Research Article

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Forward selection for multiple resistance across the non-selective glyphosate, glufosinate and oxyfluorfen herbicides in *Lolium* weed species

Pablo Fernández,[a] Ricardo Alcántara,[a] María D Osuna,[b] Martin M Vila-Aiub[c*] and Rafael De Prado[a]

Abstract

BACKGROUND: In the Mediterranean area, *Lolium* species have evolved resistance to glyphosate after decades of continual use without other alternative chemicals in perennial crops (olive, citrus and vineyards). In recent years, oxyfluorfen alone or mixed with glyphosate and glufosinate has been introduced as a chemical option to control dicot and grass weeds.

RESULTS: Dose–response studies confirmed that three glyphosate-resistant *Lolium* weed species (*L. rigidum*, *L. perenne*, *L. multiflorum*) collected from perennial crops in the Iberian Peninsula have also evolved resistance to glufosinate and oxyfluorfen herbicides, despite their recent introduction. Based on the LD_{50} resistance parameter, the resistance factor was similar among *Lolium* species and ranged from 14- to 21-fold and from ten- to 12-fold for oxyfluorfen and glufosinate respectively. Similarly, about 14-fold resistance to both oxyfluorfen and glufosinate was estimated on average for the three *Lolium* species when growth reduction (GR_{50}) was assessed. This study identified oxyfluorfen resistance in a grass species for the first time.

CONCLUSION: A major threat to sustainability of perennial crops in the Iberian Peninsula is evident, as multiple resistance to non-selective glyphosate, glufosinate and oxyfluorfen herbicides has evolved in *L. rigidum*, *L. perenne* and *L. multiflorum* weeds.

Keywords: herbicide resistance evolution; *Lolium multiflorum*; *Lolium perenne*; *Lolium rigidum*; olive groves

1 INTRODUCTION

Glyphosate has been for decades the leading and most widely used herbicide in perennial crops such as olives, citrus and grapes. With no other chemical alternatives, continuous use of glyphosate has unavoidably led to the evolution of glyphosate resistance.[1] After the first documented case found in *Lolium rigidum* from Australia,[2] a total of 32 glyphosate-resistant species have been characterised in different agroecosystems of the world.[3]

In large parts of the European Mediterranean, the widespread use of cover crops to reduce fertile soil erosion in perennial crops makes necessary the use of high glyphosate doses to control grass and dicot weeds.[4] This crop management has inevitably led to the emergence of glyphosate-resistant species within the *Lolium* genus, including *L. perenne* (Portugal) and *L. multiflorum* and *L. rigidum* (Spain).[5] Thus, the use of oxyfluorfen (PPO inhibitor) in pre-emergence and/or early post-emergence alone or in tank mixtures with glyphosate has increased significantly. Similarly, the use of glufosinate [glutamine synthetase (GS) inhibitor] in late post-emergence (from tillering to flowering stage) complements the chemical alternatives to the loss of glyphosate in these perennial crop farming systems.

Nowadays, the use of these non-selective and broad-spectrum chemical options (i.e. oxyfluorfen and glufosinate) to control glyphosate-resistant weeds has continued under no other chemical rotations, and, as expected, oxyfluorfen and glufosinate weed control efficacies have declined.

Multiple herbicide resistance evolves when various dissimilar resistance mechanisms each encoded by particular resistance genes coexist at the individual and/or population level, endowing resistance to several herbicides with different modes of action.[6] Given its importance in modern agriculture, the most serious multiple herbicide resistance scenarios are those involving glyphosate. Half of the glyphosate resistance cases include cases of multiple resistance.[3]

The aim of the present study was to characterise phenotypically the multiple resistance to non-selective glyphosate, glufosinate

* *Correspondence to: MM Vila-Aiub, IFEVA-CONICET, Facultad de Agronomía, Universidad de Buenos Aires (UBA), Av. San Martín 4453, Buenos Aires (1417), Argentina. E-mail: vila@ifeva.edu.ar*

a *Departamento de Química Agrícola y Edafología, Universidad de Córdoba, Córdoba, Spain*

b *Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX), Guadajira-Badajoz, Spain*

c *IFEVA-CONICET – Departamento de Ecología, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina*

Table 1. History of different *Lolium* weed species used in this study

Species[a]	Location[b]	Crop	Glyphosate[c]	Glufosinate[c]	Oxyfluorfen[c]
L. rigidum	Jaén/Spain	Olive groves	>15/720	5/750	>10/720
L. perenne	Douro/Portugal	Vineyard	>10/720	2/750	>10/720
L. multiflrorum	Jaén/Spain	Olive groves	>15/720	2–3/750	>10/720

[a] The susceptible populations were collected from the same locations as the resistant populations.
[b] City/country.
[c] Number of herbicide application years/herbicide field rate (g ha^{-1}).

and oxyfluorfen herbicides in several weed populations of the genus *Lolium* currently infesting different perennial crops in the Iberian Peninsula (Portugal and Spain).

2 MATERIALS AND METHODS

2.1 Collection and evaluation of putative herbicide-resistant *Lolium* spp. weeds

In 2012, seeds of different species of *Lolium* spp. (see below) were collected from plants that survived glyphosate at doses normally used in areas with perennial crops in the Iberian Peninsula (Table 1). Subsequently, farmers switched to glufosinate and oxyfluorfen herbicides alone or mixed with glyphosate to gain a better control of these *Lolium* grasses. After several years of use of these new herbicides, farmers started to report a lower herbicide weed control efficacy (Table 1). Seeds collected from 25 mature plants in the field were stored under laboratory conditions (25 °C) for 2 weeks and then in paper bags at 4 °C.

Molecular analyses (see below) revealed that field-collected plants corresponded to three *Lolium* species: *L. multiflorum*, *L. perenne* and *L. rigidum*. Two experiments were conducted in an experimental field of the University of Córdoba (Spain) in order to determine the assumed multiple resistance level to glyphosate, glufosinate and oxyfluorfen in these three species (Fig. 1).

2.1.1 First experiment: glyphosate-selected progeny (R1)

In 2013, approximately 500 seeds of these *Lolium* species were sown directly into trays (40 × 60 × 15 cm) containing a mixture of sand and peat (2:1, v/v) and placed in a greenhouse at 28/20 °C day/night under a 16 h photoperiod with 200 µmol m^{-2} s^{-1} photon flux density and 80% relative humidity. At the 3–4-leaf stage, plants of the three *Lolium* species were treated with glyphosate at 720 g ae ha^{-1} using a laboratory spray chamber equipped with a flat-fan nozzle (TeeJet 8002 EVS) with a total output volume of 250 L ha^{-1} of water at a pressure of 200 kPa. This laboratory spray chamber was employed in all subsequent experiments. Four weeks after glyphosate treatment, plant survival was estimated and seeds produced from surviving plants (progeny R1) were collected and stored in paper bags for all subsequent trials.

Seeds from *L. multiflorum*, *L. perenne* and *L. rigidum* populations never exposed to herbicides were collected from adjacent areas and used as reference control. About 500 seeds of each reference species were grown as described above and treated with glyphosate at 360 g ae ha^{-1}. No plant survival was observed beyond 4 weeks after glyphosate treatment, and thus these populations were regarded as glyphosate susceptible (S) in the following experiments.

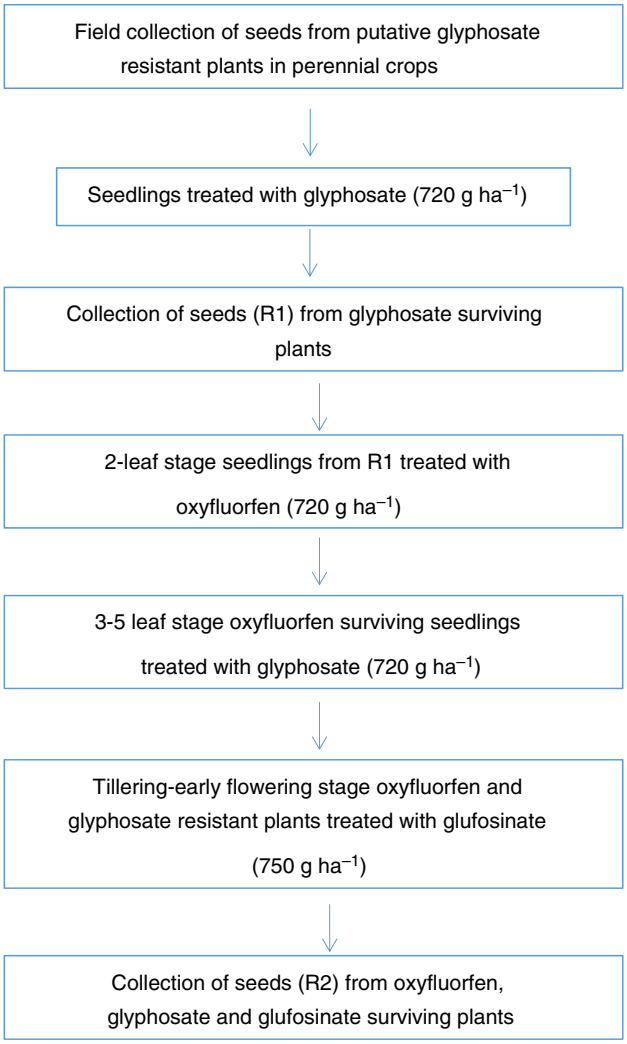

Figure 1. Selection of plant material to assess the level of multiple resistance in *L. rigidum*, *L. perenne* and *L. multiflorum* populations.

2.1.2 Second experiment: glyphosate-reselected and oxyfluorfen- and glufosinate-selected progeny (R2)

In 2015, 1000 seedlings from progeny R1 and reference population (S) of each *L. multiflorum*, *L. perenne* and *L. rigidum* were transplanted into pots containing an organic substrate. At the two-leaf stage, plants were treated with oxyfluorfen at 720 g ha^{-1} (label rate) using a Pulverex backpack sprayer with a T-coupling for the wand equipped with four flat-fan nozzles, at a spraying pressure of 200 kPa, and calibrated to deliver a volume of 200 L ha^{-1}.

During the first days after treatment (DAT), R1 and S plants stopped growing and exhibited burns on leaves. After 1 week, all S plants were controlled, while about 50% of the R1 plants survived the oxyfluorfen treatment and continued their growth. At the 3–5-leaf stage, glyphosate at label rate (720 g ae ha^{-1}) was applied to both the R1 oxyfluorfen-surviving plants and a new set of glyphosate-susceptible plants (S), following the same procedure as described above. Two weeks after treatment, all S plants were controlled, while more than 95% of the R1 oxyfluorfen-surviving plants survived the glyphosate treatment. Finally, between the tillering and early flowering stage, another set of herbicide-susceptible plants (S) and the R1 oxyfluorfen- and glyphosate-surviving plants were treated with glufosinate at label rate (750 g ha^{-1}). As in the same case of oxyfluorfen, glufosinate showed a rapid effect during the first days after treatment, with the appearance of plant chlorosis and necrosis. However, whereas more than 80% of R1 plants survived the glufosinate treatment, with new leaves being developed at 7 DAT, all S plants died at 14 DAT. Seeds from oxyfluorfen-, glyphosate- and glufosinate-resistant plants of the three *Lolium* species were collected and subjected to further resistance analysis. These seeds, hereinafter, are denoted as progeny R2 (R2 *L. multiflorum*, R2 *L. perenne* and R2 *L. rigidum*).

2.2 Identification of *Lolium* weed species using molecular markers

Identification of *Lolium* species is difficult because of similar morphological traits and genetic variation among species. In this study, AFLP markers were used as a system for identifying *Lolium* species.

The plant material used was from the reference susceptible (S) and putative resistant (R) populations (Table 1). Twelve plants (four glyphosate treated and non-treated and four reference) of each species (*L. multiflorum*, *L. rigidum*, *L. perenne*) were used for molecular analysis. Additionally, eight reference susceptible *L. temulentum* plants were included in the analysis.

DNA was extracted from the leaf tissue (50 mg), using the Speedtools DNA Extraction Plant kit (Biotools, Madrid, Spain). The quality and concentration of the DNA were evaluated by spectrophotometric analysis with 260 and 280 nm light absorption. AFLP analysis was carried out using the fluorescent AFLP IRDye kit for large plant genome analysis (LI-COR Biosciences, Lincoln, NE). Template preparation was performed following the protocol included in the kit, including digestions with *Eco*RI and *Mse*I restriction enzymes (Invitrogen, Carlsbad, CA). For selective amplification, 12 primer pairs were used: E36-M48 (E-ACC MCAC)/E36-M60 (E-ACC MCTC)/E37-M49 (E-ACG MCAG)/E38-M50 (E-ACT MCAT)/E40-M61 (E-AGC MCTG)/E35-M49 (E-ACA MCAG)/E36-M49 (E-ACC MCAG)/E35-M61 (E-ACA MCTG)/E40-M62 (E-AGC MCTT)/E32-M60 (E-AAC MCTC)/E33-M50 (E-AAG MCAT)/E35-M48 (E-ACA MCAC).

AFLP products were separated by polyacrylamide electrophoresis using an automated sequencer (LICOR 4300). Polymorphic AFLP markers and primers were identified and individuals were scored for presence or absence of AFLP fragments using the computer package SAGAMX 2 GENERATION. UPGMA analysis was performed with AFLP marker data using the computer program NTSYSpc 2.2.

2.3 Glyphosate dose–response study

Three hundred seeds of putative resistant R1, R2 and S of the three *Lolium* species were germinated in trays (12 × 12 × 6 cm) containing the same substrate as described before and placed in a growth chamber of similar controlled environmental conditions as before. At 4–5 days after germination, individual seedlings were transplanted into pots (6 × 6 × 8 cm) and grown under fluctuating 26/20 °C day/night with a 14 h photoperiod with 200 μmol m^{-2} s^{-1} photon flux density and 80% relative humidity. As glyphosate (EPSPS inhibitor) is used by farmers in early post-emergence, at the 3–5-leaf stage, R and S seedlings of the three *Lolium* species were treated with increasing glyphosate doses: 0, 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 g ha^{-1} (Roundup Energy 45% w/v, SL; Monsanto, Madrid, Spain).

2.4 Oxyfluorfen dose–response study

Oxyfluorfen (PPO inhibitor) is a pre-emergence and/or early post-emergence herbicide often used in late autumn when temperatures are relatively low compared with when glyphosate and glufosinate are used. Seed germination and seedling transplanting were carried out as described earlier. *Lolium* seedlings were grown in a growth chamber with a temperature of 22/17 °C day/night under a 12 h photoperiosd with 200 μmol m^{-2} s^{-1} photon flux density and 80% relative humidity. Preliminary tests showed a greater oxyfluorfen efficacy when sprayed in plants with one expanded leaf. Oxyfluorfen (Goal Supreme 48%, w/v, SC; Dow AgroScience, Madrid, Spain) was applied at increasing doses (0, 15, 30, 60, 120, 240, 400, 800, 1200, 1600 and 2000 g ha^{-1}) in R1, R2 and S plants at the two-leaf stage.

2.5 Glufosinate dose–response study

Seed germination of the three *Lolium* species was performed as described above. A common practice among farmers is the treatment with glufosinate (GS inhibitor) to control *Lolium* between tillering and early flowering (8–10-leaf stage). Thus, R1, R2 and S plants were treated at the mentioned growth stage with increasing glufosinate doses: 0, 12.5, 25, 50, 100, 200, 400, 600, 800, 1000, 1200 and 1400 g ha^{-1} (Finale 15% w/v, SL; Bayer, Madrid, Spain).

2.6 Experimental design and statistical analyses

For all herbicide dose–response studies, six replicates of ten pots (one plant per pot) per herbicide dose were arranged in a completely randomised block in repeated experiments ($n = 2$). Four weeks after herbicide treatment, herbicide effects on plant survival and above-ground vegetative biomass were assessed. Non-linear regression analysis was carried out to estimate the herbicide resistance parameters (LD_{50}, GR_{50}) for each R and S population and *Lolium* weed species. The assessed plant survival and biomass data from repeated experiments were pooled and fitted to a four-parameter log-logistic model:

$$Y = c + \frac{d - c}{1 + (x/xo)\,expb} \quad (1)$$

where Y is the survival or above-ground biomass at herbicide x dose, c and d are the coefficients corresponding to the lower and upper asymptotes and b is the slope of the curve at xo which is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes (i.e. LD_{50}, GR_{50}). Regression analysis was conducted using Sigmaplot 10.0 software (Systat Software Inc., San Jose, CA). Quantitative herbicide resistance levels were estimated as the resistance factor (RF) computed as the $LD_{50(R)}/LD_{50(S)}$ and $GR_{50(R)}/GR_{50(S)}$ ratios. Data were pooled, and a non-linear, log-logistic regression model [equation 1] was fitted to data.

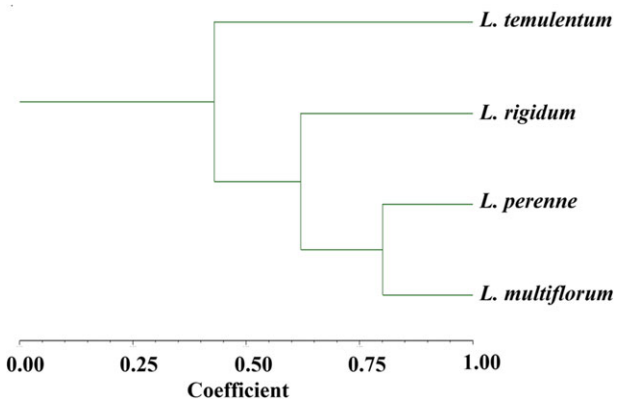

Figure 2. Dendogram of the genetic similarities among *Lolium* species after UPGMA analysis performed with AFLP marker data.

3 RESULTS

3.1 Identification of *Lolium* weed species using molecular markers

The UPGMA analysis separated the *Lolium* populations into two large clusters (Fig. 2). The first group contains individuals from the self-pollinated *L. temulentum* species. The second major cluster comprises individuals from the outcrossing *L. rigidum*, *L. multiflorum* and *L perenne* species. In this group, *L. multiflorum* and *L. perenne* are joined as a different minor cluster with the highest genetic similarity (0.80) (Fig. 2). According to these results, the *Lolium* species infesting perennial crops from the Iberian Peninsula and used in this study correspond to *L. rigidum*, *L. multiflorum* and *L perenne*.

3.2 Herbicide dose–response analysis

The effect of glyphosate, oxyfluorfen and glufosinate on the three *Lolium* species collected in the field was investigated. The herbicide effect was analysed on plant samples that were subjected to one (R1) and two (R2) herbicide selections (Fig. 1). The herbicide response of the R1 selection resembles the response of the current field *Lolium* populations, while the response of the R2 selection reflects the response of populations that underwent an extra simultaneous selection at the single plant level involving the three herbicides (Fig. 1).

3.2.1 *Glyphosate dose–response study*

Results from the dose–response experiments show that plants from the three *Lolium* species exhibit equal glyphosate resistance levels (R2 progeny selection) (Table 2 and Fig. 3). In relation to R1 plant survival and growth of surviving plants after herbicide treatment, the estimated glyphosate resistance factor (RF) ranged from nearly 5 to nearly 21 and from 4–5- to 15-fold resistance respectively (Table 2). With the exception of *L. rigidum*, which showed no difference in RF (survival) between the R1 and R2 progenies, the *L. perenne* and *L. multiflorum* RF increased about 5 times for the R2 selection compared with the R1 selection (Table 2). A similar trend was observed in RF when estimated from growth data (GR_{50}) (Table 2).

3.2.2 *Oxyfluorfen dose–response study*

At doses lower than 100 g ha^{-1} of oxyfluorfen, plants of the three sensitive *Lolium* species were totally controlled (Fig. 4). Twenty-four hours after treatment (HAT), susceptible plants showed drying of green tissues, followed by a rapid loss of pigments and later necrosis and death. Individuals from the R1 progeny selection of the three species required doses higher than 200 g ha^{-1} to display a significant mortality level, although they were totally controlled at 400 g ha^{-1}, a dose significantly lower than the recommended field label of 720 g ha^{-1} (Fig. 4). Thus, the survival and growth of R1 progeny plants under oxyfluorfen treatment accounted for a moderate to marginal RF (Table 3).

Table 2. Parameter estimates derived from the logistic analysis of plant survival (LD_{50}) and growth (GR_{50}) of *L. rigidum*, *L. perenne* and *L. multiflorum* populations under increasing glyphosate rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). S corresponds to plants from a herbicide-unselected population

Species	Population	*d*	*b*	R^2	LD_{50} (g ha^{-1})	RF
L. rigidum	S	98.5 ± 2.8	4.7 ± 0.8	0.97	179.0 ± 11.1	–
	R1	100.0 ± 0.7	27.8 ± 8.5	0.95	3846.1 ± 934.9	21.4
	R2	100.0 ± 0.6	22.2 ± 1.6	0.96	3981.6 ± 161.8	22.2
L. perenne	S	101.0 ± 4.2	1.9 ± 0.3	0.96	126.1 ± 12.3	–
	R1	100.8 ± 2.1	2.2 ± 0.4	0.97	566.0 ± 49.7	4.4
	R2	99.2 ± 3.4	3.95 ± 0.6	0.97	2205.5 ± 37.8	17.4
L. multiflorum	S	101.5 ± 4.2	2.1 ± 0.3	0.96	112.1 ± 10.2	–
	R1	100.0 ± 0.8	32.5 ± 7.3	0.99	515.5 ± 88.0	4.5
	R2	99.2 ± 0.3	3.9 ± 0.8	0.96	2205.5 ± 79.5	19.6
Species	Population	*d*	*b*	R^2	GR_{50} (g ha^{-1})	RF
L. rigidum	S	98.9 ± 0.9	4.9 ± 0.3	0.99	77.1 ± 1.3	–
	R1	98.1 ± 0.9	3.3 ± 0.6	0.97	1188.8 ± 98.2	15.4
	R2	100.8 ± 3.7	2.3 ± 0.1	0.99	1888.2 ± 78.8	24.5
L. perenne	S	100.0 ± 0.8	3.5 ± 0.4	0.99	90.9 ± 1.7	–
	R1	99.2 ± 0.6	2.9 ± 0.2	0.99	443.7 ± 17.9	4.8
	R2	103.1 ± 4.6	1.4 ± 0.1	0.99	1102.6 ± 59.4	12.1
L. multiflorum	S	100.9 ± 1.7	2.2 ± 0.1	0.98	57.1 ± 1.9	–
	R1	95.4 ± 1.3	3.4 ± 0.3	0.98	209.8 ± 6.4	3.6
	R2	101.2 ± 1.1	1.3 ± 0.2	0.98	1083.6 ± 43.8	18.9

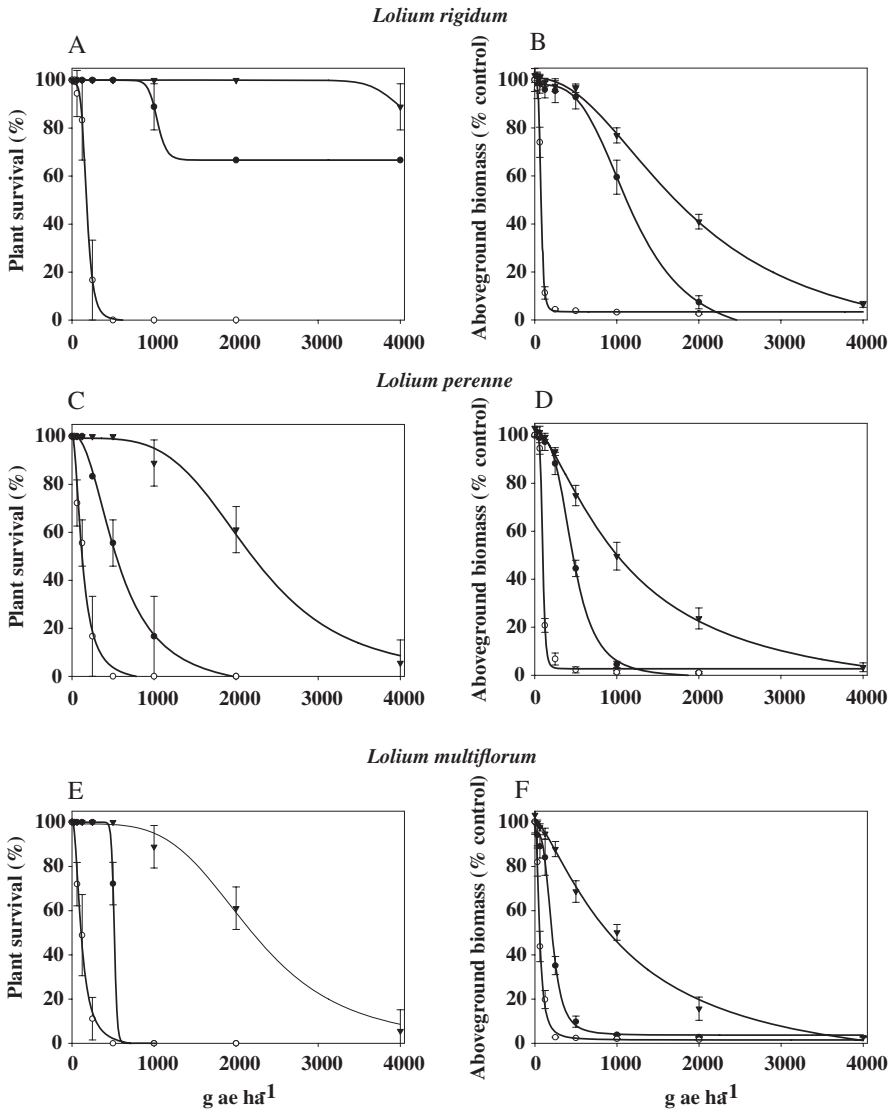

Figure 3. Plant survival (A, C, E) and above-ground biomass (B, D, F) from progenies R1 (●) and R2 (▲) and susceptible (○) *L. rigidum*, *L. perenne* and *L. multiflorum* populations in response to increasing glyphosate rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). Solid lines represent predicted values derived from non-linear regression analysis. Symbols denote mean ($n = 12$) ± standard error of the mean.

Reselection of plants with oxyfluorfen at 720 g ha^{-1} resulted in a progeny (R2) from the three *Lolium* species with significantly higher LD_{50} and GR_{50} values accounting for higher RFs (Fig. 4 and Table 3). Surviving plants of the three *Lolium* species when exposed to the oxyfluorfen dose label showed damage symptoms but recovered and reassumed growth a week after treatment. *L. rigidum* exhibited the highest LD_{50} parameter (1274 g ha^{-1}) compared with *L. perenne* (903 g ha^{-1}) and *L. multiflorum* (872 g ha^{-1}) (Table 3), which accounted for a higher RF (21 versus 19 versus 14 respectively). The same trend was observed in the RF estimated from GR_{50} data, where *L. rigidum* showed a significantly higher RF (40) than both *L. perenne* (13) and *L. multiflorum* (14) (Table 3).

3.2.3 *Glufosinate dose–response study*

The three glyphosate-susceptible *Lolium* species were also susceptible to glufosinate at 200–300 g ha^{-1} (Table 4 and Fig. 3). Glufosinate phytotoxicity was rapidly evident at 36 HAT, and plants did not show any recovery or regrowth within 21 days after herbicide treatment.

R1 populations from the three species showed nearly 100% mortality when exposed to glufosinate doses of 400–600 g ha^{-1}, accounting for marginal RF (Fig. 5 and Table 4). However, similarly to the response to oxyfluorfen, the second herbicide selection resulted in R2 progeny plants from the three *Lolium* species with higher survival and growth even at glufosinate recommended field doses (750 g ha^{-1}) (Fig. 5). This resulted in significantly higher LD_{50} and GR_{50} values accounting for a higher RF in R2 plants from the three species (Fig. 5 and Table 4). Although resistant *Lolium* plants from the three species showed some signs of phytotoxicity the first week after treatment at 750 g ha^{-1}, they completely recovered at the end of the trial.

The glufosinate LD_{50} values were similar for the resistant plants of the three species, which ranged between 891 (*L. multiflorum*) and 1095 g ha^{-1} (*L. rigidum*), as compared with those for the susceptible plants (70–105 g ha^{-1}) (Table 4). The LD_{50} values

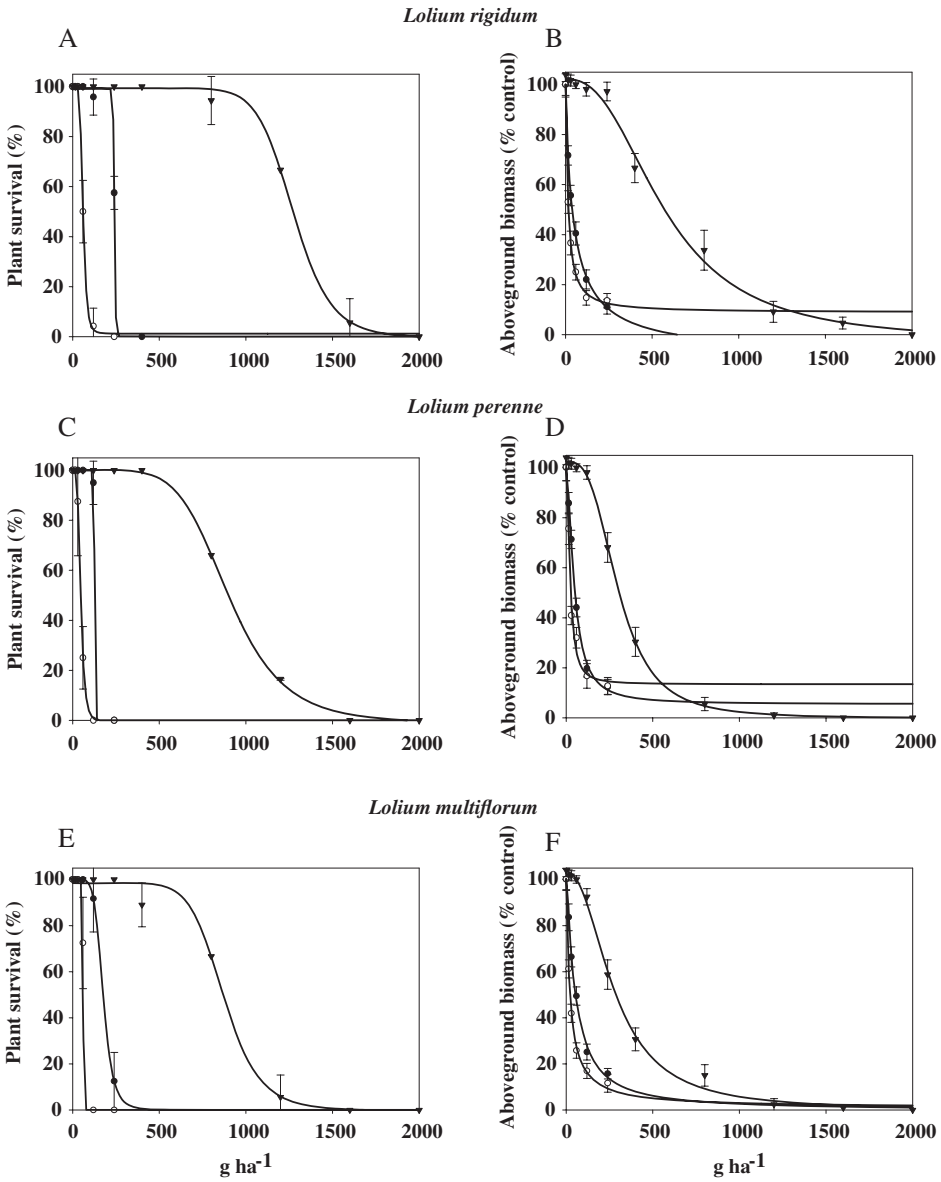

Figure 4. Plant survival (A, C, E) and above-ground biomass (B, D, F) from progenies R1 (●) and R2 (▲) and susceptible (○) *L. rigidum*, *L. perenne* and *L. multiflorum* populations in response to increasing oxyfluorfen rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). Solid lines represent predicted values derived from non-linear regression analysis. Symbols denote mean ($n = 12$) ± standard error of the mean.

accounted for a 10–12-fold resistance factor (Table 4). Similarly, the glufosinate resistance factor associated with growth (GR_{50}) was about 12–15-fold for resistant plants of the three *Lolium* species (Table 4).

4 DISCUSSION

Multiple herbicide resistance is the result of the accumulation of resistance genes each endowing resistance to herbicides with different modes of action.[7] For example, genes involved in both herbicide detoxification and target-site point mutations in herbicide target proteins have often been reported in herbicide-resistant weed populations.[8,9] The coexistence of multiple resistance genes may result from herbicide selection of independent mutational events that stack new alleles at the population and/or individual level.[10] Alternatively, multiple resistance may arise from gene flow processes (seed, pollen) between individuals from distinct populations. This is especially the case for cross-pollinated species, such as most species of the *Lolium* genus, in which individuals from interconnected populations at the agricultural landscape share male gametes as pollen and thus accumulate several herbicide resistance alleles.[11,12]

Regardless of the exact mechanism of origin, the results of the present study show that three *Lolium* species, *L. rigidum*, *L. perenne* and *L. multiflorum*, present in perennial olive groves and vineyard crops in the Iberian Peninsula, exhibit resistance to the three widely used non-selective and broad-spectrum glyphosate, glufosinate and oxyfluorfen herbicides. Given the mode of action of these three non-selective herbicides, it is highly unlikely that resistance to them is endowed by a single resistance gene, which in turn makes it possible to speculate that multiple resistance genes have been selected in populations of the three *Lolium* species.[10]

Table 3. Parameter estimates derived from the logistic analysis of plant survival (LD_{50}) and growth (GR_{50}) of *L. rigidum*, *L. perenne* and *L. multiflorum* populations under increasing oxyfluorfen rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). S corresponds to plants from a herbicide-unselected population

Species	Population	*d*	*b*	R^2	LD_{50} (g ha^{-1})	RF
L. rigidum	S	100.6 ± 2.1	5.8 ± 2.3	0.98	59.7 ± 1.4	–
	R1	100.1 ± 1.0	3.8 ± 0.8	0.99	286.9 ± 28.8	4.8
	R2	99.3 ± 0.7	11.7 ± 1.5	0.99	1274.4 ± 14.5	21.3
L. perenne	S	100.3 ± 4.1	4.3 ± 0.9	0.96	46.8 ± 3.4	–
	R1	100.0 ± 1.1	11.4 ± 0.3	0.99	155.5 ± 6.7	3.3
	R2	100.1 ± 0.4	5.7 ± 0.1	0.99	903.1 ± 3.8	19.2
L. multiflorum	S	100.0 ± 2.5	31.7 ± 2.9	0.97	61.8 ± 4.7	–
	R1	100.0 ± 1.4	6.2 ± 1.5	0.96	175.9 ± 4.5	2.8
	R2	98.4 ± 0.6	8.4 ± 1.1	0.98	872.5 ± 16.6	14.1
Species	Population	*d*	*b*	R^2	GR_{50} (g ha^{-1})	RF
L. rigidum	S	100.1 ± 1.4	1.1 ± 0.1	0.98	14.2 ± 0.8	–
	R1	99.9 ± 1.4	0.9 ± 0.0	0.98	46.4 ± 4.8	3.2
	R2	102.1 ± 0.7	2.4 ± 0.1	0.98	574.5 ± 15.1	40.5
L. perenne	S	100.9 ± 2.1	1.8 ± 0.2	0.96	22.7 ± 1.3	–
	R1	99.3 ± 1.4	1.7 ± 0.1	0.98	47.8 ± 2.5	2.1
	R2	102.3 ± 0.5	3.1 ± 0.2	0.99	302.4 ± 3.7	13.3
L. multiflorum	S	100.2 ± 1.3	1.2 ± 0.1	0.98	19.3 ± 1.1	–
	R1	99.9 ± 1.5	1.3 ± 0.1	0.98	51.9 ± 5.1	2.6
	R2	103.4 ± 0.7	2.1 ± 0.1	0.99	279.7 ± 5.9	14.4

Table 4. Parameter estimates derived from the logistic analysis of plant survival (LD_{50}) and growth (GR_{50}) of *L. rigidum*, *L. perenne* and *L. multiflorum* populations under increasing glufosinate rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). S corresponds to plants from a herbicide-unselected population

Species	Population	*d*	*b*	R^2	LD_{50} (g ha^{-1})	RF
L. rigidum	S	100.3 ± 1.6	4.2 ± 0.6	0.99	105.7 ± 2.8	–
	R1	97.8 ± 2.4	31.9 ± 6.3	0.96	313.0 ± 29.9	2.9
	R2	100.2 ± 1.7	11.1 ± 0.8	0.98	1095.2 ± 47.9	10.3
L. perenne	S	97.9 ± 3.4	7.1 ± 4.2	0.97	85.6 ± 8.4	–
	R1	100.9 ± 0.8	75.2 ± 5.2	0.98	303.8 ± 43.5	3.5
	R2	98.2 ± 1.2	8.6 ± 1.4	0.96	995.3 ± 26.1	11.6
L. multiflorum	S	97.8 ± 1.8	7.1 ± 0.8	0.99	70.7 ± 2.7	–
	R1	97.9 ± 1.9	5.0 ± 0.8	0.97	292.2 ± 11.2	4.1
	R2	98.8 ± 0.8	7.9 ± 1.2	0.97	891.4 ± 20.8	12.6
Species	Population	*d*	*b*	R^2	GR_{50} (g ha^{-1})	RF
L. rigidum	S	98.9 ± 1.4	6.9 ± 1.1	0.98	61.0 ± 2.1	–
	R1	100.2 ± 2.0	1.2 ± 0.1	0.98	169.9 ± 20.6	2.7
	R2	101.5 ± 0.8	4.5 ± 0.4	0.99	905.4 ± 26.2	14.8
L. perenne	S	98.9 ± 2.1	1.9 ± 0.1	0.97	40.7 ± 1.9	–
	R1	99.9 ± 1.9	1.2 ± 0.1	0.98	113.1 ± 11.2	2.7
	R2	98.4 ± 0.9	3.6 ± 1.2	0.98	630.7 ± 18.8	15.4
L. multiflorum	S	102.5 ± 2.6	1.9 ± 0.2	0.97	63.7 ± 3.7	–
	R1	100.1 ± 2.1	1.6 ± 0.2	0.97	202.2 ± 21.1	3.1
	R2	95.7 ± 2.4	4.7 ± 0.8	0.99	752.8 ± 29.7	11.8

The experimental approach of this study reveals that the reported herbicide resistance in the field-collected *Lolium* populations is clearly evident when subjected to two further herbicide selection cycles. Initially, a significant level of glyphosate resistance was observed in the *Lolium* populations collected from the field (R1 progeny), but with low levels of oxyfluorfen and glufosinate resistance only noticeable at doses lower than the recommended field doses for these two herbicides. However, these field-collected populations (R1) exhibit sufficient genetic variation associated with oxyfluorfen and glufosinate resistance, which manifested after a reselection cycle (R2 progeny). This result highlights the fact that herbicide resistance screening programmes should be conducted regularly to detect low resistance levels to help rotate and use herbicides with different modes of action. Future research is required to establish the extent to which this multiple non-selective herbicide resistance is present within

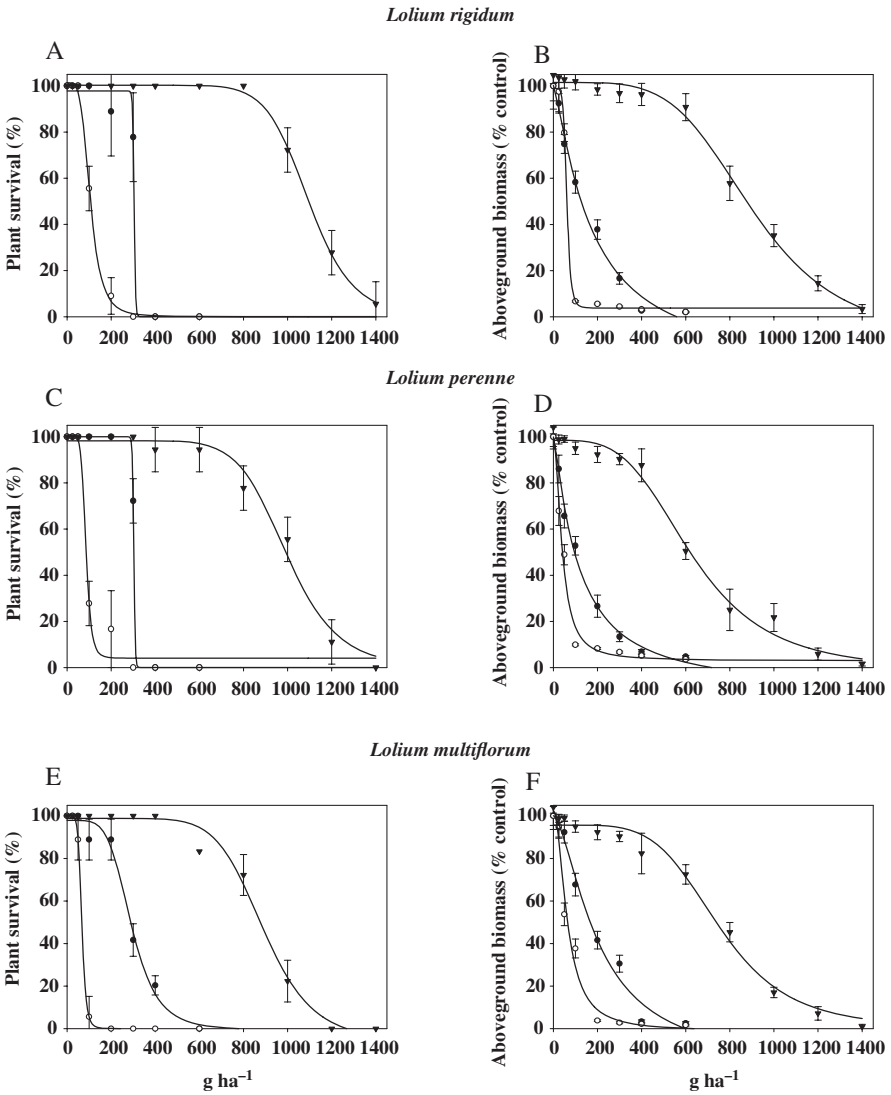

Figure 5. Plant survival (A, C, E) and above-ground biomass (B, D, F) from progenies R1 (●) and R2 (▲) and susceptible (○) *L. rigidum*, *L. perenne* and *L. multiflorum* populations in response to increasing glufosinate rates. R1 denotes glyphosate-field-selected progeny at 720 g ha^{-1}; R2 progeny both reselected with glyphosate (720 g ha^{-1}) and selected with oxyfluorfen (720 g ha^{-1}) and glufosinate (750 g ha^{-1}). Solid lines represent predicted values derived from non-linear regression analysis. Symbols denote mean ($n = 12$) ± standard error of the mean.

single individuals in each population, a result that will contribute to understanding the genetic resistance segregation for each herbicide.

Multiple resistance involving non-selective herbicides (glyphosate, glufosinate, paraquat, oxyfluorfen) is a rare trait in weed species owing to the low rate of resistance mutations associated with these herbicides.[13] In particular, glyphosate, glufosinate and oxyfluorfen are three of the most important herbicides globally, and it is known that their non-selectivity is based on dissimilar modes of action in plants. While the three herbicides are competitive inhibitors of target enzymes, glyphosate inhibits 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS), the penultimate enzyme in the shikimate pathway,[14] glufosinate inhibits glutamine synthetase (GS), a key enzyme responsible for ammonia assimilation,[15] and oxyfluorfen inhibits the protoporphyrinogen oxidase (Protox) involved in the biosynthesis of chlorophyll and group haem in plants.[16–18]

Two cases of resistance to both glyphosate and glufosinate have been reported to date. The first case corresponds to various *L. perenne* populations from Oregon (USA), in which the glyphosate and glufosinate resistance factors (both based on GR_{50}) were 3–7-fold and 2–3-fold respectively.[19] The second case has been documented in *Eleusine indica* from Malaysia, exhibiting a glufosinate GR_{50} value of 156 g ha^{-1}, accounting for a resistance factor of fivefold.[20] Multiple resistance to two non-selective herbicides including glyphosate and paraquat has also been identified in *L. rigidum*, in which the resistance mechanisms include reduced paraquat and glyphosate translocation and a target-site mutation in the *EPSPS* gene (Pro-106-Ala).[21]

Interestingly, Jalaludin *et al.*[20] found that a paraquat resistance trait is also present in the glyphosate- and glufosinate-resistant *E. indica* population, encompassing a unique case of multiple resistance to three non-selective herbicides. Our study confirms both the first report of oxyfluorfen resistance in a grass species worldwide and multiple resistance to the non-selective glyphosate and glufosinate herbicides in three species of *Lolium* genus (*L. rigidum*, *L. multiflorum* and *L. perenne*).

Multiple resistance to non-selective herbicides poses a severe threat to agriculture sustainability, especially when resistance to glyphosate is involved, given the current global scale of its use. For the particular case of *L. rigidum*, *L. perenne* and *L. multiflorum* from perennial crops in the Iberian Peninsula, an integrated management involving selective herbicides and non-chemical tools will be required.

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