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Rapid Evolution of Herbicide Resistance by Low Herbicide Dosages

Sudheesh Manalil, Roberto Busi, Michael Renton, and Stephen B. Powles*

Herbicide rate cutting is an example of poor use of agrochemicals that can have potential adverse implications due to rapid herbicide resistance evolution. Recent laboratory-level studies have revealed that herbicides at lower-than-recommended rates can result in rapid herbicide resistance evolution in rigid ryegrass populations. However, crop-field-level studies have until now been lacking. In this study, we examined the impact of low rates of diclofop on the evolution of herbicide resistance in a herbicide-susceptible rigid ryegrass population grown either in a field wheat crop or in potted plants maintained in the field. Subsequent dose–response profiles indicated rapid evolution of diclofop resistance in the selected rigid ryegrass lines from both the crop-field and field pot studies. In addition, there was moderate level of resistance in the selected lines against other tested herbicides to which the population has never been exposed. This resistance evolution was possible because low rates of diclofop allowed substantial rigid ryegrass survivors due to the potential in this cross-pollinated species to accumulate all minor herbicide resistance traits present in the population. The practical lesson from this research is that herbicides should be used at the recommended rates that ensure high weed mortality to minimize the likelihood of minor herbicide resistance traits leading to rapid herbicide resistance evolution.

Nomenclature: Diclofop; rigid ryegrass, *Lolium rigidum* Gaud. LOLRI; wheat, *Triticum aestivum* L.
Key words: Cross-resistance, field selection, reduced herbicide rate.

In most parts of the world, herbicides are the dominant technology used for the control of weeds that infest crops. Consequently, in situations of intense herbicide usage, there have been many examples of the evolution of weed populations resistant to herbicides (Heap 2010; Powles and Yu 2010). From an evolutionary perspective, many factors influence the dynamics of herbicide resistance evolution under herbicide selection (Darmency 1994; Jasieniuk et al. 1996). One crucial factor in herbicide resistance evolution is the intensity of herbicide selection, of which a major determinant is the herbicide use rate (i.e., g ha^{-1}). Herbicides, when used at the correct plant growth stage and at the registered label rate, cause very high mortality. However, there are situations where herbicides are used at rates that do not always cause such high weed mortality. Indeed, herbicide use rates can vary markedly between nations, regions, and enterprises. As one example, herbicide use rates in Australia are often only 50% of that in other parts of the world. For example, the registered use rate for the herbicide diclofop for rigid ryegrass control in Australia is 375 g ai ha^{-1} compared with 640 g ai ha^{-1} in the United States and 900 g ai ha^{-1} in France (Bayer 2010). Additionally, using herbicides at rates below the already low registered use rate (rate cutting) does occur in Australia as farm size is very large and profitability is low. Similarly, 28% of the cropped area in Canada manages weeds with reduced herbicide rates (reviewed by Beckie 2006). In addition to rate cutting, environmental variability under field conditions and decay kinetics for soil residual herbicides can result in lower-than-normal rates of herbicides being applied to target weed species (reviewed by Zhang et al. 2000). Also, if herbicide treatment occurs to bigger plants that are well past the optimum plant growth stage for control, this effectively constitutes a reduced herbicide rate (Wauchope et al. 1997). It is emphasized that where herbicides are used at low rates there can be weed survivors.

Recent studies have demonstrated that low rates of herbicides can result in the evolution of herbicide resistance. Studies (potted plants) with a herbicide-susceptible rigid ryegrass population recurrently selected with low rates of diclofop resulted in the rapid evolution of resistance (Neve and Powles 2005a,b). Similarly, recurrent selection of the same herbicide-susceptible rigid ryegrass population with low rates of glyphosate resulted in the evolution of a modest level of glyphosate resistance (Busi and Powles 2009). These laboratory-level studies demonstrate the potential for low herbicide use rates to result in herbicide resistance evolution (Neve and Powles 2005a,b; Busi and Powles 2009). However, thus far this phenomenon has not been studied in the field with crop and weeds growing in normal commercial agroecosystem field conditions. Therefore, to examine the potential for low herbicide use rates to lead to herbicide resistance evolution we have conducted experiments in a crop-field environment as well as in the laboratory environment with potted plants. We demonstrate the potential for low herbicide use rates to lead to rapid herbicide resistance evolution.

Materials and Methods

Recurrent Selection with Low Rates of Diclofop in a Pot Study. A rigid ryegrass population (WALR1) established to be susceptible to diclofop (Figure 1) and other herbicides was used in this study. In July 2006 (normal growing season), seeds (40) were sown in each of seven plastic trays (28 by 33 by 5 cm) filled with fresh potting mixture (sand and peat at 1 : 1 ratio) and maintained in the field (at the University of Western Australia) and kept well watered. When the majority of the seedlings were at the two- to three-leaf stage, they were thinned to give 200 uniform seedlings at the two- to three-leaf stage. These 200 seedlings were treated with half the Australian registered label rate (i.e., 187 g ai ha^{-1}) of diclofop (aryloxyphenoxypropionate herbicide inhibiting the enzyme acetyl-CoA carboxylase [ACCase] involved in the first step of fatty acid biosynthesis) plus 0.25% BS1000 (a nonionic surfactant) using a twin-nozzle laboratory sprayer calibrated to deliver 113 L of spray volume ha^{-1} at 210 kPa. Most plants

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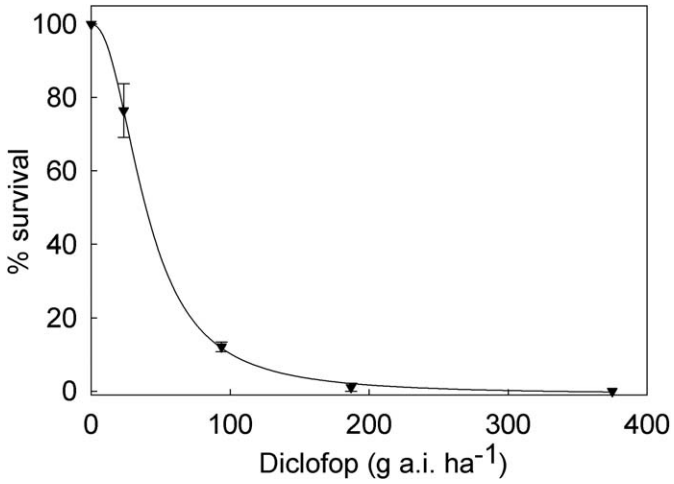

Figure 1. Dose–response curve for rigid ryegrass biotype WALR1 after application of a series of doses of diclofop in 2007. The symbols are mean observed percentage survival; error bars are ± one standard error of the mean (n = 3). The solid line is the predicted value for percentage survival.

were killed but 10%, although showing symptoms, survived and these surviving 20 seedlings were repotted (three per pot) in 250-mm plastic pots filled with fresh potting mixture and maintained in the field under well-watered conditions. These 10% survivors grew vigorously and at flowering, they were placed in pollen-proof enclosures to ensure random mating (rigid ryegrass is an obligate cross-pollinator) and to prevent the ingression of foreign pollen (watering was continued using a drip irrigation system). At maturity, seeds were harvested, cleaned, and stored in dry conditions until use. The collected seeds were designated as the 1P line, indicating the progeny of the first cycle of low-dose diclofop selection in pots.

In the next growing season (May 2007), a diclofop dose–response study was conducted with the once-low-dose diclofop-selected 1P line in comparison with its parent WALR1 line. Seeds of both lines were sown in plastic trays (28 by 33 by 5 cm) as above and when seedlings were at the two- to three-leaf stage 150 plants were treated each at rates of 23, 47, 94, 187, and 375 g diclofop ha^{-1} (with 0.25% BS1000), using the same laboratory sprayer (untreated controls were treated with water plus the surfactant). All seedlings were maintained in the field after treatment and scored for herbicide mortality after 21 d. Specifically for the 150 plants that were treated at 375 g diclofop ha^{-1}, there was 12% survival. These 18 survivors were repotted and grown to maturity for seed production (foreign pollen excluded) as described above. Seeds collected from these plants were designated as the 2P line, indicating the progeny of the second cycle of low-dose selection. In May 2008, a dose–response study with diclofop at rates of 94, 187, 375, and 750 g ha^{-1} was conducted to compare the response of the once- and twice-selected lines vs. the parent WALR1 line. After 21 d, plant survival was recorded and aboveground fresh biomass from all plants was determined and expressed as a percentage of the untreated control.

Recurrent Selection with Low Rates of Diclofop in a Crop Field (Wheat). The crop-field experiment was conducted in 2006 and 2007 at the experimental field station of the University of Western Australia (115°50′E, 31°55′S). This experimental site is separated by at least 100 km from cropping regions and it was chosen to avoid any pollen flow from herbicide-resistant rigid ryegrass, which is widespread in the cropping region (Owen et al. 2007). The density of rigid ryegrass plants growing naturally within a 500-m radius from the experimental plot was approximately 1 plant m^{-2} (400 random points assessed with a 0.25-m^2 quadrat). Measures were taken to minimize pollen or seed immigration from ryegrass plants growing adjacent to the experimental plot. For example, rigid ryegrass plants growing within the immediate 15 m of the experimental plot were manually removed or destroyed with glyphosate application in both 2006 and 2007. To assess the herbicide resistance status of the native rigid ryegrass population, seeds were collected from plants growing in the vicinity of the experimental plot (representing 500-m radius) and herbicide screened. To achieve this, seeds (40) were sown in plastic trays (28 by 33 by 5 cm) filled with potting mixture and 100 uniform seedlings at two-to three- leaf stage (three replicates) were treated with 93 g sethoxydim ha^{-1} using the laboratory sprayer, which resulted in 100% mortality.

Early in the 2006 growing season the area (0.5 ha) was irrigated and cultivated several times to stimulate rigid ryegrass germination and thus exhaust any weed seed bank. Before planting, a total of 50 soil samples (0-to 20-cm depth) were collected at the experimental plot across two main diagonal transects of the experimental area of 0.5 ha. Soil samples were weighed and distributed in 10 trays and watered well during winter and spring 2006 (July–December). Emergence of no ryegrass plants was recorded. In July 2006, this weed-free field was seeded in 18-cm rows with 60 kg ha^{-1} wheat using a commercial seeder. On the same day, 2 kg of herbicide-susceptible WALR1 rigid ryegrass seed (roughly corresponding to 600,000 seeds) was carefully hand-broadcast over the 0.5-ha experimental plot and then lightly hand-raked to achieve shallow incorporation. The experimental plot was fertilized with P 25, K 50, S 30, and Ca 55 (all kg ha^{-1}) while seeding with the seeder, and N at 180 kg ha^{-1} was broadcast as three splits, 1 wk after seeding and twice during wheat tillering. Irrigation was provided at regular intervals using an overhead sprinkler system. The broadcast-seeded herbicide-susceptible WALR1 rigid ryegrass seed emerged and established to give a density of approximately 20 seedlings m^{-2}, which is representative of the densities of rigid ryegrass infesting commercial wheat and other crops in Australia. There was uniform rigid ryegrass emergence across the field. When infesting rigid ryegrass seedlings were at the two- to three- leaf stage, a below-label 281 g diclofop ha^{-1} (75% of the Australian label rate) was applied (0.25% BS1000 surfactant) using a boom sprayer calibrated to deliver 133 L total volume ha^{-1}. The rigid ryegrass seedlings were counted before and 21, 33, and 45 d after diclofop treatment with a quadrat of 0.25 m^2 at 50 random points within the 0.5-ha plot. In total, around 100,000 rigid ryegrass seedlings within the wheat crop were diclofop treated and this caused high mortality (95% at 45 d after treatment) but leaving survivors at approximately one rigid ryegrass survivor m^{-2}. Most of these diclofop survivors (around 5,000 in total over the 0.5 ha) grew to maturity within the wheat crop, flowered, and produced seed. It is emphasized that these surviving plants showed symptoms of herbicide treatment; obviously, the growth and seed set from these surviving rigid ryegrass were not as good as rigid ryegrass growing in an ideal environment. There were no interventions; these surviving rigid ryegrass plants had to mature in the prevailing competitive environment within the crop and

flowering and pollen movement occurred naturally. Although rigid ryegrass growing within 15 m of the experimental plot were removed, there existed the possibility of pollen movement of native herbicide-susceptible rigid ryegrass growing in the vicinity of the experimental plot. As the experimental plot is at least 100 km from crop fields there was no possibility of resistance being introduced by pollen flow from resistant plants. In addition, we have done periodic inspections to ensure that there is not a flush of untreated rigid ryegrass after herbicide treatment. When the diclofop-surviving rigid ryegrass plants were mature in the crop, their seed was hand-harvested across the entire 0.5-ha experimental plot, then threshed and stored in warm and dry conditions over summer to ''after-ripen'' and overcome seed dormancy (Steadman 2004). This collected seed was designated as 1F line indicating the first cycle of selection in the field conditions. In May 2007, a dose–response study similar to that described above was conducted to compare the response of the field-selected 1F line with the susceptible WALR1 line (parent).

In 2007, the wheat crop-field selection with diclofop was repeated in the same manner on the same area as described for 2006 (on a smaller plot of 225 m^2). The 2006 seed progeny 1F (80 g, roughly corresponding to 25,000 seeds) was hand-broadcast-seeded in 2007. As previously described there was uniform rigid ryegrass emergence that resulted in a population density of 27 rigid ryegrass seedlings m^{-2}, giving a total of around 6,000 rigid ryegrass seedlings that were subsequently treated with diclofop in the wheat crop. When these rigid ryegrass seedlings were at the two- to three-leaf stage, 375 g diclofop ha^{-1} was applied with 0.25% BS1000 using the same boom sprayer. This treatment caused high mortality, but there was 11% survival. These 11% survivors grew to maturity within the wheat crop, flowered, and produced seed that was harvested, threshed, and stored and designated as the 2F line. In May 2008, the two field-selected lines were included in the final dose–response study as described above in the pot study conducted in May 2008. After 21 d, plant survival was recorded; aboveground fresh biomass was determined and expressed as the percentage of the mean of the untreated control.

Effect of Herbicides of Different Chemistries and Modes of Action. In 2008, herbicide resistance profiling of the twice-selected rigid ryegrass progenies from both the field-grown pot (2P) and the crop-field experiments (2F) was conducted in comparison with the susceptible WALR1 line to assess any change in sensitivity to various herbicides. Seeds (20) were sown in plastic pots (180-mm size) filled with fresh potting mixture and maintained in the field and watered as required. When the majority of the seedlings were at the two- to three-leaf stage, seedlings were thinned to give 48 uniform seedlings (3 replicate pots with 16 seedlings) for each herbicide treatment rate. These seedlings were treated with a series of rates of haloxyfop (0, 8, 15, 50, g ai ha^{-1}), fluazifop-P (0, 20, 40, 90 g ai ha^{-1}) (both ACCase-inhibiting aryloxyphenoxypropionates), sethoxydim (0, 15, 30, 65, g ai ha^{-1}), clethodim (0, 5, 12, 30 g $aiha^{-1}$) (both ACCase-inhibiting cyclohexanediones), chlorsulfuron (0, 8, 15, 30 g ai ha^{-1}), and imazethapyr (0, 20, 40, 90 g ai ha^{-1}) (both actolactate synthase [ALS] inhibitors). All the seedlings were maintained in the field and plant survival was assessed 21 d after herbicide application.

Statistical Analysis. A logistic model (Equation 1) was fitted to the survival data, where Y is plant

$$Y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad [1]$$

survival as a percentage and d the upper asymptotic values of Y. The parameter e is the herbicide rate producing a survival rate half way between the lower limit zero and upper limit d, the parameter x is herbicide dose, and the parameter b denotes the relative slope around e (Ritz and Streibig 2005). The same logistic model was also fitted to the biomass data. These fitted logistic models were used to estimate the rate of herbicide that causes 50% mortality (LD_{50}) and the rate that causes 50% growth reduction (GR_{50}) using the statistical software R (version 2.7) (R Development Core Team 2009) with its drc package (Knezevic et al. 2007). The null hypothesis that the LD_{50} values of the selected and unselected rigid ryegrass populations were the same was tested using the selectivity index (SI) function in the drc package. The same function was also used to test the GR_{50} values of the selected and unselected rigid ryegrass populations. The response to selection for the different selected progenies was measured as the R : S ratio (resistant : susceptible) of the estimated LD_{50} and GR_{50} values. Plant survival dose–response graphs are presented with untransformed data (not in log scale).

Results and Discussion

Confirmation of Diclofop Susceptibility of the Parent Rigid Ryegrass Population WALR1. In a preliminary herbicide screening in 2006, the rigid ryegrass population WALR1 was confirmed to be diclofop susceptible (100% mortality at 375 g diclofop ha^{-1}, the registered label rate in Australia). Further, dose–response experiments have confirmed the susceptibility of this population, with mortality ranging from 98 to 100% at the registered diclofop label rate (375 g diclofop ha^{-1}) (data not shown). The estimated LD_{50} values of this WALR1 population were 40 and 69 g diclofop ha^{-1} in the 2007 and 2008 experiments, respectively. This difference between years was mainly due to environmental variability affecting herbicide efficacy and subsequent plant survival in rigid ryegrass, as also observed by Neve and Powles 2005b. As expected, at lower diclofop rates there was plant survival, indicating phenotypic variation for diclofop sensitivity in this population (Figure 1). In the pot study, with two- to three-leaf-stage seedlings treated at 187 g diclofop ha^{-1} (50% of the registered label rate), there was 10% survival in the unselected parent WALR1 population. Similarly, in the crop-field experiment at 281 g diclofop ha^{-1} (75% of the registered label rate), there was 5% survival.

Resistance Evolution in Rigid Ryegrass Population WALR1 in Pots. Even after just one cycle of sublethal diclofop selection, there was clear evidence of diclofop resistance in the pot experiment (Table 1; Figure 2a). The LD_{50} of the once-selected 1P line (LD_{50}=150 g diclofop ha^{-1}) was substantially higher compared with its parent line (LD_{50}=69 g diclofop ha^{-1}) (Table 2); the corresponding R : S LD_{50} was 2.2. The second cycle of selection resulted in high-level diclofop resistance (Figure 2a). The LD_{50} of the twice-selected 2P individuals (1,272 g diclofop ha^{-1}) was much higher compared with the originating parent popula-

Table 1. Parameters and estimated 50% lethal dose (LD_{50}) and 50% growth reduction (GR_{50}) values from the logistic model: $Y = d/1 + \exp\{b[\log(x) - \log(e)]\}$ fitted to the dose–response data for the WALR1 biotype and the selected lines 1P, 2P,1F, and 2F with a series of doses of diclofop in 2008.

	% Survival							% Biomass						
Biotype	*d*	*e*	*b*	RMS[a]	LD_{50}	R^2 [b]	R : S ratio[c]	*d*	*e*	*b*	RMS[a]	GR_{50}	R^2 [b]	R : S ratio[d]
WALR1	100	69	2	25	69	0.98	1	100	60	0.4	40	60	0.94	1
1F	100	120	1	31	120	0.97	1.7	100	252	0.6	73	252	0.88	4.2
1P	100	150	1	49	150	0.95	2.2	100	388	0.7	65	388	0.88	6.5
2F	100	258	1	70	258	0.92	3.7	100	575	1	74	575	0.85	9.6
2P	100	1272	2	14	1272	0.90	18.4	100	1065	1	116	1065	0.84	17.8

[a] Residual mean square.
[b] Adjusted R^2.
[c] LD_{50} R : S ratios calculated as LD_{50} for selected line/LD_{50} for unselected WALR1 biotype.
[d] GR_{50} R : S ratios calculated as GR_{50} for selected line/GR_{50} for unselected WALR1 biotype.

tion (69 g diclofop ha^{-1}) (Table 2); the corresponding R : S LD_{50} was 18.4. The GR_{50} and R : S GR_{50} computed from the fresh plant biomass assessment following diclofop treatment followed a similar trend to the LD_{50} and R : S LD_{50} (Tables 1, 2). The GR_{50} of the once-selected pot 1P line was 388 g diclofop ha^{-1} compared with 60 g diclofop ha^{-1} for its parent line; the corresponding R:S GR_{50} value was 6.5. Similar to the survival data, a many-fold increase in GR_{50} was apparent with the second cycle of selection; the GR_{50} of the 2P line was 1,065 g diclofop $ha^{-1}$and the corresponding R : S GR_{50} value was 17.8.

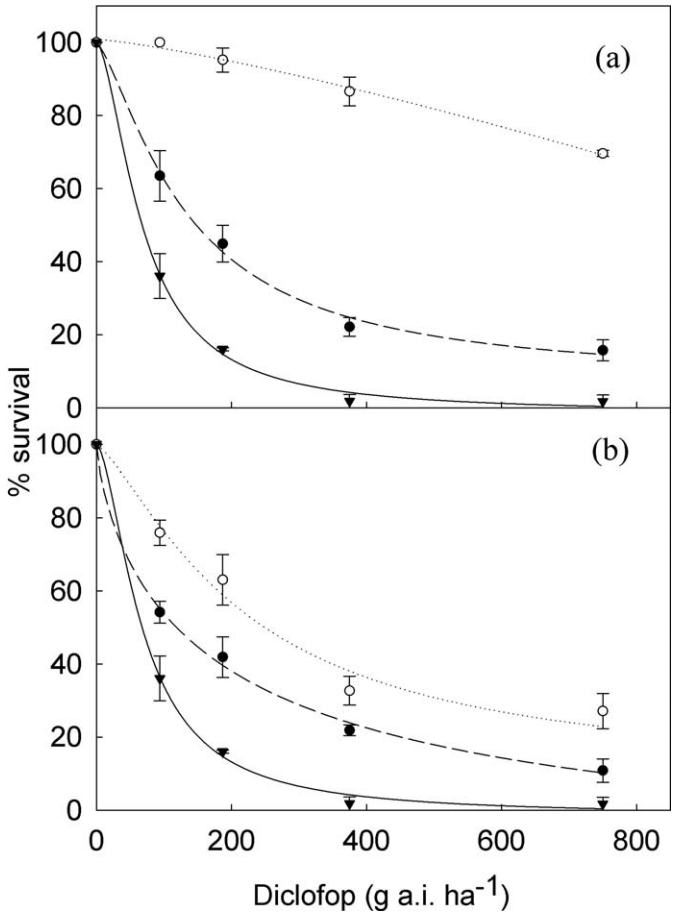

Figure 2. Dose—response curves for rigid ryegrass biotypes after the second cycle of selection in 2008 in (a) the pot experiment and (b) the crop-field experiment, following application of a series of doses of diclofop. WALR1: solid line, solid triangle; first-generation selected line: broken line, solid circle; second-generation selected line: dotted line, open circle. Symbols are mean observed percentage survival; error bars are ± one standard error of the mean ($n = 3$). Lines are the predicted values for percentage survival.

The high-level diclofop resistance that evolved in the initially herbicide-susceptible pot-selected rigid ryegrass (Figure 2a) illustrates the potential for selection at reduced rates of diclofop to result in rapid resistance evolution, confirming the results of Neve and Powles (2005a,b). This resistance evolution in susceptible rigid ryegrass (Figure 1) is undoubtedly due to the survivors possessing genetically endowed traits enabling survival at low rates of diclofop. It is emphasized that rigid ryegrass is strictly cross-pollinated, meaning that gene traits endowing low-level diclofop resistance are enriched in the progeny (Figure 2a).

Resistance Evolution in Rigid Ryegrass WALR1 in a Crop Field. As for the pot experiment (Figure 2a), the wheat-field experiment revealed resistance evolution from recurrent selection at low diclofop rate (Figure 2b). In 2008, the once and twice crop-field diclofop-selected lines 1F and 2F were compared with their unselected susceptible parent (WALR1). The LD_{50} of the once-selected line 1F line (120 g diclofop ha^{-1}) was significantly higher compared with its parent (69 g diclofop ha^{-1}) (Table 2); the corresponding R : S LD_{50} ratio was 1.7. The twice crop-field-selected 2F line showed significantly higher level of resistance with the second cycle of selection (Table 2, Figure 2b). There was an increase in the LD_{50} of the twice-selected 2F line (258 g diclofop ha^{-1}) compared with its susceptible parent (69 g diclofop ha^{-1}) (Table 1); the corresponding R : S LD_{50} was 3.7. The GR_{50} and R : S GR_{50} computed from the fresh plant biomass assessment in the crop-field selected lines indicated increased diclofop resistance as for the LD_{50} and R : S LD_{50} (Tables 1,2). The GR_{50} of the once crop-field-selected 1F line was 252 g diclofop ha^{-1} compared with 60 g diclofop ha^{-1} of its susceptible parent; the corresponding R : S GR_{50} was 4.2. Similarly, there was increase in the estimated GR_{50} of the twice crop-field-selected 2F line (575 g diclofop ha^{-1}); the corresponding R : S GR_{50} was 9.6.

The crop-field study demonstrates for the first time the herbicide resistance evolution resulting from low herbicide rate selection in a crop-field environment (Table 1, Figure 2b). The first cycle of selection was carried out at 75% of the registered label rate of diclofop, a reduced rate that is representative of that that prevailed in Australian cropping. As was found with the pot experiments, this field low-dose selection resulted in resistance evolution (Tables 1, 2). Although the resistance evolution that occurred in the field

Table 2. Estimates of 50% lethal dose (LD_{50}) or 50% growth reduction (GR_{50}) ratios and corresponding P-values from the comparison between the unselected and selected populations.

	% Survival		% Biomass	
Biotypes compared	Estimate of LD_{50} ratio[a]	P-value[b]	Estimate of GR_{50} ratio[a]	P-value[b]
1F : WALR1	1.7 (0.09)	0.001	4.2 (0.11)	< 0.0001
2F : WALR1	3.7 (0.05)	< 0.0001	9.6 (0.5)	< 0.0001
2F : 1F	2.2 (0.08)	< 0.0001	2.3 (0.13)	< 0.0001
1P : WALR1	2.2 (0.07)	< 0.0001	6.5 (0.07)	< 0.0001
2P : WALR1	18.4 (0.01)	< 0.0001	17.8 (0.03)	< 0.0001
2P : 1P	8.5 (0.02)	< 0.0001	2.8 (0.14)	< 0.0001

[a] Standard errors are shown in parentheses.
[b] The P-value indicates there is significant difference between all the populations compared.

was substantial, it was lower than that observed in the pot experiment (Table 2, Figure 2b). We attribute this difference to the conditions prevailing in a crop field. In the pot experiment, pollen flow could only occur among the herbicide survivors. In the crop-field situation, wind-borne pollen from susceptible rigid ryegrass outside the experimental area and that from late-emerging susceptible rigid ryegrass within the field could dilute pollen from herbicide survivors and thus slow the evolution of resistance.

Effect of Other Herbicides in Pot- and Crop-Field-Selected Rigid Ryegrass. In addition to the evolution of diclofop resistance (Figure 2a), there was evidence of moderate level of resistance to other ACCase herbicides haloxyfop or fluazifop-P and the ALS herbicides chlorsulfuron or imazethapyr in the pot-selected line (Table 3, Figures 3a–d). The observed resistance against all four of these herbicides was not of the magnitude observed for diclofop. The R : S LD_{50} estimated for fluazifop-P and haloxyfop was 1.5 and 1.4 respectively. There was clear evidence of resistance to chlorsulfuron and imazethapyr with R : S LD_{50} of 3.8 and 2.4 respectively. There was no evidence of increased resistance to sethoxydim and clethodim, where the response of the selected population was the same as that of the susceptible parent line (Table 3, Figures 3e,f).

Similarly to the pot-selected line (Table 3, Figures 3a–d), increased survival of the twice crop-field-selected rigid ryegrass line was observed for haloxyfop, fluazifop-P, chlorsulfuron, and imazethapyr (Table 3, Figures 3a–d). As for the pot-selected line, the response of the crop-field-selected population to sethoxydim and clethodim was the same as that of the susceptible line (Table 3, Figures 3e,f). There was moderate resistance to haloxyfop and fluazifop-P; the respective R : S LD_{50} values were 1.4 and 1.6. Similarly, there was resistance to chlorsulfuron and imazethapyr; the R : S LD_{50} values were 2.6 and 1.4 respectively.

Table 3. Parameters and estimated 50% lethal dose (LD_{50}) values from the logistic model: $Y = d/1 + \exp\{b[\log(x) - \log(e)]\}$ fitted to the dose–response data for the WALR1, 2P, and 2F lines treated with a series of doses of selected acetyl-CoA carboxylase- and acetolactate synthase-inhibiting herbicides.

Biotype	*d*	*e*	*b*	RMS[a]	R^{2b}	LD_{50}	R : S ratio[c]
Imazethapyr							
WALR1	100	13	2	15	0.99	13	1.0
2F	100	18	1	17	0.98	18	1.4[d]
2P	100	31	1	104	0.90	31	2.4[d]
Chlorsulfuron							
WALR1	100	16	1	112	0.84	16	1.0
2F	100	41	1	37	0.86	41	2.6[d]
2P	100	60	3	11	0.80	60	3.8[d]
Fluazifop-P							
WALR1	100	26	4	18	0.99	26	1.0
2F	99	41	5	146	0.91	41	1.6[d]
2P	100	40	3	131	0.91	40	1.5[d]
Haloxyfop							
WALR1	100	8	4	51	0.98	8	1.0
2F	100	11	5	49	0.97	11	1.4[d]
2P	100	11	4	53	0.96	11	1.4[d]
Sethoxydim							
WALR1	100	30	6	6	0.99	30	1
2F	101	32	4	37	0.98	32	1.1
2P	101	31	3	104	0.92	31	1
Clethodim							
WALR1	99	10	3	22	0.99	10	1
2F	98	11	3	25	0.99	11	1.1
2P	99	11	3	109	0.93	11	1.1

[a] Residual mean square.
[b] Adjusted R^2.
[c] LD_{50} R : S ratios calculated as LD_{50} for selected line/LD_{50} for unselected WALR1 biotype.
[d] Significant difference between selected line and unselected line at $P \leq 0.05$.

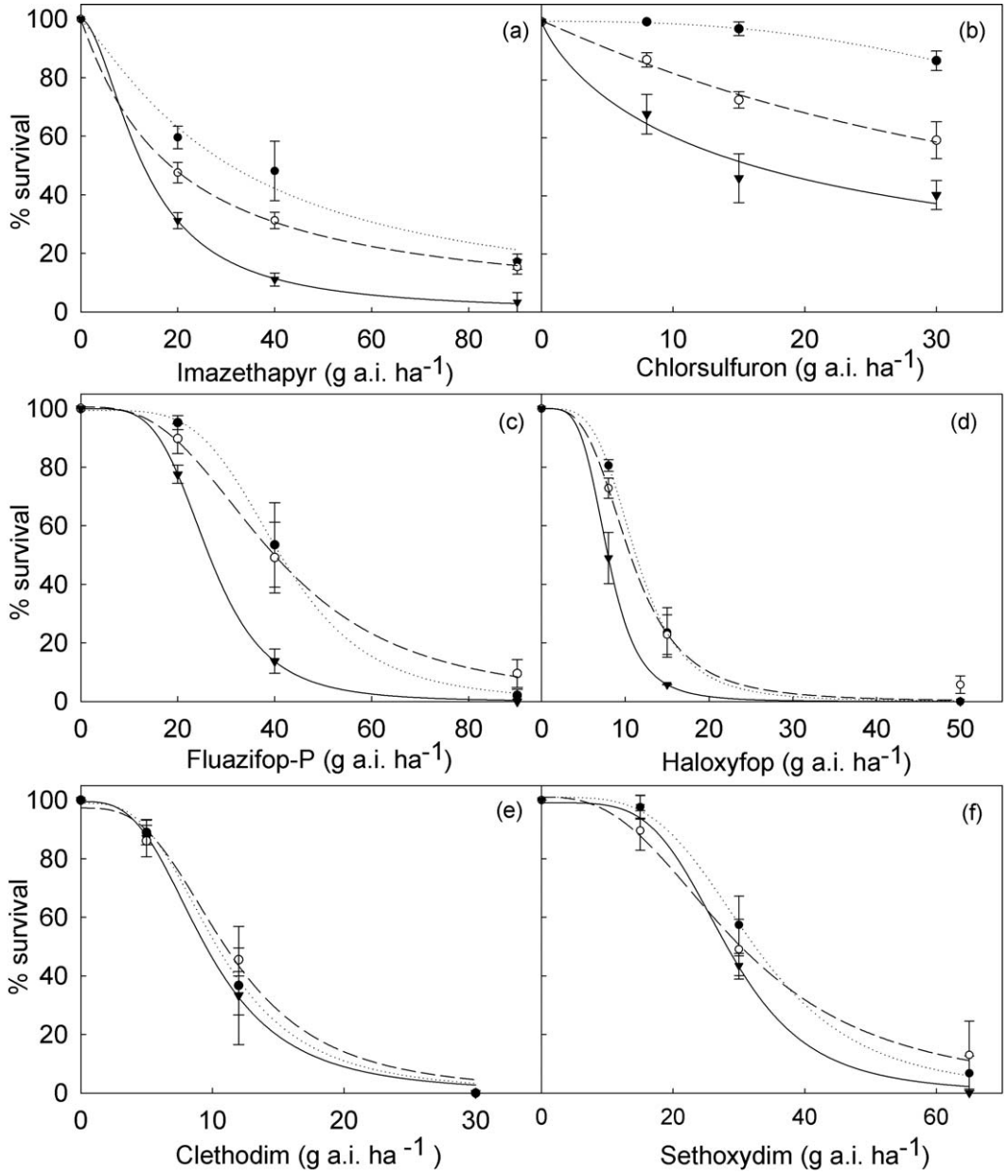

Figure 3. Dose–response curves for rigid ryegrass biotype WALR1 (solid line, black triangle), the second-generation field-selected line 2F (broken line, open circle), and the second-generation pot-selected line 2P (dotted line, solid circle) treated with a range of doses of imazethapyr (a), chlorsulfuron (b), fluazifop-P (c), haloxyfop (d), clethodim (e), sethoxydim (f). Symbols represent mean percentage survival; error bars are ± one standard error of the mean ($n = 3$). Lines are the predicted values for percentage survival.

Importantly, the diclofop-resistant lines (twice-selected 2F and 2P) from both the pot experiment and the crop-field experiment exhibited higher survival against other similar ACCase-inhibiting herbicides haloxyfop and fluazifop-P and the very dissimilar ALS-inhibiting herbicides chlorsulfuron and imazethapyr (Table 3, Figures 3a–d). This phenomenon is referred to as nontarget-site cross-resistance, where evolution of resistance to one herbicide results in resistance to herbicides of a different mode of action (Hall et al. 1994). The results of the current study (Table 3, Figures 3a–d) are in agreement with an earlier study, where cross-resistance was evident in an initially herbicide-susceptible rigid ryegrass selected with low rates of diclofop (Neve and Powles 2005b). Overall, this cross-resistance suggests that the selected herbicide resistance traits are likely metabolic rather than specific target-site-based traits. All of the herbicides for which cross-resistance was observed (Table 3, Figures 3a–d) are known to be metabolized by herbicide-resistant rigid ryegrass populations through the activity of cytochrome P450 mono-oxygenases (Christopher et al. 1994; Hidayat and Preston 2001). In addition, no cross-resistance occurred to clethodim or sethoxydim (Table 3, Figures 3e,f); these herbicides are lethal to wheat because, unlike some other herbicides (Anderson et al. 1989; Forthoffer et al. 2001), wheat cannot detoxify these herbicides by P450 mono-oxygenase metabolism. The results of the cross-resistance profile thus indicate that the herbicide resistance traits selected by low rates of diclofop are probably metabolism based.

It is emphasized that these studies were conducted with only small numbers of rigid ryegrass exposed to a herbicide

rate that left some surviving individuals. These survivors possessed weak resistance traits that were enriched in progeny through recurrent selection and cross-pollination (Figure 1). Obviously, even in a small population, genetic variability in a species like rigid ryegrass means that at low herbicide rate selection there are weak resistance gene traits present that are rapidly selected and enriched and accumulated through cross-pollination to result in resistance evolution. Similarly, insect species show rapid evolution of resistance under low-dose insecticide selection due to selection and enrichment of existing phenotypic variation within a population (Roush and McKenzie 1987). This is because minor polygenic traits are selected by low rates of pesticides (ffrench-Constant et al. 2004; McKenzie 2000). Conversely, under high-dose pesticide selection, major phenotypic traits (rare mutations with high trait value) are selected (ffrench-Constant et al. 2004; McKenzie 2000). It is important to note that in variable field conditions pesticide dose and therefore selection intensity can vary considerably and therefore it is likely that minor polygenic traits are selected and enriched (Groeters and Tabashnik 2000; McKenzie and Batterham 1994). Thus the intrinsic genetic variability of a target pest population and selection intensity (pesticide dose) are crucial factors in whether resistance evolution is monogenic (Mendelian inheritance) or polygenic (quantitative inheritance) (ffrench-Constant et al. 2004; Neve 2007; Roush and McKenzie 1987). We used a polygenic herbicide resistance model (Manalil 2010; Renton 2009) to understand the possible genetics behind resistance evolution due to recurrent selection with low doses of diclofop-methyl in rigid ryegrass. The simulated dose–response characteristics of the field experiment matched best with the field selection experiment when the genes involved are more than one (Manalil 2010). The simulations further indicated that there was no completely homozygous resistant plant in the original population (WALR1); however, a progressive increase in the resistant genotypes was observed because of recurrent selection and cross-pollination (Manalil 2010).

Current herbicide resistance understanding reveals that many resistance mechanisms can be responsible for herbicide resistance evolution (Powles and Yu 2010). Particular resistance gene traits can confer high-, moderate-, or low-level herbicide resistance (Powles and Yu 2010; Yuan et al. 2007). Especially in cross-pollinated species like rigid ryegrass (de Prado et al. 2005) or blackgrass (*Alopecurus myosuroides* Huds. ALOMY) (Letouze and Gasquez 2003), all resistance genes can be accumulated in individuals. Selection and enrichment of weak resistance mechanisms may greatly depend on the herbicide use rates (Figure 2, Neve and Powles 2005a,b; Busi and Powles 2009).

Implications of Reduced Herbicide Rate. Registration herbicide rates are set at a level designed to provide high weed mortality across a range of environmental conditions and even weed growth stages (Doyle and Stypa 2004). Use of herbicides at reduced rates is risky because herbicide efficacy depends strongly on factors such as the competitive ability of the crops, the efficiency of herbicide application, the prevailing environment, and the crop growth stage (Blackshaw et al. 2006). If a reduced herbicide rate results in substantial weed survivors then resistance evolution can follow, especially in cross-pollinated species that can accumulate resistance traits. On a herbicide-resistance management perspective, the resistance due to nontarget-site resistance mechanisms are difficult to manage by changing the herbicide as these mechanisms confer herbicide resistance across the herbicide mode of action groups (Figure 3, Powles and Yu 2010; Preston 2004; Yuan et al. 2007). The practical implications of this study are that herbicides should be used at rates that achieve high weed mortality, thus minimizing the accumulation of weak resistance gene traits in target weed populations, especially in cross-pollinated weed species.

Our study demonstrates, for the first time in the field in a commercial crop, the potential implications of herbicide rate cutting leading to rapid herbicide resistance evolution in rigid ryegrass through the selection and accumulation of minor herbicide resistance traits. The similar pattern of resistance and cross-resistance of the selected populations from the pot and the crop field indicates the similarity in the evolutionary process in both the pot and the crop field. Future work by simulation modeling is warranted to identify the effect of different herbicide rates (selection intensity) in the evolution of herbicide resistance as evidenced in the present study. This should help to identify optimal management options. The results of this study illustrate the advantage of using herbicides only at rates that cause very high target weed mortality, thus avoiding rapid evolution of herbicide resistance and cross-resistance in genetically variable rigid ryegrass.

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