Rotations and mixtures of soil-applied herbicides delay resistance

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ABSTRACT

BACKGROUND: Weed resistance to foliar herbicides has dramatically increased worldwide in the last two decades. As a consequence, current practices of weed management have changed with an increased adoption of soil-applied herbicides to restore control of herbicide-resistant weeds. We foresee metabolism-based resistance and cross-resistance to soil-applied herbicides as a potential global consequence to the increased and widespread adoption of new and old soil-applied herbicides. Thus, the aim of this study is to use computer simulation modelling to quantify and rank the risk of weeds evolving resistance to soil-applied herbicides under different usage strategies (single herbicide use, rotations and mixtures) and population genetic hypotheses.

RESULTS: Simulations indicate that without rotation, it takes twice as long to select for resistance to a particular soil-applied herbicide – trifluralin – than to any other herbicide option considered. Relative to trifluralin-only use, simple herbicide rotation patterns have no effect in delaying resistance, whereas more complex rotation patterns can delay resistance two- or three-fold. Herbicide mixtures further delay resistance up to six-fold in comparison to single use or simple herbicide rotations.

CONCLUSION: By computer modelling simulations we demonstrate that mixtures maximize herbicide effectiveness, selection heterogeneity of soil-applied herbicides and delay herbicide resistance evolution in weedy plants. Our study is consistent with previous state-of-art scientific evidence (*ie* epidemiological and modelling studies across different systems and pests) and extension efforts (*ie* ‘rotate herbicide mixtures’) to provide insight to manage the selection and evolution of weed resistance.

1. INTRODUCTION

Since crop domestication, agricultural production practices have imposed an unintended selection on crop pests such as weeds, pathogens and insects that have rapidly adapted in response to such an intensive selection pressure.^{1,2} Synthetic herbicides have been used worldwide to control weeds consequently ~~yet~~ since the 1940s, this human-imposed herbicide selection has resulted in dominant weed species resistant to specific herbicide modes of action (eg specialist resistance to ALS-, ACCase-, EPSPS-, PSII-inhibitors)³ and emergence of populations rapidly evolving multiple resistance traits (generalist cross-resistance to ALS-, ACCase-, mitosis-, VLCFA-inhibitors).^{4,5} As weeds are one of the greatest biotic constraints to crop yield, the evolution of herbicide resistance traits in strongly competitive weed species is a real threat to sustainable, global food production.⁶⁻⁸

In Australia in a highly mechanized and simplified cropping system, overreliance of post-~~emergent-emergence~~ foliar herbicides has led to rapid evolution of herbicide resistance in the damaging, resistance-prone grass weed *Lolium rigidum* (Gaudin)^{9,10}. *Lolium rigidum* ranks as the number one weed species for its ability to evolve multiple herbicide resistance due to its genetic diversity, obligate allogamous nature and likely greater frequency of resistance to different herbicide sites of action.¹¹⁻¹³ Growers have responded to weed resistance to foliar post-emergence herbicide by adoption of soil-applied pre-emergence herbicides for weed control. For example, soil-applied herbicides including trifluralin, propyzamide, prosulfocarb, and pyroxasulfone are widely used for selective control of *L. rigidum* in grain crops in Australia¹⁴. Thus, currently there is high adoption of pre-emergence herbicides to control *L. rigidum* and other grass weeds such as *Avena* sp., *Bromus* sp. and *Hordeum* sp. resistant to selective post-emergence herbicides (e.g., ACCase and ALS herbicides) as well as the rapidly escalating

problem of glyphosate resistance ^{10, 15}. Approximately 900 populations of *L. rigidum* are reported from Australia to be resistant to glyphosate ¹⁶.

Globally, resistance to pre-emergence soil-applied herbicides has evolved at a slower rate than foliar post-emergence ~~compared to other herbicides~~ modes of action ^{17, 18}. For example, resistance to trifluralin, the most widely adopted dinitroaniline herbicide, has evolved only in a few different weed genera of major grassweeds such as *Alopecurus*, *Lolium* and *Poa* and only the dicot weed *Amaranthus palmeri* ¹⁷. In Australia, trifluralin has been extensively used for pre-emergence grass weed control for 40 years with minimal resistance evolution until recently. In some regions of the Australian 'grain-belt', the incidence of trifluralin resistance in *L. rigidum* is widespread ¹⁹, whereas in other areas trifluralin remains effective ¹⁰.

Other classes of soil-applied herbicides, such as chloroacetamides and thiocarbamates, target specific enzymes involved in the first limiting steps of the elongation of very long chain fatty acids (VLCFA) in the endoplasmic reticulum ^{20, 21} and allow effective ~~pre-emergent~~ emergence grass weed control in winter cereals, corn, rice, and soybean crops worldwide. Herbicide resistance to these herbicides has remained low, only reported in a few grass weed species ¹⁴. In Australia, VLCFA elongase-inhibiting herbicides and mitosis inhibitors are used in rotation and mixture (often with trifluralin) and provide good control of multiple resistant grass weed populations ^{22, 19, 23}. In Australia, multiple resistance to chloroacetamides (eg S-metholachlor) ²⁴, dinitroanilines (trifluralin) ²⁵ and thiocarbamates (triallate) ²⁶ had been reported in field populations of *L. rigidum*, but the frequency of resistance to these soil-applied herbicides has remained low compared to the widespread resistance to selective post-emergence herbicides ^{10, 19, 27-29}. Before the global commercialisation of the soil-applied herbicide pyroxasulfone in 2012, we were able to evolve pyroxasulfone resistance in a ryegrass population by recurrent low-dose selection over three generations ³⁰. We subsequently demonstrated that pyroxasulfone low dose

selection resulted in broad cross-resistance to prosulfocarb and triallate ^{31 32}. More recently, a field population of *L. rigidum* was confirmed resistant to several pre-emergence herbicides including prosulfocarb, trifluralin, pyroxasulfone and with decreased sensitivity to propyzamide ³³.

The aim of this study is to investigate how to best deploy pre-emergence soil-applied herbicides focusing on usage patterns of four herbicides: pyroxasulfone, trifluralin, prosulfocarb and propyzamide. A modified version of the PERTH (Polygenic Evolution of Resistance To Herbicides) computer model ³⁴ was used to simulate the efficacy of different pre-emergence herbicide usage patterns to delay or avoid the evolution of resistance in the model weed species *L. rigidum* in Australia. We also investigated how the efficacy of the strategies depends on a number of factors related to the underlying genetics ³⁵, including fitness penalties ³⁶, possible negative cross-resistance between trifluralin and prosulfocarb ³², and initial resistance allele frequencies ¹¹.

The population dynamics of herbicide resistance are difficult to predict, as factors such as biological features of the target species, specifics of the herbicide molecule, mode of herbicide action and agro-ecosystem features all influence the prevailing evolutionary trajectories. Simulation modelling can distil available knowledge to simulate population dynamics in response to herbicide selection and predict the risk of weed resistance evolution to soil-applied herbicides currently in use on a global scale in wheat-based cropping systems.

2. MATERIALS AND METHODS

2.1 Experimental data for model calibration

Many of the important model parameters were based on previous empirical studies, which we summarise here. In one study, approximately 100 million *L. rigidum* herbicide-susceptible individuals were field-treated with a high dose of 400 g pyroxasulfone ha⁻¹, causing high level mortality (> 99.999%) and revealing a low initial frequency of a major resistance trait at < 10⁻⁷³⁰.. By contrast, a three-year low-dose (60% of the recommended label dose) recurrent selection experiment showed that a parental 10-fold trifluralin-resistant *L. rigidum* population²⁵ could clearly be shifted to evolve an eight-fold level of pyroxasulfone resistance after only three generations(LD₅₀ resistance / susceptible, R/S = 8)³⁰. In a subsequent study, we documented seven-fold cross-resistance to prosulfocarb (LD₅₀ R/S = 7) in the same population exposed to an additional cycle of pyroxasulfone selection³¹. In another study, we revealed the inheritance of pyroxasulfone resistance as based on one semi-dominant gene selected at incrementally higher frequency in the *L. rigidum* population under continuous pyroxasulfone pressure. When the pyroxasulfone-resistant parent was back-crossed to susceptible parental plants, we also found that resistance traits to each individual herbicide including prosulfocarb, triallate and trifluralin was consistently endowed by one single major gene(s) with incomplete dominance³⁵. A subsequent study has established that prosulfocarb and pyroxasulfone resistance-endowing trait(s) are not linked to trifluralin resistance, and trifluralin resistance levels decreased under cycles of pyroxasulfone followed by prosulfocarb selection³².

2.2 Adaptations to the PERTH model to enable it to represent multiple residual herbicides

The PERTH (Polygenic Evolution of Resistance to Herbicides) model was designed to represent the evolution of herbicide resistance in an annual weed in a cropping system with a single crop each year³⁴. The model was originally parameterised to represent *L. rigidum* in a southern Australian cropping system, but can easily be reparametrized to represent other weed species. The model is able to represent the evolution of resistance conferred by either single or multiple

139 genes (monogenic or polygenic resistance) using explicit individual-based genetics, where the
140 presence or absence of potential resistance alleles are represented separately for every plant in a
141 population, for each year of simulation. The original version represented evolution of resistance
142 to a post-emergence crop-selective herbicide, but more recently the model has been adapted to
143 represent evolution of resistance to soil-applied pre-emergence herbicides³⁷. However, to date,
144 the model has only been able to represent evolution of resistance to a single herbicide at a time.
145 To address the aims of this study, we adapted the PERTH model to enable it to simulate evolution
146 of resistance to multiple residual herbicides at the same time, while allowing for possible cross-
147 resistance. In the original model, every possible genotype was mapped to a resistance level³⁴. For
148 example, in single gene dominant resistance, susceptible SS is represented as 1, whereas RS and
149 RR are mapped to 10, indicating that heterozygous and homozygous resistant plants have 10-
150 fold resistance compared to the homozygous susceptible plants. For this current study, we
151 changed the original model so that there is now a map between genotype and resistance for each
152 herbicide. This very general approach allows us to represent any desired scenario of cross-
153 resistance between multiple herbicides, including no cross-resistance at all (complete
154 independence of resistance).

155 Many of the PERTH model parameters represent ecological (as opposed to genetic)
156 characteristics of the weed species and its interactions with its environment (Table 1). The values
157 for these parameters in this study were drawn from previous studies that aimed to represent *L.*
158 *rigidum* in a southern Australian environment. ^{34, 37-39} In reality, these values will vary in different
159 seasons, locations, and certainly for different species; however, these studies (particularly ³⁴⁻
160 have shown that the precise values of these ecological parameters have little or no effect on
161 patterns of resistance evolution and the rankings of different strategies. For this reason we focus
162 on analysing sensitivity to genetic assumptions in this study, and also predict that our results
163 regarding the relative benefits of different usage strategies are likely to be similar for other values

for these ecological parameters representing other weed species and/or other environments. The remaining genetic parameters (Table 1) have values that are either supported by empirical studies or else we analyse the sensitivity of our results to different assumptions regarding these parameters (as explained in detail below).

TABLE 1 ideally placed here

2.3 Management strategies considered:

We considered a total of 19 different strategies for rotating and/or mixing the pre-emergence herbicides (Table 2). These strategies were divided into a series of ‘strategy sets’. The first three strategy sets are based on an assumption that resistance to any of the four herbicides has not yet evolved, with the first set selected to act as a baseline, representing no or minimal herbicide rotation; the second set includes different herbicide rotation strategies; and the third set includes two different herbicide mixture strategies. The other three strategy sets are based on an assumption that resistance to the herbicide trifluralin has already evolved and thus trifluralin is no longer available as an effective herbicide, with the fourth set including different rotation strategies. The fifth set includes two different mixture strategies, and the sixth set consists of a single special strategy aimed at exploiting the empirically observed negative cross-resistance between trifluralin and prosulfocarb (Busi and Powles, unpublished).

TABLE 2 ideally placed here

2.4 Genetic assumptions and scenarios considered

The empirical data on strength of resistance and cross-resistance, together with the historical rarity of resistance evolution to these herbicides, allowed us to justify a number of assumptions regarding the genetic bases of resistance: (1) there is an initially rare semi-dominant gene (Allele 1) that confers 10-fold (specialist) resistance to trifluralin only (no cross-resistance)^{25, 26}; and (2)

there is an initially rare semi-dominant gene (Allele 2) that confers 10-fold resistance to prosulfocarb and pyroxasulfone (generalist) and no resistance to trifluralin or propyzamide. We have observed that a trifluralin-resistant population under selection with pyroxasulfone (four initial generations) and prosulfocarb (two generations) lost trifluralin resistance (Busi et al., unpublished), therefore, based on the available empirical evidence of one *L. rigidum* population studied³², we also wanted to consider a gene conferring cross-resistance to pyroxasulfone and prosulfocarb + S-metolachlor could reduce the level of resistance to trifluralin (negative cross-resistance). In the parental trifluralin-resistant population, the estimated LD₅₀ shifted from 519 g ha⁻¹ (resistance) to 21 g trifluralin ha⁻¹ after pyroxasulfone and prosulfocarb recurrently-selected progeny. In the standard reference population, we calculated a 71 g trifluralin ha⁻¹ as the LD₅₀ value. Thus, we quantified the negative cross-resistance by multiplying the LD₅₀ for trifluralin by a factor of 0.3 ($LD_{50} \text{ 21 g trifluralin ha}^{-1} / 71 \text{ g trifluralin ha}^{-1} = 0.3$) if both resistance alleles (Allele 2) are present and by 0.65 if only one (Allele 2) resistance allele is present. Since the empirical evidence relates to only one *L. rigidum* population, we considered genetic scenarios with and without this negative cross-resistance. There is also an initially rare semi-dominant gene (Allele 3) that confers 10-fold resistance to propyzamide only, with no cross-resistance to any other herbicide.

The three resistance genes are all assumed rare in susceptible populations, each with an initial allele frequency of 10^{-7} for resistant homozygous plants. Resistance to trifluralin has evolved in *L. rigidum* after several years (> 10 years) of widespread use and high selection pressures, but it remains at relatively low levels in Australia^{10, 19, 40}. Field resistance to pyroxasulfone has been recently reported in *Avena fatua* from Canada⁴¹. In *L. rigidum*, resistance to pyroxasulfone has been selected after only three generations of recurrent low-dose selection in glasshouse studies^{30, 42}, but the empirical evidence indicates it is rare in *L. rigidum*-unselected populations in the field with values possibly lower than 10^{-7} . Resistance to propyzamide has been recently

documented in *Poa annua* in USA⁴³ and Australia¹⁷ and one relatively old report of reduced propyzamide sensitivity in *A. fatua*¹⁷. However, because there is high uncertainty about the initial resistance allele frequencies, we wanted to investigate how varying this parameter would affect results. We therefore considered the effect of multiplying the standard initial allele frequencies (Table 1) by 0.01, 0.1, 1, 10, and 100.

Based on available empirical data, we assumed a 20% fitness penalty associated with Allele 1 as reported for trifluralin resistance in *Setaria viridis*⁴⁴. A similar cost of approximately 20% was detected and quantified by Vila-Aiub et al.³⁶ in trifluralin-resistant *L. rigidum* with enhanced metabolic capacity for herbicide detoxification³². Thus, we have assumed a similar 20% fitness penalty associated with Allele 2 endowing cross-resistance to prosulfocarb+ S-metolachlor and pyroxasulfone resistance in *L. rigidum*. There is, of course, no data available for propyzamide fitness penalties, and so we assumed a similar fitness penalty for Allele 3 (20%). Since the evidence for fitness penalties is not conclusive, we considered genetic scenarios with and without fitness penalties. Therefore, three genetic scenarios were considered:

Genetic scenario 1 (GS1): All assumptions as above, including fitness penalties but no negative cross-resistance.

Genetic scenario 2 (GS2): All assumptions as above, including fitness penalties and negative cross-resistance.

Genetic scenario 3 (GS3): All assumptions as above, but neither fitness penalties nor negative cross-resistance.

2.5 Modelling simulations conducted

The full combination of 19 management strategies, three genetic scenarios and five initial allele frequencies gave a total of 285 overall cases. Each of these cases was simulated 100 times to

240 account for stochastic variation. For each of these 28,500 simulation runs, we recorded the
241 number of years until resistance occurred, defined as the first year that crop yields fall below
242 75% of maximum, which is equivalent to surviving weed densities exceeding 200 plants m⁻².³⁹
243 In addition, for illustrative purposes, the 19 management strategies were simulated once each for
244 the GS1 genetic scenario and the base initial allele frequencies, and all outputs were recorded
245 and plotted.

246

3. RESULTS

The assumed herbicide usage strategy, genetic scenario and initial allele frequency clearly affected predictions for weed numbers, herbicide resistance allele frequencies, and the number of years until resistance (Figures 1, 2, Table 3). In all cases weed numbers initially declined, showing that initial control levels were adequate, but then as resistance allele frequencies increased, herbicide efficacy decreased, and weed numbers started to increase until weed densities exceeded the threshold of 200 plants m⁻² and simulations were halted (Figures 1, 2).

3.1. Starting without existing trifluralin resistance

When we assumed the GS1 with no existing resistance to trifluralin and no negative cross resistance (GS1, Fig. 1), then the best strategies for maintaining low weed numbers were clearly the strategies that employed full strength mixtures, with M12,M13,1,4 maintaining slightly lower weed numbers than M12,M13,4 (Table 3). Both mixture strategies maintain low weed numbers for at least 40 years, although for M12,M13,4 there is evidence of emerging resistance (increasing resistance allele frequencies). The next best strategies were the similar strategies 12141314 and 11241134, with negligible difference caused by the different order in which the herbicides are applied in these two strategies. Almost as good was the ‘trifluralin first’ strategy, which resulted in higher weed numbers in some years, but still maintained weed numbers below the threshold for as long as 12141314 and 11241134. Next best was 124134, followed by 1114, then 111422243334, with 1234 being the worst rotation strategy. Unsurprisingly, the worst ‘strategies’ were those with no herbicide rotation (1111 best, 2222, 3333). With no herbicide rotation, the difference in time to resistance between trifluralin (16 years) and the other herbicides (8 years) was due to the difference in LD₅₀ values observed in the susceptible standard versus the evolved resistant *L. rigidum* population that were used for model parametrization (Table 3).

3.2 With existing trifluralin resistance

When we assumed existing resistance to trifluralin, fitness penalties and no negative cross-resistance (GS1, Fig. 2), the best strategies for maintaining low weed numbers were again the strategies that employed full strength herbicide mixtures, with M12,M13,4 clearly the best, and M23,M23,4 second best. The next best was 2434, followed by CR, then 234234, with 2323 being the worst. Note that M23,M23,4 was only marginally better than 2434. Interestingly, M12,M13,4 (mixture containing trifluralin) outperformed the mixture without trifluralin, even with existing resistance to trifluralin (Table 3).

TABLE 3 ideally placed here

3.3 Varying initial resistance allele frequency

Varying initial resistance allele frequencies made a difference, with higher frequencies leading to faster resistance and lower frequencies leading to slower resistance (Table 2, GS1 versus GS3). These effects were quite consistent across rotation strategies and genetic scenarios, indicating that the effect of initial resistance gene frequency was independent of strategy or scenario. For some strategies, resistance did not evolve at all for lower allele frequencies, and so valid comparison between genetic scenarios and with other strategies can only be made for higher initial frequencies (eg M12,M13,4) (Table 3).

3.4 Including putative negative cross-resistance

Adding the negative cross-resistance to the model improved the longevity of some strategies where trifluralin is used together with prosulfocarb or pyroxasulfone in rotations or mixtures, and for some of the mixtures this improvement was substantial (Table 2, GS1cf GS2, see the bold entries in particular). Some examples of large improvements include from 15 to 33 yrs for

M12,M13,4 when there was existing trifluralin resistance and a higher initial allele frequency, and from ~26 to 44 years for M12,M13,1,4 when there was no existing trifluralin resistance and a higher initial allele frequency. For other strategies where trifluralin is used together with prosulfocarb + S-metolachlor or pyroxasulfone, it made little difference (eg. 1234 and 124134); for strategies where trifluralin is not used together with prosulfocarb + S-metolachlor or pyroxasulfone (eg. 2222 and 3333), it made no difference as expected.

3.5 Effect of fitness penalties

As expected, removing assumptions of fitness penalties from the model generally decreased the longevity of the strategies, or occasionally made no difference (Table 2, GS1 versus GS2). The biggest reductions in longevity were for the most effective strategies, especially the mixtures (e.g., from 15 to 9 years for M12,M13,4 and from 32-38 to 17-18 years for M12,M13,4).

4. DISCUSSION

4.1 Model parametrization to rank the risk of herbicide resistance

Simulation modelling allows the prediction of evolutionary rates and dynamics under different management scenarios, and thus develop preventative and adaptive strategies that take into consideration variability and change.^{45,46} In this study we compare strategies and use patterns of soil-applied herbicides (continuous use, rotation or mixture) to deliver robust long-term weed control of *L. rigidum* used as a model grass weed species. Soil-applied herbicides are currently the most efficient option to protect crops against damaging weed species such as *L. rigidum*^{19,22} and allow for sustainable profit-oriented dryland farming⁴⁷. The PERTH model, parametrized with data obtained from a decade of experimental evolution studies^{14,30-32,35,42}, predicts that herbicide mixtures are the most robust strategy to minimize the risk of resistance and cross-

resistance evolution to soil-applied herbicides. The relative benefits of rotations and particularly mixtures in delaying resistance were found to be consistent across a number of genetic and fitness-penalty scenarios and 100-fold difference in initial allele frequencies. While the model is parameterised for the important and damaging grass weed *L. rigidum*, we would expect the results to apply to other weed species infesting different agricultural systems^{45, 48-52}.

The majority of our model parametrization was based on available data. For example, we had field data indicating that resistance to pyroxasulfone is very rare ($< 10^{-7}$) and we assumed an identical initial resistance allele frequency for the four herbicides considered.³⁰ Thus far, widespread field resistance has been documented in *L. rigidum* only for trifluralin²⁵ and based on dose-response studies on susceptible and resistant populations, we assumed trifluralin had a lower LD₅₀ than prosulfocarb, pyroxasulfone and propyzamide (10% for trifluralin vs 20% in other herbicides relative to the recommended field rate). The PERTH model parametrization also reflected our experimental work documenting cross-resistance between pyroxasulfone and prosulfocarb.^{31, 42} and recent evidence of field populations of *L. rigidum* evolving resistance to the thiocarbamate herbicides such prosulfocarb and triallate and displaying a variable level of cross-resistance to pyroxasulfone and propyzamide.³³ A similar pattern of cross-resistance between pyroxasulfone and triallate has been reported in another grass weed such as *Avena fatua*⁴¹. Thus, we assumed that cross-resistance between pyroxasulfone and prosulfocarb was the norm in our modelled species *L. rigidum* despite ~~these trait(s) for field resistance to each herbicide are unique with~~ exceptions to the norm in a variable species such as *L. rigidum* could be often expected.⁵³ Additionally, resistance to propyzamide appears to be a rare phenomenon, as it has thus far only been reported at low level in grass weeds such as *Poa annua* and *L. rigidum*^{17, 33, 43}.

The simulations indicated that when single herbicides are applied annually, resistance to trifluralin occurred slower than for the other herbicide. This is due to particular model

parametrization accounting for the calculated LD₅₀ values for each herbicide (lower for trifluralin) relative to the recommended field dose at which weed resistance selection occurs^{25, 26, 30, 31}. Therefore, herbicide selection on a reduced population size is likely to result in slower increase of allele frequency and build-up for resistance. This may seem counterintuitive at first, because higher kill rates have often been correlated to higher selection pressure⁵⁴. However, the higher herbicide dosages can result in higher kill rates as they purge heterozygotes from the population or greatly suppress fitness of weak resistance phenotypes, thus keeping resistance allele frequencies lower for longer. This is analogous to the fact that higher doses can delay resistance evolution rates when resistance is polygenic resulting in a continuous distribution of individuals with weaker phenotypic resistance with lower fitness in the presence of robust full herbicide doses³⁴. As previous studies indicated monogenic inheritance for several soil-applied herbicides here tested³⁵ (ie, prosulfocarb, pyroxasulfone, triallate and trifluralin), simulations reflected the underlying genetic basis conferring resistance to trifluralin (Allele 1), prosulfocarb + S-metolachlor and pyroxasulfone (Allele 2) or propyzamide (Allele 3) at three different frequencies (0.1, 1, 100) within an evolutionary context affecting fitness and epistasis (ie negative cross-resistance). We then observed that under prosulfocarb selection, trifluralin resistance was decreased over time in a *L. rigidum* population with a calculated 70% LD₅₀ lower than a susceptible standard population⁵⁵. This observed phenomenon was incorporated into the model by a particular parametrization with trifluralin resistance lowered by a factor of 0.3 with both prosulfocarb resistance alleles or by a factor of 0.65 with one prosulfocarb resistance allele (see Table 1). With the assumption of negative cross-resistance, the simulations indicated only a modest (5-10%) delay in resistance would occur with herbicide rotation (e.g. 12141314, Table 2). On the contrary, negative cross-resistance would be best exploited with herbicide mixtures causing a substantial (from 60% up to 120%) delay in resistance evolution (Table 2).

4.2 Rotating mixtures a simple and robust message for pesticide resistance management

Pesticide mixtures at high dosages of each pesticide and/or combinations appears as the most effective measure at delaying resistance evolution, because they help ensure that pests are treated with two different pesticides^{49,56,57}. Similarly weeds resistant to one herbicide mode of action are likely to be killed by another herbicide acting atef a different site of action⁵². This simple population genetic principle can help explain our results showing that herbicide mixtures can keep both weed densities and resistance allele frequencies very low. Interestingly, our simulations suggest that mixtures containing trifluralin are less prone to select for resistance and therefore may be more robust than mixtures of prosulfocarb and pyroxasulfone even when there is some level of trifluralin resistance in a target *L. rigidum* population. The low efficacy of mixtures of prosulfocarb and pyroxasulfone reflects the cross-resistance observed in *L. rigidum* populations^{31,42} to these two herbicides and our assumption that such a mixture could be overcome by a single resistance gene³⁵. Conversely, including trifluralin in a mixture is effective because the impact of the other herbicides maintain trifluralin resistance allele frequency at around 50%, meaning that the trifluralin can continue to significantly impact weeds and allow satisfactory weed control. There is field evidence that a mixture of prosulfocarb + S-metolachlor or triallate in mixture with pyroxasulfone could deliver effective control of *L. rigidum*⁵⁸. We acknowledge the validity of this approach, but remain confident that such a strategy may not be the most effective in delaying the selection of resistance. Our parametrized version of the model PERTH predicted that even in case of trifluralin resistance, binary herbicide mixtures with trifluralin could have a greater delay in herbicide resistance than mixtures without trifluralin. Our simulations clearly suggest that the herbicide mixture of trifluralin + prosulfocarb + S-metolachlor followed by trifluralin + pyroxasulfone in the second year and propyzamide in the third year (ie M12,M13,4) is the most effective strategy to achieve good weed control and delay resistance evolution (Figure 1). This strategy is marginally more robust than using trifluralin

more frequently before propyzamide (ie M12,M13,1,4). These results are consistent with previous modelling work demonstrating that in the initial phases of resistance in small populations all individuals are likely to be susceptible to two different herbicides and resistance does not evolve ^{52, 55, 59}.

Since first documenting pyroxasulfone resistance evolution after three years of recurrent selection, there has been significant extension activity and interaction with different stakeholders to communicate an optimal strategy of herbicide usage patterns to extend herbicide longevity and minimize risk of resistance evolution. Thus far, it appears that the resistance traits delivering resistance to pyroxasulfone and prosulfocarb share some common basis ⁵⁵. Relevant literature similarly reports that thiocarbamates and chloroacetamides are generally metabolized via GST conjugation ^{14, 55, 60}. Conversely, trifluralin resistance appears to be due to different mechanisms either involving target-site mutations ⁶¹ and/or enhanced herbicide metabolism mediated by cytochrome P450 enzymes ^{32, 62}. Thus, our extension messages focus on the application of these soil-applied herbicides at the full label dosages and rotated to ensure that selection with trifluralin would not enrich for pyroxasulfone resistance and vice versa. This study shows that optimal herbicide rotation is achieved in a hypothetical eight-year cycle in which pyroxasulfone and prosulfocarb are used once every three years where trifluralin is used twice followed by propyzamide used once (i.e., 12141314). Thus, the model's prediction of resistance evolution is consistent with our extension messages.

4.3 Final concluding remarks

Soil-applied pre-emergence herbicide play a pivotal role in minimizing the detrimental effects of rapidly evolved resistance to post-emergence herbicides in the no-till wheat-based Australian agriculture ^{19, 23, 63, 64}. Trifluralin resistance has been reported to low levels in most wheat-growing regions of the southern Australian cropping system. ^{10, 19} Before significant levels of

field resistance to prosulfocarb + S-metolachlor, pyroxasulfone and propyzamide are reported in *L. rigidum* we have identified herbicide use patterns (mixtures and rotations) to achieve maximal control by complementary herbicide action and delay resistance selection. Some herbicide rotation patterns, guided by fundamental knowledge of the mechanism of resistance, proved to be effective and robust. We believe that the development of new pre-~~emergent~~emergence herbicides with a different mode of action for the control of *L. rigidum* and other major grass weeds should remain a priority for herbicide discovery programs tailored for the Australian grains industry. Foundation studies to quantifying the risk of resistance evolution before herbicide commercialization can well complement field efficacy trials. The combination of these research efforts allow proactive extension messages to guide end-users on optimal herbicide use patterns to maximize weed control efficacy and longevity of new herbicides.

435 **Authors' contributions:** RB and MR conceived the study. MR conducted modelling
436 simulations. All authors contributed to the drafts and approved the final version for publication

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Table 1. PERTH model parameters and values used in this study.

* These parameters represent ecological (as opposed to genetic) characteristics of the weed species and its interactions with its environment. The values for these parameters are drawn from previous studies that aimed to represent *L. rigidum* in a southern Australian environment.^{34,37-39} The remaining parameters represent characteristics related to resistance genetics; the values for these parameters are either based on empirical data or their impact on model results was tested using sensitivity analysis, as described in the text.

^a Under the negative cross-resistance scenario, resistance to trifluralin is modified by a factor of $21/71=0.3$ if both prosulfocarb / pyroxasulfone resistance alleles are present or by 0.65 if only one prosulfocarb / pyroxasulfone resistance allele is present.

^b One gene for trifluralin resistance, one gene for pyroxasulfone and prosulfocarb resistance, one gene for propyzamide resistance

^c Corresponding to an initial frequency of $10^{-3.5}$ for effectively heterozygous resistant plant. With strategies for situations where trifluralin resistance is assumed to have already become common (see Table 1), the initial allele frequency for Allele 1 is assumed to be 0.5 instead (0.25 for effectively resistant homozygous plants).

Parameter description	Parameter	Values used in this study
Initial seed bank	<i>isb</i>	100 m ⁻² *
Area of the field	<i>area</i>	100,000 m ² *
Summer death of seeds	<i>sd</i>	10% *
Winter death of seeds	<i>wd</i>	20% *
Germination before sowing	<i>germ1</i>	40% *
Germination after sowing	<i>germ2</i>	40% *
Survival after knockdown	<i>pre-surv</i>	1% *

Probability of weed completely escaping herbicide	<i>Put</i>	1% *
LD ₅₀ as percentage of standard dose	<i>LD₅₀</i>	10% trifluralin ^a 20% other herbicides
Variability parameter	<i>Var</i>	40% *
Number of genes	<i>Ng</i>	3 ^b
Initial resistance allele frequency (homozygous resistance)	<i>iaf</i>	10 ⁻⁷ , ^c
Maximum resistance	<i>Rmx</i>	10
Crop sowing density	<i>Pm'</i>	150 m ⁻² *
The crop plant size coefficient	<i>kp'</i>	1/11 *
The weed plant size coefficient	<i>kp</i>	1/33 *
Maximum weed seed production per unit area	<i>Psp max</i>	30,000 m ⁻² *

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459 **Table 2:** *Lolium rigidum* management strategies considered in this study. Herbicide use patterns
 460 * '1' means trifluralin (dinitroaniline, mitosis inhibitor, group K₁), '2' means prosulfocarb + S-
 461 metolachlor (thiocarbamate + chloroacetamide, VLCFA elongase inhibitor group N/K₃), '3'
 462 means pyroxasulfone (VLCFA elongase inhibitor, group K₃), '4' means propyzamide
 463 (benzamide, mitosis inhibitor, group K₁). We assume that propyzamide is only used in canola,
 464 others in wheat.

Assume	Set	Number	Herbicide use patterns *	Explanation and Justification
Trifluralin Resistance				
No	Set 1	1.1	1111	Baseline scenarios with no or minimal rotation of herbicides to represent poor practice. First four represent no diversity with single herbicide used continuously in a single continuous crop. Last one represents a wheat-wheat-wheat-canola rotation with trifluralin every year in wheat.
		1.2	2222	
		1.3	3333	
		1.4	4444	
		1.5	1114	
	Set 2	2.1	1234	Different rotation strategies in wheat, with canola every fourth or third year.
		2.2	12141314	
		2.3	124134	
		2.4	11241134	Like 2.2, but grouping trifluralin applications
		2.5	111422243334	Like 2.1, but grouping wheat herbicides (using trifluralin first)

		2.6	tr1st	Use trifluralin up first: 1114 for 12 years, then 234 for 12 years
	Set 3	3.1	M12,M13,4	Mixture strategies with canola every third or fourth year
		3.2	M12,M13,1,4	
Yes	Set 4	4.1	2323	Continuous wheat, no trifluralin
		4.2	234234	Canola every third year, no trifluralin
		4.3	2434	Canola every second year, no trifluralin
	Set 5	5.1	M23,M23,4	Mixtures in wheat, canola every third year, no trifluralin
		5.2	M12,M13,4	Mixtures in wheat including trifluralin, canola every third year
	Set 6	6.1	CR 234234234114234	Targeted strategy aimed specifically at exploiting negative cross resistance: repeat 234 three times, and then switch to 114234 (use trifluralin again)

465

466

467 **Table 3:** Years until resistance occurs (first year that crop yields fall below 75% of maximum, which is equivalent to weed densities exceeding
468 200 m²) for fifteen different rotation and mixture (M) strategies (rows) and four initial allele frequencies (1, 0.1 and 100 times the baseline
469 frequencies shown in Table 1) and three different genetic scenarios: GS1(fitness penalties and no negative cross-resistance), GS2 (fitness penalties
470 and negative cross-resistance) and GS3 (no fitness penalties and no negative cross-resistance). Results for other initial allele frequencies are not
471 shown for clarity, but follow the same trends. 'N' indicates that resistance did not occur within 60 years of simulation. In most cases the same
472 result was found in all 100 replicate runs, in which case the single result is reported, otherwise the range is reported (eg. 25-27 means resistance
473 occurred as late as 27 years and as early as 25 years), and, if relevant, the percentage of replicate runs in which resistance did not occur within 60
474 years of simulation. The rank column indicates the relative efficacy of the strategy in delaying resistance under GS1, which is not affected by allele
475 frequency GS3 vs GS1 (1 = most effective; 7 = least effective). **Bold values** indicate where GS2 makes a difference versus GS1. [#]These simulations
476 assumed existing trifluralin resistance, and are ranked separately.

477

	GS1			GS2			GS3			Rank
Herbicide use patterns	Frequency			Frequency			Frequency			
	1	0.1	100	1	0.1	100	1	0.1	100	
1111	16	20	8	16	20	9	14	18	7	6
2222	8	9	4	8	9	4	7	9	4	7
3333	8	9	4	8	9	4	7	9	4	7
4444	8	9	4	8	9	4	7	9	4	7
1114	22	29	11	22	29	11	18	23	9	4
1234	18	23	10	19	23	10	15	19	7	5
12141314	32	N	14	35	N	16	24	31-33	10	2
124134	26	32	12	26	33	12	20	26	9	3
11241134	32	50	14	34	50	16	24	30	10	2
111422243334	21	N	10	21	N	10	18	22	9	4
M12,M13,4	N	N	32-38	N	N	N	51-N 28%	N	17-18	1
M12,M13,1,4	N	N	25-27	N	N	44	N	N	16	1

Trifluralin first 'tr1st'	32	N	11	34	N	11	22	50	9	2
2323 [#]	8	10	5	8	10	5	7	9	4	6 [#]
234234 [#]	13	16	7	13	16	7	10	13	5	5 [#]
2434 [#]	15	19	7	15	19	7	12	15	6	3 [#]
M23,M23,4 [#]	16	19-23	8	16	19-25	8	13	14-17	6-7	2 [#]
M12,M13,4 [#]	28-44	N	15	N	N	33	17-20	22-N 35%	9	1 [#]
CR-234234234114234 [#]	14	20	7	19	23	7	11	14	5	4 [#]

Figure 1: Model outputs from one typical run of each herbicide rotation strategy where there was no existing resistance to trifluralin, for the first genetic scenario (GS1: fitness penalties but no negative cross-resistance), showing weed numbers at harvest each year (top) and the frequency of alleles 1, 2 and 3 in the population each year.

Figure 2. Model outputs from one typical run of each herbicide rotation strategy where there was existing resistance to trifluralin, for the first genetic scenario (GS1: fitness penalties but no negative cross-resistance), showing weed numbers at harvest each year (top) and the frequency of alleles 1, 2 and 3 in the population each year.

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Figure S1. Extension message: simple diagram for research extension suggesting herbicide rotation between the boxes which supports the modelling simulation is one of the most effective strategy in delaying resistance evolution (ie .12141314.)