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An Herbicide-Susceptible Rigid Ryegrass (*Lolium rigidum*) Population Made Even More Susceptible

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

A wild population of a plant species, especially a cross-pollinated species, can display considerable genetic variation. Genetic variability is evident in differential susceptibility to an herbicide because the population can show continuous phenotypic variation. Recent, recurrent selection studies have revealed that phenotypic variation in response to low herbicide rates is heritable and can result in rapid evolution of herbicide resistance in genetically variable cross-pollinated rigid ryegrass. In this study, the heritable genetic variation in an herbicide-susceptible rigid ryegrass population was exploited to shift the population toward greater herbicide susceptibility by recurrent selection. To enhance herbicide susceptibility, herbicide-susceptible rigid ryegrass plants were divided into two identical clones, and one series of cloned plants was treated with a low rate of herbicide (diclofop). The nontreated clones of individuals that did not survive the herbicide treatment were selected and bulk-crossed to obtain the susceptible progeny. After two cycles of selection, the overall susceptibility to diclofop was doubled. The results indicate that minor genes for resistance are present in an herbicide-susceptible rigid ryegrass population, and their exclusion can increase susceptibility to diclofop.
Nomenclature: Diclofop; rigid ryegrass, *Lolium rigidum* Gaudin. LOLRI.
Key words: Herbicide susceptibility, reduced herbicide rate, VLR1.

Weeds are an ubiquitous problem in global crop production (Oerke 2006) because of their capacity to persist despite changing selection pressure (Harper 1977). During the past 5 decades, throughout many parts of the world, herbicides have become the dominant tool for weed control. Herbicides achieve very high weed mortality, and thus, from an evolutionary perspective, herbicide treatments impose strong and persistent selection pressure. Inevitably, in response, herbicide-resistant weed populations have also evolved (Powles and Yu 2010).

The dynamics of the evolution of herbicide resistance is affected by biological, genetic, and herbicide operational factors (Darmency 1994; Jasieniuk et al. 1996; Neve 2007). Herbicide rate is an important but underappreciated operational factor affecting the intensity of selection pressure exerted on weed populations. Recent studies with the highly genetically variable, cross-pollinated weed species rigid ryegrass demonstrate that a low herbicide rate can lead to rapid resistance evolution. In this cross-pollinated weed species, low-rate herbicide selection (an herbicide rate that leaves substantial numbers of surviving individuals) causes rapid herbicide-resistance evolution (Busi and Powles 2009; Manalil et al. 2011; Neve and Powles 2005a,b). Here, we further explore the genetic variability for herbicide susceptibility within a rigid ryegrass population. Rather than selecting for herbicide resistance, the genetic variation within an herbicide-susceptible rigid ryegrass population is exploited to select for the most herbicide-susceptible individuals. Through the power of recurrent selection over two generations, we have enriched the frequency of highly herbicide-susceptible individuals in the population. Thus, in two generations, we have shifted an herbicide-susceptible rigid ryegrass population to a super-susceptible population.

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*First author: Post-Doc Researcher, School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia and Assistant Professor, Kerala Agricultural University Trissur, Kerala, India, 680 656; second author: Researh Associate, Australian Herbicide Resistance Initiative, School of Plant Biology, Institute of Agriculture, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia; third author: Assistant Professor, School of Plant Biology, University of Western Australia, 35 Sitrling Highway, Crawley 6009, Australia and Ecocsystem Sciences, CSIRO, Floreat, WA, Australia; fourth author: Professor, Director, Australian Herbicide Resistance Initiative, School of Plant Biology, Institute of Agriculture, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia. Corresponding author's E-mail: stephen.powles@uwa.edu.au

Materials and Methods

Protocol for Recurrent Selection toward Greater Herbicide Susceptibility. The study used a rigid ryegrass population ('VLR1') that is well characterized as susceptible to a wide range of herbicides (Neve and Powles 2005b). The study was conducted at the University of Western Australia, Perth (31°59′4.19″S, 115°49′8.68″E) during the normal growing season for this species. During this period (May to October), the day/night temperatures at this location ranges from 15/6 C to 21/12 C, with 6 to 7 h of average sunshine. The daytime relative humidity varies from 50 to 60% (Bureau of Meteorology 2011). In September 2006, seedlings were pregerminated (1% solidified agar), and at the one-leaf stage, 180 seedlings were transplanted into pots and were thereafter maintained outdoors. Plants were fertilized with water soluble N–P–K fertilizer (19–8.4–15.8, with Mg 1, Fe 0.1, Mn 0.05, B 0.02, Zn 0.015, Cu 0.01, and Mo 0.007, all in percentages). Twenty grams of this fertilizer was dissolved in 10 L of water and was foliar-applied (approximately 500 ml pot^{-1}) using a watering can 1 wk after planting. Watering was done at approximately 3-d intervals. These plants tillered vigorously and after 1 mo, each individual was divided into two identical clones. Each set of clones was planted in cell trays (28 by 33 by 5 cm, 20 cloned plants $tray^{-1}$) in potting mixture (50% river sand, 30% peat moss, and 20% tree bark). Labeling was used to identify the cloned counterparts in both sets. The clones were maintained outdoors in identical conditions and allowed to reestablish. After 1 wk, one set of clones was treated with a low rate (94 g ai ha^{-1}, one-quarter of the usual field rate) of the acetyl-coenzyme A carboxylase (ACCase)–inhibiting herbicide diclofop plus a surfactant (0.25% v/v of BS1000 [nonionic surfactant, Crop Care Australasia Pty. Ltd., Portal North, Unit 15, 16 Metroplex Avenue, Murarrie, QLD, Australia]) using a twin-nozzle laboratory sprayer (TeeJet XR11001 flat-fan nozzle, TeeJet Australasia Pty Ltd, 65 West Fyans Street, Newtown, Victoria 3220, Australia) calibrated to deliver a volume of 113 L water ha^{-1} at 210 kPa. After 21 d, plant survival was assessed, and as expected, at the low herbicide rate, there was only 30% plant mortality. The nonsprayed clones of only those individuals that were killed by the low herbicide rate (diclofop

Table 1. Parameters and estimated herbicide rate corresponding to 50% mortality (LD_{50}) and GR_{50} (the herbicide rate corresponding to 50% growth reduction) values from the logistic model: $Y = d/(1+\exp\{b[\log(x) - \log(e)]\})$ fitted to the rate response data for the rigid ryegrass (VLR1) biotype and the selected lines 1S (once selected) and 2S (twice selected) with a series of rates of diclofop in 2008.[a]

	% Survival								% Biomass							
Biotype	*d*	*e*	*b*	RMS	LD_{50}	R^2	R : S ratio[b]	P value[d]	*d*	*e*	*b*	RMS	GR_{50}	R^2	R : S ratio[c]	P value[d]
VLR1	101	33	3	8	33	0.99	1		100	24	2	22	24	0.97	1	
1S	99	23	3	15	23	0.99	0.7	< 0.001	100	18	1	19	18	0.98	0.8	< 0.001
2S	99	15	2	31	15	0.99	0.5	< 0.0001	100	11	1	13	11	0.98	0.5	< 0.0001

[a] Abbreviations: RMS, residual mean square; R^2, adjusted R^2; R : S, ratio of resistant to susceptible.
[b] LD_{50} R : S ratios were calculated as the ratio of the LD_{50} for the selected line to the LD_{50} for the unselected VLR1 biotype.
[c] GR_{50} R : S ratios were calculated as the ratio of the GR_{50} for the selected line to the $GR_{50}GR_{50}$ for the unselected VLR1 biotype.
[d] P value for testing the null hypothesis that R : S ratio the same.

at 94 g ai ha^{-1}) were identified and grown to maturity. Before anthesis, 24 of these individuals were placed within a pollen-proof enclosure. Rigid ryegrass is an obligate cross-pollinating species, and random mating occurred only among these 24 plants. At maturity, seed from these plants was harvested, cleaned, and stored in dry conditions. They were designated as progeny 1S, indicating the once-selected progeny toward greater herbicide susceptibility. In the following growing season (May 2007), 300 seedlings of progeny 1S were grown and cloned as described above. Similarly, one set of clones were treated with a very low rate of diclofop (47 g ha^{-1}; one-eighth the usual field rate), and mortality was assessed after 21 d. At a diclofop rate of 47 g ha^{-1}, only 18% mortality occurred; 24 of the untreated, cloned counterparts corresponding to the dead plants were grown to maturity, and seed was collected as described above. The second seed collection was designated as progeny 2S because it represented the twice-selected, low diclofop rate, rigid ryegrass progeny.

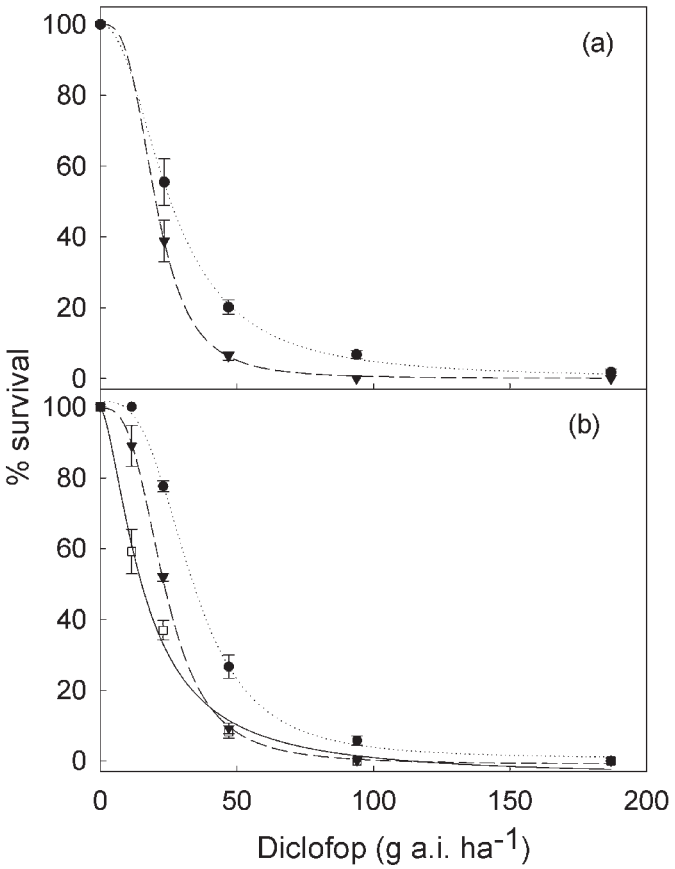

Figure 1. Rate–response curves for rigid ryegrass biotypes selected for susceptibility in (a) 2007 and (b) 2008, following application of a series of rates of diclofop. Rigid ryegrass (VLR1): dotted line, solid circle; once-selected line 1S: broken line, solid triangle; twice-selected line 2S: solid line, open box. Lines are the predicted values for the percentages of survival. Symbols are the mean observed percentage of survival; error bars are ± standard error of the mean ($n = 3$).

Susceptibility of the Selected Progenies to Diclofop and Other Herbicides. In June 2007, an herbicide-rate response study compared the response of the selected line 1S with its unselected parent population VLR1. Seeds (n = 40) of both the lines were sown in plastic trays (28 by 33 by 5 cm) filled with potting mixture (50% river sand, 30% peat moss, and 20% tree bark) and were maintained outdoors. At the two- to three-leaf stage, these seedlings were treated with diclofop at rates of 0, 23, 47, 94, 187, and 375 g ha^{-1} using the same laboratory sprayer (untreated controls were treated with water plus the surfactant). Three replicate trays with approximately 30 plants $tray^{-1}$ were treated with each herbicide rate. After 21 d, plant survival was recorded. Similarly, in July 2008, the selected line 2S was compared with its unselected parent population VLR1 with diclofop rates of 0, 11, 23, 47, 94, and 187 g ha^{-1}, the line 1S was also included to examine whether the performance of this selected line was repeatable over different years. After 21 d, plant survival was recorded, and the aboveground fresh biomass of individuals was expressed as a percentage of the untreated control. To evaluate other herbicides, field-grown seedlings of the twice-selected, rigid ryegrass progeny 2S and its unselected parent VLR1 were treated with various herbicides. For each herbicide, 60 uniform two- to three-leaf stage seedlings (three replicate pots with 20 seedlings each) were treated at a range of rates with the ACCase-inhibiting herbicides fluazifop-P (0, 7.5, 15, 30, and 70 g ai ha^{-1}), haloxyfop (0, 4, 8, 15, and 30 g ai ha^{-1}), sethoxydim (0, 7.5, 15, 30, and 60 g ai ha^{-1}), and the acetolactate synthase (ALS)–inhibiting herbicides chlorsulfuron (0, 4, 8, 15, and 30 g ai ha^{-1}) and imazethapyr (0, 10, 20, 40, and 80 g ai ha^{-1}). Plant survival was assessed 21 d after the herbicide treatment.

Data Analysis. A three parameter, logistic model was fitted to the survival and biomass data (Ritz and Streibig 2005):

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

where Y is plant survival or plant aboveground biomass (fresh wt) as a percentage of the control, d is the upper asymptotic bound on Y, e is the herbicide rate producing a survival-level halfway between the lower limit zero and the upper limit d, x

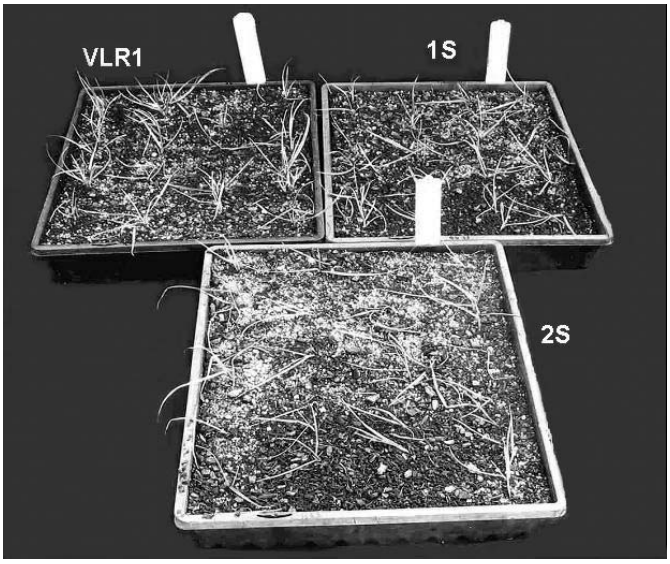

Figure 2. Rigid ryegrass (VLR1) and the selected lines 1S (once selected) and 2S (twice selected), 21 d after treatment (DAT) with diclofop at 47 g ai ha^{-1} in 2008.

is the herbicide rate, and *b* denotes the relative slope around *e*. The model was fitted to the survival and biomass data to estimate the herbicide rate corresponding to 50% mortality (LD_{50}) and the herbicide rate corresponding to 50% growth reduction (GR_{50}) using the statistical software R (version 2.7, R Foundation for Statistical Computing, Wien, Austria; R Development Core Team, 2010) with its dose–response curve (drc) package (Knezevic et al. 2007). Response to selection and the increase in herbicide susceptibility of the selected progeny over the unselected parent population VLR1 was assessed by LD_{50} or GR_{50} values ratio analysis (LD_{50} of the selected line divided by LD_{50} of the unselected line). The selectivity index function of the drc package was used to assess the difference in herbicide susceptibility level between the selected and unselected rigid ryegrass populations. The null hypothesis was that there was no difference between the estimated LD_{50} or GR_{50} values of the selected progeny vs. the unselected VLR1, or equivalently, that the ratio was equal to one. All plant survival-rate response graphs are presented with untransformed data and a linear scale.

Results and Discussion

Recurrent Selection for Greater Herbicide Susceptibility in Rigid Ryegrass. Rate-response profiles confirmed the rigid ryegrass parent population VLR1 to be susceptible to diclofop (Table 1; Figure 1). Under the carefully controlled conditions employed in this study, the Australian registered rate for diclofop (375 g ha^{-1}) always achieved 100% mortality. However, for this highly genetically variable species, individual variability is evident in the degree of diclofop susceptibility (Figure 1). Some individuals were killed by a very low diclofop rate. Using the plant-cloning technique and treating plants at a low diclofop rate, it was possible to identify and isolate the most highly diclofop-susceptible individuals within the parental population VLR1. The low rate of diclofop (94 g ha^{-1}) identified the 30% most-susceptible individuals within the population. Seed (progeny 1S) was obtained only from cross-pollination among these plants. The rate-response studies conducted in 2007 and 2008 indicated greater susceptibility of the selected line 1S over its parent VLR1 (Figure 1a and 1b). The progeny 1S LD_{50} and GR_{50} values, when compared with the parent VLR1 population, were lower, with ratios of 0.7 and 0.8, respectively (Table 1). Then, the progeny 1S was again selected with an even lower diclofop rate (47 g ha^{-1}) to identify the most susceptible individuals. The clones of these highly diclofop susceptible individuals were grown for seed (cross-pollination) to obtain the progeny 2S population. Progeny 2S was determined to have even greater diclofop susceptibility than did progeny 1S or its parent population VLR1 (Figures 1 and 2). The diclofop LD_{50} and GR_{50} in progeny 2S were lower than they were in progeny 1S, and the ratios indicating herbicide susceptibility relative to the original VLR1 parent were 0.5 (Table 1). Thus,

Table 2. Parameters and estimated herbicide rate corresponding to 50% mortality (LD_{50}) from the logistic model: $Y = d/(1+\exp\{b[\log(x) - \log(e)]\})$ fitted to the rate–response data for the rigid ryegrass (VLR1) and twice-selected (2S) lines treated with a series of rates of selected acetyl-coenzyme A carboxylase–and acetolactate synthase–inhibiting herbicides.[a]

Biotype	*d*	*e*	*b*	RMS	R^2	LD_{50}	R : S ratio[b]
				Chlorsulfuron			
VLR1	99	6	1	129	0.88	6	1
2S	99	14	1	99	0.86	14	0.4[c]
				Imazethapyr			
VLR1	100	10	2	77	0.94	10	1
2S	100	8	1.6	62	0.95	8	0.8
				Haloxyfop			
VLR1	99	4	2	117	0.92	4	1
2S	99	2	2	12	0.99	2	0.5 [c]
				Sethoxydim			
VLR1	99	10	3	49	0.97	10	1
2S	100	8	4	22	0.98	8	0.8 [c]
				Fluazifop-P			
VLR1	97	15	3	97	0.95	15	1
2S	96	15	3	50	0.97	15	1

[a] Abbreviations: RMS, residual mean square; R^2, adjusted R^2; R : S, ratio of resistant to susceptible.
[b] LD_{50} R : S ratios were calculated as the ratio of the LD_{50} for the selected line to the LD_{50} for unselected VLR1 biotype.
[c] Significant difference between the VLR1 line and 2S line at $P \leq 0.05$.

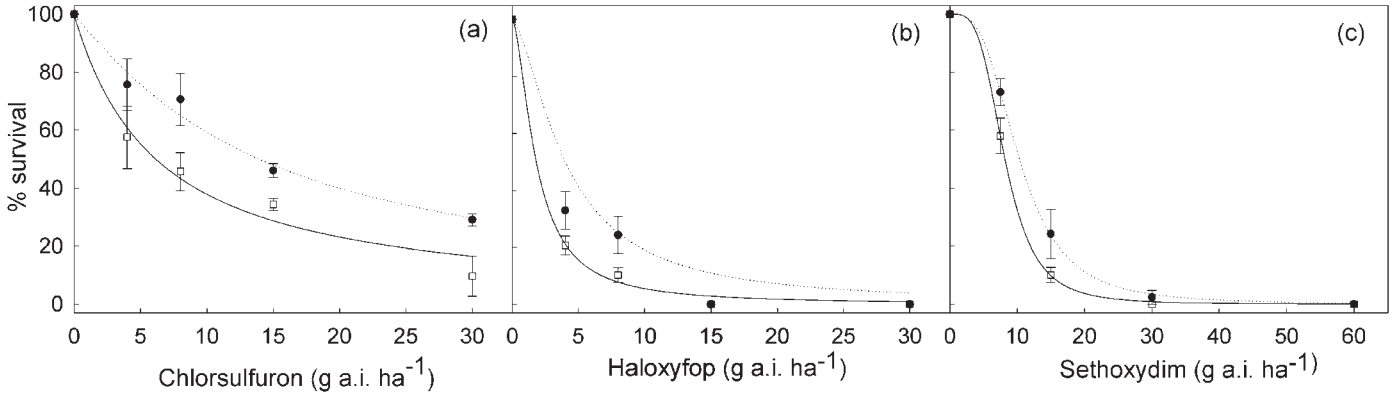

Figure 3. Rate–response curves for rigid ryegrass biotype VLR1 (dotted line, closed circle) and twice-selected susceptible line 2S (solid line, open box) treated with a range of rates of (a) chlorsulfuron, (b) haloxyfop, and (c) sethoxydim. Lines are the predicted values for the percentages of survival. Symbols are the mean percentages of survival; error bars are ± standard error of the mean ($n = 3$).

commencing with the diclofop-susceptible population VLR1 through two cycles of selection, each identifying the most diclofop-susceptible individuals, produced a population that was twice as susceptible to diclofop as was its parent (Table 1; Figure 1).

In an earlier study, the genetic variability of a glyphosate-resistant field population of tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] enabled Zelaya and Owen (2005) to select for increased sensitivity to glyphosate by recurrent selection. In this study, the existing genetic variability for herbicide susceptibility in an already-susceptible population enabled us to select for increased susceptibility by recurrent selection. This was achieved by recurrently selecting and allowing cross-pollination only among the most herbicide-susceptible individuals in the population (excluding those individuals most able to survive diclofop). The initial genetic variability in susceptibility of the parent population to diclofop enabled recurrent selection for greater susceptibility (Figure 1a and 1b). Likely, there was the removal of minor gene traits that individually or collectively confer the ability to survive a low diclofop treatment.

Increased Susceptibility to Herbicides Other than Diclofop. Herbicide rate–response studies indicate that the progeny 2S population that had been selected for greater diclofop susceptibility displayed concomitant greater susceptibility to two other ACCase herbicides, haloxyfop, and sethoxydim (Table 2; Figure 3). The LD_{50} of the twice-selected line was lowered to 2 g ha^{-1} haloxyfop from the 4 g ha^{-1} haloxyfop of the parent line (Table 2; Figure 3), and the corresponding resistant-to-susceptible (R : S) LD_{50} ratio was 0.5. There was a slight but significant increased susceptibility to the other ACCase-inhibiting herbicide sethoxydim (Table 2; Figure 3). There was increased susceptibility to the ALS-inhibiting herbicide chlorsulfuron, an herbicide with a different mode of action to the ACCase-inhibiting herbicides. Progeny 2S was found to be twice as susceptible (LD_{50} = 6 g ha^{-1} chlorsulfuron) as its parent line (LD_{50} = 14 g ha^{-1} chlorsulfuron); the corresponding R : S LD_{50} ratio was 0.4. There was no significant difference observed for the response of the 2S line over the VLR1 parent to the herbicides fluazifop-P or imazethapyr (Table 2).

Chlorsulfuron, diclofop, and haloxyfop, to which there was substantially increased susceptibility in the selected line (Table 2; Figure 3), can all be metabolized by the activity of P450 monooxygenase enzymes in rigid ryegrass (Christopher et al. 1994; Hidayat and Preston 2001). The P450 enzymes are known to be able to metabolize diclofop and chlorsulfuron to nontoxic conjugates (Werck-Reichhart et al. 2000). Thus, it is hypothesized that the selection for the most diclofop-susceptible individuals in the population removed or decreased the frequency of P450 gene capable of herbicide metabolism.

In the Illinois long-term corn (*Zea mays* L.)-selection experiment, 103 cycles of recurrent selection for high protein and high oil content in corn were performed, along with simultaneous selection for low oil and low protein content (Moose et al. 2004). Later, those selected lines were used as functional genomic tools for phenotyping and to understand the genetic architecture of the quantitative traits corresponding to oil and protein in corn (Moose et al. 2004). In a similar fashion, highly herbicide-susceptible rigid ryegrass will be a potential tool for exploring the genetic basis of resistance traits.

By exploiting genetic variability within a rigid ryegrass population, it is possible to rapidly shift a population to greater herbicide susceptibility. Conversely, rigid ryegrass populations could be rapidly shifted in the other direction to be herbicide resistant (Manalil et al. 2011; Neve and Powles 2005b). These shifts occur because the inherent genetic variability existing in the population means a continuum of individuals from highly herbicide susceptible to relatively herbicide resistant. Herbicide selection and reproduction only among individuals exclusively at either end of the herbicide susceptibility spectrum thus rapidly shifts the composition of the population. This is especially true in obligate cross-pollinated species, and it is unlikely that such rapid shifts occur in self-pollinated plant species. In these studies, there are concomitant shifts in herbicide susceptibility or resistance to herbicides of completely different modes of action and chemistry to the selecting herbicide, consistent with the involvement of one or more herbicide-detoxifying P450 genes. It is suggested that these highly herbicide-susceptible vs. herbicide-resistant populations of rigid ryegrass will be very useful for studying the role of *P450* and other genes involved in herbicide resistance.

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