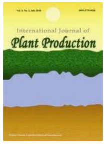

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Assessment of glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) and fleabane (*Conyza albida* Willd. ex Spreng) populations from perennial crops in Greece

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Abstract

The extended use of glyphosate resulted to its reduced efficacy against increasingly problematic weeds, such as *Conyza* spp. The objectives of this study were to determine the occurrence of glyphosate resistance in horseweed (*C. canadensis*) and fleabane (*C. albida*) populations in Greece, to evaluate the effect of weed growth stage on glyphosate efficacy under controlled environmental conditions and to study seed germination patterns of glyphosate-resistant (GR) and glyphosate-susceptible (GS) populations. Plants from 28 and 14 populations of horseweed and fleabane, respectively, sampled from five prefectures in Greece were sprayed with glyphosate at recommended rates. 68% of the tested populations of horseweed were potentially resistant or intermediate, while the relative percentage for fleabane was significantly lower (50%), probably because of the later introduction of this species. After initial screening, six populations from each species were selected and dose-response experiments were conducted. Glyphosate rates required to control some populations were 7 to 14 times greater than that for control of the reference susceptible populations. Sensitivity of GR horseweed and fleabane populations to glyphosate was strongly dependent on growth stage, with plants at the seedling stage being most sensitive to the herbicide. Moreover, when seeds of GR and GS populations from both species were subjected to different alternating temperature, germination occurring and seedling vigour did not differ between them with maximum germination at 10/20 and 15/25 ^{o}C. Consequently, various integrated management strategies should be urgently implemented in order to manage or slow the spread of glyphosate resistance in these species.

Keywords: Glyphosate resistance; Horseweed; Fleabane; Growth stages.

Introduction

Conyza spp. includes annual or short-lived perennials of North American origin that have become cosmopolitan and invasive weeds of many crops and arable lands (Michael, 1977). There are three main species of *Conyza* in Greece: hairy fleabane (*C. bonariensis* L.), horseweed (*C. canadensis* L. Cronq.) and fleabane (*C. albida* Willd. ex Spreng). Hairy fleabane and horseweed are the most common of the three species, while *C. albida* was more recently introduced (Yannitsaros, 1997; Economou et al., 2003). Today, there are many reports from Greek farmers that *Conyza* spp. have become increasingly difficult to control with several herbicides, especially in no-tillage or minimum-tillage systems (Travlos et al., 2009).

Glyphosate is widely considered the world's most important herbicide because of its many desirable characters (Baylis, 2000). During the first years of use, no cases of resistant weeds were reported and the evolution of glyphosate resistance was considered unlikely (Bradshaw et al., 1997). Glyphosate-resistant weeds have evolved and spread, currently more than 20 weed species in several countries (Heap, 2012). Adoption of glyphosate-resistant crops has increased the reliance on glyphosate. Although glyphosate use in these crops has resulted in the evolution of weed resistance (Owen and Zelaya, 2005), cultivation of glyphosate-resistant crops cannot be considered the sole cause (Dill, 2005). Repeated use of glyphosate in no-tillage systems can greatly increase the risk of glyphosate resistance, even in the absence of glyphosate-resistant crops (Urbano et al., 2007; Giannopolitis et al., 2008; Travlos and Chachalis, 2010).

Conyza spp. are prolific seed producers, with a single plant capable of producing thousands of non-dormant seeds (Weaver, 2001), which can be widely dispersed by wind (Shields et al., 2006). *Conyza* spp. became common and problematic weeds in agronomic crops (Weaver, 2001) because they adapt to periodically plant-free, undisturbed soil and establish in the absence of tillage (Brown and Whitwell, 1988). Moreover, resistance of *Conyza* spp. to herbicides with several modes of action have been reported (Heap, 2012). Resistance of *Conyza* ssp. to glyphosate has been recently reported in the Mediterranean region (Urbano et al., 2007; Travlos and Chachalis, 2010; González-Torralva et al., 2012; Heap, 2012). In Greece, the situation is rapidly ongoing and serious, since many herbicides are no longer registered, making weed control even more difficult in perennial crops.

The present study was conducted because of the many reports in Greece regarding the increasingly difficult control of *Conyza* with glyphosate. The main objectives of our study were to assess the occurrence of glyphosate-resistant (GR) populations of horseweed and fleabane in several regions of Greece, to evaluate the effect of weed growth stage of resistant plants on glyphosate efficacy and to study the seed germination patterns of GR and GS populations.

Materials and Methods

Preliminary screening

Horseweed and fleabane seeds were collected from 42 fields (populations), from November 2008 to July 2009, in the following prefectures of Greece: Argolida, Attiki, Etoloakarnania, Fthiotida and Lakonia (Table 1). Crops included alfalfa (*Medicago sativa* L.), vineyards, olive groves and orchards. Some of the sites were known as having poor control of *Conyza* spp., because of farmer complaints registered at local cooperatives. At each site, seeds from 20 plants were collected, in order to have representative samples from every location. Among the samples, there were two seed collections from populations originated in two olive groves in Attiki and Etoloakarnania prefectures that had never been treated with glyphosate and therefore were used as our reference susceptible populations for horseweed and fleabane, respectively.

Table 1. Prefecture, geographical position, crop and number of horseweed (*C. canadensis*) and fleabane (*C. albida*) accessions included in the study.

Prefecture	Code	Positions	Crops	No. of accessions	
				Horseweed (*C. canadensis*)	Fleabane (*C. albida*)
Argolida	AR	37^{o}56'-37^{o}69' N, 22^{o}64'-22^{o}80' E	Vineyards, Orchards	5	2
Attiki	AT	38^{o}10'-38^{o}12' N, 23^{o}77'-23^{o}82' E	Vineyards, Olive groves	5	3
Etoloakarnania	ET	38^{o}21'-38^{o}56' N, 20^{o}57'-21^{o}26' E	Alfalfa, Orhards, Olive groves	6	2
Fthiotida	FT	39^{o}05'-39^{o}09' N, 22^{o}12'-22^{o}21' E	Alfalfa, Vineyards Orhards	5	5
Lakonia	LA	36^{o}80'-36^{o}85' N, 22^{o}65'-22^{o}73' E	Olive groves, Orchards	7	2

Ten seeds from each plant were sown in separate 12- by 13- by 5 cm pots. An herbicide-free soil from Agricultural University of Athens (AUA) mixed with a common peat substrate (1:1, v/v) was used. Tthroughout the experiments, the pots were uniformly watered as needed and supplied with 50 ml/pot of modified Hoagland's solution (0.25%) every 10 d (Hoagland and Arnon, 1950). Seeds were placed on the substrate surface and watered. Pots were maintained in natural conditions with a photoperiod of 12-14 h and air temperatures ranging from 19 to 32 ^{o}C. Seedlings at the three- to five-leaf stage were transplanted into individual pots (15 cm diam by 30 cm deep), using the same substrate. When seedlings were at the rosette stage (7 to 12 cm diameter, 10 to 15 leaves), they were sprayed with 0.72 kg ae/ha of glyphosate (maximum recommended rate) using a custom-built, compressed-air, low pressure flat-fan nozzle experimental sprayer delivering herbicide in 300 L/ha water at 250 kPa. Of the 40 plants of each population in the growth chamber, 20 were sprayed and 20 were kept as untreated controls. Fresh weight of aboveground plant biomass was recorded 28 days after treatment (DAT) and presented as a percentage of the untreated control for each accession. The experiment was conducted twice.

Dose-response and growth stage experiments

Results of the preliminary screening trial described above were used in order to discriminate the several populations and proceed to further experiments. Similar to the classification used by Urbano et al. (2007), populations with biomass reduction of more than 70% after spraying with glyphosate at 0.72 kg ae/ha were considered ''potentially susceptible'', whereas these with biomass reductions of less than 30% were considered ''potentially resistant'' (Table 2). Two potentially susceptible, two potentially resistant and two intermediate populations from the various regions were selected for the dose-response experiment for each of the two species. The objective of these experiments was to determine the herbicide rate needed for a 50% reduction in biomass (GR_{50}). The experiments were conducted from September 2, 2009 to November 16, 2009 in the experimental field of Laboratory of Agronomy (AUA) with air temperatures ranging from 15 to 31 ^{o}C. The experiment was repeated in the greenhouse from March 4 to May 18, 2010. Sowing and transplanting were performed as described previously. When plants reached the rosette stage (7 to 12 cm diameter with 10 to 15 leaves), they were treated with glyphosate at 0, 0.09, 0.18, 0.36, 0.72, 1.44, 2.88 and 5.76 kg ae/ha. These rates correspond to 0, 1/8, 1/4, 1/2, 1, 2, 4 and 8 times the recommended rate of glyphosate. Herbicide treatments were applied as described previously and the pots were arranged in the greenhouse in a completely randomized design. Each pot (1 plant per pot) was considered a replicate and there were four replicates per treatment. The fresh weight of aboveground plant biomass was recorded 28 DAT and presented as a relative percentage of the untreated control for each population.

Table 2. Glyphosate resistance status of the sampled accessions of horseweed and fleabane after preliminary screening (potentially susceptible: biomass reduction >70% following treatment with 0.72 kg ae/ha glyphosate relative to untreated control; potentially resistant: biomass reduction <30%; intermediate: 30-70% biomass reduction).

Category	Accessions (%)	
	Horseweed	*Fleabane*
Potentially susceptible	32	50
Intermediate	43	29
Potentially resistant	25	21
Total	100	100

To study the effect of phenological stage of horseweed and broadleaf fleabane plants on response to glyphosate, an additional dose-response experiment was conducted with plants sprayed at four phenological stages: (1) seedlings (3 to 6 cm diameter or 3-5 true leaves), (2) rosettes (7 to 12 cm diameter), (3) large rosettes (13 to 17 cm diameter) and (4) bolting (20 to 30 cm in height). Two resistant populations (LA2 and FT3 for *C. canadensis* and *C. albida*, respectively) were selected based on the previous dose-response trials. For these experiments, a four by eight factorial completely randomized design was used, with four growth stages and seven glyphosate rates plus untreated control (0, 0.09, 0.18, 0.36, 0.72, 1.44, 2.88 and 5.76 kg ae/ha), with four replicates (plants) for each combination. All treatments were applied with the same equipment as described above. The reduction of fresh weight of aboveground plant biomass in comparison with the untreated control was recorded at 28 DAT.

Seed germination experiments

Horseweed seeds were collected from two orchards in Greece, one confirmed GR population in Lakonia prefecture (LA2) and a confirmed GS population in Attiki prefecture (AT5), where no glyphosate had been used in the last 20 years (Travlos et al., 2009). Fleabane seeds were also collected from two orchards, one confirmed GR population in Fthiotida prefecture (FT3) and a confirmed GS population in Argolida prefecture (AR2). Seeds of both horseweed populations were placed in Petri dishes on two sheets of Whatman No.1 paper filter disk (Whatman Ltd., Maidstone, England) saturated with 4 ml distilled water. The Petri dishes were kept at four diurnally alternating temperature regimes (0/10, 5/15, 10/20, 15/25 and 20/30 ^{o}C, 12 h each) in incubation chambers (Conviron T 38/Lb/AP) under a 12/12 h day/night light photoperiod. The light was supplied by fluorescent light tubes providing 50 µmol m^{-2} s^{-1}. Thirty seeds of each population were placed in each Petri dish, while water was subsequently added when required. The same procedure was followed for fleabane populations. Seed germination was expressed as a percentage of the total number of tested seeds (germination percentage, GP) after an incubation of 10 days. Seeds were considered germinated when the healthy, white radicle had emerged through the integument and reached more than 1 mm in length. Seedling vigor was analyzed according to a method developed by Abdul Baki and Anderson (1973). The length of the root and shoot was measured and vigor index (VI) was calculated based on percent germination (GP), mean root length (MRL) and mean shoot length (MSL) of the seedlings using the following equation:

$$VI=(MRL+MSL)\times GP$$

The experiments were conducted in a completely randomized design with split-plot arrangement (with population as main effect and temperature as sub-effect) and four replications for each treatment and was repeated.

Statistical analysis

Data obtained from the pot experiments were subjected to ANOVA using the completely randomized design combined over experimental runs. There were no significant experimental run or run by treatment interaction for both experiments and therefore data were combined across experimental runs. For the preliminary screening data, fresh weight percentage means were separated using Fisher’s protected LSD test at the significance level of P=0.05. For the dose-response data, interpolative probit analysis was conducted (Finney, 1952) to ascertain the dose resulting in a 50% reduction in plant growth (GR_{50}). In accordance with Urbano et al. (2007), the resistance index (RI) was calculated as the GR_{50} of each population divided by the GR_{50} of the reference susceptible GS populations.

Data obtained from the laboratory experiments were also analyzed by ANOVA, while mean comparison was performed using Fisher's Protected LSD test at a significance level of $P<0.05$ by means of Statistica 9.0 software package (StatSoft, Inc. 2300 East 14th Street, Tulsa, OK74104, USA). All data were tested for normality and variance before further analyses. The seed germination percentages were angular transformed and then subjected to ANOVA.

Results and Discussion

Preliminary screening

Data analysis confirmed significant differences in biomass reduction in response to glyphosate at 0.72 kg ae/ha among the 28 and 14 populations of horseweed and fleabane, respectively, collected from different locations in Greece. After review of the data, 9 horseweed and 7 fleabane populations exhibited mean plant biomass 30% lower than the untreated control biomass and were considered potentially susceptible, whereas 7 horseweed and 3 fleabane populations exhibited plant biomass 70% greater than the untreated controls and were considered potentially resistant (Table 2). In accordance with Urbano et al. (2007), the term ‘‘intermediate’’ was used for the 12 horseweed and 4 fleabane populations with a mean plant biomass of between 30 and 70% of the untreated controls. The observed differences between the two species regarding the percentage of the already resistant or intermediate populations could be attributed to the significantly earlier introduction of horseweed compared with fleabane (Travlos et al., 2009). From the screening results, the potentially resistant populations were not confined to any prefecture. In a number of instances, these populations were located very close to susceptible ones. For most of the susceptible populations, glyphosate was not recently used or used at low rates or in rotation or mixture with other herbicides (personal comments from farmers). On the other hand, in the cases of potentially resistant populations, glyphosate was the primary mean of weed control, with repeated use of the herbicide during the growing season at very high rates (two to five times greater than the recommended rate).

Dose-response and growth stage experiments

The glyphosate dose responses of the 12 selected populations from the 42 initial ones, screened previously with glyphosate at 0.72 kg/ha, are shown in Table 3. Dose-response results indicate marked differences in the response of three of the two most glyphosate-susceptible populations (AT5 and AR2) when compared with two of the most glyphosate-resistant populations (LA2 and FT3) for horseweed and fleabane, respectively. Regarding the estimated GR_{50} values for each horseweed population, it has to be noted that a glyphosate rate of 0.2 kg/ha caused 50% biomass reduction in accession AT5, whereas the same reduction was achieved with 2.86 kg/ha for accession LA2. Similarly, glyphosate rate of 0.72 kg/ha provided 93% and 11% control for the most susceptible (AR2) and resistant (FT3) fleabane population, respectively (Table 3). Glyphosate rates required to control some resistant populations (LA2 and ET3 for horseweed and FT3 and ET1 for fleabane) were about four to seven times greater than those required to control the reference susceptible populations (AT5 and AR4). These RI values were similar to those previously reported for horseweed (Van Gessel, 2001; Main et al., 2004) and hairy fleabane in Spain and Greece (Urbano et al., 2007; Travlos and Chachalis, 2010), while shikimic acid measurements for a further confirmation are going to continue in a due time (Mueller et al., 2008).

Results of the experiment examining the effect of phenological stage of populations LA2 and FT3 on sensitivity to glyphosate show a statistically significant effect of growth stage on fresh weight reduction with increasing glyphosate rate (Table 4). However, data analysis indicated no significant interaction between these two factors-growth stage and glyphosate rate. For the resistant populations tested, fresh weight reduction with increasing glyphosate rate was greatest at the seedling growth stage, with a maximum reduction of 72 and 79% at 5.76 kg/ha (eight times the maximum recommended rate). The sensitivity of plants treated at the rosette stage was intermediate, whereas sensitivity to the herbicide was least at the large rosette and bolting stages (with no significant difference between them), with a small reduction in fresh weight of approximately 31 to 38% even at eight times the recommended rate of glyphosate. This reduction of herbicide efficacy at the later growth stages (even observed for susceptible populations) is probably due to the low growth rate and dry matter accumulation of the mature plants. Our results are in accordance with recent studies on horseweed (Van Gessel, 2009) and hairy fleabane (Urbano et al., 2007; Travlos and Chachalis, 2010).

Table 3. Horseweed and fleabane biomass produced by two resistant, two susceptible and two intermediate accessions of each species in response to increasing glyphosate rate. Means followed by different case letters in each column for each species are significantly different according to Fischer's LSD test (P=0.05).

Glyphosate rate (kg ha^{-1})	Horseweed populations					
	AT5	ET2	ET3	FT5	LA2	LA3
0	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
0.09	69^{b}	77^{b}	92^{ab}	75^{b}	98^{a}	83^{ab}
0.18	52^{b}	55^{c}	90^{ab}	59^{bc}	89^{a}	72^{b}
0.36	28^{c}	32^{d}	82^{b}	46^{c}	90^{a}	58^{bc}
0.72	9^{d}	16^{c}	78^{b}	33^{cd}	90^{a}	51^{bc}
1.44	0^{e}	5^{d}	52^{c}	19^{d}	67^{b}	32^{c}
2.88	0^{e}	0^{e}	29^{d}	8^{de}	52^{b}	12^{d}
5.76	0^{e}	0^{e}	18^{d}	0^{e}	38^{b}	5^{d}
	Fleabane populations					
	AR2	AT2	ET1	FT1	FT3	LA1
0	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
0.09	77^{b}	64^{b}	92^{a}	78^{b}	98^{a}	83^{b}
0.18	56^{c}	55^{b}	88^{ab}	69^{b}	88^{a}	69^{c}
0.36	44^{c}	47^{b}	81^{b}	52^{bc}	92^{a}	58^{cd}
0.72	7^{d}	28^{c}	72^{bc}	34^{c}	89^{a}	41^{de}
1.44	0^{d}	10^{c}	56^{c}	26^{cd}	78^{ab}	31^{e}
2.88	0^{d}	0^{c}	35^{d}	15^{d}	55^{b}	18^{ef}
5.76	0^{d}	0^{c}	25^{d}	0^{d}	32^{c}	5^{f}

Table 4. Aboveground biomass reduction of GR populations of horseweed and fleabane (LA2 and FT3, respectively) at different growth stages in response to increasing glyphosate rate. Means followed by different case letters are significantly different according to Fischer's LSD test (P=0.05).

Glyphosate rate (kg ha^{-1})	GR Horseweed (LA2)				GR Fleabane (FT3)			
	Seedling	Rosette	Large rosette	Bolting	Seedling	Rosette	Large rosette	Bolting
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
0.09	10^{b}	3^{ab}	0^{a}	0^{a}	12^{b}	4^{ab}	0^{a}	0^{a}
0.18	16^{bc}	7^{ab}	8^{ab}	5^{ab}	18^{c}	12^{b}	8^{ab}	5^{ab}
0.36	20^{c}	12^{b}	13^{b}	10^{b}	16^{bc}	10^{b}	8^{ab}	5^{ab}
0.72	27^{cd}	11^{b}	9^{ab}	10^{ab}	29^{cd}	13^{b}	14^{b}	10^{b}
1.44	46^{d}	32^{cd}	28^{cd}	17^{bc}	44^{d}	22^{c}	18^{c}	12^{b}
2.88	63^{e}	48^{d}	46^{d}	31^{cd}	53^{de}	34^{cd}	28^{cd}	22^{c}
5.76	72^{ef}	54^{de}	44^{d}	38^{cd}	79^{f}	58^{e}	42^{d}	31^{cd}

Seed germination experiments

Germination percentage for GR and GS populations of both species exceeded 65% at the temperature regimes of 10/20 and 15/25 ^{o}C. Germination percentage was lowest at 0/10 ^{o}C, while the 5/15 and 20/30 ^{o}C exhibited intermediate germination percentage (31 to 51%). There were no differences between GR and GS population in terms of percentage of seed germination at any temperature regime (Table 5).

Similarly, there were no differences between the GR and GS populations in the seedling vigor. Seedling vigor was highest at 15/25 ^{o}C and lowest at 0/10 ^{o}C. It has to be noted that the vigor of seedlings grown at 15/25 ^{o}C was 20 and 24 times greater on average than when grown at 0/10 ^{o}C, for horseweed and fleabane, respectively (Table 5).

Table 5. Effect of five diurnally alternating temperature regimes (0/10, 5/15, 10/20, 15/25 and 20/30 ^{o}C) on seed germination and seedling vigor (±SE) of GR and GS horseweed and fleabane populations. Vigor index was calculated based on percent germination and mean root length and mean shoot length of the seedlings. Means followed by different case letters are significantly different according to Fischer's LSD test (P=0.05).

Temperature treatment (^{o}C)	Horseweed			
	Germination (%)		Vigor Index	
	GR	GS	GR	GS
0/10	8^{a}	10^{a}	32^{b}	36^{b}
5/15	31^{b}	36^{b}	184^{e}	196^{e}
10/20	66^{c}	69^{c}	612^{fg}	586^{f}
15/25	82^{d}	86^{d}	654^{g}	632^{fg}
20/30	42^{bc}	45^{bc}	234^{e}	254^{e}
	Fleabane			
	Germination (%)		Vigor Index	
	GR	GS	GR	GS
0/10	4^{a}	6a	28^{b}	24^{b}
5/15	36^{b}	42^{b}	212^{e}	234^{e}
10/20	68^{c}	71^{c}	546^{f}	576^{fg}
15/25	88^{d}	84^{d}	684^{h}	641^{gh}
20/30	51^{bc}	49^{bc}	246^{e}	231^{e}

Previous reports on *Conyza* spp. and other weed species showed that seed germination was stimulated by alternating temperatures, therefore we have tested a wide range of alternating temperatures (Karlson and Milberg, 2007; Travlos et al., 2009). In the present study, germination increased with temperature, but declined at 20/30 ^{o}C. The present study also revealed no difference in seed germination or seedling vigor between GR and GS biotypes at the tested temperature regimes. Our results are in accordance with previous studies of Alcocer-Ruthling et al. (1992) and Travlos and Chachalis (2010) reported no significant difference in seed germination of R populations of other weeds.

Conclusions

Glyphosate-resistant populations of horseweed and fleabane have been confirmed in Greece. Resistance has been shown under controlled conditions through a preliminary screening and following dose-response experiments. About 70% of the tested populations of horseweed were potentially resistant or intermediate, while the relative percentage for fleabane was significantly lower (50%), probably because of the earlier introduction of horseweed in the country. Glyphosate rates required to control some populations were up to 14 times greater than that for control of the reference susceptible populations. The level of resistance found in our study is similar to or slightly higher than the corresponding values reported for *Conyza* spp. in other studies. Glyphosate efficacy on a glyphosate-resistant accession was enhanced when applied at the three- to five-leaf stage. Moreover, it seems that there is not any cost regarding seed germination and seedling vigor of GR populations. Consequently, the role of several integrated management strategies is crucial for the control of the further spread of glyphosate resistance in these species. The comparative evaluation of mixtures, herbicides with different mode of action or residual activity and other integrated methods should clearly be among the main goals of future research (Travlos, 2012; Weber and Kieloch, 2013).

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