

ORIGINAL ARTICLE

Evolution of glyphosate resistance in a *Lolium rigidum* population by glyphosate selection at sublethal doses

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The majority of the documented cases of field-evolved herbicide-resistant weed biotypes established that single major genes confer glyphosate resistance. However, the contribution of minor genes endowing substantial plant survival at sublethal herbicide doses may be a potential complementary path to herbicide resistance evolution in weed populations under selection. Here, we subjected a number of susceptible individuals of *Lolium rigidum* to recurrent glyphosate selection to test the potential for sublethal glyphosate doses to additively select for glyphosate resistance. After 3–4 cycles of glyphosate selection in two distinct environments, the progenies of the initially susceptible population were shifted toward glyphosate resistance. The results indicate progressive enrichment of minor gene trait(s) contributing toward plant survival in the glyphosate-selected progenies. After three generations of selection, the estimated LD₅₀ values

were doubled compared with the original population and up to 33% plant survival was obtained in the glyphosate-selected progeny at the recommended glyphosate label rate. This level of resistance probably was the maximum shift achievable with sublethal glyphosate dose selection in this small population. Cross-pollination was a crucial factor enabling the rapid rate of accumulation of minor glyphosate resistance gene trait(s) that are likely to be present at a relatively high frequency in a small susceptible population. The mechanistic basis of the moderate glyphosate resistance level selected by sublethal glyphosate doses remains unknown and warrants future research. Studying the main factors influencing the evolution of resistant weed populations is crucial for understanding, predicting and managing herbicide resistance. *Heredity* (2009) **103**, 318–325; doi:10.1038/hdy.2009.64; published online 3 June 2009

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Introduction

Single major gene traits endow resistance in the great majority of documented cases of field-evolved herbicide-resistant plants (reviewed by Darmency, 1994). Herbicide selection of large populations of initially herbicide-susceptible plant species selects for the rare individuals expressing resistance genes (reviewed by Jasieniuk *et al.*, 1996). Therefore, it is generally accepted that the dynamics of herbicide resistance evolution are strongly influenced by the initial frequency of major resistance genes in weed populations under herbicide selection (Preston and Powles, 2002). However, a potential concomitant evolutionary path to herbicide resistance in weed populations under selection could be the recurrent enrichment of several additive minor genes, which, in combination, endow sufficient resistance for a substantial number of individuals to survive at the recommended (label) herbicide use rates (Neve and Powles, 2005b). Such enrichment of minor genes would be favored if

herbicide selection occurred at a relatively low herbicide dose, such that there would be plant survivors that subsequently produce viable seeds. The rate of herbicide resistance evolution in weed populations subjected to recurrent selection at sublethal doses would rely on additive genetic variation for resistance, the frequency of such minor genes, and the capacity of surviving plants to respond to selection (that is, incremental accumulation toward polygenic resistance) (Holsinger, 2000). In addition, empirical results (Brotherton *et al.*, 2007) and recent modeling simulations of glyphosate resistance evolution (Neve, 2008) suggest the plant reproductive system (cross- vs self-pollination) is a crucial factor in sublethal dose resistance evolution. In cross-pollinated species such as *Lolium rigidum* Gaud. or *Amaranthus palmeri* L., gene accumulation or amplification can occur at the individual plant level attributable to herbicide selection after a few generations (Neve and Powles, 2005b; Gaines, 2009).

Although considerable literature exists on the use of sublethal herbicide doses to provide sufficient weed control (reviewed by Zhang *et al.*, 2000; O'Donovan *et al.*, 2007), very few studies have analyzed the subsequent genetic evolutionary consequences of such a low-dose selection (Gressel, 1995). If a herbicide is used at a sublethal dose, then the (low) selection pressure acting within the phenotypic variation is evident in the population and there will likely be survivors. It has been

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suggested that sublethal herbicide doses could enable resistance alleles to be progressively enriched through generations resulting in a polygenic-based shift toward more resistant individuals (Gressel, 2002). Evidence of sublethal drug dose selection for polygenic resistance is known for bacteria (Olofsson and Cars, 2007), fungi (Shaw, 2006) and insects (Roush and McKenzie, 1987). A recent study documented the evolution of resistance in an initially herbicide susceptible *L. rigidum* population subjected to recurrent selection at sublethal herbicide doses for three generations (Neve and Powles, 2005b). The selection and subsequent enrichment of gene traits progressively led to polygenic resistance to diclofop-methyl and concomitant low-level cross-resistance to some other herbicide modes of action. In a separate study, even a single cycle of selection with diclofop-methyl moved susceptible *L. rigidum* populations (five) toward resistance (Neve and Powles, 2005a). Notwithstanding these results, there is still an ongoing discussion on the effect and relevance of sublethal herbicide doses leading to polygenic herbicide resistance evolution by the accumulation of several minor genes (genes of small effect) vis-à-vis resistance due to a single major gene with a large phenotypic effect (typically a discrete Mendelian gene).

Glyphosate is a potent herbicide that inhibits the enzymatic activity of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) in the shikimate pathway in plants (Duke and Powles, 2008). Since 1996, because of a very high level of adoption of genetically modified glyphosate-resistant crops (that is, 80 million Ha in 2006 in Argentina, Brazil, Canada and USA), glyphosate has been intensively used across important agricultural regions (reviewed by Dill *et al.*, 2008). In a relatively short period of time, large-scale usage of glyphosate as a principal tool for weed control in glyphosate-resistant crops without diversity has resulted in glyphosate-resistant weeds and shifts in weed communities (reviewed in Owen, 2008; Powles, 2008). Indeed, studies on evolved glyphosate-resistant weeds such as *Conyza Canadensis* L., *Eleusine indica* L. and *L. rigidum* established that single major genes confer glyphosate resistance (Lorraine-Colwill *et al.*, 2001; Ng *et al.*, 2004; Zelaya *et al.*, 2004). However, the contribution of minor genes to additional plant survival at a sublethal dose of glyphosate has also been reported in *L. rigidum* (Lorraine-Colwill *et al.*, 2001).

L. rigidum is an obligate cross-pollinated species characterized by high genetic diversity (Balfourier *et al.*, 1998). Therefore, even a small *L. rigidum* population subjected to genetic bottlenecks through recurrent selection can still respond to selection because considerable genetic diversity is retained in the selected progeny (Cooper, 1959; Falconer, 1981). Here, we examined whether a small, glyphosate-susceptible population of *L. rigidum* could evolve glyphosate resistance when exposed to selection by sublethal doses of glyphosate over consecutive generations.

Materials and methods

Plant material

This study was conducted with the *L. rigidum* biotype VLR1, which has never received herbicide selection and

is known to be susceptible to all herbicides registered for its control. Seed stocks of this herbicide-susceptible population have been maintained and multiplied since 1985 in the absence of selection and without introgression of any herbicide resistance genes.

Dose–response bioassays and recurrent selection

Cycles of recurrent selection at sublethal doses of glyphosate were conducted on glyphosate-susceptible population of VLR1 in two separate pot experiments conducted in two different environments (Table 1). In one experiment, plants were grown in plastic pots in a controlled environment room (CER) under fluorescent light (300 μmol quanta m⁻² s⁻¹) with a photoperiod of 16 h at 20/12 °C (light phase coinciding with the warm phase). In a second experiment, plants were grown during the normal growing period (May–August) in a natural outdoor environment simulating field conditions (hereinafter referred to as field). Plants were kept well watered and fertilized. Plants were treated at the two-leaf stage with 0, 38, 75, 150, 250, 350, 450, 550 and 700 gHa⁻¹ glyphosate (recommended rate 450 gHa⁻¹) (RoundUp Power Max as potassium salt, 540 g l⁻¹ a.e. SL; Nufarm, Laverton North, VIC, Australia). For each herbicide dose, there were at least three replicates. After 15 days, survivors were counted, and fresh and dry aboveground biomass evaluated. Dose–response bioassays were repeated at least once. Those plants that survived the glyphosate treatment at a certain dose, and grew vigorously after biomass assessment were transplanted and grown to maturity, then confined for cross-pollination only with other survivors at that specific dose. The glyphosate selection intensity was based on plant survival ratio at specific sublethal glyphosate doses. In CER experiments, analogous selection intensities were applied to subsequent generations by increasing glyphosate doses. Conversely, in the field experiments, surviving plants were selected at a fixed glyphosate dose (Table 1). The effective population size (N_e) was calculated as suggested by Falconer (1981) at each generation (Table 1). For a t number of generations, the effective population size (N_e) is given by the harmonic mean of the number of plants selected (N_i):

$$\frac{1}{N_e} = \frac{1}{t} \sum_{i=1}^t \frac{1}{N_i} \quad (1)$$

At flowering, the selected surviving plants were engaged in a pollen-proof enclosure to ensure random cross-pollination (panmixia). The seed obtained from these selected plants represented the selected progeny. In CER selection experiments, the harvested seeds were maintained for at least 3 weeks in a warm (37 °C) and dry environment to release seed dormancy before the next cycle of selection was initiated. Seed germination rates greater than 60% were always achievable, and two consecutive cycles of selection per year were conducted. At each cycle of selection, the selected progeny was immediately evaluated and compared in dose–response studies to the unselected VLR1 population. The selection process was repeated four times, as previously described. Field selection experiments necessarily involved only one generation per year and were conducted for three consecutive growing seasons (3 years); then, all the

Table 1 Seed progeny collected from a number of selected plants (N_i) surviving a specific sublethal glyphosate dose (g Ha^{-1}) resulting in average selection intensity

Progeny	Environment	Glyphosate dose (g Ha^{-1})	Selection intensity (1-survival ratio)	Plants selected (N_i) ^a	Effective population size (N_e)
1a	CER	150	0.71	32	32
1b	Field	150	0.57	176	176
1c	Field	250	0.82	124	124
1d	Field	350	0.92	44	44
2a	CER	250	0.88	13	18
2b	Field	150	0.33	22	39
2c	Field	250	0.76	23	39
2d	Field	350	0.83	23	30
3a	CER	250	0.87	15	17
3b	Field	150	0.30	20	30
3c	Field	250	0.66	20	30
3d	Field	350	0.82	20	26
4a	CER	350	0.90	7	13

Abbreviation: CER, controlled environment room.

The effective size of the population (N_e) is calculated for each cycle of selection.

^aPlants grown to maturity that effectively produced seed after herbicide selection.

selected progenies were evaluated in final dose–response bioassays.

Statistics applied to dose–response studies

Data sets from repeated experiments were pooled, fitted to a non-linear logistic model and analyzed by a lack-of-fit F -test. If differences between repeated experiments were not significant, pooled data were used for subsequent non-linear regression analysis. Analysis of variance (ANOVA) and regression assumptions were held under square root data transformation (that is, Box–Cox transformation lambda, $\lambda = 0.5$) (Box and Cox, 1964). The herbicide doses causing 50% plant mortality (LD_{50}) or growth reduction (GR_{50}) in the selected and unselected populations at each generation were estimated by using the logistic model:

$$Y = c + [(d - c) / [1 + (x/G)^b]] \quad (2)$$

Y denotes plant survival or biomass expressed as a percentage of the untreated control, d and c are upper and lower asymptotic values of Y , respectively, b the slope of the curve, G the herbicide dose at the point of inflection halfway between the upper and the lower asymptotes, and x the herbicide dose (Streibig *et al.*, 1993). The response to selection for the different selected progenies was measured as the R/S (resistant/susceptible) ratio of estimated LD_{50} or GR_{50} values. Mortality dose–response graphs are presented with untransformed data.

Results

Response of the susceptible population VLR1 to glyphosate

As expected, the unselected VLR1 population was confirmed to be susceptible to glyphosate. For CER-grown plants, only 2% of individuals survived 450 g Ha^{-1} glyphosate (that is, the recommended label rate in Australia) in several different dose–response studies (96

and 100% mortality at 350 and 550 g Ha^{-1} , respectively). Similarly, for plants grown in field conditions 93, 98, and 99% mortality was observed at 350, 450 and 550 g Ha^{-1} glyphosate, respectively. However, as expected, there were survivors (phenotypic variation) at lower glyphosate doses (Figures 1 and 2). Glyphosate susceptibility (pooled data from several experiments) of plants grown in the CER vs the field environment was not significantly different ($P = 0.381$), with calculated LD_{50} values of 123 and 129 g Ha^{-1} , respectively.

Response to recurrent selection with glyphosate sublethal doses

Glyphosate selection of CER-grown plants: Glyphosate dose–response bioassays compared the glyphosate-selected progeny 1a and 2a to the unselected originator population (VLR1). The LD_{50} R/S ratio showed a shift of 11% toward resistance in one generation, followed by a further 9% in the second generation. In progeny 2a, the resistance level continued to increase, and the estimated LD_{50} values obtained were higher than those obtained with progeny 1a (data not shown). An effective population size of 18 individuals (Table 1) after two selection cycles retained substantial genetic variation, because a significant additional shift toward glyphosate resistance occurred between the second and third selected progeny (Table 2). After three cycles of recurrent selection, progeny 3a clearly showed a level of glyphosate resistance: there was $20 \pm 1.7\%$ survival at the registered glyphosate use rate of 450 g Ha^{-1} (Figure 1a). The dose–response studies to quantify the level of glyphosate resistance in progeny 3a were repeated three times and the LD_{50} R/S ratios obtained ranged from 1.67 to 2.08 (Table 2). This resistance was also evident in the biomass measurements of these plants (Table 3 and Supplementary Figure S2). The level of resistance observed in progeny 3a was confirmed in progeny 4a (Figure 1b). Similarly, the dose–response studies were repeated three times. In two experiments

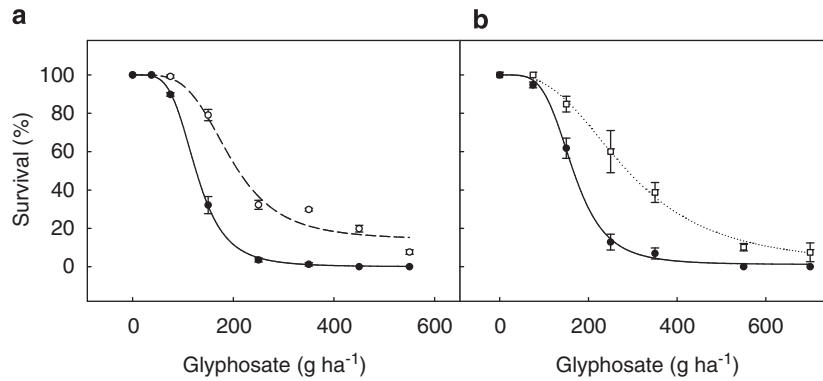


Figure 1 Glyphosate dose–response curves (survival) of glyphosate-selected progenies in CER. Solid circles and solid line represent the unselected original population (VLR1); (a) Open circles and dashed line, the three-time glyphosate-selected progeny 3a; (b) Open squares and dotted line, the four-time glyphosate-selected progeny 4a. Symbols are mean \pm 1 s.e., $n = 3$, lines are predicted values for percentage survival.

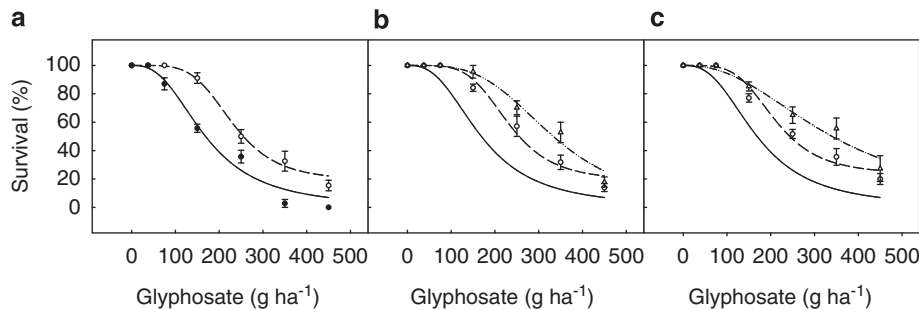


Figure 2 Glyphosate dose–response curves (survival) of glyphosate-selected progenies in field conditions. Solid circles and solid lines represent the unselected original population (VLR1); Open triangles and dashed lines are the once glyphosate-selected progenies, open triangles and dash-dotted lines the twice glyphosate-selected progenies. (a) Once-selected progeny at 150 g glyphosate ha^{-1} (progeny 1b, refer to Table 1); (b) Once- and twice-selected progeny at 250 g glyphosate ha^{-1} (progeny 1c, 2c); (c) Once- and twice-selected progeny at 350 g glyphosate ha^{-1} (progeny 1d, 2d). Symbols are mean \pm 1 s.e., $n = 3$, lines are predicted values for percentage survival.

Table 2 Parameters of the log–logistic model [$Y = c + (d - c) / (1 + (x/G)^b)$] used to calculate the LD_{50} values of selected and unselected progeny and their R/S ratios from dose–response studies (CER)

Progeny	Experiments	d	c	G	b	RMS ^a	LD_{50} (g Ha^{-1})	R/S
VLR1	1–2	100	0	126	4	25	126	—
3a	1–2	100	14	194	4	41	210	1.67
VLR1	3	100	0	77	3	2	76.6	—
3a	3	100	0	158	3	24	158	2.08
VLR1	1–2	100	1	165	5	62	165	—
4a	1–2	100	0	288	3	166	288	1.73
VLR1	3	100	2	121	3	188	123	—
4a	3	100	0	291	2	148	291	2.37

ANOVA analysis conducted for each non-linear regression is highly significant ($P < 0.001$) and adjusted- $R^2 > 0.84$.

^aResidual mean square.

(pooled data for analysis), the LD_{50} values for both the selected and unselected populations were higher than in the preceding bioassays. However, a consistent difference was maintained between the unselected population and glyphosate-selected progeny 4a. The highest R/S value was obtained in the third repetition of the herbicide bioassay (Table 2). At the substantial dose of 550 g Ha^{-1} glyphosate, the observed plant survival was 12 ± 2.7 . Consistent results were obtained by assessing fresh and dry biomass to evaluate plant growth reduction in progeny 4a (Table 3). It is important to note that, when grown to maturity, the glyphosate-

selected survivors (progeny 4a) produced tillers and subsequent viable seed, whereas those VLR1 plants surviving the same glyphosate dose produced fewer tillers and significantly less seed (Supplementary Figure S1). Due to the recurrent selection acting on a small number of individuals, the effective size of the population in the selected progeny 4a was low (Table 1). However, no significant reduction was observed in plant biomass of untreated check plants. Pooled data from the three bioassays carried out on progeny 4a showed no significant difference in either fresh or dry plant weights ($P \geq 0.407$) between the

Table 3 Parameters of the log-logistic model [$Y = c + (d - c) / (1 + (x/G)^b)$] used to calculate the GR_{50} values of selected and unselected progeny and their R/S ratios from dose-response studies (CER)

Progeny	Experiments	<i>d</i>	<i>c</i>	<i>G</i>	<i>b</i>	RMS ^a	GR_{50} (g Ha ⁻¹)	R/S
VLR1	1-2	100	2	82	3	38	82.9	—
3a	1-2	100	8	150	5	217	155	1.87
VLR1	3	100	7	31	2	9	33.6	—
3a	3	100	8	72	2	197	77.6	2.31
VLR1	1-2	100	7	78	4	81	80.8	—
4a	1-2	100	14	125	3	137	139	1.72
VLR1	3	100	4	67	3	35	68.9	—
4a	3	100	0	116	2	124	116	1.68

ANOVA analysis conducted for each non-linear regression is highly significant ($P < 0.001$) adjusted- $R^2 > 0.89$.

^aResidual mean square.

Table 4 Parameters of the log-logistic model [$Y = c + (d - c) / (1 + (x/G)^b)$] used to calculate the LD_{50} values of selected and unselected progeny, and their R/S ratios from dose-response studies (Field)

Population	<i>d</i>	<i>c</i>	<i>G</i>	<i>b</i>	RMS ^a	LD_{50} (g Ha ⁻¹)	R/S
VLR1	100	0	169	3	92	169	—
1b	100	18	231	5	50	255	1.51
3b	100	27	226	5	28	268	1.59
1c	100	0	269	3	40	269	1.59
2c	100	0	333	4	71	333	1.97
3c	100	0	325	3	137	325	1.92
1d	100	1	257	2	37	260	1.54
2d	100	0	340	2	84	340	2.01
3d	100	31	237	3	80	315	1.87

ANOVA analysis conducted for each non-linear regression is highly significant ($P < 0.001$) and adjusted- $R^2 > 0.83$.

^aResidual mean square.

Table 5 Parameters of the log-logistic model [$Y = c + (d - c) / (1 + (x/G)^b)$] used to calculate the GR_{50} of selected and unselected progeny and their R/S ratios from dose-response studies (Field)

Population	<i>f</i> ^a	<i>d</i>	<i>c</i>	<i>G</i>	<i>b</i>	RMS ^b	GR_{50} (g Ha ⁻¹)	R/S
VLR1	1	100	-12	71	2	113	133	—
1b	0	99	7	172	5	72	179	1.35
3b	12	100	-40	28	2	238	252	1.90
1c	4	100	-243	78	1	114	133	1.00
2c	4	100	-88	77	2	560	241	1.82
3c	0	98	12	154	3	155	214	1.62
1d	1	100	11	95	3	139	152	1.15
2d	9	100	-104	30	1	173	170	1.28
3d	1	100	14	126	4	287	193	1.45

ANOVA analysis conducted for each non-linear regression is highly significant ($P < 0.001$) and adjusted- $R^2 > 0.87$.

^aWhere a stimulation of plant growth at low doses (hormesis) was observed this log-logistic model was used $Y = c + [(d - c) + (f^*x)] / [1 + (x/G)^b]$. In the equation, *f* is proportional to the hormetic behavior.

^bResidual mean square.

selected progeny 4a and the unselected VLR1 population.

Glyphosate selection of field-grown plants: Glyphosate dose-response studies were conducted to compare the one-, two- and three-time glyphosate-selected progenies vs the unselected originator population (VLR1). It is emphasized that in this final experiment, all plants were grown in the same field environment at the same time. The once-selected progenies showed higher survival to glyphosate compared with unselected VLR1 (Table 4, Figures 2a-c). An increased level of resistance was observed in the three-time selected populations, but no further incremental shift toward glyphosate resistance was observed in the thrice-selected

progenies. The maximum resistance levels were observed in populations 2c and 2d, twice-selected at the highest doses (250 and 350 g Ha⁻¹ glyphosate, respectively) (Tables 4 and 5, Figures 2a-c and Supplementary Figure S3). Up to 33% ± 4.3 plant survival was obtained in the progeny 3d at the recommended field rate of 450 g Ha⁻¹.

Discussion

Effect of recurrent selection at sublethal doses of glyphosate

Herbicide resistance is the evolved and inherited capacity of a plant population to withstand the normal recommended herbicide dose (registered label rate) that

had been previously effective in controlling the same population (Hall *et al.*, 1994). Here, we show that with an initially glyphosate-susceptible *L. rigidum* population, three to four generations of recurrent selection at sublethal glyphosate doses (in two different environments) are sufficient to cause a shift toward glyphosate resistance (Tables 2–5, Figures 1 and 2, Photo Supplementary Figure S1). It is emphasized that this recurrent glyphosate selection study commenced with a population (VLR1) highly characterized as susceptible to a wide range of herbicides, including glyphosate (Yu *et al.*, 2007). The results we obtained with glyphosate selection are similar to earlier experiments in which the same *L. rigidum* population evolved resistance from recurrent selection at sublethal doses of diclofop-methyl (Neve and Powles, 2005b). In a field-evolved glyphosate-resistant population of *L. rigidum* the contribution of complementary minor genes for glyphosate resistance at a low glyphosate rate (225 g Ha⁻¹) could not be excluded because of higher-than-expected survival in back-cross families (Lorraine-Colwill *et al.*, 2001). The glyphosate-selected progenies reported here showed a progressive increase in survival after each generation of recurrent glyphosate selection, suggesting the involvement and progressive accumulation of minor gene trait(s). However, the genetic basis remains to be determined. Preliminary analysis by using the polygenic platform *q*-gene (Podlich and Cooper, 1998), under a simplified range of genetic assumptions suggests the potential involvement of one or two minor additive genes (Supplementary Figure S4b, c, f, g).

The selected glyphosate resistance was consistent under different environmental conditions (CER vs field) and at different stages of plant development illustrating the potential for a herbicide-resistant population to be selected by the use of sublethal herbicide doses in variable field conditions (Supplementary Table S1). The level of resistance to glyphosate after three or four generations of recurrent selection at sublethal glyphosate doses (Figures 1 and 2) was much lower than the level of diclofop-methyl resistance evident after three cycles of recurrent selection with diclofop-methyl (Neve and Powles, 2005b). Glyphosate resistance from recurrent selection at a sublethal glyphosate dose is moderate when compared with LD₅₀ values obtained in field-evolved glyphosate-resistant populations of *L. rigidum* (Powles *et al.*, 1998; Yu *et al.*, 2007) or glyphosate-resistant biotypes in other species (reviewed in Gustafson, 2008; Vila-Aiub *et al.*, 2008).

It is clear that for glyphosate, the phenotypic variation in glyphosate sensitivity displayed in even a small number of individuals is sufficient and when exposed to recurrent selection at sublethal glyphosate doses, this can result in a shift toward resistance within a few generations. However, the magnitude and the rate of these evolutionary phenomena are dependent on the specifics of the herbicide molecule, the biology of the plant species and any potential resistance mechanisms present in the population under selection. Allopatric (obligate cross-pollinated) species such as *Lolium* are particularly responsive because all existing minor resistance gene trait(s) may be additively enriched through cross-pollination among surviving plants. This does not occur in self-pollinated species or it may be a much slower evolution depending on the out-crossing rate

(Holsinger, 2000). For example, no shift in glyphosate resistance has been observed in a susceptible population of self-pollinated *Avena fatua* subjected to recurrent low-dose glyphosate selection (R Busi and SB Powles, unpublished). Similarly, the visual inspection of glyphosate-treated seedlings failed to identify a shift toward glyphosate resistance in self-pollinated *Arabidopsis thaliana* L. after seven cycles of recurrent selection at low doses of glyphosate (Brotherton *et al.*, 2007). Therefore, we conclude that cross-pollination is a significant factor in herbicide resistance evolution from recurrent selection, as cross-pollination enables the progressive accumulation of minor genes (Neve and Powles, 2005a, b).

Population size and evolution of herbicide resistance after recurrent selection

The genus *Lolium* is genetically variable (Balfourier *et al.*, 1998), and a small population subjected to a severe bottleneck still retains genetic variation (Cooper, 1959). Substantial herbicide resistance evolution occurred in both this experiment and the selection study reported by Neve and Powles (2005b). In this study, a sufficient number of surviving plants at each generation ensured that adequate population size and genetic variation were maintained throughout the course of the recurrent selection (Polans and Allard, 1989) (Table 1). Probably, there was a constant level of heritability at each cycle of recurrent selection, due to similar environmental conditions obtained in both CER and the field environment. However, there was only a small or negligible increment in R/S ratios obtained with three- (field) or four-time (CER) glyphosate-selected progenies. This phenomenon, together with a declining effective population size (Table 1) because of a progressive genetic bottleneck imposed by recurrent selection, suggests that the shift in glyphosate resistance achieved in three or four generations may correspond to the maximum level achievable (Falconer, 1981). However, no substantial effects of genetic stress by restricting the population size were observed on the biomass of untreated plants (comparing unselected VLR1 and selected populations). We conclude that the variable phenotypic responses evident at sublethal herbicide doses means that even in a small susceptible *L. rigidum* population, these minor gene trait(s) can be enriched and channeled by recurrent selection to result in a degree of herbicide resistance (Neve and Powles, 2005a, b).

Potential mechanistic basis for low—dose-selected glyphosate resistance

In field-evolved glyphosate-resistant weed biotypes, two distinct glyphosate resistance mechanisms have been identified (reviewed by Powles and Preston, 2006). In *L. rigidum*, a reduced glyphosate translocation resistance mechanism and a mutation in the EPSPS target gene have been documented (Yu *et al.*, 2007; Preston and Wakelin, 2008). These two mechanisms are each single gene traits (Powles and Preston, 2006; Wakelin and Preston, 2006a, 2006b). It is very unlikely that these rare single genes were present in our originator susceptible population VLR1, given the very small number of plants that we had exposed to glyphosate selection (Yu *et al.*, 2007). Aside from these two single-gene-endowed glyphosate resistance mechan-

isms, reduced foliar retention of glyphosate and differential uptake have also been reported in *Lolium* (Michitte *et al.*, 2007). In our low-dose glyphosate-selected population, the mechanistic basis of resistance remains to be identified. We presume it may be multi-faceted because a gradual enrichment and accumulation of minor gene trait(s) was observed.

Conclusions

Identifying the main factors influencing the evolution of resistant weed populations is crucial for understanding, predicting and managing herbicide resistance. Our study highlights that selection at low (sublethal) glyphosate doses in cross-pollinated, genetically variable *Lolium* can lead to glyphosate resistance evolution. The results presented here are of special significance because of the importance of glyphosate in world food production and the recent evolution of several glyphosate-resistant weed biotypes (Powles, 2008). Moreover, these data might be relevant to the continued evolution of herbicide resistance crop technology toward the development of new breeding stacks for multiple herbicide-resistant crops combining resistance to several modes of action. We believe that herbicides should be used at the recommended rate to achieve high weed mortality and therefore minimize the possibility of accumulation of minor gene traits (creeping resistance) that may endow a level of resistance over a few generations. The rapid evolution of glyphosate (Figure 2) and diclofop-methyl resistance (Neve and Powles, 2005b) due to the recurrent use of low (sublethal) herbicide doses warrants future research on other herbicide modes of action. The genetic control and resistance mechanisms enriched from a susceptible small population remain unknown and should be investigated to identify the basis of low-dose-selected resistance. Substantial precautions need to be taken by the industry to ensure that herbicides are used at the registered recommended rates. This should contribute to the sustainability and longevity of herbicide molecules in world agriculture.

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Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)