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Shikimate leaf disc assay for early detection of glyphosate resistance in *Conyza canadensis* and relative transcript levels of EPSPS and ABC transporter genes

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Summary

Twenty-two biotypes of *Conyza canadensis* (Canadian fleabane, horseweed) from a conventional orchard in Crete displayed varying degrees of reduced glyphosate susceptibility in standard whole plant assays. A refined shikimate leaf disc assay was developed to precisely determine the resistance levels, permitting early detection of resistance evolution and integrated management of the weed. The 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) homologue genes (1 and 2) were sequenced for three different biotypes (one of reduced susceptibility from Crete, one resistant from mainland Greece and one resistant from the USA), and no amino acid substitution of Pro106 was found. Real-time qRT-PCR was used to study the expression profiles for EPSPS and the M10 and M11 ABC transporter genes, following glyphosate application. The expression levels of the EPSPS genes were not significantly altered following glyphosate application in any biotype, but both M10 and M11 were found to be highly upregulated in glyphosate-treated reduced susceptibility or resistant biotypes and not in a susceptible biotype. These results are in accordance with data recently reported by other researchers, supporting a role of the M10 and M11 ABC transporter genes in glyphosate resistance in *Conyza canadensis*, because of reduced translocation.

Keywords: ABC transporters, Canadian fleabane, EPSPS expression, herbicide resistance, horseweed, shikimate accumulation, weed management.

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Introduction

Conyza canadensis (L.) Cronq. (Canadian fleabane, horseweed) is the most abundant species of the genus *Conyza* (family Asteraceae), occurring worldwide in the temperate zone and constituting a persistent problem in many crops, particularly under conditions of reduced tillage (Weaver, 2001). On the island of Crete (Greece), *C. canadensis* is a major weed in vineyards, citrus and olive orchards, where it can grow throughout the year, favoured by the mild winter conditions and summer irrigation practices. Following the withdrawal of all residual pre-emergence herbicides by the European Union, the importance of *C. canadensis* in perennial crops has increased dramatically, as repeated applications of post-emergence herbicides are required for its

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control. Glyphosate is the best available post-emergence herbicide and is used exclusively on the island for weed control in conventional orchards.

Furthermore, *C. canadensis* is the first broad-leaved weed species reported to have evolved resistance to glyphosate (VanGessel, 2001). After the first documented case in Delaware (USA), glyphosate-resistant populations have been observed in twenty-one other US States. *Conyza canadensis* is now regarded as the most widespread resistant species worldwide, with resistant populations identified in Brazil, China, Spain and the Czech Republic (Heap, 2012). On mainland Greece, there have been reports of reduced efficacy of glyphosate against *Conyza* species in orchards (Giannopolitis *et al.*, 2008), and a case of glyphosate resistance in the related species of *C. bonariensis* was recently described (Travlos & Chachalis, 2010).

Regarding the mechanism of glyphosate resistance in *C. canadensis,* studies have shown that reduced translocation plays a major role in highly resistant populations from the USA, but it has not been ascertained whether a single mechanism is responsible for the resistance observed in populations of this weed worldwide (Feng *et al.*, 2004; Koger & Reddy, 2005; Dinelli *et al.*, 2006; Shaner, 2009). The reduced translocation can be explained by the rapid sequestration of glyphosate in the vacuole, observed by Ge *et al.* (2010) with resistant *C. canadensis* plants. A single amino acid substitution at position 106 of the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS), the target site of glyphosate, has been identified as the main cause of moderate levels of resistance in a number of other weed species (Baerson *et al.*, 2002; Perez-Jones *et al.*, 2005; Wakelin & Preston, 2006; Jasieniuk *et al.*, 2008).

Monitoring for the early detection of resistance evolution in areas like Crete is important, because of the spread of *C. canadensis* resistance to glyphosate worldwide. This monitoring can lead to timely readjustments in control strategies that may avoid herbicide failure, offering significant financial and other savings, and should form a part of an integrated approach to the management of *C. canadensis*.

Shikimic acid has been shown to accumulate in plant tissues following glyphosate treatment, with the amount accumulated usually being higher in susceptible than in resistant plants (Henry *et al.*, 2007; Mueller *et al.*, 2008). A leaf disc assay would appear to be the most promising rapid method to detect glyphosate resistance in *Conyza* and other plant species (Koger *et al.*, 2005; Shaner *et al.*, 2005). Here, we report a shikimate assay suitable for the routine monitoring of glyphosate resistance levels and evaluate the upregulation of two ABC transporter genes in resistant *C. canadensis* biotypes from outside Crete and those with reduced susceptibility from Crete. The work aimed to evaluate the non-target mechanism of glyphosate resistance in *Conyza canadensis* and the potential role of ABC transporters in vacuolar sequestration.

Materials and methods

Origin and collection of seeds

Single plant accessions (here referred to as biotypes) were obtained by random sampling the population of the weed in selected orchards. Twenty-two *C. canadensis* plants (rosette or bolting stages) were collected in October 2009 from a conventional citrus orchard (Lake Agia, Chania, Crete) in which glyphosate had been applied 4–5 times annually for a period of at least 10 years. The collected plants (designated as P1-P22) were transplanted and grown to maturity in a glasshouse (supplemental light to a 12-h photoperiod, temperature 20–24°C, r.h. 75–85%), and ripe seeds were separately collected from each plant. Plants P14, P15 and P16 failed to reach maturity and were not further included. Seeds were also directly collected from mature plants from the same orchard (designated as biotypes P23, P24 and P25). Finally, seeds from the same area were collected from representative mature plants of the same species from two organic orchards (one citrus and one olive) designated as biotypes O and O1 respectively. Donated seeds of the same species from confirmed glyphosate-resistant plants originating from the Peloponnese, Greece (R1 and R2) or Delaware, USA (U), were used as the resistant standards.

Plant growing conditions and whole plant experiments

Seeds were germinated in plastic pots ($9 \times 9 \times 9$ cm) containing a Pot-ground P soil mixture (Klasmann – Deilmann GmbH, Geeste, Germany). The seedlings (cotyledon stage) were individually transplanted into new pots ($5 \times 5 \times 6$ cm) containing the same soil mixture. Plants to be used at the bolting stage were transplanted to larger (12-cm-diameter) pots when they had developed 12–15 leaves. All plants were grown under standard conditions (24°C, r.h. 75% and a 12-h photoperiod). Response to glyphosate was examined at the 5- to 6-leaf stage (seedling stage), 12- to 13-leaf stage, 5–8 cm diameter (rosette stage) and at 30–35 cm height (bolting stage).

To evaluate the response of the various *C. canadensis* biotypes to glyphosate, three separate experiments were conducted. In the first experiment, all Cretan biotypes (from conventional and organic orchards) and the non-Cretan-resistant biotypes were sprayed with the 1× and 3× recommended rate of glyphosate (0.548 and 1.644 kg

glyphosate a.e. ha^{-1}) at the seedling stage, the stage of highest reported susceptibility to glyphosate. In the second experiment, plants of most biotypes from the conventional orchard and one from the organic orchard (O) were sprayed with the 1× and 3× rate of glyphosate at the rosette stage, to further elucidate the variability in response within the conventional orchard biotypes. Finally, in the third experiment, selected plants from the conventional (P23, P24 and P25) and the organic (O) orchards were compared with a resistant standard (R2) after spraying with 0.548 and 1.644 kg a.e. ha^{-1} at the seedling, rosette and bolting stages, as it is known that *C. canadensis* response varies with the growth stage (VanGessel *et al.*, 2009). For each experiment, a randomised complete block design was used with 10 or 15 single plant replications for each treatment (untreated control, 0.55 and 1.64 kg glyphosate a.e. ha^{-1}). A herbicide formulation (Roundup 36 SL, 360 g a.e. L^{-1}, Monsanto) of the isopropylamine salt of glyphosate was used. Spraying was performed with a 5-L hand-operated sprayer (Blue Dot), equipped with a pressure gauge and a flat fan-type nozzle and calibrated to deliver 507 L ha^{-1} at a pressure of 200 kPa. The treated plants, maintained under the standard conditions, were observed weekly for toxicity symptoms, and the number of surviving plants was recorded 28 days after treatment (DAT). The number of leaves on 0 and 28 DAT was recorded when appropriate.

Shikimate leaf disc assay

Unsprayed plants of biotypes selected as having varying levels of glyphosate susceptibility or resistance in the previous whole plant experiments were subjected to the procedure described by Koger *et al.* (2005) with several modifications. For each biotype, three plants grown to the rosette stage were sampled by excising four leaf discs (5 mm diameter) from between main veins of the youngest fully expanded leaves of each plant. The leaf discs from each biotype, in groups of three (derived from the three different plants), were placed in disposable Petri dishes containing a glyphosate solution at one of the four different concentrations. Preliminary experiments in this study had shown that the most appropriate concentrations of glyphosate for the leaf disc treatment were 0, 0.9, 1.8 and 3.6 mg a.e. L^{-1}. The glyphosate solutions were in 10 mM NaH_2PO_4 (pH 4.4) with 0.1% Tween 20 surfactant. The Petri dishes were sealed with Parafilm to reduce evaporation and incubated in a growth chamber (25°C, r.h. 75%) for 16 h under continuous light (200 μmol m^{-2} s^{-1}). All discs in each dish were then transferred into a 1.5-mL Eppendorf tube and ground in liquid nitrogen. Shikimate was extracted by adding 1 mL of 1.25 M HCl to each tube. The samples were mixed vigorously for 5 min and incubated under agitation for 30 min at 37°C. A second extraction was occasionally performed to verify complete recovery.

The concentration of shikimate was measured according to the procedure of Cromartie and Polge (2002). After centrifugation at 12 000 ***g*** for 10 min, three 250-μL replicate aliquots of the supernatant were transferred into vials containing 1 mL of 0.25% periodic acid with 0.25% metaperiodate solution. Samples were incubated for 60 min at 37°C, before 1-mL aliquot of 0.6 M sodium hydroxide with 0.22 M sodium sulphite solution was added to each. The optical density at 383 nm was measured using a Specord 205 spectrophotometer (Analitik Jena) within 30 min against a shikimate standard curve (r^2 = 0.999) made by adding known amounts of shikimic acid to HCl solution (1.25 N). The background shikimate level of discs treated with buffer without glyphosate was subtracted from the resulting levels in the respective glyphosate-treated samples. The experiment was repeated three times, and the values presented represent the average shikimate accumulation.

EPSPS gene sequencing and determination of transcript levels using real-time qRT-PCR

Specific oligonucleotides were used to amplify two EPSPS genes (EPSPS1 Acc.: AY545666; EPSPS2 Acc.: AY545667), and RT-PCR products of the expected size (approximately 1500 bp) were cloned into the pGEM-T easy vector. The EPSPS 1 and 2 genes from biotypes R1 (R), U (R) and P24 (RS) were RT-PCR-amplified using specific oligonucleotide primers EPSP1_For: 5′-ATGG CAGCTACTCACATTAACAC-3′, EPSP1_Rev: 5′-TT AATGCTTGGCAAATCTTTG-3′, EPSPS2_For: 5′-ATGGCAGTTCACATCAACAACT-3′ and EPSPS2_Rev: 5′-TTAATGCTTAGTGTATCTTTCA-3′ (TIB – Molbiol Berlin). Total RNA was extracted using TRIzol reagent, and reverse transcription and PCR amplification were carried out using PrimeScript Reverse Transcriptase and LA-Taq polymerase (TaKaRa Bio. Inc., Otsu, Japan), respectively, according to the manufacturer's instructions. Amplification was conducted using an automated DNA thermal cycler (Labnet, Edison, NJ, USA) for 35 cycles consisting of denaturation for 45 s at 96°C, annealing for 45 s at 50°C and elongation for 2 min at 72°C, with a 10-min final extension at 72°C. Products of PCR were gel extracted and cloned into the pGEM-T vector (Promega, Madison, WI, USA), and three plasmid transformants were sequenced twice in both orientations, using labelled primer cycle sequencing with the Sequitherm EXCEL II DNA Sequencing Kit-LC [Epicentre Biotechnologies (Madison, WI, USA); in both insert 66-cm gel; Li-Cor Long Read IR2 4200].

M13, T7 and SP6 promoter universal oligonucleotides (labelled with IRD700 or IRD800) were used in these procedures. Sequence data were assembled and compared with databases using BLAST (NCBI Web server) and ClustalX program (Thompson *et al.*, 1997).

Total RNA was isolated from leaves (rosette stage) of both glyphosate-treated (24 h after herbicide application) and untreated plants from three different biotypes, namely O (S), P24 (RS) and R1 (R), and the target genes were amplified using specific oligonucleotides. Leaves were harvested and ground in liquid nitrogen. Total RNA was isolated (NucleoSpin® RNA Plant) and quantified by spectrophotometry and agarose gel electrophoresis. First-strand cDNA was reverse-transcribed from 2 μg of total RNA. Samples were denatured at 65°C for 5 min followed by quick chill on ice. The reaction mixture (12 μL in volume) contained 500 ng oligo (dT) 18 mer and 1 μL of 10 mM dNTPs. After the addition of 4 μL of 5× PrimeScript™ buffer (TaKaRa), 1 μL (40 units) of human placental ribonuclease inhibitor (HT Biotechnology Ltd., Cambridge, UK), 1 μL (200 units) of PrimeScriptTM RT (TaKaRa) and water to a 20-μL final volume, the reaction mixture was incubated at 42°C for 50 min, followed by heat inactivation at 70°C for 15 min. Target cDNAs were amplified using gene-specific oligonucleotides (Table 1) designed from the transcribed region of each gene using Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR was performed on the Stratagene MX3005P using Power iTaqTM SYBR® Green supermix with ROX (Biorad, Hercules, CA, USA). The primer pair used to amplify the EPSPS genes was designed from common regions of three EPSPS genes: EPSPS1 (Acc.: AY545666), EPSPS2 (Acc.: AY545667) and EPSPS3 (Acc.: AY545668), and the primer pairs used to amplify the M10 and M11 genes were designed according to Peng *et al.* (2010). The reaction mixture (10 μL) contained gene-specific oligonucleotides at a final concentration of 0.2 μM each and 1 μL of the cDNA as template. PCR cycling started with the initial polymerase activation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 20 s. The primer specificity and the formation of primer dimers were monitored by dissociation curve analysis. The expression levels of a *C. canadensis* actin gene were used as internal standards to normalise small differences in cDNA template amounts. Relative transcript levels of the gene of interest (X) were calculated as a ratio to the actin gene transcripts, as E^{Ct} (actin)/E^{Ct} (gene x). PCR efficiency (E) for each amplicon was calculated by employing the linear regression method on the log (fluorescence) per cycle number data, using the LinRegPCR software (Ramakers *et al.*, 2003). All real-time qPCR was performed on three biological replications.

Statistical analyses

For growth comparisons, the standard deviation (from the mean number of leaves) was calculated for each biotype. For survival comparisons for each growth stage, one-way ANOVA was performed and the significance level of the difference from the susceptible standards was determined. For survival comparisons among the three growth stages, an analysis of frequencies was performed and the Pearson chi-square values were determined. For shikimate accumulation, one-way ANOVA was performed and the LSD 0.05 value was determined for each glyphosate concentration, while a regression analysis was performed and the respective coefficients of linear determination (r^2) calculated for each biotype over all glyphosate concentrations. The SPSS statistical pack PASW Statistics 18 was used for all statistical analyses. Statistical analysis for the real-time qPCR was performed by t-test at a 95% level of significance using the SigmaStat 3.5 software (Statcon, Germany).

Results

In the first experiment (plants at the seedling stage), biotypes Ol and O (from organic orchards) did not survive either of the herbicide concentrations tested; most of the plants died within 7–10 DAT and they can therefore be regarded as susceptible standards. Plants of biotypes R1 and R2 survived the 3× recommended dose, verifying their designation as resistant standards. Plants from the conventional orchard (biotypes P1-P25 group) did not survive the 3× rate, but displayed variable survival rates at the recommended rate (Table 2). Nine of the 22 biotypes from this orchard displayed a significantly ($P < 0.05$) higher survival rate, compared with the susceptible standards, and for three, the

Table 1 Oligonucleotide primers used for real-time qRT-PCR

Target gene	Forward primer	Reverse primer
EPSPS	5′-TTACTTCTTAGCTGGTGCTG-3′	5′-GGCATTTTGTTCATGTTCACATC-3′
M10	5′-TTGGCTCAACTTCGTGGTATTGGG-3′	5′-CCAAGAAATTCCAAGCGGAACCCT-3′
M11	5′-ATGCTGTCTTCTTTTACCTTTGC-3′	5′-CGACTTCCCACTACCAGTTCTTC-3′
Actin	5′-GTGGTTCAACTATGTTTCCCTG-3′	5′-CTTAGAAGCATTTCCTGTGG-3′

Table 2 Survival and regrowth of *C. canadensis* seedlings treated with the recommended amount of glyphosate (0.548 kg a.e. ha^{-1})

Biotype	Sprayed plants (0 DAT) Mean number of leaves ± SD†	Survived plants (28 DAT) Mean number of leaves ± SD†	%‡
P1	5.6 ± 0.49	7.0 ± 0.63	50*
P2	5.4 ± 0.49	6.0 ± 0.00	10
P3	5.8 ± 0.60	8.9 ± 0.78	80*
P4	5.5 ± 0.50	8.3 ± 0.90	100*
P5	5.9 ± 0.70	8.5 ± 0.96	60*
P6	5.9 ± 0.54	–	0
P7	5.9 ± 0.54	–	0
P8	6.0 ± 0.63	–	0
P9	5.7 ± 0.46	10.0 ± 2.35	40*
P10	5.7 ± 0.64	9.0 ± 2.45	30*
P11	5.8 ± 0.60	–	0
P12	5.8 ± 0.40	–	0
P13	5.8 ± 0.40	10.2 ± 1.21	60*
P17	5.6 ± 0.49	9.0 + 1.26	50*
P18	5.9 ± 0.70	11.0 ± 3.00	20
P19	5.7 ± 0.49	–	0
P20	5.4 ± 0.49	–	0
P21	5.5 ± 0.25	–	0
P22	5.9 ± 0.70	–	0
P23	5.8 ± 0.75	–	0
P24	5.9 ± 0.54	8.6 ± 0.83	90*
P25	6.0 ± 0.63	–	0
R1	5.7 ± 0.46	10.0 ± 0.77	100*
R2	5.8 ± 0.75	9.3 ± 0.90	100*
O	5.8 ± 0.60	–	0
O1	6.5 ± 0.50	–	0

†Mean number of leaves ± SD obtained from 10 single plant replications per biotype.
‡The survival percentage was calculated by considering as alive the plants that had produced new leaves and continued growing 28 DAT. Values marked with (*) differ significantly from those of the susceptible controls O and Ol at $P < 0.05$.

percentage survival was above 80%. Therefore, *C. canadensis* plants from the conventional orchard in Crete were either susceptible (S) or exhibited some degree of reduced susceptibility (RS), indicating that glyphosate resistance is probably in the process of developing in this population.

In the second experiment (plants at the rosette stage), again most biotypes from the conventional orchard (P1-P22) exhibited a high survival rate, at the recommended rate of application, behaving as RS and only two of them (P19 and P20) behaving as S (Table 3).

In the third experiment (plants at three growth stages), only plants of biotype R2, regardless of the growth stage, survived the 3× rate, as expected. Survival percentages at the recommended rate (Fig. 1) for the three conventional orchard biotypes (P23, P24 and P25), at the two more advanced growth stages, were in the range of 80–100%, closer to the survival rates of the resistant standard R2 and much higher than those of the susceptible standard O, further supporting the presence

Table 3 Survival of *C. canadensis* plants treated with the recommended rate of glyphosate (0.548 kg a.e. ha^{-1}) at the rosette stage

Biotype	Sprayed plants (0 DAT) Mean number of leaves ± SD†	Survived plants (28 DAT) %‡
P1	11.9 ± 0.96	100*
P2	12.1 ± 1.23	100*
P3	12.1 ± 0.93	100*
P4	12.6 ± 0.95	100*
P5	12.3 ± 1.30	93.3*
P6	12.2 ± 1.22	80*
P7	12.5 ± 1.09	100*
P8	12.9 ± 1.29	93.3*
P9	12.9 ± 1.58	100*
P10	12.5 ± 1.09	100*
P11	11.9 ± 1.39	100*
P12	12.5 ± 1.14	100*
P13	12.5 ± 1.02	100*
P17	12.7 ± 1.06	93.3*
P18	12.3 ± 1.43	100*
P19	12.1 ± 1.26	53.3
P20	12.4 ± 1.14	53.3
P21	12.2 ± 0.96	86.7*
P22	11.9 ± 1.06	93.3*
O	12.3 ± 1.23	33.3

†Mean number of leaves ± SD obtained from 15 single plant replications per biotype.
‡The survival percentage was calculated by considering as alive the plants that continued growth and had started bolting 28 DAT. Values marked with (*) differ significantly from those of the susceptible control O at $P < 0.05$.

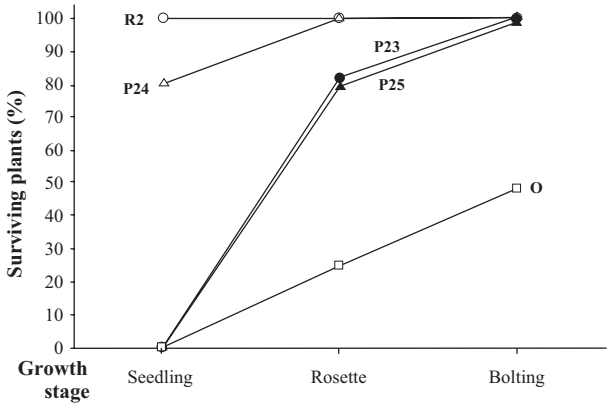

Fig. 1 Influence of the growth stage (seedling, rosette or bolting) on the survival percentage of five *C. canadensis* biotypes (P23, P24, P25 from the Cretan conventional orchard; O susceptible biotype from the Cretan organic orchard; R2 Greek glyphosate-resistant biotype) treated with the recommended dose (0.548 kg a.e. ha^{-1}) of glyphosate. LSD 0.05 between biotype means 3.26, 4.82 and 2.56 at the seedling, rosette and bolting stages respectively.

of RS biotypes in the *C. canadensis* population from the conventional orchard. The statistical analysis of these data (Table 4) indicated that survival frequencies over the three growth stages varied significantly ($P < 0.05$)

Table 4 Pearson chi-square values and two-sided asymptotic significance for data shown in Fig. 1: survival frequencies for each of the five biotypes treated with the recommended dose of glyphosate (0.548 kg a.e. ha^{-1}) over the three growth stages (seedling, rosette and bolting)

	Statistical value	
Biotype†	χ^2	Significance*
P23	26.124	0.00
P24	4.286	0.12
P25	23.333	0.00
O	6.477	0.04
R2‡	–	–

†The results were obtained from 10 single plant replications per biotype per growth stage 28 DAT.
‡No statistical values calculated because of constant survival frequency of resistant biotype R.
*The significance value (two-sided asymptotic significance) presents the dependency level, where <0.01 is highly significant and >0.10 is not significant.

for biotypes P23, P25 and O, but not for biotype P24. Biotype P24 was therefore shown to be the most distinct RS biotype from the conventional orchard population and was selected to be used in subsequent EPSPS gene sequencing and transcript determination experiments.

Results regarding the shikimate assay are shown in Table 5 and indicate that leaf discs from the two resistant biotypes (R2 and U) accumulated the lowest concentration of shikimate, while leaf discs from the two RS biotypes (P4 and P24) accumulated intermediate concentrations of shikimate, significantly lower than those measured in leaf discs from the four susceptible biotypes. Furthermore, it was observed that concentration of shikimate correlated linearly with the concentration of glyphosate, because coefficients of determination values near 1 (r^2 = 0.85–0.99) were obtained for all eight biotypes. Linear fitness was even better (r^2 = 0.92–1.00) when the logarithm of glyphosate concentration was used. By using the line equations of shikimate concentration vs. the logarithm of glyphosate concentration, therefore, biotypes could be characterised more precisely according to the line slopes or intercepts. The intercepts seem to be a very convenient basis for characterising the eight biotypes examined in this study, with the R biotypes giving an intercept value <1, the RS biotypes a value $>1 < 10$ and the S biotypes a value >10. It is worth noting that, although characterised as S, biotypes P6, P13 and P17 from the Cretan conventional orchard accumulated slightly more shikimate and gave a lower intercept value than biotype O (the susceptible standard), thus appearing to have some degree of reduced susceptibility, an observation in agreement with the results from the whole plant treatment at the rosette stage.

Three individual transformants were sequenced in both orientations to allow comparisons at nucleotide and amino acid level of the EPSPS 1 and 2 of biotypes P24 (RS), R1 (R) and U (R). A mutation at the Pro106 residue in EPSPS, reported to confer 2- to 3-fold glyphosate resistance in other weed species, was not detected in the examined biotypes (data not shown).

The expression profiles of the EPSPS, M10 and M11 genes after glyphosate application were subsequently tested using a real-time qRT-PCR. The expression levels of the three EPSPS genes were unaltered following glyphosate application in all tested biotypes (Fig. 2A), indicating that none of the EPSPS genes is involved in the glyphosate resistance mechanism in *C. canadensis*. However, expression of both M10 and M11 ABC transporter genes was highly upregulated in glyphosate-treated plants of P24 and R1 biotypes (Fig. 2B,C). In P24-treated plants, gene transcripts were 11-fold and

Table 5 Shikimate accumulation in leaf discs of eight selected *Conyza* biotypes

		Glyphosate (mg a.e. L^{-1})			Shikimate vs. linear glyphosate		Shikimate vs. logarithmic glyphosate	
		0.9	1.8	3.6				
Conyza biotype	Resistance category	Shikimate accumulation* (μg mL^{-1} HCl solution)			Equation†	r^2	Equation	r^2
R2	R	0.13	0.60	1.06	$y = 0.33x - 0.10$	0.96	$y = 0.67\text{Ln}(x) + 0.20$	1.00
U	R	0.13	3.17	6.98	$y = 2.48x - 1.77$	0.98	$y = 4.94\text{Ln}(x) + 0.52$	1.00
P4	RS	7.23	14.45	21.57	$y = 5.12x + 3.67$	0.96	$y = 10.34\text{Ln}(x) + 8.34$	1.00
P24	RS	6.96	16.21	20.69	$y = 4.71x + 4.72$	0.86	$y = 9.90\text{Ln}(x) + 8.80$	0.96
P6	S	12.29	15.30	24.57	$y = 4.63x + 7.65$	0.99	$y = 8.85\text{Ln}(x) + 12.18$	0.92
P13	S	13.17	17.37	23.24	$y = 3.70x + 10.18$	0.99	$y = 7.33\text{Ln}(x) + 13.65$	0.99
P17	S	13.37	20.70	24.34	$y = 3.77x + 11.55$	0.86	$y = 7.91\text{Ln}(x) + 14.82$	0.96
O	S	13.46	23.52	28.26	$y = 5.07x + 11.09$	0.85	$y = 10.67\text{Ln}(x) + 15.47$	0.96
LSD 0.05		2.26	3.24	1.84				

*Values are means from three replications (three plants sampled per biotype).
†y = shikimate concentration (μg mL^{-1}), x = glyphosate concentration (mg a.e. L^{-1}).

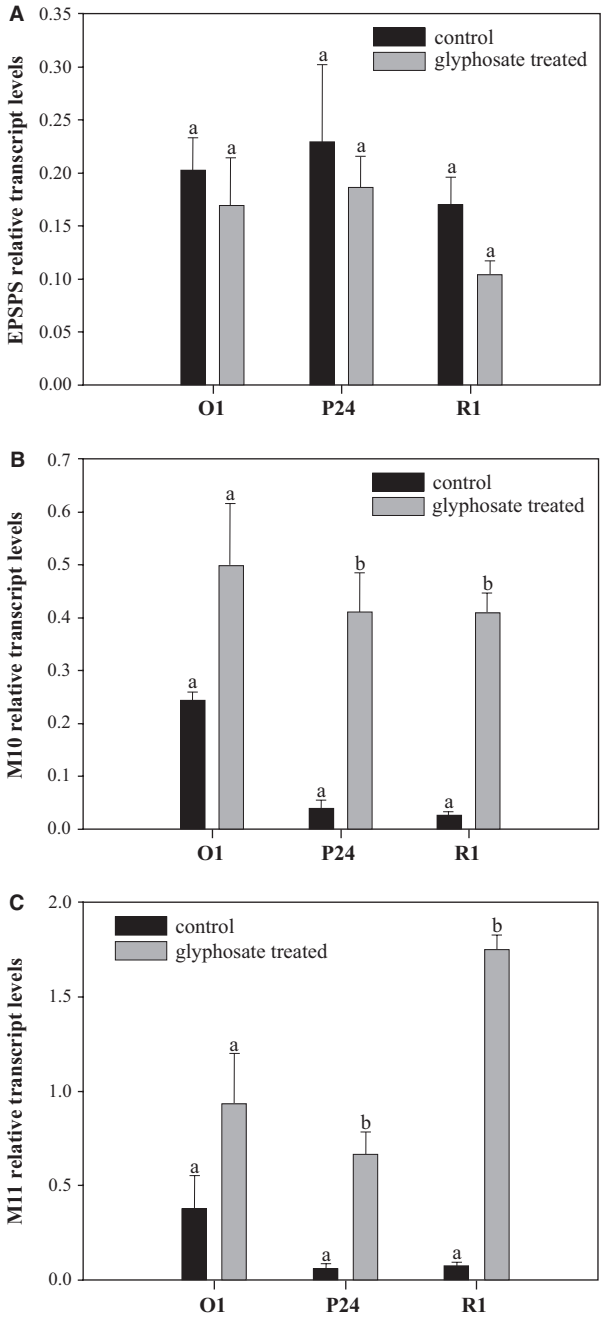

Fig. 2 Accumulation of (A) EPSPS, (B) M10 and (C) M11 gene transcripts in control and glyphosate-treated plants of three different *C. canadensis* biotypes (Ol: susceptible, P24: of reduced susceptibility and R1: resistant). Total RNA was isolated from leaves of control and glyphosate-treated (24 h post-application) plants, reverse-transcribed to cDNA and subjected to RT-qPCR. Relative mRNA level was calculated with respect to the level of actin transcripts. Bars represent means (+SE) of three biological replications. Significant differences ($P = 0.05$) are indicated by different letters.

12-fold, for M10 and M11, respectively, higher than in untreated plants. Similarly, in R1-treated plants, gene transcripts were 16-fold and 23-fold, for M10 and M11, respectively, higher than in untreated plants. Moreover, both M10 and M11 gene expression was not affected by glyphosate application in the susceptible biotype (O1) (Fig. 2B,C). Overall, these results may well indicate a relationship between induction of the M10 and M11 genes and resistance to glyphosate.

Discussion

For an assay to be useful in the early detection of resistance, it should allow the detection of changes in susceptibility and quantification of existing response variability within a weed population. Studies using the shikimate leaf disc assay to date (e.g. Koger *et al*., 2005; Shaner *et al*., 2005; Gulpepper *et al*., 2006) have demonstrated the capacity to differentiate resistant from susceptible plants, but no attempts have been made to quantify susceptibility. The availability of a *C. canadensis* population with reduced susceptibility and significant glyphosate response variability in the Cretan conventional orchard, as established with the standard whole plant procedure over three growth stages in this study, provided a basis to test a modified shikimate leaf disc assay for its suitability to this purpose.

Attention was paid to the selection of suitable glyphosate concentrations for the leaf disc pre-treatment as it has been noted that shikimate accumulates in a glyphosate dose-dependent manner. Koger *et al*. (2005), assessing the leaf disc assay for the detection of glyphosate resistance in *C. canadensis,* found that shikimate accumulated in the leaf discs of all biotypes at high glyphosate concentrations (>21.1 mg a.e. L^{-1}), while only in those of susceptible biotypes at low concentrations (<10.6 mg L^{-1}). The modified assay presented in this study, which used even lower concentrations of glyphosate, was capable of identifying biotypes with varying levels of resistance or susceptibility. For the leaf disc pre-treatment, the three glyphosate concentrations used resulted in a linear accumulation of shikimate, facilitating comparisons. This assay in its present form therefore represents a convenient and rapid method for routine monitoring aimed at the early detection of an evolving resistance.

Shaner *et al*. (2005), working with a variety of plant species, questioned the ability of the shikimate assay to detect glyphosate resistance not associated with an alteration in EPSPS. Further work with *C. canadensis* by Koger *et al*. (2005), however, provided evidence that this assay can detect not only target site–based resistance, but also resistance because of reduced absorption and translocation. The results of this study confirm the capacity of the assay to detect glyphosate resistance in *C. canadensis* that is not target-site-based.

For a number of weeds, a target-based mechanism for resistance to glyphosate has been reported, namely

a point mutation in EPSPS, leading to the substitution at amino acid 106 of proline by serine or threonine (Ng *et al*., 2003; Wakelin & Preston, 2006) and recently of proline by leucine or a variety of other substitutions (Collavo & Sattin, 2012). In our experiments, this mutation was not detected in either EPSPS1 or EPSPS2 from both the Cretan RS biotypes and the non-Cretan R biotypes examined. Furthermore, investigation of EPSPS transcript levels in this study showed that EPSPS gene transcription was not significantly altered in three *C. canadensis* biotypes (of differing susceptibility), following glyphosate treatment.

Recent reports have suggested reduced translocation as one mechanism for glyphosate resistance in *C. canadensis* (Feng *et al*., 2004; Koger *et al*., 2005), in which ABC transporters might be involved (Yuan *et al*., 2007). Furthermore, the preferential movement of glyphosate from the cytosol to the vacuole, in the resistant but not in the susceptible plants, suggested the presence of a transporter for glyphosate either specific to resistant or at a substantially greater concentration in the resistant than in the susceptible plants (Ge *et al*., 2010). Peng *et al*. (2010) have reported that glyphosate treatment leads to upregulated transcription of the M10 and M11 ABC transporter genes. In the present study, the relative transcription levels of M10 and M11 genes were significantly increased in glyphosate-treated resistant and reduced susceptibility biotypes, but in the susceptible biotype it did not change significantly. It is interesting to note, however, that the levels of M10 and M11 RNA transcripts were relatively elevated in the untreated susceptible plants, an observation that restricted our statistical comparisons within each biotype. It becomes therefore apparent that under selection pressure *C. canadensis* biotypes, either resistant or with reduced levels of susceptibility, upregulate the expression of specific ABC transporter genes, providing an indication for their possible involvement in *C. canadensis* glyphosate resistance. A plausible mechanism could be that the resultant overexpressed ABC transporters participate in the sequestration and excretion of toxic compounds into the vacuole in the context of a reduced translocation type of resistance. Remarkably, using genetic engineering, overexpression of a pea ABC transporter gene (psNTP9) conferred multiherbicide resistance in *Arabidopsis* (Windsor *et al*., 2003). Future application of reverse genetics to overexpress or eliminate M10 and M11 genes could shed further light onto the mechanism of *C. canadensis* glyphosate resistance.

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