Glyphosate-resistant *Lolium multiflorum* in Chilean orchards

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Summary

Lolium multiflorum (Italian ryegrass) seeds suspected of being resistant to glyphosate were collected from fruit orchards at two locations, San Bernardo (SB) and Olivar (OL), Chile, that had been treated an average of three times per year with the isopropylamine salt of glyphosate during the previous 8–10 years. Laboratory experiments were conducted for each orchard population and a susceptible population, a commercial cultivar called Tama (TM), using Petri dishes containing filter paper saturated with 5 mL of glyphosate solution (0–160 mg a.e. L^{-1}). Pot dose–response experiments were also conducted in the greenhouse. The three *L. multiflorum* populations were treated with glyphosate (0.00–4.32 kg a.e. ha^{-1}). The dose needed to reduce shoot length (Petri dish experiment) and fresh weight (pot dose–response experiment) by 50% was determined for each population. Compared with the TM population, the Petri dish experiment found that the SB and OL populations were five- and sixfold, respectively, more resistant to glyphosate, whereas the pot dose–response experiment found that the SB and OL populations were two- and fourfold, respectively, more resistant to glyphosate. These results confirm a new case of glyphosate resistance in a novel species, *L. multiflorum*, and correspond to the first case of glyphosate resistance reported from South America.

Keywords: Italian ryegrass, herbicide resistance.

Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] is a herbicide that was introduced in 1974. Since its introduction, glyphosate has established itself as a leading post-emergence, systemic, non-selective herbicide for the control of annual and perennial weeds and volunteer crops in a wide range of different situations. Although it was initially a non-crop and plantation crop herbicide, it is now widely used in no-till crop production, precision agricultural systems and, more recently, for weed control in herbicide-resistant transgenic crops, particularly in soyabean and cotton (Baylis, 2000; Caseley & Copping, 2000; Woodburn, 2000).

Herbicide resistance is defined as the inherited ability of a weed population to survive a herbicide application that is normally lethal to the vast majority of individuals of that species (Powles *et al.*, 1997). Weed populations evolve herbicide resistance through selection pressure imparted by frequent use of one or more herbicides with the same mode of action or metabolic degradation pathway on one location over an extended period of time (Christoffers, 1999). To date, more than 250 biotypes resistant to herbicides have been documented in more than 150 species (Heap, 2001).

Glyphosate has been used worldwide since 1974 and, despite its widespread and long-term use, no case of evolved resistance to glyphosate under field conditions had been identified by 1993 (Holt *et al.*, 1993), the first case being documented in 1996 (Pratley *et al.*, 1996). Since then, very few cases have been reported (Heap, 2001). A naturally tolerant biotype of *Convolvulus arvensis* L. (field bindweed) was documented by DeGenaro & Weller (1984), although the low susceptibility of this biotype did not evolve as a result of selection pressure. According to Bradshaw *et al.* (1997), the unique properties of glyphosate, such as its mode of action, metabolism, chemical structure and lack of residual activity in soil, may explain the lack of evolution of weed resistance to this herbicide. To date, evolved resistance to glyphosate has been identified and documented in two accessions of *Lolium rigidum* Gaudin

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(rigid ryegrass) in Australia (Powles *et al*., 1998; Pratley *et al*., 1999) and one accession of *Eleusine indica* L. Gaertn. (goosegrass) in Malaysia (Lee & Ngim, 2000). Also, one accession of *L. rigidum* in South Africa, one accession of *L. rigidum* in California (USA) and one accession of *Conyzia canadensis* L. Cronq. (horseweed) in Delaware (USA) have been mentioned as resistant to glyphosate (Heap, 2001).

In late 1999, growers from central Chile reported poor control of *Lolium multiflorum* Lam. (Italian ryegrass) in fruit orchards after glyphosate application. Glyphosate has been used widely throughout this region during the past 8–10 years, resulting in intense selection pressure and the potential for evolution of glyphosate resistance. In 1999, we initiated a study to examine the cause of glyphosate treatment failure, and we document the results of this investigation here.

Materials and methods

Seed types

Lolium multiflorum seeds were hand collected during the summer of 2001 from fruit orchards at two locations, San Bernardo (SB) and Olivar (OL), both located in Region VI of Chile. Both locations were selected because poor response to commercial applications of glyphosate had been observed recently after successful herbicide use during the previous 8–10 years. Commercial products containing the isopropylamine salt of glyphosate had been applied an average of three times per year at 1.08–1.44 kg a.e. ha^{-1} combined with MCPA (2-methyl-4-chlorophenoxyacetic acid) amine salt at 1.5 kg a.e. ha^{-1} to improve broad-leaved weed control. Seeds were collected only from within previously treated areas in each location to ensure that only seeds from suspected resistant plants were used. *Lolium multiflorum* cv. Tama (TM) was obtained from a commercial source and was known from previous studies to be susceptible to glyphosate (data not shown).

Petri dish experiments

Seed bioassays have been reported to be used successfully for documenting *L. rigidum* resistance to diclofop-methyl and fluazifop-butyl (Heap & Knight, 1986), *Setaria viridis* L. Beauv. (green foxtail) resistance to trifluralin (Beckie *et al*., 1990), *Avena fatua* L. (wild-oat) resistance to triallate (O'Donovan *et al*., 1996), *A. fatua* resistance to fenoxaprop-P-ethyl and sethoxydim (Murray *et al*., 1996), *L. rigidum* resistance to diclofop and chlorsulfuron (Kotoula-Syka *et al*., 2000) and *L. rigidum* and *Alopecurus myosuroides* Huds. (blackgrass) resistance to diclofop, fenoxaprop-P and clodinafop (Tal *et al*., 2000).

Petri dish experiments were conducted for each *L. multiflorum* population to confirm and determine the level of herbicide resistance in the laboratory. Seeds from each *L. multiflorum* population were selected and treated with 0.5% Vitavax Flo (17% carboxim + 17% thiram; BASF, Chile) solution to inhibit fungal growth. The experiments were conducted using 9-cm-diameter glass Petri dishes, containing two sheets of filter paper (MFS, grade no. 2) in a randomized design with two replicates per treatment. Before placing the seeds in the Petri dishes, aliquots of 5 mL of aqueous glyphosate (Roundup, 360 g a.e. L^{-1}; Monsanto, Argentina) solution were applied at 0, 10, 20, 40, 80 and 160 mg a.e. L^{-1}. After treatment, 20 seeds were placed in each Petri dish for germination in the presence of herbicide, and dishes were transferred to a growth chamber at 22 °C with 100 μmol m^{-2} s^{-1} photosynthetically active radiation and a 12-h photoperiod. Relative humidity ranged between 30% and 50%. The lids of the Petri dishes were not sealed to allow gas exchange and avoid an anaerobic environment. Eight days after treatment (DAT), shoot length was measured from the point of attachment to the seed to the tip of the coleoptile. Some studies have considered root length, as well as shoot length, in the diagnosis of herbicide resistance (Murray *et al*., 1996; Kotoula-Syka *et al*., 2000; Tal *et al*., 2000). However, root growth was less sensitive to glyphosate and more variable than shoot growth and, therefore, was not used as a seedling growth parameter in this study. The Petri dish experiment was repeated for each population, and the results were averaged for the two replicate experiments.

Pot dose–response experiments

Three series of pot dose–response experiments were conducted in the greenhouse for each *L. multiflorum* population (TM, SB and OL) to confirm herbicide resistance to glyphosate and to determine the level of resistance. The experiments were performed from May to September 2001, with a 28/18 ± 5 °C day/night temperature. The photoperiod was 14 h, and natural sunlight was supplemented by high-pressure sodium lamps yielding a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Relative humidity was not controlled, but was ≈ 50%. These conditions corresponded approximately to normal field conditions when glyphosate is applied. Seeds from each population were selected and treated with 0.5% Vitavax Flo solution to inhibit fungal growth. After fungicide treatment, nine seeds were sown per pot in 1-L plastic pots filled with potting soil (50% loamy soil, 40% perlite, 10% peat). Fifteen days after sowing (DAS), each pot was thinned to six uniform plants per pot. Pots were watered throughout

the experiment as required. At the initiation of tillering (25 DAS), the *L. multiflorum* populations were treated with glyphosate (Roundup, 0.36 kg a.e. L^{-1}; Monsanto, Argentina) (0.0, 0.36, 0.72, 1.44, 2.88 and 4.32 kg a.e. ha^{-1}), which was applied using a backpack sprayer equipped with a manometer and an 80-02 flat-fan nozzle calibrated to deliver a spray volume of 179 L ha^{-1} at 200 kPa. Herbicide rates corresponded to approximately half, one, two, four and six times the field rate recommended in Chile to control *L. multiflorum*. Treatments were arranged in a completely randomized design with three replicates per treatment. Twenty-one DAT, shoots were harvested by cutting the plants at the soil surface level, and fresh weight for each pot (including any dead leaf tissue) was recorded. After evaluation, pots were maintained in the greenhouse to regrow and, 21 days later, the number of living plants per pot that regrew was recorded to determine percentage plant mortality. Plant mortality resulting from the different glyphosate treatments was estimated as the difference in plant mortality between each glyphosate treatment and its respective control.

Statistical analysis

As shoot lengths and fresh weight of untreated plants varied among populations, data are expressed as percentages of untreated controls to standardize comparisons between populations for their response to glyphosate. Data from all experiments were fitted to a log-logistic regression model (Streibig, 1988; Streibig *et al*., 1993; Seefeldt *et al*., 1995):

$$y = C + \frac{D - C}{1 + \exp[b * \ln(x/GR_{50})]}$$

where y represents shoot length or fresh weight (% of control), C is the mean response at very high herbicide rates (lower limit), D is the mean response when the herbicide rate is zero (upper limit), b is the slope of the line at the GR_{50}, GR_{50} is the herbicide rate at the point of inflection halfway between C and D, which means the herbicide dose required to inhibit growth by 50%, and x is the herbicide dose. To estimate the parameters of the log-logistic response curve, a non-linear regression routine (procedure NLIN) was used with the SAS software system (Schabenberger, 2001). Parameter estimates were not considered to be statistically significant at the 0.05 level when the 95% confidence interval included zero. To assess the quality of fit of the model, the coefficient of determination (R^2) was calculated for each regression according to Schabenberger *et al*. (1999). To determine whether *L. multiflorum* populations varied significantly in their response to glyphosate, the sum of squares reduction test described by Schabenberger *et al*. (1999) was used to compare parameter values between the different dose–response curves. This test fits two versions of the model. One is considered to be the full model and has more parameters, and the other is considered to be the reduced model and includes fewer parameters. Then the test statistic F_{obs} is calculated:

$$F_{obs} = \frac{SS_R^{II} - SS_R^I / D.F._R^{II} - D.F._R^I}{MS_R^I}$$

where SS_R, $D.F._R$ and MS_R represent sum of squares, degrees of freedom and mean square of residual, respectively, of the full (I) and reduced (II) models. The calculated F_{obs} is compared with the cut-offs from an F distribution with $D.F._R^{II}$–$D.F._R^I$ numerator and $D.F._R^I$ denominator degrees of freedom.

The level of resistance, also called resistant index (RI), for each population was determined calculating the ratio GR_{50} (resistant)/GR_{50} (susceptible). In addition, the results were combined across experiments and subjected to analysis of variance (ANOVA) to test significant differences between the main effects of population (TM, SB and OL), treatment (glyphosate concentration or rate) and interaction terms. Data from repeated experiments were pooled because interactions between experiments by treatments were not detected by ANOVA (Tables 1 and 2).

Results and discussion

Petri dish experiments

The responses of the three populations, TM, SB and OL, to increasing concentrations of glyphosate are presented

Table 1 Analysis of variance to test significant differences between the main effects of population (TM, SB and OL), glyphosate concentration and interaction terms in Petri dish experiments

Source	d.f.	*F*-value	*P*-value
Experiment	1	2.50	0.1144
Population	2	172.83	< 0.0001
Concentration	5	354.47	< 0.0001
Population × concentration	10	23.36	< 0.0001

Table 2 Analysis of variance to test significant differences between the main effects of population (TM, SB and OL), glyphosate rate and interaction terms in pot dose–response experiments

Source	d.f.	*F*-value	*P*-value
Experiment	2	2.46	0.0912
Population	2	131.96	< 0.0001
Rate	5	396.03	< 0.0001
Population × rate	10	24.45	< 0.0001

in Fig. 1. The non-linear model provided a good description of the relationship between *L. multiflorum* shoot length and glyphosate concentration, obtaining R^2 values of 0.75 or higher (TM = 0.88; SB = 0.75; OL = 0.80). For each population, there was a general decrease in shoot length relative to its respective untreated controls as glyphosate concentration increased (Fig. 1). The SB and OL populations showed a different response from the TM population. TM commercial seeds were the largest, and initial growth of TM was more vigorous than for the SB or OL populations. At 20 mg a.e. glyphosate L^{-1}, TM shoot growth was totally inhibited, whereas the SB and OL shoot growths were greatly and slightly reduced respectively (Fig. 2).

The sum of squares reduction test indicated that parameters of the model representing the relationship between *L. multiflorum* shoot length and glyphosate concentration were significantly different ($P < 0.0001$) between populations (Table 3). The calculated GR_{50} values indicated that shoot length was less affected by glyphosate in the SB and OL populations than in the TM population (Table 4). The RI values showed that, when compared with the susceptible population TM, the SB and OL populations were five- and sixfold more resistant to glyphosate respectively. The OL population therefore showed the greatest level of glyphosate resistance (Table 5). The seed bioassay technique used in this experiment was a simple, comparatively quick, inexpensive and accurate method of identifying glyphosate-resistant *L. multiflorum* populations.

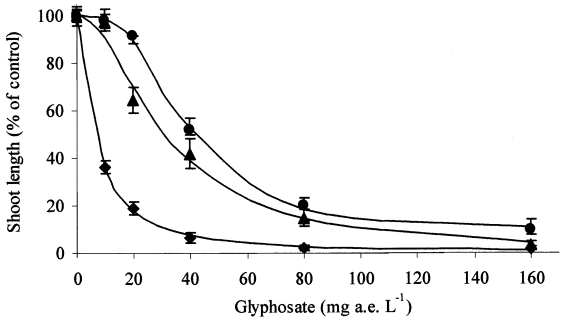

Fig. 1 Shoot length of susceptible TM (filled diamonds) and resistant SB (filled triangles) and OL (filled circles) *L. multiflorum* populations as influenced by glyphosate concentration (mg a.e. L^{-1}) 8 DAT. Data are the means of two Petri dish experiments with two replications. Symbols and lines represent actual and estimated responses respectively. Vertical bars represent ± standard errors of the mean. Refer to Table 4 for parameter estimates.

Pot dose–response experiments

The responses of the three populations, TM, SB and OL, to increasing rates of glyphosate are presented in Fig. 3. The non-linear model provided a good description of the relationship between *L. multiflorum* fresh weight and glyphosate rate, obtaining R^2 values of 0.90 or higher (TM = 0.97; SB = 0.90; OL = 0.94). For each population, there was a general decrease in fresh weight relative to its respective untreated control as the glyphosate rate increased (Fig. 3). The SB and OL populations showed a

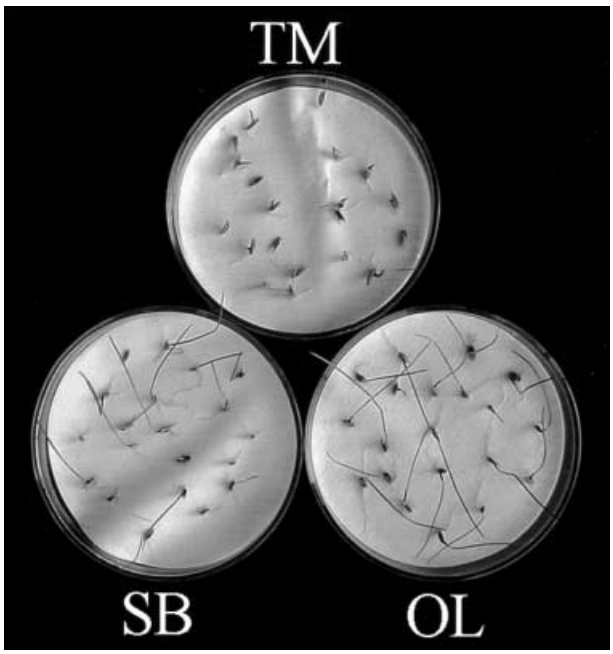

Fig. 2 Effect of glyphosate (20 mg a.e. L^{-1}) on shoot length of susceptible TM and resistant SB and OL *L. multiflorum* populations 8 DAT.

Table 3 Sum of squares reduction test fitting the two versions of the model to test for differences in Petri dish experiments between *L. multiflorum* populations (TM, SB and OL)

Populations	SS_R*		$D.F._R$†		MS_R‡	F_{obs}	*P*-value
	Full	Reduced	Full	Reduced			
TM vs. SB	104 766.57	207 938.00	328	332	319.41	80.75	< 0.0001
TM vs. OL	81 814.43	250 538.71	328	332	403.18	169.11	< 0.0001
SB vs. OL	132 241.76	142 521.00	328	332	403.18	6.37	< 0.0001

*Sum of squares of residual.
†Degrees of freedom of residual.
‡Mean square of residual.

Table 4 Parameter estimates*, standard errors and 95% confidence intervals describing shoot length of three *L. multiflorum* populations (TM, SB and OL) as influenced by glyphosate concentration (mg a.e. L^{-1})

Population†	Parameter	Estimate	Asymptotic standard error	Asymptotic 95% confidence intervals	
				Lower	Upper
TM	*D*	99.99	2.432	95.19	104.79
	C	–0.03‡	2.647	–5.26	5.20
	b	1.45	0.311	0.83	2.06
	GR_{50}	6.80	0.775	5.27	8.33
SB	*D*	102.44	3.913	94.71	110.17
	C	–0.54‡	6.341	–13.06	11.98
	b	1.85	0.331	1.19	2.50
	GR_{50}	31.56	3.615	24.41	38.70
OL	*D*	100.31	2.667	95.04	105.57
	C	10.56	3.890	2.88	18.24
	b	3.04	0.578	1.90	4.18
	GR_{50}	39.42	2.448	34.58	44.25

*Refer to Materials and methods section for a description of model fitted.
†TM, commercial *L. multiflorum* Tama cultivar; SB, San Bernardo population; OL, Olivar population.
‡Parameter estimate not considered to be statistically different from zero ($P > 0.05$).

Table 5 Calculated RI values derived from logistic regression equations fitted to data shown in Figs 1 and 3 respectively

Population	RI Petri dish (shoot length)	RI pot dose–response (shoot fresh weight)
TM	1.0	1.0
SB	4.6	2.2
OL	5.8	4.2

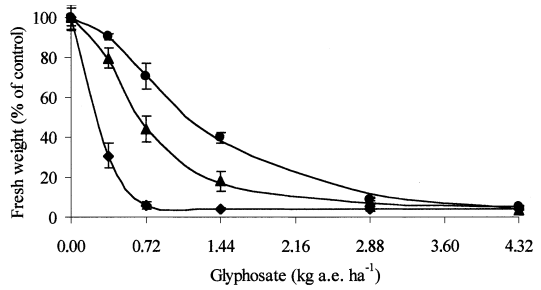

Fig. 3 Above-ground fresh weight of susceptible TM (filled diamonds) and resistant SB (filled triangles) and OL (filled circles) *L. multiflorum* populations as influenced by glyphosate rate (kg a.e. ha^{-1}) 21 DAT. Data are the means of three pot dose–response experiments with three replicates. Symbols and lines represent actual and estimated responses respectively. Vertical bars represent ± standard errors of the mean. Refer to Table 7 for parameter estimates.

different response from the TM population. At 0.72 kg a.e. ha^{-1}, TM shoot growth was totally inhibited, whereas the SB and OL shoot growths were moderately and slightly reduced respectively (Fig. 4).

The sum of squares reduction test indicated that parameters of the model representing the relationship between *L. multiflorum* shoot growth and glyphosate rate were significantly different ($P < 0.0001$) between populations (Table 6). The calculated GR_{50} values indicated that fresh weight was less affected by glyphosate rate in the SB and OL populations compared with the TM population (Table 7). The RI values showed that the SB and OL populations were two- and fourfold more resistant to glyphosate, respectively, compared with the susceptible population TM. Again, this indicated a higher level of resistance in the OL population (Table 5). The level of resistance found for each field population was less in this experiment than in results obtained through bioassays in the Petri dish experiments.

Percentage plant mortality after harvest for the three populations (TM, SB and OL) as a result of the glyphosate treatments is presented in Fig. 5. After herbicide application and evaluation, only those plants that still had active meristems survived and regrew. At 1.44 kg a.e. ha^{-1} or higher, none of the plants belonging to the susceptible population (TM) survived, whereas plants from the SB and OL populations were still alive after they were treated with glyphosate rates up to 2.88 and 4.32 kg a.e. ha^{-1} respectively (Fig. 5).

The pot dose–response experiment was an effective technique to identify glyphosate-resistant populations and to determine the level of resistance. However, it took more time and space and was more expensive than the Petri dish experiments when several populations had to be evaluated. Nevertheless, bioassays using Petri dish experiments should always be accompanied by pot dose–response experiments for diagnosing herbicide-resistant weeds. Pot dose–response experiments provide more realistic RI values, as the plant growth stage at the time of herbicide application and the application rate in such experiments is more comparable with field conditions.

Fig. 4 Effect of glyphosate (0.72 kg a.e. ha^{-1}) applied at the initiation of tillering (25 DAS) on growth of susceptible TM and resistant SB and OL *L. multiflorum* populations 21 DAT.

Table 6 Sum of square reduction test fitting the two versions of the model to test for differences in pot dose–response experiments between *L. multiflorum* populations (TM, SB and OL)

	SS_R*		$D.F._R$†				
Populations	Full	Reduced	Full	Reduced	MS_R‡	F_{obs}	*P*-value
TM vs. SB	6235.98	18 211.12	64	68	97.44	30.73	< 0.0001
TM vs. OL	4407.76	31 241.47	64	68	132.34	97.41	< 0.0001
SB vs. OL	8469.61	12 291.28	64	68	132.34	7.22	< 0.0001

*Sum of squares of residual.
†Degrees of freedom of residual.
‡Mean square of residual.

Table 7 Parameter estimates*, standard errors and 95% confidence intervals, describing above-ground fresh weight of three *L. multiflorum* populations (TM, SB and OL) as influenced by glyphosate rate (kg a.e. ha^{-1})

			Asymptotic standard	Asymptotic 95% confidence intervals	
Population†	Parameter	Estimate	error	Lower	Upper
TM	*D*	100.00	2.379	95.15	104.85
	C	4.00	1.433	1.08	6.92
	b	4.17	2.046	0.01	8.34
	GR_{50}	0.29	0.032	0.220	0.352
SB	*D*	100.27	5.131	89.82	110.73
	C	3.64‡	4.848	−6.23	13.52
	b	2.21	0.493	1.21	3.21
	GR_{50}	0.63	0.072	0.47	0.77
OL	*D*	99.75	3.946	91.71	107.79
	C	–4.14‡	8.291	−21.03	12.74
	b	1.92	0.393	1.12	2.72
	GR_{50}	1.18	0.159	0.84	1.49

*Refer to Materials and methods section for a description of model fitted.
†TM, commercial *L. multiflorum* Tama cultivar; SB, San Bernardo population; OL, Olivar population.
‡Parameter estimate not considered to be statistically different from zero ($P > 0.05$).

Recently, a quick test has been developed to determine the resistance status of weeds during the year in which the herbicide failure occurs (Boutsalis, 2001). It uses cuttings taken from suspected plants and does not depend on seed availability, eliminating the need for seed set and collection.

A *L. rigidum* population from an orchard in New South Wales, Australia, that was resistant to glyphosate in pot dose–response experiments, exhibited seven- to 11-fold resistance (LD_{50} = 600–1800 g a.e. ha^{-1}) compared with a susceptible population (LD_{50} = 59–174 g a.e. ha^{-1}) (Powles *et al.*, 1998), whereas a

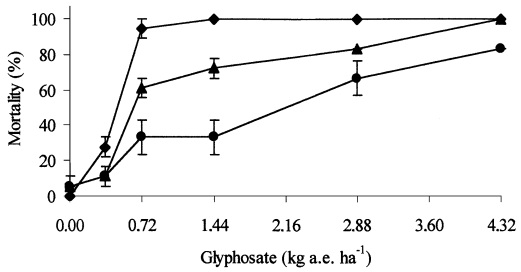

Fig. 5 Percentage mortality of susceptible TM (filled diamonds) and resistant SB (filled triangles) and OL (filled circles) *L. multiflorum* populations as influenced by glyphosate rate (kg a.e. ha^{-1}) Vertical bars represent ± standard errors of the mean.

population from a field in northern Victoria, Australia, exhibited nearly 10-fold resistance (LD_{50} = 796–1140 g a.e. ha^{-1}) when compared with a susceptible population (LD_{50} = 81–124 g a.e. ha^{-1}) (Pratley *et al*., 1999). In this study, the OL population, which showed the highest level of resistance in pot dose–response experiments, exhibited fourfold resistance (GR_{50} = 840–1490 g a.e. ha^{-1}) compared with a susceptible population (TM) (GR_{50} = 220–350 g a.e. ha^{-1}). Unfortunately, it is not possible to compare the three studies because of the different conditions under which the herbicide was applied. Powles *et al*. (1998) demonstrated that timing and formulation of glyphosate both influenced LD_{50} values, but had little impact on the comparative resistance. Moreover, glyphosate resistance was reported in Australian *L. rigidum* populations in two different places after 15 years of successful herbicide use, whereas it took only 10 years of glyphosate use in Chile for *L. multiflorum* populations to evolve resistance. This suggests that either there may be more intensive selection pressure at the Chilean sites, given the local glyphosate strategies applied, or there may be a higher frequency of resistant individuals in Chilean *L. multiflorum* populations compared with Australian *L. rigidum* populations.

It is essential to know whether or not a lack of weed control after glyphosate applications results from herbicide resistance or other reasons. Using the identification of herbicide-resistant weeds as a first step in resistance management demands an efficient and effective screening test. Only after accurate diagnosis can the nature, distribution and abundance of resistant weed populations be monitored. Appropriate seed sampling techniques, protocols for screening weeds from different locations for herbicide resistance, interpretation of results and distribution of information to growers have been reviewed recently (Beckie *et al*., 2000).

Glyphosate resistance has been confirmed and documented in very few species. The results presented in this paper confirm a new case of glyphosate resistance in a novel species, *L. multiflorum*, and is the first case of weed resistance to glyphosate in South America. Chilean orchard growers should begin to implement strategies to minimize the chance of widespread resistance before new cases appear. A more integrated weed management programme should be adopted.

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