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First Case of Glufosinate-Resistant Rigid Ryegrass (*Lolium rigidum* Gaud.) in Greece

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Abstract: Repeated applications of the same herbicide(s), which are characterized by the same mode of action, increase selection pressure, which in turn favours the evolution of herbicide-resistant weeds. Glufosinate is a broad-spectrum non-selective herbicide being used for weed control for many years around the world. Rigid ryegrass (*Lolium rigidum* Gaud.) is an economically important grass weed in Greece. Recent complaints by growers about control failure of rigid ryegrass with glufosinate require further investigation and have been the basis of this study. The objectives of this study were to confirm the existence of glufosinate-resistant *L. rigidum* in Greece and evaluate the effect of *L. rigidum* growth stage on glufosinate efficacy. Twenty populations of rigid ryegrass from Greece were sampled from five regions, and whole plant dose–response studies were conducted for five populations under controlled conditions with eight rates of glufosinate (0.0, 0.098, 0.187, 0.375, 0.75, 1.5, 3.0, and 6.0 kg a.i. ha^{-1}). Glufosinate resistance was confirmed in three out of five populations with the level of resistance ranging from three-to seven-fold compared with the susceptible populations based on above-ground biomass reduction. Results also revealed that the level of glufosinate-resistance of rigid ryegrass was dependent on the growth stage at which it was applied.

Keywords: Dose response; glufosinate resistance; herbicide; growth stage

1. Introduction

Glufosinate (2-amino-4-[hydroxyl (methyl) phosphoryl] butanoic acid) is a non-selective, post-emergence, contact herbicide [1] which is commonly used in minimum-tillage farming systems, including orchards and vineyards. Glufosinate is the most efficacious inhibitor of glutamine synthetase (GS) [2]. GS constitutes an essential enzyme that converts L-glutamic acid to L-glutamine in the presence of ammonia and adenosine triphosphate (ATP) [3]. GS inhibition in higher plants results in ammonia accumulation within their cells. Intracellular ammonia accumulation, followed by reduced photosynthesis, leads to the subsequent death of treated plants [4].

Glufosinate can effectively control a wide spectrum of weeds, including several grass and broadleaf weed species [5–7]. Globally, the wide usage of glufosinate led to the first case of glufosinate resistance in 2009 in a Malaysian *Eleusine indica* population [8,9]. Currently, species including *E. indica* L., *Lolium perenne* L., and *L. perenne* ssp. *multiflorum* are reported to have evolved glufosinate-resistant biotypes [10].

Despite the fact that glufosinate-resistant crops have been widely available at a global level, there has been increasing concern about their impact on the evolution of weed resistance to herbicides, since applications are increasingand the selection pressure is higher. For instance, glufosinate-resistant rice volunteers pose a risk for severe weed-resistance problems [11,12]. In particular, monoculture cropping systems that depended on glufosinate-resistant rice for weed control were associated with the presence of resistant weeds within a period ranging from 3 to 8 years [13]. However, repeated use of glufosinate in minimum- or no-tillage systems can greatly increase the risk of glufosinate resistance, even in the absence of glufosinate-resistant crops.

Rigid ryegrass (*L. rigidum* Gaud.), belonging to the *Poaceae* family, is considered to be one of the major grass weed species in Mediterranean-climate regions [14,15]. Herbicide resistance of *Lolium* species has been reported in several habitats, such as agricultural fields, orchards, vineyards, and road sides [10], while rigid ryegrass is considered to be one of the most economically important weeds in some other countries, especially Australia [16]. Moreover, in the US, resistance to glufosinate has been confirmed in Italian ryegrass (*L. perenne* ssp. *multiflorum*) populations [10]. The rapid development of resistance in *L. rigidum* indicates the necessity for integrated weed management strategies which will reduce the accelerated evolution of resistance. The control of the rigid ryegrass populations in Greece was usually based on the intensive use of acetyl-CoA carboxylase ACCase inhibitors, acetolactate synthase ALS inhibitors, and glyphosate. Further to the evolution of resistance, glufosinate became one of the most widespread alternative chemical choices for farmers [17]. Unfortunately, glufosinate resistance and multiple resistance have already been reported in *L. rigidum* L. due to the accumulation of several resistance mechanisms [18,19]. Rigid ryegrass's ability to evolve different resistance mechanisms is attributed—among other contributory factors—to the following characteristics: its widespread distribution within cropping regions, prolific seed set, cross-fertilization, and, finally, its significant genetic variability and phenotypic plasticity [20].

The present study was conducted after receiving some complaints by Greek farmers regarding control failure of *Lolium* spp. by applying glufosinate to their fields. The objectives of this study were to confirm glufosinate-resistant rigid ryegrass in several regions of Greece and to evaluate the potential effect of the *Lolium* spp. growth stage on glufosinate efficacy.

2. Materials and Methods

2.1. Preliminary Screening

Putative glufosinate-resistant rigid ryegrass seeds were collected from 20 fields from the following regions of Greece: Attiki (AT), Etoloakarnania (ET), Fthiotida (FT), Korinthia (KO), and Viotia (VI) during the period of 2016–2017 (Table 1). AT, VI, and FT are located in the eastern and central part of the country, ET in the western part, and KOin the southern part of Greece. Crops include olives, vines, citrus, apple, and apricot. Some of the sites were reported to have poor rigid ryegrass control based on farmers' complaints, while five of the sites had never been treated with glufosinate in the past and had been characterized as susceptible (S). Seeds from 20 plants were collected from each field, as obtaining representative samples was a prerequisite for the experiments. It has to be noted that the weed was not restricted to field margins, while the above-mentioned 20 plants were sampled from a wider area of each vineyard and orchard, and not from a single patch of the weed.

Table 1. Region, geographical position, crop, and number of *L. rigidum* accessions included in the study.

Prefecture	Code	Positions	Crops	No. of Accessions
Attiki	AT	37°56′–37°59′ N, 23°53′–23°57′ E	Vineyards, Olive orchards	2
Etoloakarnania	ET	38°23′–38°32′ N, 21°15′–21°28′ E	Olive orchards, Orchards	9
Fthiotida	FT	39°08′–39°09′ N, 22°16′–22°27′ E	Vineyards, Orchards	5
Korinthia	KO	37°54′–37°57′ N, 22°41′–22°51′ E	Orchards, Vineyards	2
Viotia	VI	38°19′–38°20′ N, 23°05′–23°15′ E	Vineyards, Olive orchards	2

According to the procedure described in previous studies [21], five seeds from each accession were sown in 12 × 13 × 5 cm pots. A mix of herbicide-free soil from the field of the Agricultural University of Athens and a common peat substrate (1:1, *v*/*v*) was used. During the experiment, the pots were uniformly watered as needed and supplied with 50 mL pot^{-1} of modified Hoagland's solution (0.25%) every 10 days [22]. The position of the pots was changed every 3 days in order to ensure the necessary randomization. Plants were grown under 25/13 °C day/night temperature and natural sunlight. At the two- to three-leaf stage, the plants were sprayed with the recommended rate of glufosinate (0.75 kg a.i. ha^{-1}) in the formulation of Basta 15 SL® (glufosinate-ammonium, 150 g L^{-1}, Bayer CropScience AG, Leverkusen, Germany) using a custom-built, compressed-air, low-pressure flat-fan nozzle experimental sprayer, calibrated to deliver 300 L ha^{-1} at 250 kPa. Out of the 60 plants of each accession (5 plants × 12 pots), 30 were sprayed and 30 were kept as untreated. Plants were cut just above the soil surface and the dry weights of above-ground biomass were determined 21 days after treatment (DAT) after drying the samples at 60 °C for 48 h and presented as a percentage of the untreated control for each accession. The experiment was conducted twice.

2.2. Dose–Response Experiments

Results of the preliminary screening trial described above were used to discriminate the twenty accessions and proceed to further experiments. Similarly to the classification used by Travlos and Chachalis [21], populations showing a mean above-ground biomass lower than 40% of the untreated control biomass were considered "potentially susceptible", whereas populations exhibiting a mean above-ground biomass higher than 80% of the untreated control were considered "potentially resistant". Furthermore, populations with a mean above-ground biomass between 40% and 80% of the untreated control can be characterized as intermediate.

One susceptible, one intermediate, and three potentially resistant accessions from the various regions were selected for dose–response experiments. One accession from each region was chosen. The objective of this experiment was to determine the herbicide rate for a 50% reduction in biomass (GR_{50}). The experiment was conducted from 6 November 2016 to 7 January 2017 and was repeated from 13 December 2016 to 21 February 2017. Sowing was performed as described above. At the two- to three-leaf stage, the plants were sprayed with glufosinate at 0.0, 0.094, 0.187, 0.375, 0.75, 1.5, 3.0, and 6.0 kg a.i. ha^{-1}. These rates correspond to zero, one-eighth, one-quarter, one-half, one, two, four, and eight times the recommended rate of glufosinate for rigid ryegrass. Herbicide treatments were applied as described above, with pots (12 × 13 × 5 cm) arranged in a completely randomized design. Each pot (one plant per pot) was considered as a replicate and there were eight replicates per treatment. Plants were cut just above the soil surface and the dry weights of above-ground biomass were determined 21 days after treatment (DAT) after drying the samples at 60 °C for 48 h. The results were presented as a percentage of the untreated control for each accession.

The effect of phenological stage of rigid ryegrass plants on response to glufosinate was studied with an additional dose–response experiment which was conducted with plants sprayed at three phenological stages: (1) 2- to 3-leaf stage, (2) tillering stage, and (3) 6- to 8-leaf stage. One accession was selected on the basis of the previous dose–response experiment. Sowing was performed as previously described. For this experiment, a three-by-seven factorial completely randomized design was used, with three growth stages and six glufosinate rates plus the untreated control (0.0, 0.187, 0.375, 0.75, 1.5, 3.0, and 6.0 kg a.i. ha^{-1}), with eight replicates (plants) for each combination. All treatments were applied by using the same equipment as described above. The above-ground biomass was recorded 21 DAT and presented again as a percentage compared with the untreated control.

2.3. Statistical Analysis

Data obtained from the pot experiments were subjected to ANOVA and combined across experimental runs. Means were pooled over time and they were separated using Fisher's Protected LSD test at the $p = 0.05$ significance level. Furthermore, the level of resistance to glufosinate and its

effectiveness were estimated using interpolative probit analysis [23] to ascertain the concentration causing a 50% reduction of plant growth (GR_{50}) [24]. Resistance index (RI) was also estimated on the basis of the 50% growth reduction (GR_{50}) values from the susceptible and the potentially resistant population and computed as the $GR_{50(R)}/GR_{50(S)}$ ratio. For the experiment on the different growth stages, regression analysis was carried out. The regression analysis used arcsine-transformed data of(percentage of the control) and natural log of glufosinate rates [25]. The data were fitted to a four parameter logistic curve (Equation: y = min + (max − min)/ 1 + (x/EC50)^−Hillslope),using SigmaPlot, v.11 (Systat Software, Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. Preliminary Screening

Preliminary screening revealed significant differences in biomass reduction among the 20 populations collected from different locations of Greece, confirming presence of glufosinate-resistant rigid ryegrass in Greece. It has to be noted that the resistant accession was found in the area near susceptible accessions. Such an observation is in agreement with the findings of previous studies for other weed species [21,26]. Even if the field history was not known for all sites, conversation with growers from the orchards and vineyards with the resistance issues provided history of continuous glufosinate use at twoto fourtimes higher than the labeled rate. Thus, a continuous usage of glufosinate provided selection pressure and probably resulted in the evolution of glufosinate-resistant rigid ryegrass in Greece. Furthermore, almost half (45%) of the studied accessions had intermediate level of glufosinate resistance (Table 2). The frequent but not exclusive use of glufosinate was reported in some of these sites, and therefore, integrated weed management during the next years will be crucial to avoid further selection pressure of glufosinate.

Table 2. Glufosinate resistance status of the sampled accessions of rigid ryegrass after preliminary screening (potentially susceptible: biomass reduction >60% following treatment with 0.75 kg a.i. ha^{-1} glufosinate relative to untreated control; potentially resistant: biomass reduction <20%; intermediate: 20–60% biomass reduction).

Category	Accessions (no.)	Accessions (%)
Potentially susceptible	6	30
Intermediate	9	45
Potentially resistant	5	25
Total	20	100

3.2. Dose–Response Experiments

For the further determination of the level of glufosinate resistance by calculating GR_{50} (glufosinate rate required to kill 50% rigid ryegrass population) value, the trials were carried out with five accessions selected from the 20 accessions screened previously with glufosinate at 0.75 kg a.i. ha^{-1}. Dose–response regression curves (Figure 1) indicate significant differences in the response of the S population (AT2) when compared with the most R populations (e.g., ET6 and FT3). The estimated GR_{50} values for each of the selected accessions are reported in Table 3. A glufosinate rate of 0.52 kg ha^{-1} caused 50% of biomass reduction in accession AT2, whereas the same reduction was achieved with 3.84 kg ha^{-1} in population ET6, indicating a resistance index of 7.38. It is also noticeable that the recommended rate of glufosinate (i.e., 0.75 kg a.i. ha^{-1}) provided 95% and 16% control for AT2 and ET6 populations, respectively. In the present study, glufosinate rates required to control resistant accessions were about threeto seventimes higher than those required to control the susceptible accessions. These values were either similar to or lower than those reported for *L. rigidum* in Australia and Spain, respectively [19,27]. Moreover, the differences found between glufosinate-susceptible and -resistant rigid ryegrass biotypes

were either equal to or higher than the corresponding values previously confirmed in other gramineous species such as *L. perenne* and *E. indica* [8,9,28].

Table 3. Glufosinate dose resulting in a 50% reduction in above-ground plant biomass (GR_{50}) for the five selected accessions (SE are given in parentheses). AT2 is the chosen susceptible accession. Resistance index (RI) is also given for each accession (GR_{50} of accession/GR_{50} of AT2).

	AT2	ET6	KO2	FT3	VI2
GR_{50} [a]	0.52 (0.22) c	3.84 (0.61) a	0.61 (0.13) c	2.8 (0.48) ab	1.6 (0.36) b
RI	1.00 c	7.38 a	1.17 c	5.38 ab	3.08 b

[a] Values followed by different letters in each row are significantly ($p \leq 0.05$) different according to Fisher's Protected LSD test.

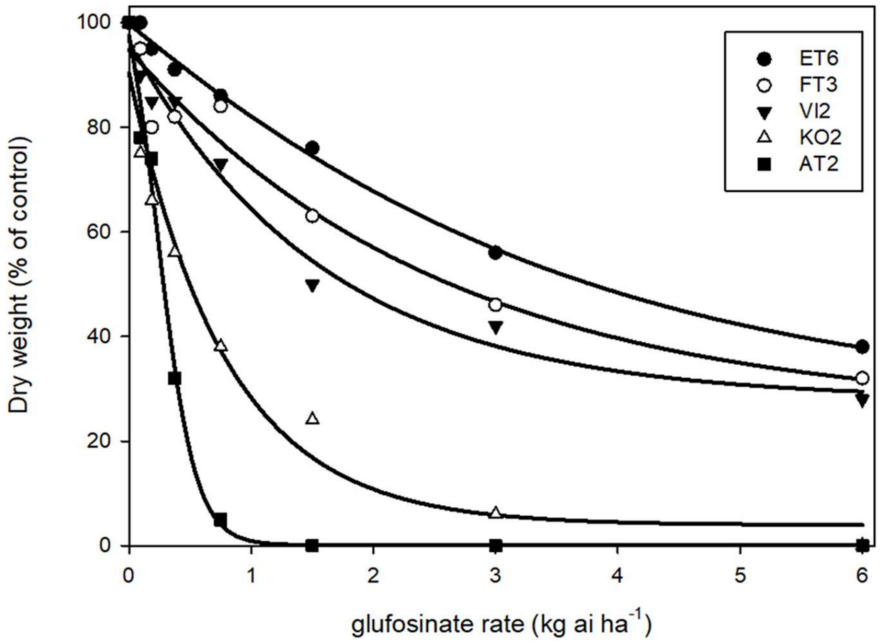

Figure 1. Ryegrass biomass of five accessions (AT2, ET6, KO2, FT3, and VI2) in response to increasing glufosinate rates.

Our experiments regarding the potential effect of phenological stage on efficacy reveal a clear growth stage by glufosinate efficacy interaction (Figure 2). In the intermediate population tested, dry weight reduction was highest for the youngest growth stage (2–3 leaves), with a maximum reduction of 72% at 6 kg ha^{-1} of glufosinate. The response to glufosinate was less for the tillering and 6–8 leaves stages, with a maximum reduction in dry weight of approximately 34–50% even at 8× the recommended rate of glufosinate. Reduced efficacy of glufosinate at the later growth stages is probably due to the following factors: glufosinate being a contact herbicide, its poor translocation, along with the low growth rate and dry-matter accumulation of the mature plants. In addition, previous studies with glufosinate indicate that its efficacy depends on both environmental conditions and weed species [29,30], while farmers often apply glufosinate after tillering and pose a further risk on its efficacy [19]. Our results are also in accordance with other recent studies [21,25]. For instance, glyphosate-resistant horseweed treated with glyphosate at three different stages of growth was more responsive at the seedling stage than at the large rosette or bolting stages. Furthermore, the survival and seed production of these potentially resistant plants due to the late treatment will probably result in an increase of the ratio of resistant plants in the accession [25].

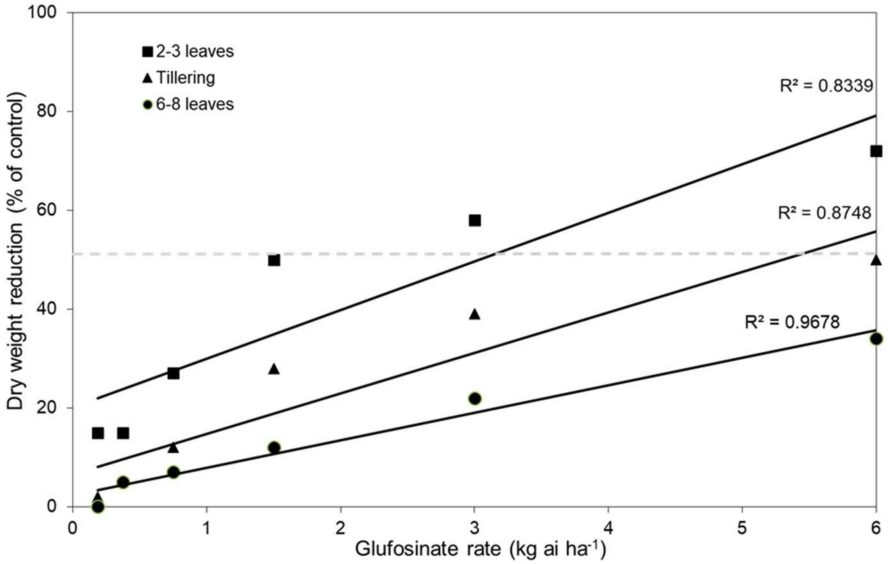

Figure 2. Regression of aboveground biomass response (accession VI2) of *L. rigidum* at different growth stages to increasing glufosinate rate. Untransformed data are shown, although regression analysis was performed using the natural log of glufosinate rate and arcsine-transformed fresh weight values (coefficient of determination, R^2 values are also shown).

4. Conclusions

The results of the present study confirm the first report of glufosinate-resistant weed in Greece with the specific case of *L. rigidum* meeting all the relevant criteria for confirmation of herbicide-resistant weeds [10]. This weed already constitutes a problem for the farmers when using glufosinate at the recommended rate and consequently our timely survey will allow the awareness and appropriate management. Integrated weed management practices should be adopted in regions of existence or potential existence of glufosinate-resistant *Lolium* spp. including tillage, mowing, and cover crops [31]. Moreover, the results of this study also highlight the importance of the growth stage in order to achieve maximum glufosinate efficacy and minimize the risk of resistance. Further herbicide resistance screening and monitoring should be conducted on a systematic basis and in a range of herbicides, since multiple resistance is another challenge and an additional risk [19,32].

Finally, equally crucial is the necessity for several pro-active measurements to be taken by the farmers along with the proper use of glufosinate (e.g., in terms of stage, rates, and conditions) in order to avoid the spread of herbicide resistance issues and keep sustainable the use of pesticides in a balance with cultural practices [33]. Proactive herbicide resistant management may include the early detection of resistance through monitoring fields, management of weed seed bank, usage of herbicides with different mechanisms of action in the same spray tank, and the appropriate and accurate application of herbicides. It is noteworthy to mention that weed characteristics should also be taken into account [31].

The resistance management principles outlined above will help delay or prevent resistance from occurring as well as prove beneficial in managing *L. rigidum* glufosinate or multiple resistance.

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Conflicts of Interest: The authors declare no conflict of interest.

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