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Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: field selection at high doses versus recurrent selection at low doses

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Summary

Pyroxasulfone is a new pre-emergence herbicide that provides effective control of *Lolium rigidum*, including populations with evolved resistance to multiple herbicide modes of action. Prior to its commercialisation, the potential of resistance evolution was examined with two separate studies: (i) a field screening with a high pyroxasulfone dose causing mortality >99.999% of 100 million *L. rigidum* herbicide-susceptible individuals to establish the frequency of major gene resistance mechanisms and (ii) a 3-year low-dose recurrent selection experiment of a herbicide-susceptible (S) and a multiple herbicide-resistant (MR) *L. rigidum* population. The field screening indicated that no major-effect resistance genes were present in 100 million *L. rigidum* individuals. By contrast, pyroxasulfone resistance was obtained by recurrent low-dose pyroxasulfone selection of multiple herbicide-resistant *L. rigidum*. The multiple-resistant MR population showed a clear capacity to evolve pyroxasulfone resistance with >30% plant survival at 240 g ha^{-1} (2.4-fold the recommended rate) after three generations of recurrent pyroxasulfone selection. For the first time, information regarding the potential for resistance evolution is available prior to herbicide commercialisation. Persistent pyroxasulfone use at low dose has the potential to rapidly lead to herbicide resistance evolution in L. *rigidum* field populations. Effective stewardship programmes should be developed to encourage pyroxasulfone use at the full label rate to minimise the possibility of rapid low-dose-induced resistance evolution and to ensure pyroxasulfone sustainability.

Keywords: *Lolium rigidum*, genetic variation, herbicide resistance, plant adaptation, pyroxasulfone, major genes, minor genes.

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Introduction

Global cropping demands high productivity with minimal human labour and maximum input-use efficiency (Pingali & Heisey, 1999). Weedy plants are the greatest biological constraint to annual crop yields in agricultural systems worldwide, and herbicide use for weed control is often essential, with positive impacts on crop yields (Oerke, 2006). However, intense and recurrent herbicide selection pressure exerted on weedy plants has led to the evolution of herbicide resistance in weed populations in many agro-ecosystems (Powles & Yu, 2010).

Many examples of evolved herbicide resistance in plants are monogenic, endowed by a major-effect

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resistance gene conferring high-level resistance (Darmency, 1994; Jasieniuk *et al*., 1996). Molecular characterisation of such major resistance genes generally reveals that a point mutation results in a structural change in the encoded target-site enzyme (herbicide site of action) to prevent or reduce herbicide binding (Tranel & Wright, 2002; Délye, 2005; Powles & Yu, 2010). Modelling simulations show that initially rare major resistance genes can be rapidly enriched in crop-field weed populations under herbicide selection (Gressel, 1978; Neve *et al*., 2003; Renton *et al*., 2011). In addition to monogenic resistance traits, examples of polygenic quantitatively inherited resistance are well known for insecticide resistance (Roush & McKenzie, 1987) and for heavy metal tolerance in plants (Macnair, 1993). Quantitatively inherited polygenic herbicide resistance has rarely been reported. However, it may particularly occur where herbicides are used at low dose (Busi *et al*., 2011, 2012). Insufficient attention has been given to herbicide resistance evolution as a progressive quantitative shift towards resistance, caused by repeated herbicide selection acting upon existing population genetic variability for herbicide response (Neve *et al*., 2009). Genetically diverse, cross-pollinated weed genera such as *Lolium* are particularly responsive to herbicide selection. Studies with *Lolium rigidum* (Gaud.) have shown that recurrent low-dose herbicide selection can lead to rapid and substantial herbicide resistance evolution (Neve & Powles, 2005a; Busi & Powles, 2009, 2011; Manalil *et al*., 2011). Therefore, it is important to recognise that herbicide resistance evolution is dependent on the strength of herbicide selection (dose), the population size (the presence or absence of initially rare, major resistance genes), the plant breeding system (cross- vs. self-pollinated species) and inter-/intrapopulation genetic variation (phenotypic effect endowed by gene(s) presence) in response to a specific herbicide mode of action (Neve *et al*., 2009; Renton *et al*., 2011).

Herbicide resistance in *L. rigidum* and other weedy *Lolium* species is very extensive in Australia (Broster & Pratley, 2006; Owen *et al*., 2007; Boutsalis *et al*., 2012) and prominent in other parts of the world (Sherwood & Jasieniuk, 2009; Kaundun *et al*., 2011; Heap, 2012). Since herbicide resistance was first reported (Heap & Knight, 1982), *L. rigidum* in Australia has repeatedly evolved resistance to many herbicides and now exhibits multiple resistance. Both widespread herbicide resistance and adaptation to many varied agro-ecosystems are partly due to high levels of genetic diversity, cross-pollination and high seedbank density of *L. rigidum* (Powles & Matthews, 1992; Gill, 1996; Owen *et al*., 2011a,b).

New solutions are needed to control *Lolium* and other weed species. Pyroxasulfone is a novel pre-emergence herbicide with introduction planned from 2012 in Australia, USA/Canada and some other countries. Pyroxasulfone inhibits multiple steps in the elongation of very-long-chain fatty acids (VLCFA) in susceptible seedlings, with excellent selectivity in several crops such as wheat, maize and soybean (Tanetani *et al*., 2009). Pyroxasulfone provides effective control of *L. rigidum*, including populations with resistance to multiple herbicide modes of action (Walsh *et al*., 2011). Given the imminent introduction of pyroxasulfone, the widespread presence of *Lolium* weeds in many agro-ecosystems, and the demonstrated ability of this genus to evolve herbicide resistance, we have assessed the potential for *L. rigidum* to evolve pyroxasulfone resistance. Field selection with pyroxasulfone at a very high dose on a large number (100 million) of *L. rigidum* herbicide-susceptible individuals was conducted to examine for any rare major genes able to confer immediate pyroxasulfone resistance. In a separate study, we have subjected two distinct *L. rigidum* populations to pyroxasulfone recurrent selection, using a dose-permitting selection within the range of existing population genetic variation. We have detected resistance evolving following low pyroxasulfone dose selection, and we discuss the observed evolutionary dynamics of pyroxasulfone resistance in *L. rigidum* populations.

Materials and methods

High-dose pyroxasulfone field screening

Two commercially available herbicide-susceptible diploid *L. rigidum* cultivars Dargo (Downes, 1996) and Safeguard (McKay, 2004) were screened during the 2009 winter growing season at the University of Western Australia research station (31°57′3.12′S; 115°47′41.88′E). These cultivars have Western Australian ecotypes in their pedigrees and contain much of the genetic diversity present in weedy *L. rigidum* populations. These populations were confirmed via dose–response studies to be similarly pyroxasulfone susceptible to the standard susceptible *L. rigidum* population VLR1 (S) (Walsh *et al*., 2011), and >95% germination of seeds was observed in untreated control pots (Fig. 1). To field screen over 100 million *L. rigidum* individuals, a 30 m by 90 m field plot was irrigated, then cultivated with rotary tillage equipment and hand-sown with 75 kg of Dargo in an 18 m by 90 m section and 50 kg of Safeguard in a 12 by 90 m section. The number of seeds planted was estimated by measuring individual seed weight, and germination was estimated based on our observed germination rates (>95%), resulting in an average planting density of 20 000 seeds m^{-2} and a total of 50 million estimated germinating seeds. The experiment was conducted twice in the same growing season,

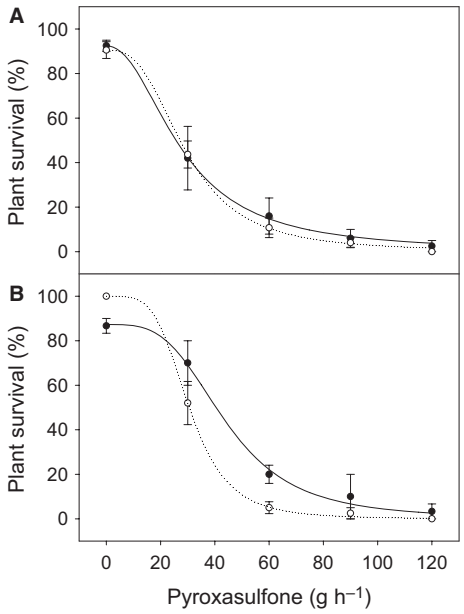

Fig. 1 Mean plant survival (emergence) and standard errors in pyroxasulfone dose response of diploid *Lolium rigidum* cultivars Dargo, Safeguard and progeny of Dargo and Safeguard individuals that survived field pyroxasulfone treatment. (A), Dargo, filled circles and solid line; survivor progeny, open circles and dotted line. (B), Safeguard, filled circles and solid line; survivor progeny, open circles and dotted line. Symbols are observed means ± SE (n = 3). Lines represent the fitted log-logistic model (Eqn 1).

once in June and again in September (2009) on the same 30 m by 90 m plot. Following hand sowing, seeds were incorporated through the moist topsoil (1–5 cm) using a rolling harrow. Within 4 h, a commercial formulation of pyroxasulfone (3-[5-(difluoromethoxy)-1-methyl-3-(trifluoromethyl) pyrazol-4-ylmethylsulfonyl]-4,5-dihydro-5,5-dimethyl-1,2-oxazole) (Sakura 85WG, 850 g kg^{-1}, WG, Bayer Crop Science) was applied to the soil surface at the high dose of 400 g ha^{-1} (four times the recommended label dose in Australia), in 100 L ha^{-1} water volume at 210 kPa using a boom sprayer fitted with flat-fan nozzles. This high pyroxasulfone dose was chosen to ensure high *L. rigidum* mortality, enabling easy identification of any (rare) surviving individuals. Non-treated control areas were established by physically covering four random 1 m^2 plots prior to pyroxasulfone treatment in each cultivar block, permitting the assessment of pyroxasulfone activity in relation to untreated areas. Good conditions for pyroxasulfone activity occurred as rainfall incorporation of pyroxasulfone occurred within 7 days of treatment and growing conditions were representative of normal field conditions.

High-dose pyroxasulfone field survivors

Very few (n = 86) *L. rigidum* individuals emerged in the pyroxasulfone-treated soil. These 86 surviving seedlings were assessed 50 days after treatment and then carefully extracted from the field, removing all soil and then transplanted in trays (300 mm by 400 mm by 100 mm) containing potting mix (50% composted pine bark, 25% peat and 25% river sand). These survivors were then moved to a 20°C glasshouse along with non-treated Dargo and Safeguard individuals (36 of each) as controls. All transplants were maintained with adequate water (>80% field capacity) and fertilisation for one week. Some post-emergence activity is known for the pre-emergence herbicide pyroxasulfone when used at a high dosage (C White pers. comm., Bayer CropScience). Thus, when the transplants were at the 3- to 4-leaf stage, they were treated with 300 g of pyroxasulfone ha^{-1} and 0.25% v/v non-ionic surfactant, applied in 110 L ha^{-1} spray volume at 210 kPa in a laboratory spray cabinet, followed 14 days later (5- to 6-leaf stage) by a second 300 g of pyroxasulfone ha^{-1} foliar treatment. All treated individuals were assessed for survival, and surviving individuals were transplanted to 4 L plastic pots. To confirm that survival could be due to resistance, traits endowing resistance must be heritable, and therefore, vegetative clones of surviving individuals were used to produce seed for progeny tests. Two tillers from surviving individuals were vegetatively cloned to produce the following two types of seed populations for progeny tests: (i) all surviving individuals were paired with a clone of their non-treated parental cultivar (individual paired crosses) and (ii) all surviving individuals were cross-pollinated with clones of all other survivors from their respective cultivar as a group (bulk crosses). At the onset of reproductive development, all crosses were placed in enclosures for controlled pollination and seeds were harvested at maturity.

Progeny evaluation

The progeny obtained from bulk crosses of pyroxasulfone-surviving individuals were screened in dose–response studies during the following winter growing season to investigate any change in sensitivity to pyroxasulfone (relative to the original cultivar). Seeds were germinated on 0.8% w/v agarose, and when the primordial root was visible, they were transplanted into seedling trays containing potting mix. Seeds were covered with 0.5 cm of soil and treated with pyroxasulfone at 0, 30, 60, 90, and 120 g ha^{-1} and placed on benches outdoors to reproduce similar environmental conditions to the original screening procedure. Three replications of 10 germinated seeds per progeny group were screened at each dose, and the experiment was conducted three times. Dargo and Safeguard individuals were included as susceptible controls. The number of surviving plants was counted, and biomass was determined following

drying for 7 days at 60°C. Data from repeated experiments were pooled for statistical analysis.

Estimation of resistance gene frequency

A 95% confidence interval for the estimated frequency of major-effect pyroxasulfone resistance in a susceptible *L. rigidum* population was calculated using the Wilson binomial confidence interval method in R 2.11.1 (R, 2010), using the total number of resistant individuals confirmed and the total number of *L. rigidum* individuals screened in the field study.

Low-dose pyroxasulfone recurrent selection

Two very different *L. rigidum* populations were subjected to recurrent selection with pyroxasulfone. Population VLR1 (hereinafter referred to as S) has never received herbicide selection and is known to be susceptible to all herbicides (Christopher *et al.*, 1991). The multiresistant population SLR31 (hereinafter referred to as MR) has an extensive herbicide selection history (Burnet *et al.*, 1994) and exhibits multiple resistance to herbicides with different modes of action. Most individuals display enhanced rates of metabolism of certain herbicides, likely mediated by cytochrome P450 enzymes (Christopher *et al.*, 1991, 1992). The population has been purified to include only those individuals with a metabolism-based resistance mechanism, in a subpopulation described in the study by Vila-Aiub *et al.* (2005). This material was used for recurrent pyroxasulfone selection.

Cycles of recurrent selection at low pyroxasulfone doses were conducted with the S and MR population (Table 1). Selection was conducted over three consecutive seasons during the normal winter growing period (May–August) in a natural outdoor environment simulating field conditions. Seeds were germinated on 0.6% agarose and then planted into 2 L pots. Seeds were covered with 0.5 cm of commercial potting soil (50% peat moss, 25% sand and 25% pine bark). Pyroxasulfone was applied at the soil surface 1 day after planting at 0, 15, 30, 60 or 120 g ha^{-1}. Three replications per dose and at least 15 germinated seeds per replication were used. Pots were kept well watered (> 80% field capacity) and fertilised. Nitrogen (as NH_4NO_3) was applied (50 mg kg^{-1}) at weekly intervals over the course of the experiment. At 21 days after treatment, plant survival was recorded. Those plants of both populations that survived the pyroxasulfone treatment at a certain dose (Table 1) were transplanted into 10 L pots (5 plants per pot) and grown to flowering initiation. At flowering, surviving plants within a population were confined in a pollen-proof enclosure to ensure cross-pollination (panmixia) only among survivors at that specific pyroxasulfone dose. The seed obtained from these selected plants represented the selected bulk-crossed progeny (Table 1). The following season the selected progeny of each population was evaluated by a dose–response study and compared to the original unselected S or MR parent populations to monitor resistance evolution. As described and detailed in Table 1, the pyroxasulfone selection process was repeated over three consecutive generations for both S and MR populations.

Table 1 Pyroxasulfone dose–response results on diploid *Lolium rigidum* cultivars Dargo and Safeguard, and the progeny of individuals surviving the pyroxasulfone field experiment. Parameter estimates following non-linear regression analysis: LD_{50} and GR_{50} values, resistance index (RI) and probability values (*P*) of difference between LD_{50} or GR_{50} values of the parental and selected progeny calculated by selectivity index (*SI*) function in the *drc* package of the statistical software R 2.11.1 (R, 2010)

Population	*n**	LD_{50} (g ha^{-1})†	RI	*P*	GR_{50} (g ha^{-1})†	RI	*P*
Dargo	230	27.7 (4.0)	–		22.0 (4.0)	–	
Progeny	510	29.3 (2.3)	1.1	0.75	23.2 (2.4)	1.1	0.79
Safeguard	180	44.0 (4.7)	–		31.1 (3.4)	–	
Progeny	230	30.6 (2.0)	0.7	0.0007	23.0 (5.9)	0.7	0.2

*Number of individuals evaluated.
†Standard errors are shown in parentheses.

Final dose–response study

At the end of the 3-year recurrent selection experiment, both parental S and MR populations and all the respective once-, twice- and thrice-selected progenies were evaluated in dose–response experiments under identical growing conditions. Seeds were planted into 2 L pots as described above. The pots were treated 1 day after planting with 0, 15, 30, 60, or 90 g of pyroxasulfone ha^{-1} for S and all S-selected progenies and with 0, 30, 60, 120, 240, or 480 g ha^{-1} for MR and MR-selected progenies. After 21 days, assessments were made and emerged plants cut at the soil surface above the meristematic zone were oven-dried to determine aboveground plant biomass. For each herbicide dose, there were four replicates and 15 plants per replicate. The final dose–response study was conducted twice.

Herbicide cross-resistance in selected progeny

Given the potential for enhanced metabolism to confer herbicide cross-resistance in *L. rigidum* (Hall *et al.*, 1994), an additional study was conducted to evaluate the changes in sensitivity to different herbicide modes of action in the recurrent selected progeny. Dose–response experiments were conducted with particular emphasis on herbicides that can be metabolised by wheat and/or

L. rigidum plants (Shimabukuro *et al*., 1979; Christopher *et al*., 1991; Tardif *et al*., 1993). Atrazine, chlorsulfuron, diclofop-methyl, S-metolachlor, pinoxaden, prosulfocarb and trifluralin were used to compare selected progenies and parental populations across a range of doses (Table 2). Conversely, glyphosate, paraquat, sethoxydim and sulfometuron usually cannot be metabolised by wheat and/or *L. rigidum* and were used at a single discriminating dose, because it was expected they would have killed plants from both parental and selected progeny (Table 2).

Statistical analysis

Plant survival data sets obtained in dose–response studies above were expressed by dividing the number of surviving plants by the total number of treated seeds. Survival values ranged between 0 and 1, and a binomial distribution of errors was adopted in the non-linear regression analysis. Biomass data were expressed as a per cent of the untreated control and analysed with the same model assuming Gaussian continuous distribution of errors in non-linear regression analysis (Ritz & Streibig, 2005). Data from repeated experiments were pooled for analysis. The herbicide doses causing 50% plant mortality (LD_{50}) or 50% growth reduction (GR_{50}) in the selected and unselected populations at each generation were estimated by using the three-parameter log-logistic model:

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

where parameter d is the upper limit, b is the slope of the curve, x is the herbicide dose, and e is the dose producing a 50% reduction in response. The response to selection in the selected progenies was measured as the resistance index (RI). RI is the resistant/susceptible ratio, here defined as the ratio of estimated LD_{50} or GR_{50} values between each selected progeny and the unselected parental population. Statistical difference in estimated LD_{50} or GR_{50} values of the each selected progeny and the unselected parental population was assessed by using the selectivity index (*SI*) function in the *drc* package of the statistical software R 2.11.1 (R, 2010).

Table 2 Herbicide treatments to assess cross-resistance in pyroxasulfone-selected progeny from S and MR populations. MR population (unselected parent) is resistant to diclofop-methyl, chlorsulfuron, trifluralin and S-metolachlor (Burnet *et al*., 1994)

Herbicide	Mode of action*	Dose (g ha^{-1})	Populations
Atrazine	Inhibitor of PSII	0, 960, 1920, 3840	MR, MR-P1, MR-P2, MR-P3
Chlorsulfuron	Inhibitor of ALS	0, 10, 20, 40	S, S-P2, S-P3
Diclofop-methyl	Inhibitor of ACCase	0, 125, 250, 500	S, S-P2, S-P3
S-metolachlor	Inhibitor of VLCFAs	0, 120, 240, 480	S, S-P2, MR, MR-P3
Pinoxaden	Inhibitor of ACCase	0, 7.5, 15, 30, 45	S, S-P2, MR, MR-P3
Prosulfocarb	Inhibitor of lipid synthesis	0, 500, 1000, 2000	S, S-P2, MR, MR-P2, MR-P3
Trifluralin	Cell division inhibitor	0, 230, 460, 920	S, S-P2
Glyphosate	Inhibitor of EPSPS	450	S, S-P2, MR, MR-P3
Paraquat	Inhibitor of PSI	200	S, S-P2, MR, MR-P3
Sethoxydim	Inhibitor of ACCase	186	S, S-P2, MR, MR-P3
Sulfometuron	Inhibitor of ALS	15	S, S-P2, MR, MR-P3

*ACCase, acetyl-coenzyme A carboxylase; ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PSI, Photosystem I; PSII, Photosystem II; VLCFAs, very-long-chain fatty acids.

Results

High-dose pyroxasulfone field screening and progeny evaluation

Over 100 million germinable *L. rigidum* seeds were field-screened at a high (400 g ha^{-1}) pyroxasulfone dose. As expected, pyroxasulfone caused very high mortality (>99.999%), such that only a very small number of seedlings survived. Despite these very few individuals surviving pyroxasulfone, their progeny was found to be completely pyroxasulfone susceptible (Table 1, Fig. 1 A,B). Similarly, when 19 of the survivors were individually crossed with their original parental cultivar (three individuals did not produce seed), the resulting progeny were as sensitive to pyroxasulfone as the parental cultivars (data not shown). Thus, there were no confirmed resistant individuals in the progeny of survivors from the 100 million *L. rigidum* individuals screened in the field. We combined these data from both *L. rigidum* cultivars to calculate the Wilson binomial confidence interval. The upper 95% confidence limit for a major-effect pyroxasulfone resistance gene in a susceptible *L. rigidum* population, if present, was calculated to be very rare at a frequency below 4×10^{-8}.

Dose response of parental unselected multiresistant (MR) and susceptible (S) populations with pyroxasulfone

The herbicide-susceptible S population was well controlled at 90 g of pyroxasulfone ha^{-1} (only 2% plant

survival) (Fig. 2). However, the multiresistant MR population was discernibly less affected by pyroxasulfone, exhibiting 10% plant survival at 120 g ha^{-1} (Fig. 3). The estimated LD_{50} values for these two populations were significantly different ($P < 0.001$), indicating evident interpopulation phenotypic difference in response to pyroxasulfone.

Response to pyroxasulfone recurrent selection of the S population

For the S population, 90% mortality occurred at a relatively low dose of 60 g of pyroxasulfone ha^{-1}, leaving 10% (±1.1) survivors in the initial selection. The same dose of 60 g of pyroxasulfone ha^{-1} applied to their progeny S-P1 resulted in high pyroxasulfone mortality with only 14% (±5.3) plant survival. The following year the progeny S-P2 suffered 98% plant mortality from 60 g of pyroxasulfone ha^{-1}. Thus, surviving plants (n = 33) of the S-P2 progeny treated with a lower dose (30 g of pyroxasulfone ha^{-1}) were grown to seed, constituting progeny S-P3 (Table 3). In experiments conducted over different years, differences in herbicide efficacy were observed, probably affected by environmental conditions. Thus, the selected progenies obtained from survivors of three generations (years) were compared with the original unselected S parent in final dose–response studies under identical conditions. The analysis of LD_{50} and GR_{50} values of parental S versus the selected progeny indicates a significant pyroxasulfone resistance shift in progeny S-P2 (Table 4). At the highest tested rate of pyroxasulfone (90 g ha^{-1}), the progeny S-P2 exhibited 17% plant survival. A further shift towards resistance was not evident after the third cycle of recurrent pyroxasulfone selection (Fig. 2, Table 4). Estimated LD_{50} and GR_{50} values of S-P3 progeny were significantly lower than those of S-P2 progeny and not different to the original S parent (Table 4).

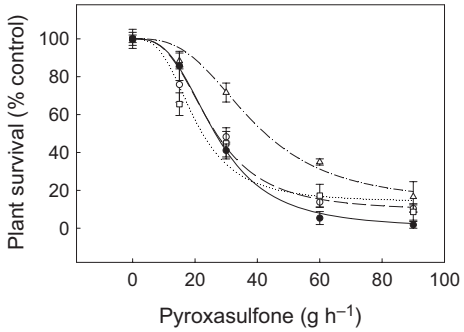

Fig. 2 Herbicide dose–response study of the S *Lolium rigidum* population and seed progenies following three generations of recurrent selection. S, continuous line and full circles; progeny selected once at 60 g ha^{-1} (S-P1), empty circles and dashed line; progeny selected twice at 60 g ha^{-1} (S-P2), empty triangles and dashed-dotted line; progeny selected twice at 60 g ha^{-1} and once at 30 g ha^{-1} (S-P3), empty squares and dotted line. Symbols are observed means ± SE (n = 4). Lines represent the fitted log-logistic model (Eqn 1).

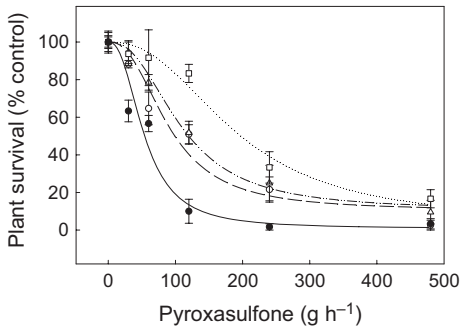

Fig. 3 Herbicide dose–response study of MR *Lolium rigidum* population and progenies selected in three generations of recurrent selection. MR, continuous line and full circles; progeny selected once at 60 g ha^{-1} (MR-P1), dashed line and empty circles; progeny selected once at 60 g ha^{-1} and once at 120 g ha^{-1} (MR-P2), empty triangles and dashed-dotted line; progeny selected once at 60 g ha^{-1} and twice at 120 g ha^{-1} (MR-P3), empty squares and dotted line. Symbols are observed means ± SE (n = 4). Lines represent the fitted log-logistic model (Eqn 1).

Response to pyroxasulfone recurrent selection of the MR population

The MR population, resistant to several herbicides but never selected with the new herbicide pyroxasulfone, had a mean plant survival of 38% (±4.8) at 60 g of pyroxasulfone ha^{-1} in the initial dose–response study. The seed progeny MR-P1 obtained from those surviving plants (n = 32) was then selected at 120 g of pyroxasulfone ha^{-1} which caused 80% (±3.1) mortality (Table 3). The seed progeny MR-P2 treated with 120 g of pyroxasulfone ha^{-1} exhibited 53% (±6.8) plant survival. Survivors were kept to generate the three time-selected progeny MR-P3 (Table 3). In a final

Table 3 Progeny of populations S and MR selected at a specific dose (g ha^{-1}) of pyroxasulfone at each cycle of recurrent selection with average selection intensity (1 - survival ratio) and total number of selected plants (n)

Year of selection	Population/ selected progeny	Pyroxasulfone dose selection (g ha^{-1})	Selection intensity	Plants selected (n)
–	S			
2006	S-P1	60	0.90	9
2007	S-P2	60	0.86	13
2008	S-P3	30	0.57	33
–	MR			
2006	MR-P1	60	0.62	32
2007	MR-P2	120	0.80	9
2008	MR-P3	120	0.47	20

Table 4 Parameter estimates following non-linear regression analysis: LD_{50} and GR_{50} values, resistance index (RI) and probability values (*P*) of difference between the LD_{50} or GR_{50} of the unselected parental population S or MR and each selected progeny calculated by selectivity index (*SI*) function in the *drc* package of the statistical software R 2.11.1 (R, 2010)

Selection cycle	Population	LD_{50} (g ha^{-1})*	RI†	*P*	GR_{50} (g ha^{-1})*	RI†	*P*
0	S	26.5 (2.0)	–		18.9 (1.9)	–	
1	S-P1	27.4 (2.6)	1.0	0.77	16.1 (2.6)	0.9	0.43
2	S-P2	44.7 (3.4)	1.7	<0.01	25.4 (3.2)	1.3	0.03
3	S-P3	23.8 (2.7)	0.9	0.46	11.4 (2.5)	0.6	0.10
0	MR	46.9 (4.7)	–		24.0 (5.4)	–	
1	MR-P1	106 (16)	2.2 (4)	<0.01	91.7 (9.6)	3.8 (5)	<0.01
2	MR-P2	127 (14)	2.6 (5)	<0.01	75.0 (8.2)	3.1 (4)	<0.01
3	MR-P3	208 (24)	4.2 (8)	<0.01	192 (16.3)	8.0 (10)	<0.01

*Standard errors are shown in parentheses.
†Resistance indexes of MR pyroxasulfone-selected progenies relative to S parental population are shown in parentheses.

experiment, all three selected progenies were compared with the original MR parental population and a clear shift towards pyroxasulfone resistance was evident (Fig. 3). The analysis of estimated LD_{50} values indicated that the level of pyroxasulfone resistance significantly increased with each generation of pyroxasulfone selection (Fig. 3, Table 4). The progeny MR-P3 was clearly pyroxasulfone resistant, exhibiting 33% (±8.3) survival at a high pyroxasulfone dose (240 g ha^{-1}) (Fig. 3). By the analysis of LD_{50} values, there was a 4- and 8-fold increase in pyroxasulfone resistance in the progeny MR-P3 compared with the unselected parents MR and S respectively (Table 4). The progeny MR-P3 also had an 8- or 10-fold increase in the GR_{50} value compared with the unselected MR or S (Table 4).

Herbicide cross-resistance in pyroxasulfone-selected progeny from S and MR populations

Eleven herbicides across seven different modes of action were evaluated on the unselected parents (S and MR) and their pyroxasulfone-selected progenies (Table 2).

Despite there being only a modest shift towards pyroxasulfone resistance in the S-P2 progeny, there was clear evidence of cross-resistance to both chlorsulfuron (ALS-inhibiting herbicide) and diclofop-methyl (ACCase-inhibiting herbicide), with plants surviving herbicide application higher than the recommended field dose (Fig. 4A,B). Thus, two cycles of pyroxasulfone recurrent selection were sufficient to select for cross-resistance identified by a statistical increase in LD_{50} values ($P < 0.01$) and substantially different survival observed at the recommended herbicide rate. There was a 4- and 5-fold increase in chlorsulfuron and diclofop-methyl resistance, respectively, between the progeny S-P2 and the unselected parent S (Table 5). Progeny S-P2 also exhibited a significant decrease in susceptibility to S-metolachlor, a pre-emergence herbicide with a similar mode of action to pyroxasulfone (inhibition of VLCFA synthesis) (Fig. 4C; Table 5). By contrast, progeny S-P3 displayed similar level of susceptibility to both chlorsulfuron and diclofop-methyl as the original parent S (Fig. 3A,B, Table 5). No significant change in sensitivity was observed with the herbicides pinoxaden,

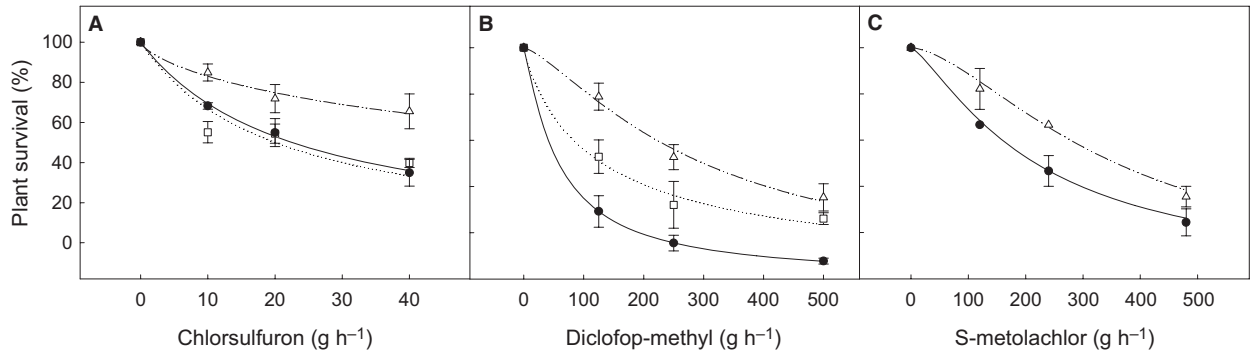

Fig. 4 Herbicide cross-resistance as a result of recurrent selection with pyroxasulfone in *L. rigidum*. (A) Chlorsulfuron, (B) Diclofop-methyl and (C) *S*-metolachlor. (A, B) S, black line and circles; S-P2, dashed-dotted line and empty triangles; S-P3, dotted line and empty squares. (C) S, black line and circles; S-P2, dashed-dotted line and empty triangles. Symbols are observed means ± SE ($n = 3$). Lines represent the fitted log-logistic model (Eqn 1).

Table 5 Cross-resistance profile with different herbicide modes of action by the analysis of estimated LD_{50} values, relative resistance index (RI) and probability values (*P*) of difference between the unselected parental populations S and the respective pyroxasulfone-selected progenies calculated by selectivity index (*SI*) function in the *drc* package of the statistical software R 2.11.1 (R, 2010)

Herbicide	Populations	LD_{50} (g ha^{-1})*	RI	*P*
Chlorsulfuron	S	23 (4)	–	
	S-P2	91 (50)	4.0	<0.01
	S-P3	20 (7)	0.9	0.74
Diclofop-methyl	S	58 (31)	–	
	S-P2	293 (57)	5.1	<0.01
	S-P3	130 (41)	2.2	0.056
S-metolachlor	S	210 (36)	–	
	S-P2	347 (38)	1.7	<0.01

*Standard errors are shown in parentheses.

prosulfocarb or trifluralin in the pyroxasulfone-selected progenies from the S population (data not shown).

The MR-P3 progeny displaying pyroxasulfone resistance showed some degree of evolved cross-resistance to prosulfocarb, a pre-emergence herbicide with a different mode of action to pyroxasulfone (lipid synthesis inhibition) (data not shown). No significant phenotypic shift was found to the herbicides atrazine, S-metolachlor or pinoxaden (data not shown). Both unselected parental S and MR populations and their respective pyroxasulfone-selected progenies remained susceptible to several other herbicides including glyphosate, paraquat, sethoxydim and sulfometuron (none of which can be metabolised by wheat).

Discussion

Major-effect pyroxasulfone resistance genes are likely to be rare in susceptible Lolium rigidum populations

High-dose pyroxasulfone screening of 100 million *L. rigidum* seeds did not reveal any resistant individuals (Table 1). The progeny of a very small number of individuals surviving a high pyroxasulfone dose was pyroxasulfone susceptible. Thus, if there are major gene traits endowing resistance to high doses of pyroxasulfone in *L. rigidum* populations, these traits are very rare or have low penetrance. Based on our field experiment, the frequency in *L. rigidum* is likely to be $< 4 \times 10^{-8}$.

Dynamics and factors involved in low-dose pyroxasulfone resistance evolution

We have demonstrated the capability of two distinct *L. rigidum* populations (MR vs. S; Figs 2–3), with different initial levels of pyroxasulfone susceptibility, to evolve pyroxasulfone resistance over three consecutive generations of recurrent low-dose pyroxasulfone selection. Commencing with the parental *L. rigidum* MR population, which is resistant to multiple herbicides by enhanced rates of herbicide metabolism, recurrent pyroxasulfone selection for three consecutive generations clearly resulted in highly pyroxasulfone-resistant progeny (Fig. 3). In contrast, commencing with a fully herbicide-susceptible *L. rigidum* population (S), there was only a modest shift towards pyroxasulfone resistance with generations S-P1, P2 or P3, still controlled at expected pyroxasulfone label rate of 100 g ha^{-1} (Fig. 2). The results from this study with two highly characterised *L. rigidum* populations can help predict the possible evolutionary dynamics of pyroxasulfone resistance in field populations when this herbicide becomes widely used in world agriculture (from 2012 onwards). The field recommended label rate of pyroxasulfone in Australia is 100 g ha^{-1} (Bayer CropScience), and we found that a MR and S *L. rigidum* population were effectively controlled at this pyroxasulfone dose (Figs 2 and 3) (see also Walsh *et al.*, 2011). Importantly, these selection experiments revealed that selection of field *L. rigidum* populations at pyroxasulfone doses lower than the recommended dose poses a risk for pyroxasulfone resistance evolution (Fig. 3; Table 4). The evolution of pyroxasulfone resistance, evidenced here by low-dose pyroxasulfone selection, is similar to other examples of rapid resistance evolution experimentally observed in *L. rigidum* (Neve & Powles, 2005a,b; Busi & Powles, 2009, 2011; Manalil *et al.*, 2011). Such rapid resistance evolution has been documented in other pests under persistent low-dose pesticide selection (Roush & McKenzie, 1987; DeRyke *et al.*, 2006; Martinez *et al.*, 2007; Meihls *et al.*, 2008).

The unselected parental *L. rigidum* populations MR and S were chosen for this study because they ranked at the lowest and highest extreme of interpopulation susceptibility in response to pyroxasulfone (R Busi and SB Powles, unpublished). The initial higher pyroxasulfone survival of the MR relative to the S population prior to pyroxasulfone selection suggests that previous historical field selection with herbicides has enriched for genetic trait(s) serendipitously effective against pyroxasulfone. We emphasise that pyroxasulfone had not been commercialised where these experiments were conducted, and thus, field populations had never been selected with pyroxasulfone. In the obligate cross-pollinated *L. rigidum*, any gene traits endowing a level of pyroxasulfone survival can be quantitatively accumulated by recurrent pyroxasulfone selection and cross-pollination among survivors. A substantially greater level of resistance evolved in the MR, relative to the S population, following three cycles of low-dose pyroxasulfone selection. Thus, the structure of heritable

genetic variation in response to pyroxasulfone in the MR versus S population was substantially different. Pyroxasulfone recurrent selection in MR was conducted at higher herbicide doses in the last two cycles, because the inherited levels of pyroxasulfone resistance in the progeny were incrementally higher. By contrast, the opposite occurred in the S population and the last cycle of selection occurred at a very low dose (30% the recommended label rate), which resulted in a significant regression of resistance and cross-resistance in the progeny S-P3. As shown in a multiyear recurrent selection study, variability and fluctuations in response to selection can be expected (Dudley & Lambert, 2004). This could help explain the poor response to selection observed in the progeny S-P3. Alternatively, lower selection intensity might have decreased the heritability of those genetic trait(s) under herbicide selection in the population S. Given the limited number of individuals under pyroxasulfone recurrent selection, it is also possible that genetic drift had an impact on pyroxasulfone resistance evolution, with random loss of favourable resistance-endowing gene(s) (Gillespie, 2004). Thus, there is a range of herbicide-use doses at which heritability for resistance-endowing genetic traits under selection is high. From this study, we can conclude that if low-dose pyroxasulfone is persistently used to control *L. rigidum* field populations, heterogeneous levels of pyroxasulfone resistance will evolve because of the high population variability in *L. rigidum* in response to pyroxasulfone. Heterogeneity in geographical distribution and frequency of herbicide resistance is well known in populations of *L. rigidum* (Owen *et al*., 2007) and other weed species with similar biology (Délye *et al*., 2010).

Analysis of herbicide resistance evolution prior to herbicide commercialisation

This is the first ever documentation and analysis of herbicide resistance risk before commercialisation of a new herbicide. Understanding the resistance potential prior to commercial introduction of a new herbicide provides valuable information (Délye *et al*., 2011) and contributes towards an evolutionary understanding of herbicide resistance (Neve *et al*., 2009). Equally, this study provides for the first time a unique opportunity to model evolution of herbicide resistance, using an empirical estimate of initial major-effect gene frequency from field screening a large population prior to commercialisation and widespread use of a new herbicide. Predictive models simulating the evolution of herbicide resistance are very sensitive to the initial resistance gene frequency (Neve, 2008). Our results demonstrate that major gene resistance to pyroxasulfone should be very rare in susceptible *L. rigidum* populations, in contrast to single gene mutations conferring resistance to other herbicide groups, such as ALS-inhibiting herbicides, which have previously been measured in susceptible *L. rigidum* at frequencies between 2.2×10^{-5} and 1.2×10^{-4} (Preston & Powles, 2002).

The relative rarity of major pyroxasulfone resistance genes found in this study does not exclude the potential for other resistance mechanisms to be enriched in weed populations via repeated herbicide use and recurrent selection for any traits that may confer increased survival, as has been previously observed in *L. rigidum* (Neve & Powles, 2005a; Busi & Powles, 2009; Manalil *et al*., 2011). The parental MR population, although initially pyroxasulfone susceptible, was already resistant to ACCase- and ALS-inhibiting herbicides, S-metolachlor (VLCFAs) and trifluralin (microtubule assembly) because of enhanced capacity for herbicide metabolism (Christopher *et al*., 1991). Recurrent pyroxasulfone selection shifted this population to be pyroxasulfone resistant (VLCFAs). Pyroxasulfone is highly selective in wheat, probably because wheat can metabolise pyroxasulfone (Tanetani *et al*., 2009). Thus, under pyroxasulfone selection, a 'wheat-like' likely metabolism-based resistance mechanism was quantitatively enriched in this *L. rigidum* population. The observed resistance and cross-resistance patterns in S and MR following recurrent pyroxasulfone selection (Fig. 4, Table 5) and previous studies conducted on these two highly characterised *L. rigidum* populations (Christopher *et al*., 1991, 1992) consistently suggest that enhanced rates of herbicide metabolism can be the mechanistic basis of the selected pyroxasulfone resistance. Thus, the use of herbicide modes of action that can be metabolised by wheat can select for herbicide-resistant plants with the same broad-spectrum metabolic capacity, and the subsequent management of these resistant plants is difficult (Preston, 2004).

In conclusion, this study investigated resistance evolution dynamics in *L. rigidum* following two very distinct selection regimes: a single application of a very high pyroxasulfone dose versus recurrent selection at low pyroxasulfone doses. We have established with a multiple-resistant *L. rigidum* population that resistance evolution can rapidly occur from low-dose recurrent pyroxasulfone selection (Fig. 3). Across disciplines, it has been shown that an evolutionary approach may help to reduce the rate of pest adaptation, provide better understanding of resistance management and give insights into predicting resistance evolution in agroecosystems (Roush & McKenzie, 1987; Palumbi, 2001; Martinez *et al*., 2007; Neve *et al*., 2009; Thrall *et al*., 2011). Herbicide resistance studies have also typically reacted *a posteriori* to problems reported from the field (Délye *et al*., 2011). Our results make it possible to

predict genetic changes in weed populations under pyroxasulfone selection before this herbicide is commercialised and gives very clear directions that pyroxasulfone should be used only at doses that give high weed mortality and with diversity in weed control practices.

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