EVALUATION OF HERBICIDE-RESISTANCE STATUS ON POPULATIONS OF LITTLESEED CANARYGRASS (*PHALARIS MINOR* RETZ.) FROM SOUTHERN GREECE AND SUGGESTIONS FOR THEIR EFFECTIVE CONTROL

*Ilias S. Travlos**

Agricultural University of Athens, Faculty of Crop Science
Laboratory of Agronomy, 75, Iera Odos St., 11855 Athens, Greece

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Abstract: In 2010, a survey was conducted in the wheat fields of a typical cereal-producing region of Greece to establish the frequency and distribution of herbicide-resistant littleseed canarygrass (*Phalaris minor* Retz.). In total, 73 canarygrass accessions were collected and screened in a field experiment with several herbicides commonly used to control this weed. Most of the weed populations were classed as resistant (or developing resistance) to the acetyl-CoA varboxylase (ACCase)-inhibiting herbicide diclofop, while resistance to clodinafop was markedly lower. The results of the pot experiments showed that some of the canary populations were found to have a very high level of diclofop resistance (resistance index up to 12.4), while cross resistance with other herbicides was also common. The levels of resistance and cross resistance patterns among populations varied along with the different amounts and times of selection pressure. Such variation indicated either more than one mechanism of resistance or different resistance mutations in these weed populations. The population which had the highest diclofop resistance level, showed resistance to all aryloxyphenoxypropinate (APP) herbicides applied and non-ACCase inhibitors. Alternative ACCase-inhibiting herbicides, such as pinoxaden remain effective on the majority of the tested canarygrass populations, while the acetolactate synthase (ALS)-inhibiting herbicide mesosulfuron + iodosulfuron could also provide some solutions. Consequently, there is an opportunity to effectively control canarygrass by selecting from a wide range of herbicides. It is the integration of agronomic practices with herbicide application, which helps in effective management of *P. minor* and particularly its resistant populations.

Key words: canarygrass, survey, diclofop, herbicide resistance

INTRODUCTION

Phalaris minor Retz. (littleseed canarygrass) is a native weed of Mediterranean origin, and has spread to many parts of the world (Anderson 1961; Baldini 1995; Singh *et al*. 1999). Holm *et al*. (1979) reported that it is now a serious grass weed of wheat and barley in USA, Canada, Africa, Australia, India and elsewhere. Globally, *P. minor* has been reported in more than 60 countries of the world, widely covering all the continents except for the polar regions. It is one of the most troublesome grassy weeds in wheat (Holm *et al*. 1979; Singh *et al*. 1999; Jabran *et al*. 2010). In many cases, cereal fields are heavily infested by *P. minor* which emerges with the germinating wheat crop, competes for water and nutrient requirement, and significantly reduces the grain yield (Malik and Singh 1995; Afentouli and Eleftherohorinos 1996).

Unfortunately, the control of canarygrass is almost totally dependent on early post-emergence, crop-selective herbicides, with acetyl-CoA carboxylase (ACCase)-inhibiting herbicides being of highest importance, followed by some acetolactate synthase (ALS)-inhibiting herbicides (Beckie *et al*. 2002). Both aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD) herbicides inhibit acetyl-CoA carboxylase and have been widely used to control grass weed species, such as wild oat and canarygrass. Pinoxaden is a newly registered grass weed herbicide, which belongs to the phenolpyrazolines chemical group and also inhibits the activity of acetyl-CoA carboxylase and lipid synthesis. The use of Pinoxaden results in the control of sterile grass weeds (Zand *et al*. 2007). The increasing reliance on target-site-specific herbicides such as acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors is certainly exacerbating the resistance risk. Resistant biotypes of weeds are constantly spreading (Heap 2012). It seems that in Europe, the situation is evolving faster in Mediterranean regions, where a wider variety of biotypes, herbicides and cropping systems are involved (Sattin 2005; Travlos *et al*. 2012). Resistance has previously been reported for biotypes of *Avena sterilis* L. and other weeds from Greece (Travlos and Chachalis 2010; Travlos *et al*. 2011). Many *P. minor* populations have been reported to have evolved

*Corresponding address:
htravlos@yahoo.gr

resistance to ACCase inhibitor herbicides or even, in some cases, cross- or multiple resistance patterns (Afentouli and Georgoulas 2002; Heap 2012). The situation is getting even worse, where in some instances, wheat growers were forced to harvest their immature crop as fodder in the absence of effective alternative herbicides (Malik and Singh 1995).

Phalaris in Greece is considered to be one of the most frequent weeds in wheat and is represented mainly by three species; *P. paradoxa*, *P. brachystachys* and *P. minor* (Vassiliou *et al*. 2006). It has been reported that certain biotypes of *P. brachystachys* and *P. paradoxa* might have developed resistant biotypes to herbicides such as fenoxaprop-p-ethyl. These are herbicides which have been applied for at least eight years in regions of northern Greece (Afentouli and Georgoulas 2002; Vassiliou *et al*. 2006). However, there is significantly less evidence concerning *P. minor*. The present study was conducted because many reports show that *P. minor* is becoming increasingly difficult to control in the major cereal-producing regions of Greece. Information on the quantification of the extent of the resistance problem could be crucial for the implementation of integrated weed management practices, especially in some target-regions.

The first objective of the present study was to evaluate the effectiveness of several herbicides registered for littleseed canarygrass control in Greece (clodinafop-propargyl, diclofop methyl, fenoxaprop-p-ethyl, pinoxaden, and iodosulfuron + mesosulfuron methyl) against *P. minor* populations from a typical wheat-producing prefecture. The second objective was to investigate patterns of herbicide resistance and cross-resistance using both field and pot experiments.

MATERIALS AND METHODS

Plant material collection and seed pretreatment

The prefecture of Viotia, which is a typical cereal producing area in Greece, was selected for the littleseed canarygrass survey, and as the location for the collection of seeds. Previous studies in Greece have revealed that canarygrass infestations can be composed of mixtures of up to 3 species: *P. brachystachys*, *P. minor*, and *P. paradoxa* (Afentouli and Eleftherohorinos 1996). All five regions (Cheronia, Orhomenos, Aliartos, Mouriki and Thiva) were selected since they were already known to have histories of difficulties with grass weed control (mainly wild oat and canarygrass). The problems were known from farmer complaints registered at local cooperatives (Travlos *et al*. 2011). The surveys were conducted during a three week period at the beginning of maturity, from May 22 to June 12, 2010. For a better distribution of the sampling sites, each region was divided into 3 distinct subregions and 4 to 6 fields were sampled in each subregion. Seeds were mainly collected from herbicide-treated wheat fields along with some fields that had never been treated with herbicides (in order to use them as susceptible control populations). In total, more than 90 wheat fields were visited at random in the five selected regions. Each surveyed field was walked through on the two diagonals, and records were kept of the canarygrass species present. In each field, panicles and seed were collected from 10 plants and transferred to the Laboratory of Agronomy (Agricultural University of Athens) after *in situ* determination of species. The seeds were separated, air-dried, and stored in paper bags in room temperature. In Table 1, the number of *P. minor* populations included in the study is shown for each region.

In order to promote germination, seeds of each of the collected canarygrass populations were imbited for 24 h in distilled water at 20°C (Om *et al*. 2003). Then, the seeds were sown in the field or pots.

Field experiment

A field experiment was conducted during 2010 and 2011 in the experimental field of the Laboratory of Agronomy in the Agricultural University of Athens (37° 59′ N, 23° 42′ E). The soil was sandy clay loam (52% sand, 13% clay and 35% silt), with pH (1:2 H_2O) 7.4, $CaCO_3$ 14 g/kg, an organic matter content of 18 g/kg, 0.195% total nitrogen, a medium supply of available phosphorus (P-Olsen 16 ppm), and a good supply of potassium (630 ppm). The previous crop was maize (*Zea mays* L.). The experiment was conducted in a randomized complete block design with split-plot arrangement and four replications for each treatment (herbicide x canarygrass population). The population of *P. minor* was the main plot and herbicide treatment was done on the sub-plot. The experimental plots were 2 m x 1 m and an untreated control was also included. The planting date was 19 November 2010. All weeds, except those which had been sown, were removed by hand-hoeing. For all accessions, germination and seedling survival were high (> 80%), ensuring that 80 individual seedlings in each accession were screened with each herbicide. When seedlings reached the three to five leaf stage (BBCH 13–15), they were treated with the maximum recommended doses of a range of the most widely used herbicides against *P. minor* in Greece (Table 2). A motorized backpack sprayer was used to deliver 300 l/ha spray solution at 2.5 kg/cm^{-2} pressure through flat-fan spray nozzles.

Table 1. Geographical position and number of *P. minor* accessions included in the present study

Origin	Positions	No. of accessions
Cheronia (CH)	38°21′–38°31′ N, 22°48′–22°49′ E	12
Orhomenos (OR)	38°28′–38°32′ N, 22°57′–23°01′ E	17
Aliartos (AL)	38°24′–38°25′ N, 23°05′–23°07′ E	14
Mouriki (MO)	38°24′–38°26′ N, 23°18′–23°22′ E	13
Thiva (TH)	38°19′–38°22′ N, 23°16′–23°22′ E	17

Table 2. Preparations of herbicides (commercial formulations) and adjuvant applied at their upper recommended rates during our field experiments, at littleseed canarygrass Zadoks stages 13–15

Chemical class	Active substance [a.s.]	Concentration [g a.s./l]	Upper recommended rate [g a.s./ha]	Trade name
Aryloxyphenoxypropionate	clodinafop-propargyl	240	40.8	Topik 240 EC
	diclofop-methyl	284	945	Illoxan 28 EC
	fenoxaprop-P-ethyl	69	82.8	Puma S 6.9 EW
Phenylpyrazolin	pinoxaden	100	40	Axial 100 EC
Sulfonylureas	mesosulfuron-methyl + iodosulfuron-methyl sodium	7.5+7.5	7.5+7.5	Hussar maxx OD

For all the herbicides, 0.5% of paraffinic oil (AtPlus Oil formulation, 600 g/l paraffinic oil) was used as an adjuvant

Herbicide effect was assessed by counting seedling mortality 21 days after herbicide treatment (DAT). In accordance with Travlos *et al*. (2011), littleseed canarygrass populations were classed as resistant if 20% or more of the individuals in the accession survived the herbicide. Where there was 2–19% survival, the population was classed as developing resistance. Where there was less than 2% survival, the population was classed as susceptible.

Pot experiments

After the previously described field screening, seven of the most resistant accessions from the five surveyed regions and one susceptible control population (OR4) were selected for the dose-response experiment. This experiment was performed to determine the herbicide dose needed for a 50% reduction in biomass (GR_{50}) and was conducted twice in the Agricultural University of Athens. On September 11 and October 6, 2011, ten seeds from each accession were sown in separate pots (12x13x5 cm). Herbicide-free soil from the field of the Laboratory of Agronomy was mixed with a common peat substrate (1:1, V/V). Throughout the experiments the pots were uniformly watered as needed and supplied with 50 ml/pot of modified Hoagland's solution (0.25 strength) every 10 days. All pots were placed outdoors, arranged in a randomized complete block design (four replicates for each treatment) and randomized each week in order to achieve uniform growth conditions for all plants.

Clodinafop, diclofop, fenoxaprop, pinoxaden and mesosulfuron + iodosulfuron were applied at Zadoks stage 13–15 and at seven doses corresponding to 0, 0.25X, 0.5X, X, 2X, 4X and 8X of recommended label rate (X). Herbicide treatments were applied with the same experimental sprayer which was described above. At 21 DAT, control of weed accessions was assessed by determining the fresh weight of all plants which had survived in each pot. Preliminary measurements showed no significant differences in the water content of plants of the different accessions and treatments, (data not shown). Data were expressed as percentage of the untreated control for each accession.

Statistical analysis

Data obtained from the field and pot experiments were analyzed by ANOVA. Fisher's protected least significant difference (LSD) test at a 5% probability level was used to separate means. Because the ANOVAs indicated no significant treatment by time interaction, means were averaged over the repeated experiments.

The weed biomass data are expressed as a percentage of the untreated control. The GR_{50} values were obtained by nonlinear regression using the following log-logistic equation (Seefeldt *et al*. 1995):

$$y = c + < (d - c) / 1 + \exp\{b[\log (x) - \log (GR_{50})]\} > [1]$$

where: y represents shoot fresh wt (percentage of the untreated control) at herbicide dose x, *c* and *d* denote the lower and upper limits, respectively, GR_{50} is the herbicide dose centered between the asymptotic values and *b* is the slope of the response curve. The level of resistance was expressed by means of the resistance index (RI), which was calculated as the ratio of the GR_{50} of each resistant (R) accession by the GR_{50} of the most susceptible (S) biotype (Travlos and Chachalis 2010; Travlos *et al*. 2011).

RESULTS

Screening of the canarygrass populations collected from the five regions revealed widespread resistance to the wheat-selective ACCase-inhibiting herbicide diclofop. It is important to note that about 60% of the screened accessions displayed some level of diclofop resistance (Table 3). The level of resistance to the other ACCase-inhibiting herbicides (fenoxaprop and clodinafop) was lower than diclofop but still concerned about 40% of the tested accessions. On the other hand, over 85% of the studied canarygrass biotypes were generally susceptible to the other herbicides. The recently commercialized (2008) pinoxaden showed the highest susceptibility, since less than 10% of the accessions showed resistance to it.

The survey and the field experiments also revealed high resistance of *P. minor* to one or more herbicides, especially in some of the surveyed regions. Indeed, over 20% of the canarygrass accessions originating from the prefectures of Thiva and Mouriki were already highly resistant to diclofop (Table 4) and/or other herbicides (data not shown). Although markedly fewer than those previously mentioned, some of the collected accessions from the three other regions (Cheronia, Aliartos and Orhomenos) were also resistant to diclofop.

Table 3. Percentage of herbicide-resistant sterile littleseed canarygrass populations in each category for each herbicide

Herbicide	Susceptible	Developing resistance	Resistant	Total resistance
	[%]			
Clodinafop	64 a[1]	29 b	7 c	36 b
Diclofop	38 e	43 e	19 f	62 d
Fenoxaprop	59 g	30 h	11 i	41 gh
Pinoxaden	91 j	8 k	1 k	9 k
Mesosulfuron + Iodosulfuron	86 l	14 m	0 n	14 m

[1] two means not sharing a letter in common in each row differ significantly at $p \leq 0.05$
Populations were classed as resistant (20% or more survival), as developing resistance (2–19% survival), or as susceptible (less than 2% survival)

Table 4. Percentage of diclofop-resistant littleseed canarygrass populations in each category for each surveyed region

Herbicide	Susceptible	Developing resistance	Resistant	Total resistance
	[%]			
Cheronia	58 a[1]	25 b	17 b	42 ab
Orhomenos	41 d	41 d	18 e	59 c
Aliartos	36 g	50 fg	14 h	64 f
Mouriki	46 ij	31 jk	23 k	54 i
Thiva	35 mn	41 m	24 n	65 l

[1] two means not sharing a letter in common in each row differ significantly at $p \leq 0.05$
Populations were classed as resistant (20% or more survival), as developing resistance (2–19% survival), or as susceptible (less than 2% survival)

Table 5. Fresh weight and GR_{50} values of selected littleseed canarygrass populations after diclofop application

Population	GR_{50} [g a.s./ha]	Fresh weight [% of untreated]						
		0	1/4X*	1/2X	X	2X	4X	8X
CH2	464	100	78	66	46	40	26	14
OR7	963	100	83	74	62	54	37	23
AL11	714	100	82	70	64	47	35	19
MO3	843	100	79	72	62	52	41	24
MO9	421	100	76	67	51	35	26	11
TH4	1066	100	88	77	65	56	41	27
TH11	568	100	81	70	53	44	29	15
S	86	100	28	12	5	2	0	0

S – susceptible population (OR4) collected from organic fields which had never been exposed to herbicides
* is the recommended label rate of diclofop

Table 6. Resistance index of selected littleseed canarygrass populations according to the dose response experiments

Population	Clodinafop	Diclofop	Fenoxaprop	Pinoxaden	Mesosulfuron + Iodosulfuron
CH2	1.5	5.4	3.6	1.6	0.7
OR7	4.6	11.2	7.2	0.8	1.0
AL11	6.1	8.3	3.8	2.5	1.2
MO3	2.4	9.8	2.8	0.3	0.6
MO9	2.1	4.9	4.8	0.6	0.4
TH4	4.3	12.4	6.6	1.1	1.3
TH11	7.4	6.6	4.1	3.4	3.1
S	1.0	1.0	1.0	1.0	1.0

The data is based on GR_{50} values calculated from fresh weight data
S – susceptible population (OR4) collected from organic fields which had never been exposed to herbicides

The data of the dose-response experiment for the seven selected populations (CH2, OR7, AL11, MO3, MO9, TH4 and TH11) showed that some of them exhibited higher levels of resistance than other accessions. When treated with diclofop at 1/4 the recommended field dose, the fresh weight reduction was about 17–24% for most of the selected accessions, while in TH4 and OR7 the corresponding value was less than 15% (Table 5). It is noticeable that even at the recommended diclofop dose, the biomass reduction of the most resistant population (TH4) was only 35%, while at the same time the reduction for the susceptible accession (OR4) was 95%. Moreover, almost all resistant accessions had biomass reductions of less than 50%.

The resistance indices for clodinafop, diclofop, fenoxaprop, pinoxaden and mesosulfuron + iodosulfuron are given in table 6. Resistance index for diclofop was between 4.9 and 12.4. It is also noticeable that one population was more resistant to clodinafop than diclofop, while resistance index for fenoxaprop ranged between 2.8 and 7.2. Resistance indices for pinoxaden and mesosulfuron + iodosulfuron did not exceed 3.4 and 3.1, respectively, while most biotypes were clearly susceptible to these herbicides (R/S ≤ 1).

DISCUSSION

Our results revealed a widespread resistance of the collected canarygrass populations to the wheat-selective ACCase-inhibiting herbicide diclofop-methyl. All together, 19% of the collected accessions were found to be already resistant, with a further 43% in the developing resistance category. It should be emphasised that this survey was conducted very late in the growing season. Almost all the fields had probably received early-season herbicide treatments, though it is difficult to know the precise treatment history of each field. The present survey also highlighted that resistance to other ACCase-inhibiting herbicides was markedly lower than that found for diclofop-methyl. Field screening of *P. minor* accessions with the herbicides clodinafop and fenoxaprop, showed significantly fewer canarygrass accessions to be resistant to clodinafop and fenoxaprop compared with diclofop. Similar differences have also been reported in other countries (Om *et al*. 2004; Chhokar *et al*. 2008) and are very likely due to the lengthy period of selection pressure imposed from the much greater historical use of diclofop. This higher overall use of diclofop could be explained by its earlier registration (more than two decades before the other two herbicides) and its broad weed spectrum (Owen *et al*. 2007).

The present study also revealed that resistance is lower to pinoxaden, compared with the tested APP herbicides. Only 1% of the accessions exhibited resistance to pinoxaden. One population was classed as resistant and a further six accessions as developing resistance. It should be pointed out that these accessions were resistant to most other ACCase-inhibiting herbicides tested. Pinoxaden had been used in Greece only a year before the present survey, and its resistance had probably been selected by the use of other ACCase-inhibiting herbicides (Travlos *et al*. 2011). For the ALS-inhibiting herbicide mesosulfuron + iodosulfuron, most of the littleseed canarygrass populations were also susceptible (86%). Only 11 populations (14%) were classed as developing resistant.

The frequency of herbicide resistance across different ACCase-inhibitors was found to be variable. Many biotypes (21%) were found to be resistant (or developing resistant) only to the ACCase-inhibiting herbicide diclofop, however, more than 60% of the diclofop-resistant biotypes displayed resistance to other ACCase-inhibiting herbicides. In the case of the littleseed canarygrass accessions with resistance to other ACCase-inhibiting herbicides, these accessions were, in most cases, (> 85%) also resistant to diclofop. Using seven selected accessions we further characterized the observed resistance level and cross-resistance patterns. All the examined populations were regarded as resistant to diclofop with a resistance index which ranged between 4.9 and 12.4. It also has to be noted that the most resistant accessions, TH4 and OR7 were collected from wheat monoculture where herbicides have been used for over 20 years. Lack of crop and herbicide rotations has been considered the main cause of evolved resistance in many parts of the world (Tal *et al*. 1996; Travlos and Chachalis 2010). Many populations resistant to diclofop were also resistant to clodinafop or fenoxaprop. Nonetheless, the resistance level in these accessions was different in response to these two herbicides, with a resistance index in most cases higher for fenoxaprop compared with clodinafop. In our study, and according to the classification by Bourgeois *et al*. (1997), the most resistant biotypes would be classified as type A concerning their cross-resistance pattern (high resistance to APP herbicides and no resistance to CHD herbicides), and were also previously reported in other grass weeds (Mansooji *et al*. 1992; Travlos *et al*. 2011).

In addition, about half of the diclofop-resistant accessions, were at least equally or more sensitive to pinoxaden and mesosulfuron + iodosulfuron than the sensitive accession used as a standard in this study. It is also true, that crop fields among regions or in each region sometimes receive different herbicide (and non-herbicidal) weed control treatments and therefore, each field receives a somewhat unique evolutionary selection pressure. As a result, individual field canarygrass populations under herbicide selection will usually be different from each other (Travlos *et al*. 2011). This could be due to potentially different resistance mechanisms (enhanced metabolism and/or one or more different ACCase mutations) being present in different *P. minor* populations as well as other weed species (Maneechote *et al*. 1994; Travlos *et al*. 2011).

Conclusively, this survey has revealed a noticeable littleseed canarygrass resistance to the ACCase-inhibiting herbicide diclofop across five regions of a typical wheat producing prefecture of Greece. The resistance is closely associated with monoculture and lack of herbicide rotation. However, resistance to other ACCase-inhibiting herbicides was lower. Alternative herbicides, namely pinoxaden and mesosulfuron + iodosulfuron remained effective on more than 85% of *P. minor* accessions tested. Many biotypes are in the developing resistance category. This categorisation means that resistance evolved in these and other accessions seems to be still in progress, since the current weed management is still continuing. Thus, currently there is an opportunity to effectively control canarygrass by selecting from a diverse range of herbicides and other cultural practices. Unfortunately, the lack of new herbicide modes of action and the withdrawal of many of the available active ingredients due to the impact of European Union (EU) legislation on pesticides are aggravating the situation. The diversity of available chemical options is reduced, making resistance a major threat to the sustainability of several European cropping systems. Resistance is especially true where crop and herbicide rotation is absent or limited. However, there are still realistic chances for the reduction of selection pressure against R populations. Alternative herbicide, herbicide mixture, crop rotation and other agronomic practices such as early crop sowing, competitive cultivars, and an increased seeding rate can provide crops with a competitive edge over weeds (Gressel 1990; Wrubel and Gressel 1994; Cavan *et al*. 2000; Om *et al*. 2004; Travlos *et al*. 2009).

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