

# Cross-resistance to prosulfocarb + S-metolachlor and pyroxasulfone selected by either herbicide in *Lolium rigidum*

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## Abstract

**BACKGROUND:** Weeds can be a greater constraint to crop production than animal pests and pathogens. Pre-emergence herbicides are crucial in many cropping systems to control weeds that have evolved resistance to selective post-emergence herbicides. In this study we assessed the potential to evolve resistance to the pre-emergence herbicides prosulfocarb + S-metolachlor or pyroxasulfone in 50 individual field *Lolium rigidum* populations collected in a random survey in Western Australia prior to commercialisation of these pre-emergence herbicides.

**RESULTS:** This study shows for the first time that in randomly collected *L. rigidum* field populations the selection with either prosulfocarb + S-metolachlor or pyroxasulfone can result in concomitant evolution of resistance to both prosulfocarb + S-metolachlor and pyroxasulfone after three generations.

**CONCLUSIONS:** In the major weed *L. rigidum*, traits conferring resistance to new herbicides can be present before herbicide commercialisation. Proactive and multidisciplinary research (evolutionary ecology, modelling and molecular biology) is required to detect and analyse resistant populations before they can appear in the field. Several studies show that evolved cross-resistance in weeds is complex and often unpredictable. Thus, long-term management of cross-resistant weeds must be achieved through heterogeneity of selection by effective chemical, cultural and physical weed control strategies that can delay herbicide resistance evolution.

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**Keywords:** agriculture; evolution; herbicide resistance; prosulfocarb; pyroxasulfone; weeds

## 1 INTRODUCTION

Highly mechanised agricultural systems sustain efficient large-scale food and fibre production. Crop productivity in these systems requires pesticides for crop protection.<sup>1</sup> Persistent reliance on pesticides has resulted in the global evolution of pesticide-resistant populations of many target pest species. The evolution of resistance in target pests threatens the sustainability of agroecosystems globally and thus world food security.<sup>2–4</sup>

Weeds are a primary constraint to crop production, as potential crop losses due to weeds are greater than the combination of losses due to other pests and pathogens.<sup>5</sup> On a global scale, herbicides in field crops are an effective tool to remove weeds, yet issues related to herbicide resistance have escalated, with numerous cases of herbicide-resistant weeds reported in the last 40 years.<sup>6</sup>

Herbicide resistance evolution in weeds is driven by various factors, including heritable genetic variation for resistance trait(s) at a given frequency in weed populations, the biological features of a weed species, herbicide chemical structure, the biochemical interactions between herbicide and site of action within a plant and the prevailing agroecological conditions that determine the intensity of selection and subsequent fitness of individuals in the presence of the herbicide.<sup>4,7,8</sup> Given the complexity associated with the evolution of resistant weeds, it is a major challenge to predict the evolutionary dynamics of resistance and develop diagnostic methods and tools.

Herbicide resistance is usually diagnosed in agricultural fields following commercial failure of previously effective herbicide treatments. Random geographical surveys (see, for example, Beckie *et al.*<sup>9</sup> and Owen *et al.*<sup>10</sup>) provide benchmark information on the current distribution and levels of phenotypic herbicide resistance to different herbicide modes of action, allow inference on coevolution of target- versus non-target site resistance<sup>11,12</sup> and can allow retrospective analysis to correlate herbicide use history and evolved resistance levels.<sup>13,14</sup> However, the retrospective nature of these studies does not allow prediction of the potential for resistance evolution to a new herbicide. Computer modelling can provide such a prediction and simulation of the risk of herbicide resistance evolution in weed populations under different scenarios.<sup>15</sup> However, simulated long-term predictions of herbicide resistance evolution necessarily rely on incomplete empirical knowledge of biological, genetic, environmental and other factors.<sup>16</sup> Uniquely, we have proactively assessed the potential risk

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of weed populations to evolve resistance to the novel herbicide pyroxasulfone, before its commercialisation.<sup>17</sup>

*Lolium rigidum* (Gaud.) is a genetically diverse, cross-pollinated crop weed that is widespread in southern Australian cropping regions where it has evolved resistance to several different herbicide modes of action.<sup>18</sup> *Lolium* has been established to be a resistance-prone global weed genus.<sup>19,20</sup> In Australia, post-emergent selective control of *L. rigidum* in crops commenced from 1978 with the first of the ACCase-inhibiting herbicides, diclofop-methyl, and in 1983 with the first of the ALS-inhibiting herbicides, chlorsulfuron. ACCase herbicide resistance evolution quickly followed,<sup>21</sup> with unexpected cross-resistance to ALS herbicides.<sup>22</sup> Now, *L. rigidum* with multiple resistance to ACCase- and ALS-inhibiting herbicides is widespread across the vast southern Australian cropping regions.<sup>10,23,24</sup>

A management response to widespread evolution of resistance in *L. rigidum* to post-emergence herbicides is the reliance on pre-emergence soil-applied herbicides, including trifluralin (microtubule assembly inhibitor) used in Australia since the 1960s, prosulfocarb + *S*-metolachlor (very-long-chain fatty acid elongase inhibitor) introduced in Australia in 2008 and pyroxasulfone (very-long-chain fatty acid elongase inhibitor) commercialised in 2012 to control *L. rigidum* in crops.<sup>25</sup> Prosulfocarb + *S*-metolachlor and pyroxasulfone are inhibitors of the biosynthesis of very-long-chain fatty acids (VLCFAs) [chloroacetamides (K<sub>3</sub>) and thiocarbamates (N)] and are globally adopted to control grass weed species in pre-emergence in major field crops.<sup>25</sup>

Before commercialisation of the new herbicide pyroxasulfone, we demonstrated *L. rigidum* pyroxasulfone resistance evolution by recurrent low-dose pyroxasulfone selection over three generations.<sup>17</sup> We have also shown that pyroxasulfone low dose selection resulted in cross-resistance to prosulfocarb + *S*-metolachlor and triallate in one multiresistant *L. rigidum* population,<sup>26</sup> with this population also resistant to trifluralin.<sup>27,28</sup>

Here, we have assessed the potential for pyroxasulfone or prosulfocarb + *S*-metolachlor resistance evolution by screening 50 crop field *L. rigidum* populations collected in a 2003 random survey.<sup>29</sup> These *L. rigidum* populations had never been selected with prosulfocarb + *S*-metolachlor or pyroxasulfone, as these herbicides were introduced after 2003. Each of the 50 populations was assessed at the recommended dose of prosulfocarb + *S*-metolachlor or pyroxasulfone, and a number of populations were subsequently subjected to independent three-generation recurrent selection. In this study we report the evolution of prosulfocarb + *S*-metolachlor and pyroxasulfone cross-resistance in one specific population of *L. rigidum* by independent selection with prosulfocarb + *S*-metolachlor or pyroxasulfone and discuss possible management options to delay evolution of cross-resistance.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

*L. rigidum* seed samples were collected from randomly selected crop fields in a large geographical survey conducted in Western Australia in 2003 (see Owen *et al.*<sup>29</sup> for methods). The survey was conducted in cropping areas dominated by wheat and barley (90%) and canola (10%). Seeds of populations individually collected from 50 cropped fields in different agronomic regions of Western Australia were randomly chosen to be subjected to recurrent herbicide selection either with the pre-emergence herbicide prosulfocarb + *S*-metolachlor or pyroxasulfone. The agronomic

regions for crop suitability are broadly defined by rainfall (high, 450–750 mm 'H'; medium, 325–450 mm 'M'; low, <325 mm 'L'), latitude and longitude (see Owen *et al.*<sup>10</sup>). A multiple-resistant population, hereinafter referred to as 'MR' (resistant to multiple herbicide modes of action, including groups A, B, K1 and K3 as per the HRAC classification), with documented ability to evolve resistance to pyroxasulfone recurrent selection (see Busi *et al.*<sup>17</sup>), was used as a control in prosulfocarb + *S*-metolachlor or pyroxasulfone recurrent selection experiments. A herbicide-susceptible standard (susceptible to all herbicides used for *L. rigidum* control, including pyroxasulfone), hereinafter referred to as 'S' (see Owen *et al.*<sup>17</sup>), was used as control in herbicide dose–survival response experiments. The population MR P6, resistant to both prosulfocarb and pyroxasulfone, was used as a standard resistant control in herbicide dose–survival response studies to assess/compare the level of resistance obtained in field populations after recurrent selection. Population MR P6 was obtained by six consecutive cycles of recurrent selection with pyroxasulfone at 60 g ha<sup>-1</sup> (first generation), 120 g ha<sup>-1</sup> (second generation), 120 g ha<sup>-1</sup> (third generation), 240 g ha<sup>-1</sup> (fourth generation), then further subjected to two consecutive selections at 1000 g prosulfocarb ha<sup>-1</sup> (fifth generation) and 2000 g prosulfocarb ha<sup>-1</sup> (sixth generation) (see also Busi and Powles<sup>26</sup>).

### 2.2 Prosulfocarb + *S*-metolachlor or pyroxasulfone survival in *L. rigidum* field populations

The study was conducted in each of three recurrent normal winter growing seasons (May–August) in a natural outdoor field environment. Commencing in 2010, viable seeds of each of the 50 different *L. rigidum* populations were germinated on 0.6% (v/w) solidified agar and planted into 2 L pots containing commercial potting mixture (50% peatmoss, 25% sand and 25% pine bark) when the primordial root was visibly erupting from the seed coat. Pyroxasulfone at the recommended label dose of 100 g ha<sup>-1</sup> or the mixture prosulfocarb at the recommended label dose of 2000 g ha<sup>-1</sup> + 300 g *S*-metolachlor ha<sup>-1</sup> was applied to the soil surface of pots. Prior to herbicide treatments, pots were gently watered so that approximately 1 mm of potting mix covered the transplanted germinating seeds. Approximately 1 h later, the herbicide treatments were applied as prosulfocarb (Boxer Gold® 80% prosulfocarb + 12% *S*-metolachlor EC; Syngenta Crop Protection Pty Ltd, Macquarie Park NSW, Australia) or pyroxasulfone (Sakura® 85% pyroxasulfone WDG; Bayer CropScience Pty Ltd, Gordon NSW, Australia) with a cabinet track sprayer mounted with twin flat-fan nozzles and delivering a water volume of 120 L ha<sup>-1</sup> per pass at a pressure of 2 bar. For each herbicide there were two replicated pots for a total of approximately 50 herbicide-treated seeds and at least one untreated control pot (refer to Table 1). After the herbicide treatments, all pots were covered with 0.5 cm of fresh potting mix to simulate field conditions with herbicide incorporation by sowing.

After 21 days, emerged plants evidently growing well and producing new leaves were assessed as survivors. The total number of treated versus surviving plants was summed across the two replicates, and the percentage survival was calculated for each population and herbicide. At least ten surviving plants were selected for each of ten different *L. rigidum* populations that displayed the highest plant survival with a >20% survival threshold in the first two generations of selection with either herbicide (data not shown), transplanted into 10 L pots and grown to flowering initiation (refer to Table 1). At flowering, all the selected

**Table 1.** Survival response of a *L. rigidum* field population (#20) collected from the agronomic region M2 of the Western Australia wheatbelt and a well-characterised multiresistant population (MR) exposed to pyroxasulfone or prosulfocarb + *S*-metolachlor selection. Individuals surviving the initial selection in 2010 were grown to seed to constitute the first selected progeny (P1). P1 seeds were exposed to herbicide selection in 2011, and the two-time selected progeny was obtained from survivors (P2). P2 was exposed to herbicide selection in 2012, and the three-time selected progeny was obtained from survivors (P3)

Herbicide	Dose (g ha <sup>-1</sup> )	Population	Seeds treated	Survival (%)		
				2010 – P1	2011 – P2	2012 – P3
<i>Prosulfocarb selection</i>						
Prosulfocarb + <i>S</i> -metolachlor	2000 + 300	#20	40, 40, 52	12	36	13
Prosulfocarb + <i>S</i> -metolachlor	2000 + 300	MR	40, 40, 52	29	29	20 <sup>a</sup>
Prosulfocarb + <i>S</i> -metolachlor	2000 + 300	S	40, 40, 52	10	8	9
<i>Pyroxasulfone selection</i>						
Pyroxasulfone	100	#20	40, 40, 52	25	8	14
Pyroxasulfone	100	MR	40, 40, 52	31	10	29
Pyroxasulfone	100	S	40, 40, 52	0	4	4

<sup>a</sup> Owing to a severe fungal attack during the reproductive stage, plants did not produce sufficient viable seed.

surviving plants within each population and herbicide treatment were isolated in pollen-proof enclosures to ensure random cross-pollination. The seed obtained from these selected plants represented the first selected progeny.

### 2.3 Prosulfocarb + *S*-metolachlor or pyroxasulfone recurrent selection in *L. rigidum* populations

After harvest, the seed was after-ripened over summer in hot, dry conditions to break seed dormancy. These seeds were then treated with prosulfocarb or pyroxasulfone as previously described. The surviving seedlings were maintained outdoors during the normal winter growing season, and at anthesis each population was isolated to produce seed. This procedure was repeated over three consecutive generations, constituting three cycles of recurrent herbicide selection. Several herbicide dose–response studies were conducted across the 3 years of selection to assess the response to selection of the ten populations selected from the initial 50 *L. rigidum* populations. Recurrent selection was not continued for herbicide-selected progenies that appeared not to respond progressively to selection or had a survival of <20% to the full herbicide dose in the first two generations. Only one parental *L. rigidum* population appeared consistently to respond to prosulfocarb or pyroxasulfone recurrent selection and evolve a level of herbicide resistance. This *L. rigidum* population (hereinafter referred to as population #20) was never exposed to field-selection of either herbicide used in this study and was field-collected from the mid-rainfall agronomic region M2 of the Western Australian wheatbelt in 2003 and characterised for resistance to a number of herbicide modes of action.<sup>29</sup> We had previously subjected to recurrent selection the two standard parental populations S and MR with pyroxasulfone and fully characterised their ability to respond to low-dose pyroxasulfone selection.<sup>17</sup> Thus, we resubjected to recurrent selection the parent MR plants to be able to compare the evolutionary outcomes obtained from parental populations collected in 2003 in the Western Australian grain belt with those reobtained with our reference MR *L. rigidum* population. This concomitant reselection was an important comparative standard to monitor the progress of the several *L. rigidum* populations under recurrent herbicide selection.

### 2.4 *L. rigidum* population response to prosulfocarb + *S*-metolachlor or pyroxasulfone recurrent selection

At the end of the three-year recurrent selection, parental populations #20 and MR and their respective selected progenies were evaluated with a single effective prosulfocarb or pyroxasulfone treatment. The S population was the herbicide-susceptible control. As previously described, seeds were germinated on 0.6% water-solidified agar and planted into 2 L pots. Pots were treated with 1000 + 150 g prosulfocarb + *S*-metolachlor ha<sup>-1</sup> or 50 g pyroxasulfone ha<sup>-1</sup>. For each herbicide treatment there were two replicates and 25 viable germinated seeds treated per replicate. Survival was assessed 21 days after herbicide treatments. The experiment was repeated.

The three-time selected progeny of population #20 P3 and the sixth progeny MR P6 were subjected to comprehensive herbicide dose–survival response studies. In these experiments, the herbicide-selected progenies #20 P3 and MR P6 and the S population were assessed with a full dose–response experiment at 0, 250 + 37.5, 500 + 75, 1000 + 150, 2000 + 300 or 4000 + 600 g prosulfocarb + *S*-metolachlor ha<sup>-1</sup> or 0, 12.5, 25, 50, 100 and 200 g pyroxasulfone ha<sup>-1</sup>. For each herbicide dose there were three replicates and 25 viable germinated seeds treated per replicate. Survival was assessed 21 days after herbicide treatments. The herbicide dose–response experiments were repeated.

### 2.5 Statistical analysis

#### 2.5.1 Response to prosulfocarb or pyroxasulfone selection

Plant survival datasets obtained in dose–response studies were analysed by fitting a three-parameter log-logistic model to the survival data, expressed by dividing the number of surviving plants by the total number of treated plants. Survival values ranged between 0 and 100% and were subjected to non-linear regression analysis.<sup>30</sup> Data from repeated experiments for herbicide dose–plant survival response to prosulfocarb of progenies selected with prosulfocarb or pyroxasulfone or herbicide dose–plant survival response to pyroxasulfone of the progeny selected with pyroxasulfone or prosulfocarb were pooled prior to statistical analysis. The herbicide doses causing 50% plant mortality (LD<sub>50</sub>) in the selected and unselected populations at each generation were estimated by using

the three-parameter log-logistic model

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

where  $Y$  denotes the plant survival or biomass relative to the untreated control,  $d$  is the upper asymptotic value of  $Y$ ,  $b$  is the slope of the curve,  $e$  is the herbicide dose at the point of inflection halfway between the upper and lower asymptotes and  $x$  is the herbicide dose.<sup>30</sup> The response to selection in the selected progenies was measured as the resistance index (RI). RI is the resistant/susceptible ratio, whereas here it was defined as the ratio of estimated LD<sub>50</sub> values between the selected progeny and the unselected parental population  $S$ . The statistical difference in estimated LD<sub>50</sub> values of each selected progeny and unselected parental population was assessed by using the selectivity index (SI) function in the drc package of the statistical software R 2.14.1 (R Foundation for Statistical Computing). A  $t$ -test was conducted under the null hypothesis of no difference between the estimated LD<sub>50</sub> values of the selected progeny versus the unselected parent, or equivalently, that their ratio was equal to 1.<sup>31</sup>

### 2.5.2 Correlation between survival to post- and pre-emergence herbicides

Correlation analysis between phenotypic plant survival to diclofop-methyl, prosulfocarb +  $S$ -metolachlor and pyroxasulfone in unselected parental populations of *L. rigidum* was performed to understand whether evolved resistance to a wheat-selective post-emergence herbicide such as the ACCase inhibitor diclofop-methyl could be linked to prosulfocarb or pyroxasulfone resistance. For *L. rigidum*, survival to prosulfocarb or pyroxasulfone is most likely endowed by non-target-site traits, although the exact resistance mechanism(s) remain unknown.<sup>25</sup> Conversely, resistance to diclofop-methyl is known to be conferred by specific amino acid substitutions caused by mutations in the ACCase target gene,<sup>32</sup> as well as by non-target-site enhanced herbicide metabolism endowed by P450 enzymes.<sup>33</sup> We hypothesised that *L. rigidum* individuals resistant to diclofop-methyl, with the exception of target-site ACCase mutations at position 2041,<sup>34</sup> owing to putative enhanced herbicide metabolism capacity (P450), remain susceptible to non-metabolisable herbicides such as sethoxydim.<sup>35,36</sup> Thus, to be able to ascertain whether non-target-site resistance to diclofop-methyl could correlate with non-target-site resistance to prosulfocarb or pyroxasulfone, plant survival to the recommended dose of diclofop-methyl (target-site + non-target-site resistance) was corrected by subtracting the percentage survival to sethoxydim (target-site resistance) applied on diclofop-methyl-surviving plants. Diclofop-methyl was applied at the recommended label dose of 375 g ha<sup>-1</sup> to 50 *L. rigidum* plants at the two-leaf stage. Diclofop survivors were subsequently treated with the label rate of 186 g sethoxydim ha<sup>-1</sup>. Methods and details are described by Owen *et al.*<sup>37</sup>

GraphPad Prism (GraphPad Software, La Jolla, CA) was used to calculate Pearson's correlation coefficient ( $r$ ), 95% confidence intervals and two-tailed  $P$ -values for 50 pairwise combinations of survival between values of plant survival to diclofop-methyl, prosulfocarb or pyroxasulfone recorded from initial screening of the 50 parental *L. rigidum* populations under investigation.

## 3 RESULTS

### 3.1 Correlation between survival to prosulfocarb + $S$ -metolachlor, pyroxasulfone and diclofop-methyl

As expected, as reported by Owen *et al.*,<sup>10</sup> we confirmed that 92% of the 50 randomly collected WA grain-belt *L. rigidum* populations were diclofop-methyl resistant (survival  $\geq 20\%$ ), and only four populations were found to be diclofop-methyl susceptible, with  $< 20\%$  survival. The overall mean survival to diclofop-methyl was  $> 50\%$  (Fig. 1A). This level of diclofop resistance is putatively due to the combined effect of coexisting target-site ACCase mutations and metabolic non-target-site resistance-conferring mechanisms in single individual plants.<sup>12</sup> In contrast, all 50 *L. rigidum* populations were susceptible to prosulfocarb and pyroxasulfone (Fig. 1A). Thus, there was no correlation between diclofop-methyl resistance and prosulfocarb +  $S$ -metolachlor or pyroxasulfone survival (Figs 1B and C). Conversely, significant correlation was found in the degree of plant survival response to the full dose of prosulfocarb +  $S$ -metolachlor or pyroxasulfone (Fig. 1D). Consistent with results reported by Owen *et al.*,<sup>29</sup> these populations were found to be resistant to 15 g ha<sup>-1</sup> of the ALS-inhibiting herbicide sulfometuron (78% mean survival) and susceptible to the recommended label dose of atrazine, glyphosate and trifluralin ( $< 1\%$  survival).

### 3.2 Single dose – survival response to assess prosulfocarb + $S$ -metolachlor-selected *L. rigidum* population #20

The original parental *L. rigidum* population  $S$ , MR and #20 were susceptible to a dose of 1000 g prosulfocarb ha<sup>-1</sup> + 150 g  $S$ -metolachlor ha<sup>-1</sup>, with  $> 84\%$  mortality across all populations (Figs 2A and C). Conversely, the prosulfocarb-selected progenies of population #20 were substantially less affected than the original parental population, with 34% plant survival in the #20 P2 progeny and 26% in the #20 P3 respectively. There were no survivors in the parental plants at the same dose (Fig. 2A). Importantly, prosulfocarb +  $S$ -metolachlor selection shifted the selected progenies towards cross-resistance to pyroxasulfone (Fig. 2B).

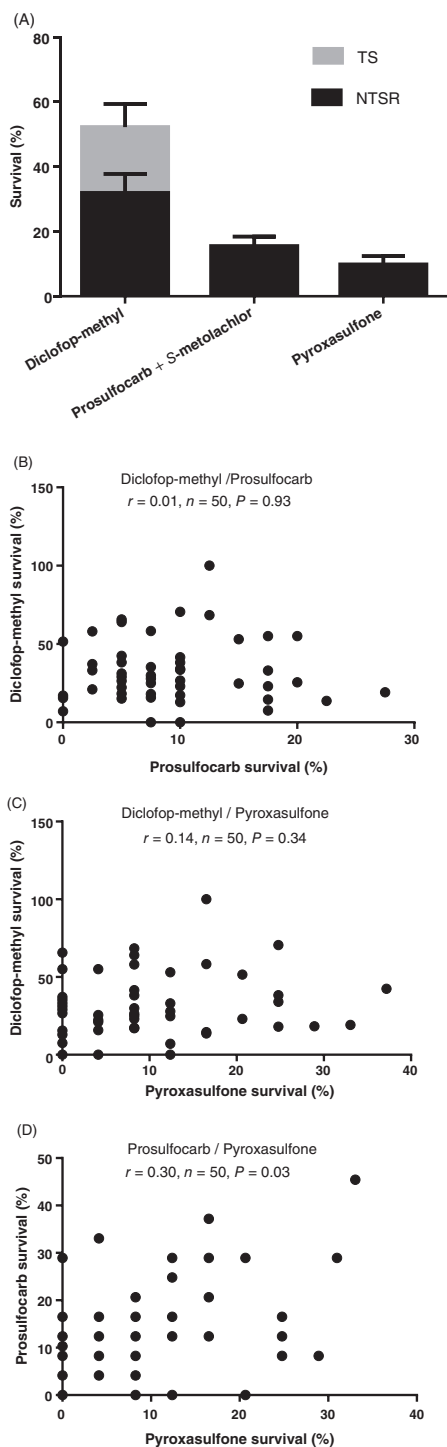
### 3.3 Single dose – survival response to assess pyroxasulfone-selected *L. rigidum* population #20

As expected, all three pyroxasulfone-susceptible *L. rigidum* parent populations ( $S$ , MR and #20) were further confirmed to be pyroxasulfone susceptible ( $> 84\%$  mortality at 50% of the label dose of 100 g ha<sup>-1</sup>) (Figs 2B to D). However, after three generations of low-dose pyroxasulfone selection, the progeny of the multiresistant MR and #20 populations were substantially less affected by pyroxasulfone treatment, indicating a clear progressive shift towards pyroxasulfone resistance for population #20. Greater survival to pyroxasulfone was generally exhibited by successive pyroxasulfone-selected progeny (Fig. 2D). Pyroxasulfone-selected progeny #20 P3, MR P3 and MR P6 exhibited 36, 38 and 58% plant survival in response to 50 g pyroxasulfone ha<sup>-1</sup> respectively (Fig. 2D). Greater survival to 1000 g prosulfocarb ha<sup>-1</sup> was also observed in pyroxasulfone-selected progenies of populations #20 and MR (Fig. 2C). As previously reported,<sup>26</sup> pyroxasulfone recurrent selection shifted the parental populations #20 and MR towards cross-resistance to prosulfocarb +  $S$ -metolachlor.

### 3.4 Herbicide dose – response study to quantify LD<sub>50</sub> values and coevolved response to selection of *L. rigidum* population #20

The progenies selected with either prosulfocarb or pyroxasulfone from the *L. rigidum* population #20 exhibited a similar





**Figure 1.** (A) Mean plant survival response as percentage of untreated control (error bars are 95% confidence intervals) observed in 50 original *L. rigidum* populations in response to the recommended dose of three wheat-selective herbicides used for *L. rigidum* control; the superimposed grey bar is target-site resistance (TS) + non-target-site resistance (NTSR) to diclofop-methyl; the black bar is NTSR only. Survival to the recommended dose of diclofop-methyl (target-site + non-target-site resistance) was corrected by subtracting the percentage survival to sethoxydim (target-site resistance) applied on diclofop-methyl surviving plants. (B) Correlation between survival to the recommended rate of 375 g diclofop-methyl ha<sup>-1</sup> and the recommended rate of 2000 g prosulfocarb + 300 g S-metolachlor ha<sup>-1</sup>. (C) Correlation between survival to 375 g diclofop-methyl ha<sup>-1</sup> and the recommended rate of 100 g pyroxasulfone ha<sup>-1</sup>. (D) Correlation between survival to 2000 g prosulfocarb + 300 g S-metolachlor ha<sup>-1</sup> and 100 g pyroxasulfone ha<sup>-1</sup>.

and symmetrical increase in survival response to each of the two herbicides pyroxasulfone or prosulfocarb + S-metolachlor (Figs 2A to D). The increase in survival to pyroxasulfone or prosulfocarb + S-metolachlor appeared to be independent of the herbicide used in the recurrent selection. The estimated LD<sub>50</sub> values in progeny #20 P3 in response to pyroxasulfone following prosulfocarb + S-metolachlor or pyroxasulfone recurrent selection for three generations were not statistically different ( $P = 0.93$ ). Similarly and symmetrically, LD<sub>50</sub> values in response to a range of prosulfocarb doses were not statistically different in those #20 P3 progenies obtained with 3 year recurrent pyroxasulfone or prosulfocarb selection ( $P = 0.33$ ). Thus, data were pooled to assess resistance level response to pyroxasulfone selection or cross-resistance to pyroxasulfone following prosulfocarb + S-metolachlor selection and, vice versa, prosulfocarb + S-metolachlor resistance in response to the same selecting agent or cross-resistance following pyroxasulfone selection (Figs 3 and 4).

#### 3.4.1 Prosulfocarb + S-metolachlor resistance relative to populations MR and S

The estimated prosulfocarb LD<sub>50</sub> values for the prosulfocarb-selected progeny #20 P3 were significantly greater ( $P < 0.01$ ) than for the standard herbicide-susceptible population S, indicating an evident shift towards resistance to prosulfocarb following prosulfocarb recurrent selection (Fig. 3 and Table 2). The estimated LD<sub>50</sub> values calculated for the progeny #20 P3 were significantly lower than those for the standard resistant progeny MR P6 ( $P = 0.04$ ). Prosulfocarb selection also caused a substantial 2.8-fold shift in pyroxasulfone cross-resistance in the progeny #20 P3 compared with S (Fig. 4 and Table 2).

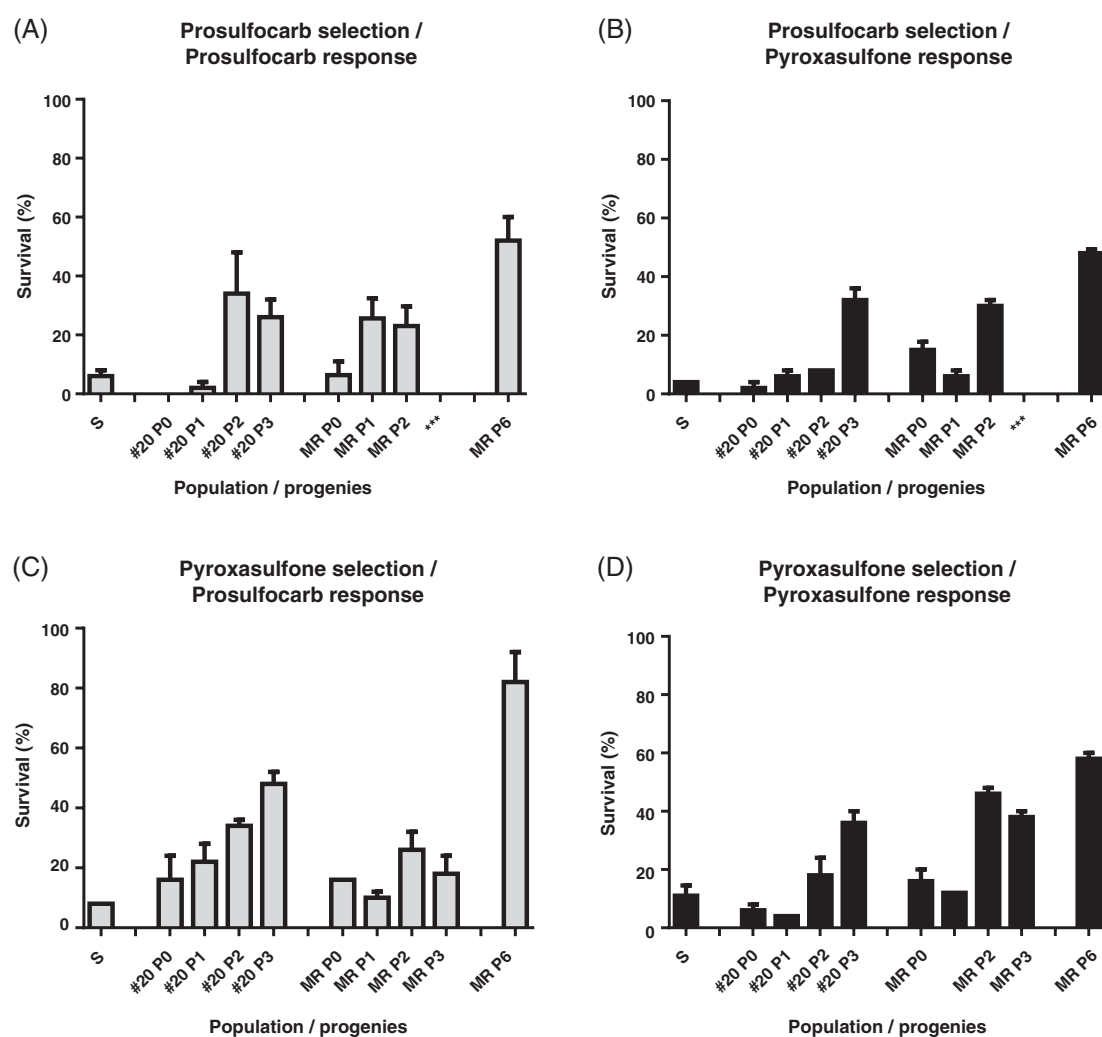
#### 3.4.2 Pyroxasulfone resistance relative to populations MR and S

The estimated LD<sub>50</sub> values for the #20 P3 and the resistant standard pyroxasulfone-selected MR P6 populations were significantly different ( $P < 0.001$ ) from the herbicide-susceptible population S. As already observed with the single-dose study (Fig. 2), this study confirmed the evident shift towards pyroxasulfone resistance (2.8-fold) following pyroxasulfone recurrent selection in the progeny of the population #20 (Fig. 4). As expected, the MR P6 progeny exhibited the highest level of pyroxasulfone resistance (Table 2). The LD<sub>50</sub> value calculated for the progeny MR P6 was significantly greater than in #20 P3 ( $P = 0.034$ ). Herbicide dose-plant survival response studies confirmed that the pyroxasulfone-selected progeny #20 P3 was cross-resistant to prosulfocarb + S-metolachlor (Fig. 3). The estimated pyroxasulfone LD<sub>50</sub> for progeny #20 P3 was 2.8-fold greater than for the S population (Table 2).

## 4 DISCUSSION

### 4.1 Coevolution of prosulfocarb and pyroxasulfone resistance

This study has investigated the risk of resistance evolution in 50 crop field populations of *L. rigidum* susceptible and never exposed to the pre-emergence wheat-selective herbicides prosulfocarb + S-metolachlor or pyroxasulfone. These *L. rigidum* populations were collected years before prosulfocarb or pyroxasulfone were commercialised in Australia. However, in one of these 50 parental *L. rigidum* populations (population #20), recurrent selection with prosulfocarb + S-metolachlor or pyroxasulfone for three generations resulted in coevolution of both prosulfocarb + S-metolachlor and pyroxasulfone resistance.



**Figure 2.** Survival response to 1000 g of prosulfocarb + 150 g *S*-metolachlor ha<sup>-1</sup> (grey bars) or 50 g pyroxasulfone ha<sup>-1</sup> (black bars) of *L. rigidum* parental (P0) populations #20 and MR and their herbicide-selected progenies (P1, P2, P3) subjected to recurrent selection with 2000 g prosulfocarb ha<sup>-1</sup> (A, B) or 100 g pyroxasulfone ha<sup>-1</sup> (C, D) for one, two or three recurrent selection cycles respectively. Bars are the mean survival, and error bars are the calculated 95% confidence intervals of the mean. MR P6 corresponding to a six times selected progeny was selected by four consecutive generations with pyroxasulfone at 60 g ha<sup>-1</sup> (MR P1), 120 g ha<sup>-1</sup> (MR P2), 120 g ha<sup>-1</sup> (MR P3) and 240 g ha<sup>-1</sup> (MR P4), and with 1000 g prosulfocarb + 150 *S*-metolachlor ha<sup>-1</sup> (MR P5) and 2000 g prosulfocarb + 300 g *S*-metolachlor ha<sup>-1</sup> (MR P6). \*\*\* MR P3 prosulfocarb-selected plants failed to produce viable seed owing to severe damage caused by pathogens at the plants' reproductive stage, and herbicide testing could not be conducted.

We emphasise that thus far in Australia *L. rigidum* populations have been controlled with prosulfocarb + *S*-metolachlor (introduced in 2008) or pyroxasulfone (introduced in 2012), with no known cases of field-evolved resistance.<sup>38,39</sup> However, this study shows that resistance traits to these herbicides were present prior to the commercialisation of prosulfocarb + *S*-metolachlor and pyroxasulfone in at least one among 50 random populations analysed. It is possible that previous field use of other pre-emergence VLCFA inhibitors (e.g. triallate) had enriched for genetic trait(s) somewhat effective against prosulfocarb + *S*-metolachlor or pyroxasulfone.

Our previous studies established that recurrent low-dose pyroxasulfone selection could rapidly lead to pyroxasulfone resistance in a *L. rigidum* population,<sup>17</sup> and that in a multiresistant *L. rigidum* population (MR in this study) there was also cross-resistance to thiocarbamate herbicides prosulfocarb and triallate.<sup>26</sup> An inheritance study revealed the involvement of single semi-dominant genetic traits conferring cross-resistance to different herbicide modes of action.<sup>27</sup> Here, with a different *L. rigidum* field population

(#20), we confirm that concomitant evolution of cross-resistance to prosulfocarb and pyroxasulfone rapidly evolved within three generations when only a small number of individuals (40 viable seeds) were initially selected with either herbicide. In this population, the frequency of resistance to the ALS herbicide sulfometuron was 85%, and 20% individuals were found to be resistant to the ACCase herbicide diclofop-methyl. However, prosulfocarb or pyroxasulfone selection did not shift towards greater resistance to the ALS herbicide chlorsulfuron or ACCase herbicide diclofop-methyl (data not shown).

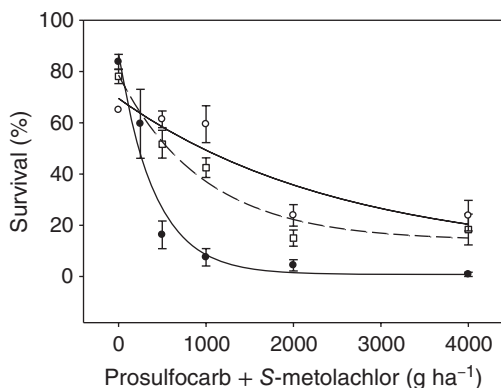
#### 4.2 Insights to achieve long-term weed control and minimise cross-resistance evolution

In Australia, ACCase herbicide resistance evolution in *L. rigidum* has been known since the early 1980s<sup>21</sup> following the introduction in 1978 of the first ACCase grass wheat-selective herbicide diclofop-methyl. Cross-resistance to the ALS herbicide chlorsulfuron in *L. rigidum* was unexpected and yet appeared to be correlated with diclofop-methyl herbicide selection.<sup>22</sup> In *L. rigidum*,

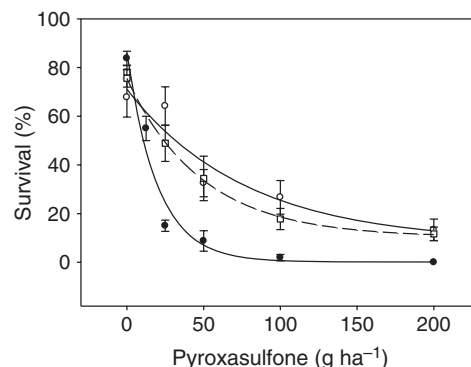
**Table 2.** Pooled data from herbicide dose–plant survival response studies to assess resistance and cross-resistance to pyroxasulfone and prosulfocarb + *S*-metolachlor in the three-time selected progeny #20 P3. Estimated LD<sub>50</sub> values expressed as pyroxasulfone or prosulfocarb g ha<sup>-1</sup> with standard errors in parentheses and resistance index (RI) compared with the standard herbicide-susceptible *S* population. MR P6 progeny were used as a resistance standard as they were documented as displaying cross-resistance to pyroxasulfone and prosulfocarb elsewhere.<sup>26</sup> Probability values (*P*) of difference between parental and selected populations in response to pyroxasulfone or prosulfocarb were assessed by the SI function in the *drc* package in the software program R v.2.14.1 (R Foundation for Statistical Computing). Parameters *b*, *d* and *e* in equation 1 are given for each population tested

Population	Herbicide selection	LD <sub>50</sub> (g ha <sup>-1</sup> )	<i>b</i>	<i>d</i>	<i>e</i>	RI <sup>a</sup>	<i>P</i>
<i>Response to prosulfocarb</i>							
<i>S</i>	None	319 (41)	2.8 (1)	84 (5)	319 (41)	–	–
#20 P3	Prosulfocarb or pyroxasulfone	934 (168)	1.3 (0.3)	80 (5)	934 (168)	2.9	<0.01
MR P6	Pyroxasulfone followed by prosulfocarb	1798 (276)	1.7 (0.4)	70 (6)	1796 (276)	5.6	<0.01
<i>Response to pyroxasulfone</i>							
<i>S</i>	None	15 (3)	2.5 (1)	84 (5)	15 (3)	–	–
#20 P3	Pyroxasulfone or prosulfocarb	42 (8)	1.2 (0.3)	75 (5)	42 (8)	2.8	0.01
MR P6	Pyroxasulfone followed by prosulfocarb	65 (13)	1.5 (0.4)	69 (5)	65 (13)	4.4	<0.01

<sup>a</sup> Resistance index is referred to the standard susceptible population *S*.



**Figure 3.** Herbicide dose–response study of *L. rigidum* populations. Prosulfocarb + *S*-metolachlor survival response of a standard herbicide-susceptible population (*S*) and a characterised prosulfocarb-resistant population (MR P6) compared with the third generation (#20 P3) of population #20 selected three consecutive times with 2000 g prosulfocarb ha<sup>-1</sup> + 300 g *S*-metolachlor ha<sup>-1</sup> or 100 g pyroxasulfone ha<sup>-1</sup>. *S*, continuous line and full circles; #20 P3 progeny selected three times with pyroxasulfone or prosulfocarb, empty quadrats and dashed line; MR P6 progeny selected 6 times, empty circles and continuous line. Symbols are observed means ± SE (*n* > 6). Lines show the fit of equation (1) to the dose–survival response data.



**Figure 4.** Pyroxasulfone survival response of a standard herbicide-susceptible *L. rigidum* population (*S*) and a characterised pyroxasulfone-resistant population (MR P6) compared with the third generation of population #20 P3 selected three consecutive times with 100 g pyroxasulfone ha<sup>-1</sup> or three consecutive times with 2000 g prosulfocarb ha<sup>-1</sup> + 300 g *S*-metolachlor ha<sup>-1</sup>. *S*, continuous line and full circles; #20 P3 progeny selected three times with pyroxasulfone or prosulfocarb, empty quadrats and dashed line; MR P6 progeny selected 6 times, empty circles and continuous line. Symbols are observed means ± SE (*n* > 6). Lines show the fit of equation (1) to the dose–survival response data.

non-target-site cross-resistance to these herbicide modes of action is often polygenic and endowed by enhanced rates of herbicide metabolism.<sup>18</sup> For example, it was shown that resistance to the ACCase herbicide diclofop-methyl can be synergistically reversed by amitrole which can inhibit diclofop metabolism,<sup>40</sup> and that enhanced metabolic resistance to the ALS herbicide chlorsulfuron is likely mediated by P450 genes.<sup>41,42</sup> Subsequent studies confirmed that in *L. rigidum* ACCase and ALS resistance P450 traits are linked,<sup>43</sup> and that resistance to the ALS herbicide chlorsulfuron can rapidly coevolve by recurrent field selection with the ACCase diclofop-methyl.<sup>44</sup>

In other grass weed and crop species it appears that the detoxification of residual pre-emergence herbicides such as chloroacetamide, thiocarbamate and triazines requires the involvement of both P450 and GST enzymes.<sup>45,46</sup> As ACCase- and ALS-resistant grass weed populations remain susceptible to a range of residual pre-emergence herbicides, it may be

that herbicide detoxification is endowed by different traits.<sup>47</sup> For example, residual pre-emergence herbicides (e.g. chloroacetamides, dinitroanilines, thiocarbamates and triazines) remain an important component to manage multi ACCase- and ALS-resistant weeds of *Alopecurus*, *Avena*, *Echinochloa* and *Lolium* genera,<sup>25,47,48</sup> as well as other glyphosate-resistant weed species.<sup>49</sup> Consistently in this study we did not find in *L. rigidum* any evident correlation between evolved resistance to the post-emergence herbicide diclofop-methyl and survival to the pre-emergent prosulfocarb or pyroxasulfone. The patterns of evolved herbicide cross-resistance in weeds often appear as a mosaic of selected resistance-endowing traits,<sup>47</sup> and our understanding of these complexities, especially related to non-target-site resistance traits, remains only partial.<sup>18,50</sup> Thus, a current research challenge is to elucidate the precise biochemical and genetic basis of non-target-site-based cross-resistance to different herbicide modes of action (e.g. resistance traits to pre-emergence versus post-emergence herbicides) to inform effective use patterns.<sup>4,51</sup>

This study showed a correlation between survival to prosulfocarb and pyroxasulfone in unselected parental *L. rigidum* populations, suggesting no evidence of selection from either herbicide in parental populations and also the possibility of some common traits for cross-resistance to these herbicides. As in one *L. rigidum* population cross-resistance to both herbicides evolved by selection with either herbicide, we suggest that rotation between prosulfocarb and pyroxasulfone may have a limited effect in reducing the risk of resistance evolution to either herbicide.

Studies have shown that mixtures of herbicide modes of action can be more effective than herbicide rotation in reducing the occurrence of resistance.<sup>52–54</sup> Similarly, in *Chlamydomonas reinhardtii* it has been shown that high doses of mixtures help to reduce resistance evolution,<sup>55</sup> whereas herbicide rotation in some instances could accelerate cross-resistance evolution at a greater cost.<sup>56</sup> However, modelling simulations suggest that binary herbicide mixtures, as opposed to herbicide rotation, are only effective in delaying resistance if weed populations are susceptible to both components.<sup>57</sup>

In Australia, it is common practice to use binary herbicide mixtures of the dinitroaniline herbicide trifluralin and pyroxasulfone or trifluralin and prosulfocarb, as dinitroanilines can improve weed control in conditions of limited soil moisture.<sup>58</sup> Thus, we believe that there is an urgent need to conduct simulation modelling research to explore use patterns (mixtures versus rotation) of pre-emergence residual herbicide to establish which practices can deliver the most effective control and best delay the evolution of resistance in *L. rigidum*.

We emphasise that, although pre-emergence residual herbicides remain effective tools to provide satisfactory weed control in many cropping systems, the implementation of a diverse array of chemical, cultural and physical weed removal practices is very important to maximise the heterogeneity of selection pressures in order to minimise resistance evolution, achieve satisfactory control of weed populations and allow sustainable long-term herbicide use.

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