1XPCR Buffer, 50 ng/μL of cDNA, 0.4 μM of each forward and reverse primer, 0.2 mM dNTP mix, 1 U Taq DNA Pol., and 1.5 mM MgCl₂ in a 50-μL total volume. The PCR conditions were initial denaturation for 3 min at 97 °C; 35 cycles of 15 s at 97 °C, 45 s at 60 °C, and 2 min at 72 °C; and a final extension of 5 min at 72 °C. PCR products

were purified using Pure Link PCR Purification Kit (Invitrogen) based on the manufacturer's protocol. Purified PCR products were analysed by Genoxs (Turkey) to obtain the DNA and peptide sequences of the GR and GS *Conyza* biotypes. The sequences were then analysed using BioEdit, aligned with the CLUSTAL W program, and finally compared with the respective *EPSPS* sequence of *Conyza* (GenBank accession numbers AY545667, AY545666, AY545668, EF200069, EF200070, EF200071, EF200072, EF200073, and EF200074) and *Arabidopsis thaliana* (NM_130093.2).

Real-time PCR

The acetolactate synthase (*ALS*) and cDNA expression levels of four biotypes were used to calculate the *EPSPS* genomic copy number. Primer efficiency curves were generated for each primer set using a 1×, 1/5×, 1/25×, and 1/125× dilution series of GR genomic DNA. The primer sets *EPSPSF* (5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3'), *EPSPSR* (5'-TGATTTCCTCCAGCAACGGCAA-3') (195-bp product) (Gaines et al., 2010), *ALSF2* (5'-GCTGCTGAAGGC TACGCT-3'), and *ALSR2* (5'-GCGGGACTGAGT CAAGAAGTG-3') (118-bp product) (Gaines et al., 2010; Tranel et al., 2004) were used for quantitative PCR. The *ALS* gene was used as a low-copy control gene with known monogenic inheritance. Real-time PCR was employed on a Rotor-Gene Q 5plex HRM System using GoTaq@qPCR Master Mix (Promega). Negative controls consisting of the template with no primers and primers devoid of template were added. The data from the quantitative PCR experiments were analysed according to the relative quantification (RQ) with a modification of the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen (2001). An increase in the *EPSPS* copy number was considered as $\Delta\Delta C_t$ because RQ of *EPSPS* was $\Delta C_t = (C_t, ALS - C_t, EPSPS)$. The average increase in the *EPSPS* copy number and its standard deviation were calculated for each individual sample run as three biological replicates (Gaines et al., 2010).

Results and discussion

Result of the screening test

Conyza biotypes suspected to be GR constituted less than a quarter of all the accessions (Table 1).

Table 1 Distribution of the suspected glyphosate-resistant *Conyza* spp. populations in citrus orchards and vineyards

Crop	Province	<i>Conyza canadensis</i>	<i>Conyza bonariensis</i>	<i>Conyza sumatrensis</i>	Population Number
Orchard	Adana	5	7	-	12
	Hatay	2	5	1	8
	Mersin	7	5	-	12
Vineyard	Mersin	8	5	-	13
Total		22	22	1	45

According to the screening results, 22 horseweed biotypes, 23 hairy fleabane biotypes, and 1 tall fleabane biotype were suspected to have glyphosate resistance. The origins of these accessions were limited to only three provinces of the Mediterranean region, although samples were collected from 11 provinces in the Aegean and Mediterranean regions. The screening test showed that all the accessions collected from the Aegean region were susceptible to glyphosate. Among the *Conyza* spp. collected from the two regions, *C. sumatrensis* was the only species that had not yet lost sensitivity to glyphosate. Mersin province had the highest number of accessions in terms of possible glyphosate resistance, while suspected GR tall fleabane was only found in Hatay province.

Orchard and vineyard fields of the Mediterranean region harboured GR *Conyza* accessions at a high rate. In orchard fields, one-third of *Conyza* accessions had lost their sensitivity to glyphosate. The rate of resistant horseweed accessions was high in Adana and Mersin, but resistant hairy fleabane had a higher rate than other *Conyza* accessions. In vineyard fields of Mersin, most of the *Conyza* accessions were found to be resistant to glyphosate. All hairy fleabane accessions were resistant, while only one-third of horseweed accessions were susceptible.

Horseweed was identified as the first GR *Conyza* sp. in 2000, and the first GR tall fleabane biotype was found in South Africa 3 years later (Heap, 2021). The first GR hairy fleabane was discovered more than 6 years after GR tall fleabane. To date, 43 horseweed, 13 hairy fleabane, and 13 tall fleabane biotypes have been reported to be resistant to glyphosate from many countries (Heap, 2021). In Turkey, Mengüç (2019) found that 10 of 121 *Conyza* accessions were not effectively controlled by glyphosate applied at double

the recommended rate. He also reported that glyphosate resistance was more frequent in horseweed and tall fleabane biotypes than in hairy fleabane biotypes. In another study, İnci et al. (2019) found three GR tall fleabane accessions in peach orchards of Çanakkale Province, Turkey.

Results of the dose–response studies

The ED₅₀ values of hairy fleabane populations varied from 690 to 3912 g a.i. ha⁻¹ and from 547 to 3330 g a.i. ha⁻¹ based on the logarithmic rates of the herbicide applied to the accessions collected from orchard and vineyard fields (F₁) and the F₂ populations, respectively (Table 2). In F₂ populations, seven accessions stored in ambient conditions lost their germination ability and three accessions were more susceptible to glyphosate than the previous generation;

therefore, their dose–response parameters were not calculated. The ED₅₀ value of GS accessions belonging to the F₁ generation was 710 g a.i. ha⁻¹, while the ED₅₀ of GS accessions of F₂ was 423 g a.i. ha⁻¹. As an informative indicator of herbicide resistance, the RI of F₁ varied from 1.0 to 5.5, whereas it was 1.3 to 7.9 in F₂. These values accord with the results of Urbano et al. (2007), who found that resistant biotypes were controlled by 7 to 10 times the amount of glyphosate needed to control susceptible biotypes; Travlos and Chanchalis (2010), who indicated that RI values varied from 4 to 7 depending on the accessions; and Mylonas et al. (2019), who reported values of 5 to 11.6. In another study, Mengüç (2019) calculated RI values of 1.6 and 9, which were similar to our results. Based on the origin of the accessions, the lowest RI values were obtained from Adana, whereas the highest were associated with the accessions

Table 2 ED₅₀ and RI values of hairy fleabane (*C. bonariensis*) accessions collected from orchard and vineyard fields of Adana, Mersin, Hatay

Population		F1 Generation		F2 Generation	
Code	Coordinate	ED ₅₀	RI ¹	ED ₅₀	RI ¹
MT16 S	36 40 0.5664 N, 34 24 2.7576 E	710	1,0	96	1.0
AT13	36 52 42.1248 N, 35 20 47.058 E	785	1,1	-	-
AT2	36 55 5.2536 N, 35 19 4.5588 E	882	1,2	*	*
AT30	37 24 58.6404 N, 35 45 16.7328 E	847	1,2	*	*
AT40	37 1 35.166 N, 35 10 38.0604 E	1.764	2,5	201	2.1
AT5*	36 49 10.0164 N, 35 12 32.6376 E	688	1,0	-	-
AT7*	36 44 54.5172 N, 35 6 20.3868 E	869	1,2	-	-
HT12	36 54 12.6252 N, 36 7 41.0016 E	2.192	3,1	418	4.4
HT16	36 49 4.6416 N, 36 12 1.7604 E	1.323	1,9	402	4.2
HT17	36 49 25.932 N, 36 12 59.868 E	3.912	5,5	325	3.4
HT8	36 57 38.7144 N, 36 10 9.8292 E	3.537	5,0	124	1.3
HT9	36 58 15.2472 N, 36 8 45.906 E	1.813	2,6	174	1.8
MB12	36 57 45.1368 N, 34 44 59.4924 E	2.121	3,0	755	7.9
MB18	36 58 10.3368 N, 34 44 54.5568 E	2.624	3,7	734	7.6
MB19	36 58 59.574 N, 34 43 59.8332 E	1.222	1,7	-	-
MB20	36 58 45.516 N, 34 44 32.7948 E	1.649	2,3	272	2.8
MB21	37 0 27.9324 N, 34 46 26.1012 E	1.896	2,7	-	-
MB5	36 59 0.222 N, 34 47 1.3488 E	1.182	1,7	442	4.6
MT17	36 40 0.5664 N, 34 24 2.7576 E	2.337	3,3	190	2.0
MT2	36 34 8.2632 N, 34 15 22.6008 E	1.345	1,9	366	3.8
MT21	36 51 38.3544 N, 34 38 7.9764 E	1.773	2,5	-	-
MT22	36 51 25.1892 N, 34 38 30.9048 E	1.949	2,7	*	*
MT24	36 52 50.5092 N, 34 48 57.5964 E	803	1,1	-	-

¹ RI was calculated by dividing the ED₅₀ values of GR accessions by the ED₅₀ values of MT16 S

*ED₅₀ was not calculated because of high sensitivity of the accession

-ED₅₀ was not calculated because the seeds did not germinate

collected from Hatay. There were no meaningful differences between the accessions obtained from the orchard fields and the vineyard fields in Mersin in terms of RI values.

The accessions obtained from Adana had only a slight degree of glyphosate resistance, whereas the accessions from Hatay had higher resistance against glyphosate. The RI values calculated from the F₂ populations were higher than those for the F₁ populations for three accessions, while the ED₅₀ for F₂ was lower in five accessions than for F₁.

The ED₅₀ values of horseweed accessions of F₁ varied from 525 to 3669 g a.i. ha⁻¹ glyphosate, whereas the values obtained from F₂ ranged between 565 and 3308 g a.i. ha⁻¹ glyphosate (Table 3). For susceptible populations, ED₅₀ values of F₁ and F₂ were 406 and 340 g a.i. ha⁻¹, respectively. Compared with GR and GS hairy fleabane, GR and GS horseweed accessions had higher ED₅₀ values for both generations. RI values for F₁ and F₂ generations ranged from 1.3 to 9.0 g a.i. ha⁻¹ and from 1.7 to 9.7 g a.i. ha⁻¹ glyphosate, respectively. The RI values for horseweed were higher than for hairy fleabane, but there was no meaningful increase or decrease between the F₁ and F₂ generations. Kumar et al. (2017) found RI values of 3.5–4.0 for GR horseweed biotypes from Montana, USA. Koger et al. (2004) reported that GR horseweed biotypes from Mississippi, USA, were controlled by 8- to 12-fold higher levels of glyphosate compared with those needed for susceptible biotypes. In a study from Delaware, USA, VanGessel (2001) indicated that the RI for horseweed varied from 8 to 13. Mengüç (2019) found that the glyphosate rate to control GR horseweed accessions was 4.9 to 12.8 times higher than the rate needed to control GS horseweed accessions.

The accessions obtained from orchard fields in Mersin were more resistant to glyphosate than those from vineyards, except for one accession. The biotypes collected from Hatay could be controlled by a higher glyphosate rate than those from Adana for both generations.

Shikimic acid results

SA content in GS and GR accessions rapidly increased after glyphosate treatment regardless of the accession. SA content of GS hairy fleabane accessions rose from 106 µg g⁻¹ in untreated accessions

Table 3 ED₅₀ and RI values of horseweed (*C. canadensis*) accessions collected from orchard and vineyard fields of Isparta, Adana, Mersin, Hatay

Population		F1 Generation		F2 Generation	
Code	Coordinate	ED ₅₀	RI ¹	ED ₅₀	RI ¹
IB7	37 79 69.1941 N, 30 5 41.1986 E	406	1.0	77.1	1.0
AT11	36 42 37.3572 N, 35 6 28.3212 E	2.540	6.3	129	1.7
AT12	36 47 16.0152 N, 35 18 36.094 E	1.852	4.6	372	4.8
AT16	36 35 19.8870 N, 35 18 28.0404 E	1.279	3.2	297	3.9
AT4	36 50 34.368 N, 35 14 28.276 E	1.985	4.9	706	9.2
AT42	37 4 9.8436 N, 36 6'37.8684 E	3.330	8.2	439	5.7
AT8	36 44 54.5172 N, 35 6 20.3868 E	2.245	5.5	128	1.7
HT13	36 52 40.7172 N, 36 8 54.6288	2.179	5.4	649	8.4
HT18	36 47 56.6196 N, 36 13 28.7688	3.665	9.0	566	7.3
MB11	36 57 51.4908 N, 34 45 16 6176	1.658	4.1	-	-
MB15	36 56 42.8676 N, 34 43 19.8588	1.424	3.5	-	-
MB16	36 55 51.8664 N, 34 42 41.742	3.669	9.0	750	9.7
MB6	36 58 41.2032 N, 34 47 13.8444	1.028	2.5	634	8.2
MB7	36 58 17.0616 N, 34 47 8.6856	525	1.3	-	-
MB9	36 57 28.1844 N, 34 46 32 1852	2.156	5.3	375	4.9
MT1	36 34 30.6156 N, 34 15 37.08 E	1.495	3.7	-	-
MT14	36 37 53.8356 N, 34 20 41.8776 E	1.910	4.7	203	2.6
MT20	36 51 1.4336 N, 34 38 22.4592 E	1.385	3.4	66	0.9
MT23	36 51 27.4392 N, 34 45 26.6004 E	1.032	2.5	248	3.2
MT25	36 54 46.6776 N, 34 54 42.2064 E	2.417	6.0	402	5.2
MT26	37 0 30.726 N, 35 5 20.7852 E	992	2.4	-	-
MT27	37 1 38.8884 N, 34 45 54.306 E	1.707	4.2	*	*

¹ RI was calculated by dividing the ED₅₀ values of GR accessions with the ED₅₀ values of MT16 S

*ED50 was not calculated because of high sensitivity of the accession

-ED50 was not calculated because the seeds did not germinate

to 11,446 $\mu\text{g g}^{-1}$ following glyphosate applied at a rate of 464 g a.i. ha^{-1} (data not shown). In contrast, the SA content in GR accessions was changed at the wide range from 44–109 to 2428–4911 $\mu\text{g g}^{-1}$. SA relative content of GR accessions was 10–41.6 after glyphosate was applied at 29 g a.i. ha^{-1} , whereas the SA content varied from 39.7 to 84.2 when glyphosate was applied at 1.856 kg a.i. ha^{-1} (Fig. 1). The change in SA relative content was generally higher in GS hairy fleabane biotypes than in GR biotypes.

Alterations in the SA content in GS and GR horseweed accessions were very similar to those in GS and GR hairy fleabane accessions. SA accumulation in the GS accession was significantly higher than in the GR accessions. The increase in SA was generally greater as the dose increased. The SA content of untreated

horseweed accessions varied from 42 to 96 $\mu\text{g g}^{-1}$. The SA content in the GS accession was 9169 $\mu\text{g g}^{-1}$ when glyphosate was applied at 464 a.i. ha^{-1} , whereas the content was 1579–3554 $\mu\text{g g}^{-1}$ in GR accessions (data not showed). Following glyphosate application at logarithmic rates, the SA relative content in the GS horseweed accession was 42–101 $\mu\text{g g}^{-1}$ (Fig. 2). The increase in SA content generally continued in the GR horseweed accessions after glyphosate application, but this increase was limited compared with the GS accession. The least and the highest SA relative content of GR accessions varied from 4.6 to 21.5 $\mu\text{g g}^{-1}$ and from 36 to 105.3 $\mu\text{g g}^{-1}$, respectively.

Relative SA content in most GR hairy fleabane and horseweed accessions accorded with the ED_{50} values for MT2, MB5, MB6, and HT16. This conformity

Fig. 1 Shikimic acid relative content (relative to the untreated control) accumulation 2–3 DAT in hairy fleabane leaves from plants treated with various glyphosate concentrations

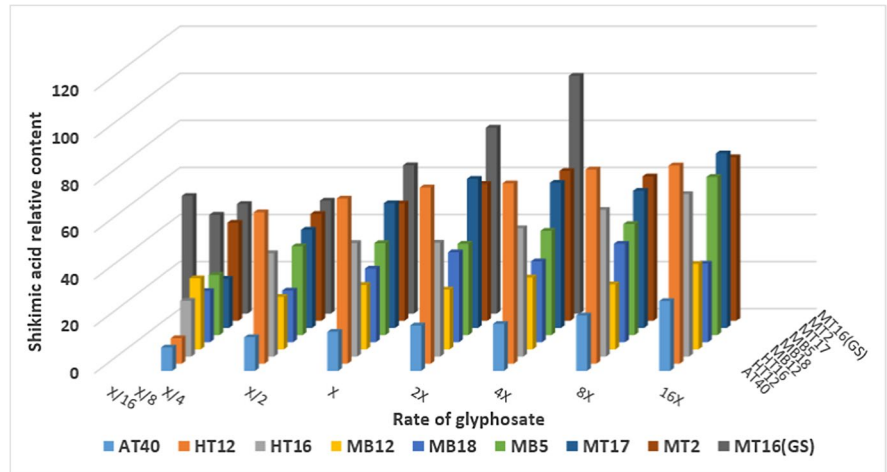
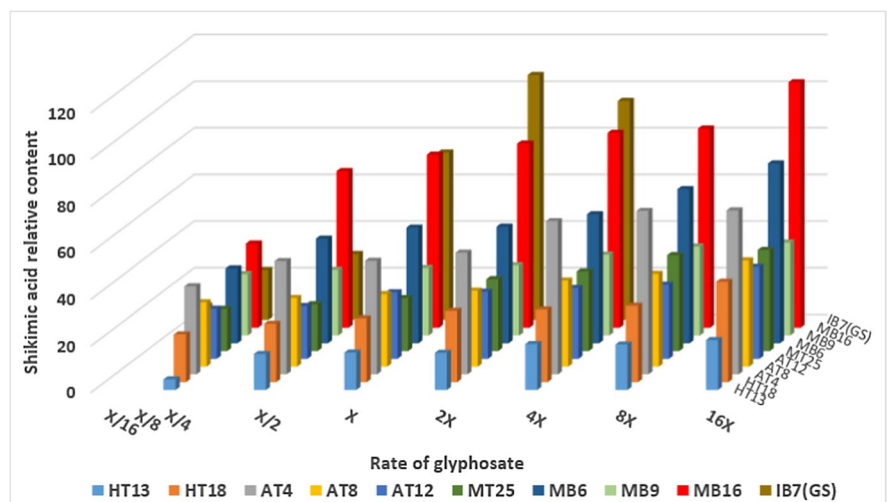


Fig. 2 Shikimic acid relative content (relative to the untreated control) accumulation 2–3 DAT in horseweed leaves from plants treated with various glyphosate concentrations



may have resulted from SA accumulation following glyphosate application and was similar to previously described results of Beres et al. (2018). Changing SA content may be caused by a high level of basal EPSPS mRNA or by a relative transcript level of *EPSPS* gene, similar to findings by Dinelli et al. (2008). Relative SA content of some hairy fleabane and horseweed accessions, including MT17, HT12, MB18, MB12, HT18, AT4, AT8, AT12, MT25, MB9, and MB16, were below the expected values because their ED_{50} values were higher. Although EPSP enzyme inhibition is described as the main mode of action of glyphosate, recent studies have revealed that glyphosate has additional effects on plant physiology. Glyphosate may trigger other unknown mechanisms in these accessions, creating a toxic effect on the plant (Singh et al., 2020). Relative SA content of some hairy fleabane accessions, including AT40 and HT13, was higher than expected, although their ED_{50} values were low compared with the previous group of GR accessions. This unexpected difference may be caused by other GR mechanisms such as reduced glyphosate translocation, vacuole sequestration, metabolism (Wakelin et al., 2004; Ge et al., 2010; Gonzalez-Torralva et al., 2012), altered metabolism (Pline-Srnic, 2006), or inhibition of root-to-shoot translocation of Fe and Mn (Eker et al., 2006) instead of the well-known resistance mechanism, EPSP inhibition (Beres et al., 2018).

Molecular studies

Glyphosate resistance in *Conyza* spp. is generally conferred by two mechanisms: target site and non-target site. Although target-site resistance is conferred by a

mutation in the key herbicide target gene (*EPSPS*) in many weeds (Perez-Jones et al., 2007; Simarmata & Penner, 2008; Wakelin & Preston, 2006), in *Conyza* spp. it results from overexpression of the *EPSPS* gene (Dinelli et al., 2006, 2008; Tani et al., 2015). In this study, any mutation in Pro106, which is known to confer glyphosate resistance in many weed species, was not found according to sequence analysis in the GR cDNA. A polymorphism (changed from CCG to CCC) did occur in the GR *C. bonariensis* biotype, but it was not related to resistance (Fig. 3).

Real-time PCR was used to evaluate the genomic copy number of *EPSPS* and the expression level of cDNA based on the *ALS* gene (Gaines et al., 2010; Tranel et al., 2004). Gene copy numbers of the GR *C. canadensis* and *C. bonariensis* accessions were 5.02 ± 2.2 and 4.6 ± 1.8 , while the numbers for the GS accessions were 1.2 ± 0.3 and 0.9 ± 0.2 , respectively. The gene copy number in the GR *C. canadensis* and *C. bonariensis* accessions was increased 4.18- and 5.11-fold compared with GS accessions, respectively. Mennan et al. (2021) indicated that the increase in the genomic *EPSPS* copy number in resistant *Amaranthus palmeri* biotypes from Turkey was 34- to 37-fold; however, the number in some GR *A. palmeri* accessions was 1.6 to 6.4 for one of the resistant biotypes and 6 to 8.5 for another resistant biotype (Mohseni-Moghadam et al., 2013).

The *EPSPS* expression relative to *ALS* 8 h after treatment of GR accessions varied from 1.8 ± 0.2 to 1.01 ± 0.1 , whereas it varied from 0.8 ± 0.1 to 0.5 ± 0.2 in GS accessions when glyphosate was not used. However, expression of *EPSPS* in the GR *C. canadensis* accessions increased by 2.8 times after glyphosate application compared with GS

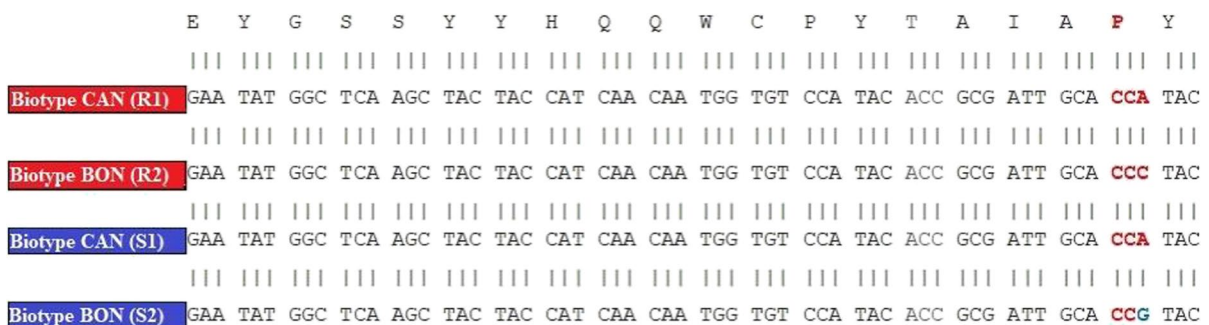


Fig. 3 Amino acid sequences of GR and GS *Conyza* accessions. Biotype CAN(R1), resistant *C. canadensis*; Biotype BON(R2), resistant *C. bonariensis*; Biotype CAN(S1), susceptible *C. canadensis*; Biotype BON(S2), susceptible *C. bonariensis*

accessions. The increase for the GR *C. bonariensis* accessions was 4.6-fold compared with GS accessions. Gaines et al. (2010) reported that the EPSPS expression relative to ALS changed on average 0.8–35.0 between GS and GR *A. palmeri* accessions when glyphosate was not used. In another study, Dinelli et al. (2008) reported that the relative transcript level of the EPSPS gene in hairy fleabane varied from 1.1 to 2.5.

Conclusion

Our results showed that there are three different *Conyza* spp. in citrus orchards and vineyards in the Mediterranean and Aegean regions of Turkey, and some samples had a degree of resistance to glyphosate. Among the 252 populations collected from orchards and vineyards with heavy *Conyza* infestations, 45 biotypes were suspected to be resistant to glyphosate, but resistance was confirmed in only 32 biotypes. This confirmation was done by both dose–response experiments and SA assays. Molecular studies showed that target-site mechanisms including mutations or expression of the EPSPS gene did not contribute to the mechanism of glyphosate resistance in *Conyza* biotypes from Turkey. The results derived from the study indicate that glyphosate resistance in *Conyza* spp. studied was associated with non-target resistance. Since most seed samples were collected from orchards and vineyards where *Conyza* species were dominant or even sole weed species, resistance should be confirmed with more biotypes than were assessed here. It is possible that there are problems other than resistance also affecting *Conyza* control like the effectiveness of glyphosate applied at various growth stages. To explore this, further studies should investigate the sensitivities to glyphosate of *Conyza* spp. at different growth stages.

Funding This research was financially supported in part by the Scientific and Technological Research Council of Turkey, Project number: 115O179.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

- Beres, Z. T., Ernst, E. E., Ackley, B. A., Loux, M. M., Owen, M. D. K., & Snow, A. A. (2018). High Levels of Glyphosate Resistance in *Conyza canadensis* from Agricultural and Non-Agricultural Sites in Ohio and Iowa. *Science and Reports*, 8, 10483. <https://doi.org/10.1038/s41598-018-28163-w>
- Bordelon, B. P., & Weller, S. C. (1997). Preplant cover crops affect weed and vine growth in first-year vineyards. *HortScience*, 32, 1040–1043.
- Byrne, M. E., & Howell, G. S. (1978). Initial response of Baco noir grapevine to pruning severity, sucker removal, and weed control. *American Journal of Enology and Viticulture*, 29, 192–198.
- CABI (2018). <https://www.cabi.org/isc/datasheet/15250>
- Cerdeira, A. L., Gazziero, D. L., Duke, S. O., & Matallo, M. B. (2011). Agricultural impacts of glyphosate-resistant soybean cultivation in South America. *Journal of Agricultural and Food Chemistry*, 59(11), 5799–5807. <https://doi.org/10.1021/jf102652y>
- CWMT (2019). Citrus: World Markets and Trade. <https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf>
- Dinelli, G., Marotti, I., Bonetti, A., Minelli, M., Catizone, P., & Barnes, J. (2006). Physiological and molecular insight on the mechanisms of resistance to glyphosate in *Conyza canadensis* (L.) Cronq. biotypes. *Pesticide Biochemistry and Physiology*, 86, 30–41.
- Dinelli, G., Marotti, I., Bonetti, A., Catizone, P., Urbano, J. M., & Barnes, J. (2008). Physiological and molecular bases of glyphosate resistance in *Conyza bonariensis* biotypes from Spain. *Weed Research*, 48, 257–265.
- Eker, S., Ozturk, L., Yazici, A., Erenoglu, B., Romheld, V., & Cakmak, I. (2006). Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. *Journal of Agricultural and Food Chemistry*, 54(26), 10019–10025. <https://doi.org/10.1021/jf0625196>
- Fogliatto, S., Ferrero, A., & Vidotto, F. (2020). Current and future scenarios of glyphosate use in Europe: Are there alternatives? *Advances in Agronomy*, 163, 219–278.
- Gaines, T. A., Zhang, W., Wang, D., Bukuna, B., Chisholm, S. T., Shaner, D. L., Nissen, S. J., Patzoldt, W. L., Tranel, P. J., Culpepper, A. S., Grey, T. L., Webster, T. M., Vencill, W. K., Sammons, R. D., Jiang, J., Preston, C., Leach, J. E., & Westra, P. (2010). Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proc. Natl. Acad. Science*, 107, 1029–1034.
- Ge, X., d'Avignon, D.A., Ackerman, J.J., & Sammons, R.D. (2010). Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. *Pest Management Science*, 66, 345–348.
- Gonzalez-Torraiva, F., Rojano-Delgado, A. M., Luque de Castro, M. D., Mulleder, N., & de Prado, R. (2012). Two non-target mechanisms are involved in glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes. *Journal of Plant Physiology*, 169, 1673–1679.
- Heap, I. (2021). The International Herbicide-Resistant Weed Database. Online. Thursday, February 25, 2021. Available www.weedscience.org

- Henry, W. B., Koger, C. H., & Shaner, D. L. (2005). Accumulation of Shikimate in Corn and Soybean Exposed to Various Rates of Glyphosate. *Crop Management*, 4, 1–7. <https://doi.org/10.1094/CM-2005-1123-01-RS>
- HH (2014). Herbicide handbook. Weed Science Society of America Tenth Ed. ISBN: 978–0–615–98937–2.
- Işık, D., Dok, M., Kaya Altop, E., & Mennan, H. (2015). Applicability of early tillage and glyphosate together with pre and post emergence weed control in corn production. *Journal of Agricultural Science*, 21, 596–605.
- İnci, D., Galvin, L., Al-Khatib, K., & Uludağ, A. (2019). Sumatran fleabane (*Conyza sumatrensis*) resistance to glyphosate in peach orchards in Turkey. *HortScience*, 54, 873–879. <https://doi.org/10.21273/HORTSCI13749-18>.
- Koger, C. H., Poston, D. H., Hayes, R. M., & Montgomery, R. F. (2004). Glyphosate-resistant horseweed (*Conyza canadensis*) in Mississippi. *Weed Technology*, 18, 820–825.
- Kudsk, P., & Mathiassen, S. (2020). Pesticide regulation in the European Union and the glyphosate controversy. *Weed Science*, 68(3), 214–222. <https://doi.org/10.1017/wsc.2019.59>
- Kumar, V., Jha, P., & Jhala, A. (2017). Confirmation of glyphosate-resistant horseweed (*Conyza canadensis*) in Montana cereal production and response to post herbicides. *Weed Technology*, 31(6), 799–810. <https://doi.org/10.1017/wet.2017.49>
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, 25(4), 402–408. <https://doi.org/10.1006/meth.2001.1262>
- Loux, M., Stachler, J., Johnson, B., Nice, G., Davis, V., & Nordby, D. (2006). Biology and management of horseweed. Purdue Extension Publication GWC-9. West Lafayette, IN: Purdue Extension. Pp. 1–11.
- Mengüç, Ç. (2019). Researches on determination of glyphosate resistance of *Conyza* species in orchards and vineyards of South Marmara Region. Unpublished PhD thesis, Ankara University Graduate School of Natural and Applied Sciences. Ankara, Turkey.
- Mennan, H., Kaya Altop, E., Belvaux, X., & Brants, I., Zandstra, B., Jabran, K., & Uysal, M. (2021). Investigating glyphosate resistance in *Amaranthus palmeri* biotypes from Turkey. *Phytoparasitica*, 1-10. <https://doi.org/10.1007/s12600-021-00910-2>
- Mohseni-Moghadam, M., Schroeder, J., & Ashigh, J. (2013). Mechanism of resistance and inheritance in glyphosate resistant palmer amaranth (*Amaranthus palmeri*) populations from New Mexico, USA. *Weed Science*, 61(4), 517–525. <https://doi.org/10.1614/WS-D-13-00028.1>
- Moss, S. R., Clarke, J. H., Blair, A. M., Culley, T. N., Read, M. A., Ryan, P. J., & Turner, M. (1999). The occurrence of herbicide-resistant grass-weeds in the United Kingdom and a new system for designating resistance in screening assays. *Proceedings Brighton Crop Protection Conference - Weeds*, 1999, 179–184.
- Mylonas, P. N., Giannopolitis, C. N., Efthimiadis, P. G., Menexes, G. C., & Eleftherohorinos, I. G. (2019). Dose-response and growth rate variation among glyphosate resistant and susceptible *Conyza albida* and *Conyza bonariensis* populations. *Journal of Plant Protection Research*, 59(1), 32–40.
- OIV (2018). OIV Statistical Report on World Vitiviniculture. International Organisation of Vine and Wine. <http://www.oiv.int/public/medias/6782/oiv-2019-statistical-report-on-world-vitiviniculture.pdf>
- Patzoldt, W. L., Hager, A. G., McCormick, J. S., & Tranel, P. J. (2006). A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proceedings of the National Academy of Sciences of the United States of America*, 103(33), 12329–12334. <https://doi.org/10.1073/pnas.0603137103>
- Peng, Y., Abercrombie, L. L., Yuan, J. S., Riggins, C. W., Sammons, R. D., Tranel, P. J., & Stewart, C. N., Jr. (2010). Characterization of the horseweed (*Conyza canadensis*) transcriptome using GS-FLX 454 pyrosequencing and its application for expression analysis of candidate non-target herbicide resistance genes. *Pest Management Science*, 66, 1053–1062. <https://doi.org/10.1002/ps.2004>
- Perez-Jones, A., Park, K. W., Polge, N., Colquhoun, J., & Mallory-Smith, C. A. (2007). Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. *Planta*, 226, 395–404.
- Pline-Srnica, W. (2006). Physiological mechanisms of glyphosate resistance. *Weed Technology*, 20(2), 290–300.
- PPPT (2019). Plant Protection Products in Turkey. <https://bku.tarim.gov.tr/> Accessed 14 December 2019.
- R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Ritz, C., & Streibig, J.C. (2005) 'Bioassay Analysis using R. J. Statist. Software, 12, 5. <https://doi.org/10.18637/jss.v012.i05>
- Sanguankeo, P. P., Leon, R. G., & Malone, J. (2009). Impact of weed management practices on grapevine growth and yield components. *Weed Science*, 57, 103–107.
- Shrestha, A., Fidelibus, M. W., Alcorta, M. F., & Cathline, K. A. (2010). Threshold of horseweed (*Conyza canadensis*) in an established 'Thompson Seedless' vineyard in the San Joaquin Valley of California. *International Journal of Fruit Science*, 10, 301–308.
- Simarmata, M., & Penner, D. (2008). The basis for glyphosate resistance in rigid ryegrass (*Lolium rigidum*) from California. *Weed Science*, 56, 181–188.
- Singh, S., Kumar, V., Datta, S., Wani, A. B., Dhanjal, D. S., Romero, R., & Singh, J. (2020). Glyphosate uptake, translocation, resistance emergence in crops, analytical monitoring, toxicity and degradation: A review. *Environmental Chemistry Letters*, 18, 663–702. <https://doi.org/10.1007/s10311-020-00969-z>
- Statista (2019). Global leading grape producing countries in 2018/2019 <https://www.statista.com/statistics/739149/global-top-grape-producing-countries/>
- Tani, E., Chachalis, D., & Travlos, S. T. (2015). A glyphosate resistance mechanism in *Conyza canadensis* involves synchronization of EPSPS and ABC-transporter genes. *Plant Molecular Biology Reporter*, 33, 1721–1730. <https://doi.org/10.1007/s11105-015-0868-8>
- Tranel, P. J., Jiang, W. L., Patzoldt, W. L., & Wright, T. R. (2004). Intraspecific variability of the acetolactate synthase gene. *Weed Science*, 52, 236–241.

- Travlos, I. S., & Chachalis, D. (2010). Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) is reported in Greece. *Weed Technology*, *24*, 569–573.
- Travlos, I. S., & Chachalis, D. (2013). Relative competitiveness of glyphosate-resistant and glyphosate-susceptible populations of hairy fleabane (*Conyza bonariensis* L.). *Journal of Pest Science*, *86*, 345–351.
- TUIK (2021). Turkish Statistical Institute. <https://biruni.tuik.gov.tr/medas/?locale=tr>
- Urbano, J. M., Boggeo, A., Torres, V., Leon, J. M., Jimenez, C., Dinelli, G., & Barnes, J. (2007). Glyphosate-resistant hairy fleabane (*Conyza bonariensis*) in Spain. *Weed Technology*, *21*, 396–401.
- VanGessel, M. J. (2001). Glyphosate-resistant horseweed from Delaware. *Weed Science*, *49*, 703–705.
- Wakelin, A. M., & Preston, C. (2006). Inheritance of glyphosate resistance in several populations of rigid ryegrass (*Lolium rigidum*) from Australia. *Weed Science*, *54*, 212–219.
- Wakelin, A. M., Lorraine-Colwill, D. F., Preston, C. (2004). Glyphosate resistance in four different populations of *Lolium rigidum* is associated with reduced translocation of glyphosate to meristematic zones. *Weed Research*, *44*, 453–459. <https://doi.org/10.1111/j.1365-3180.2004.00421.x>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.