Research Article

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Herbicide resistance across the Australian continent

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Abstract

BACKGROUND: *Lolium rigidum* is the weed of greatest economic impact in Australia due to its formidable capacity to evolve herbicide resistance. In this study, 579 field-sampled *L. rigidum* populations were tested for resistance to 21 herbicides applied at the recommended rate. Nine herbicide treatments were binary mixtures.

RESULTS: A total of 15 876 individual resistance tests were conducted by screening two million seeds at the recommended label rate. The overall frequency of resistant populations was 31%, 14%, 71%, 6% and 0% in response to the post-emergence herbicide treatments clethodim, clethodim + butroxydim, imazamox + imazapyr, glyphosate and paraquat, respectively. The resistance frequency to stand-alone pre-emergence wheat-selective herbicides ranged from 10% to 34%. Conversely, the levels of resistance to pre-emergence mixtures or stand-alone propyzamide were significantly lower, ranging from 6% to 0%. In winter, the responses to glyphosate, paraquat, cinmethylin, prosulfocarb, pyroxasulfone and trifluralin were reassessed, with 7%, 0%, 0%, 21%, 21% and 28% as the respective resistance frequencies. South Australia and Victoria are identified as epicenters for *L. rigidum* population resistance to pyroxasulfone, whereas populations in New South Wales have the greatest resistance to glyphosate and in Western Australia to clethodim.

CONCLUSIONS: For the first time, resistance levels to stand-alone herbicides and binary mixtures are geographically ranked across the Australian continent by benchmark statistical analysis of resistance frequencies and distribution. The extension of these results will raise awareness of rapidly emerging patterns of herbicide resistance, encouraging the adoption of cost-effective modes of action and integration of diverse strategies for weed resistance management.

Supporting information may be found in the online version of this article.

Keywords: herbicide mixtures; herbicide resistance; herbicide selection; herbicide technology; resistance test; resistance survey; weed control

1 INTRODUCTION

Weeds are a major biotic constraint that can cause crop yield losses greater than those for other agricultural pests.[1–3] A continuous effort is required to keep weed infestation levels to a minimum as weeds are generally very prolific plants. Since its inception, herbicide technology has been increasingly used by farmers worldwide as the most cost-effective weed control measure. Within strict guidelines and defined-use patterns so as not to harm the applicator or the environment, herbicides continue to be the most common tool to provide the most advantageous level of crop protection against weeds.[4–6] As predicted, weeds have evolved resistance to highly effective herbicides deployed for their control and new cases of resistance are increasing steadily worldwide.[2] As the evolution of herbicide resistance is directly linked to the frequency of herbicide use, weed resistance has been more frequently selected in cropping systems characterized by little diversity in weed control tactics and herbicide overreliance.[7–9]

In Australia, *Lolium rigidum* Gaud. (annual ryegrass) is considered the most damaging weed, with an agronomic cost estimated

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to be $100 million annually.[10] To date, *L. rigidum* is the best example of weedy plant capability for evolving resistance under continuous herbicide selection. Some *L. rigidum* populations have been reported to be resistant to multiple herbicide modes of action (MOA)[2]; this is a consequence of persistent herbicide selection leading to a steady increase of the frequency of cross-resistance and multiple-resistance to post-emergence (herein referred to as POST) herbicides, particularly the acetolactate synthase (ALS) and acetyl CoA carboxylase (ACCase)-inhibiting herbicides.[11,12] In the last two decades, several studies have documented the levels of herbicide resistance evolution locally in New South Wales (NSW),[13] South Australia (SA),Victoria (VIC),[14] Tasmania (TAS)[15] and Western Australia (WA).[12,16–18] Two studies have summarized herbicide resistance levels and trends at the national level, with a predominant focus on POST herbicides.[19,20] Due to the rapid selection of resistance to POST herbicides, farmers' choice has increasingly shifted to the adoption of pre-emergence (PRE) herbicides that are much less prone to resistance[14,15] For example, soil-applied herbicides including trifluralin, triallate, propyzamide, prosulfocarb, and pyroxasulfone are widely used for selective control of *L. rigidum* in wheat crops in Australia.[21–23]

Table 1. Analysis of the number of resistance traits found in *L. rigidum* populations

Herbicide resistance to individual herbicide treatments (*n*)	Number of populations	Frequency (%)	HRAC MOA affected by resistance
0	59	10.2	—
1	143	24.7	1, 2, 3, 9, 15, 30
2	93	16.1	1, 2, 3, 9, 15, 30
3	81	14.0	1, 2, 3, 9, 15, 30
4	70	12.1	1, 2, 3, 9, 15, 30
5	48	8.3	1, 2, 3, 9, 15, 30
6	32	5.5	1, 2, 3, 9, 15, 30
7	21	3.6	1, 2, 3, 9, 15, 30
8	17	2.9	1, 2, 3, 9, 15, 30
9	7	1.2	1, 2, 3, 15, 30
10	5	0.86	1, 2, 3, 9, 15, 30
11	3	0.52	1, 2, 3, 15, 30

Herbicide resistance to binary herbicide mixtures (*n*)	Number of populations	Frequency (%)	Binary combinations of MOA affected by resistance
0	360	62.2	—
1	134	23.1	1 + 1, 15 + 3, 15 + 15, 30 + 3, 30 + 15
2	50	8.6	1 + 1, 15 + 3, 15 + 15, 30 + 3, 30 + 15
3	24	4.1	1 + 1, 15 + 3, 15 + 15, 30 + 3, 30 + 15
4	8	1.4	1 + 1, 15 + 3, 15 + 15, 30 + 3, 30 + 15
5	3	0.52	1 + 1, 15 + 3, 15 + 15, 30 + 3

The number of herbicide resistance traits found in corresponding herbicide-resistant populations (total of 579 tested) is reported with the calculated frequency (%) in response to individual herbicide treatments tested and in response to binary (tank) herbicide mixtures tested.

Despite alarming trends of increasing herbicide resistance incidence in the southern Australian cropping regions due to the proven ability of *L. rigidum* to evolve broad patterns of cross- and multiple-resistance to PRE and POST herbicides, there has been insufficient recognition of the role of herbicide mixtures to help mitigate resistance as shown with fungicides and insecticides.[24,25] A recent study in WA has clearly shown that the level of resistance to herbicide mixtures is significantly reduced or negligible.[26]

A new herbicide MOA, cinmethylin,[27] was introduced in Australia in 2020 as a 'resistance breaker' for the control of multiple-resistant *L. rigidum* populations.[28] Prior to cinmethylin first use, *L. rigidum* seed samples were collected in a comprehensive field survey study conducted at the end of the 2019 growing season from problematic fields across the Australian continent. The study aimed at proactively investigating the frequencies of resistance, profiling cross- and multiple-resistance patterns and then ranking the efficacy of a very large number of herbicide options across different Australian states. The main hypothesis is that herbicide resistance would occur at different frequencies depending on the herbicide MOA (PRE applied to seed *vs.* foliar POST), use pattern (herbicide mixture *vs* stand-alone) and geography, with geographical variation in resistance distribution reflecting seasonal and/or historical herbicide usage in the field. Herbicide resistance was determined by treating plants with recommended dosages of stand-alone PRE and POST herbicides in direct comparison with binary (tank) mixtures. We report current levels of resistance to stand-alone *versus* herbicide mixtures and discuss the observed ability of *L. rigidum* populations to stack resistance traits in the context of an integrated effort for weed resistance mitigation.

2 MATERIALS AND METHODS

2.1 Weed sample collection and preparation

The study was planned as a large observational survey, where a total of 579 individual seed samples of *L. rigidum* field populations were collected from 298 farms (approximately two samples per farm) at different locations across Australia at the end of the 2019 growing season: NSW (25 field-collected populations), SA (98 populations), VIC (104 populations) and WA (352 populations). Fields were chosen according to the grower or consultant's interests to better understand the herbicide resistance status of particular *L. rigidum* infestations. Thus, selected fields were characterised by control failures or lower-than-expected control of *L. rigidum* by specific herbicides (e.g. glyphosate application along fence lines, clethodim use in canola or PRE herbicide used in cereals). Weed seeds were collected from October 2019 to January 2020 depending on the length of the growing season across different Australian states. Seed collection in each field was conducted as previously described by Boutsalis *et al.*,[14] with each seed sample consisting of at least 100 *L. rigidum* heads collected across an area equivalent to approximately 2 ha per field sampled. After samples were received at the University of Western Australia, seed heads were threshed and the cleaned seeds were then stored under dry conditions at room temperature for at least 2 months before being tested with herbicides. Such a period allowed for sufficient dormancy release via seed after-ripening. The experiments were conducted from February to March 2020 during the Australian summer–autumn season, with plants grown in a cooled glasshouse (25 °C average temperature). Plants were grown in 20-cell trays with each tray containing 20 individual square cells (65 × 65 mm). Cell trays were filled with commercial potting mix (50% river sand, 25% pine bark, 25% peat moss), kept well-watered (>80% field capacity) and fertilized once a week with nitrogen (50 mg kg^{-1}) applied as NH_4NO_3. Two hundred seeds of each population were placed in individual cells (20 populations per tray) prior to PRE herbicide treatments, which were applied directly onto the seeds. Herbicide-treated seeds were immediately covered with a layer of 0.5 cm of potting mix. Fifty seeds were sown in each cell prior to POST herbicide treatments, which were applied to a known number (≤50) of emerged two-leaf seedlings counted prior to herbicide treatment (see Table 2). The herbicide treatments were chosen as those usually deployed to control *L. rigidum* during different phases of the growing season: from knock-down applications prior to sowing to PRE or POST herbicide treatments in the presence of the crop. In the summer–autumn experiment, the 21 herbicide treatments were individually tested at the same time under the same environmental conditions. Each herbicide treatment was delivered by using a twin-nozzle laboratory sprayer calibrated to deliver 110 L water ha^{-1} at 210 kPa and mounted with flat spray tips (Teejet XR) producing fine droplets.

The study was repeated in winter with the rationale of assessing herbicide efficacy at a lower temperature (16 °C average recorded during the winter study) for six key herbicides including two POST (glyphosate and paraquat) and four PRE (cinmethylin,

Table 2. Herbicides, HRAC classification, use (PRE, POST, formulated or tank mixtures), dosages (g active ingredient ha^{-1}) and overall survival % (and median values) observed in the 579 populations of *Lolium rigidum* (annual ryegrass) tested in summer–autumn or winter at the University of Western Australia resistance testing centre

Herbicide	HRAC MOA	Use	Dose active ingredient (g ha^{-1})	Mean survival % (median)	GROUP
Summer–autumn testing (POST)					
Clethodim	1	POST (stand-alone)	120	7.4 (0)	h
Clethodim + butroxydim	1 + 1	POST (tank mixture)	120 + 45	2.8 (0)	g
Imazamox + imazapyr	2 + 2	POST (formulated mixture)	25 + 11	19.6 (18)	i
Glyphosate	9	POST (stand-alone)	1080	1.1 (0)	cdefg
Paraquat	22	POST (stand-alone)	250	0 (0)	a
Summer–autumn testing (PRE)					
Prosulfocarb	15	PRE (stand-alone)	2400	4.4 (3.3)	f
Prosulfocarb + trifluralin	15 + 3	PRE (tank mixture)	2400 + 960	1.2 (0)	b
Triallate	15	PRE (tank mixture)	1500	3.1 (1.7)	cde
Prosulfocarb + *S*-metolachlor	15 + 15	PRE (formulated mixture)	2000 + 300	3.0 (2.2)	cd
Metazachlor	15	PRE (stand-alone)	900	1.8 (0)	c
Propyzamide	3	PRE (stand-alone)	500	0.01 (0)	a
Cinmethylin	30	PRE (stand-alone)	375	2.9 (0)	cd
Cinmethylin + pyroxasulfone	30 + 15	PRE (tank mixture)	375 + 100	0.8 (0)	b
Cinmethylin + prosulfocarb	30 + 15	PRE (tank mixture)	375 + 2400	0.8 (0)	b
Cinmethylin + triallate	30 + 15	PRE (tank mixture)	375 + 1500	0.04 (0)	a
Cinmethylin + trifluralin	30 + 3	PRE (tank mixture)	375 + 960	0.7 (0)	b
Pyroxasulfone	15	PRE (stand-alone)	100	3.5 (0)	ef
Pyroxasulfone + triallate	15 + 15	PRE (tank mixture)	100 + 1500	0.8 (0)	b
Pyroxasulfone + trifluralin	15 + 3	PRE (tank mixture)	100 + 960	0.9 (0)	b
Trifluralin	3	PRE (stand-alone)	960	3.7 (0)	def
Triallate + trifluralin	15 + 3	PRE (tank mixture)	1500 + 960	0.8 (0)	b
Winter testing (POST)					
Glyphosate + 1% ammonium sulfate	9	POST (stand-alone)	1080	1.7 (0)	b
Paraquat	22	POST (stand-alone)	250	0 (0)	a
Winter testing (PRE)					
Prosulfocarb	15	PRE (stand-alone)	2400	4.1 (3)	c
Cinmethylin	30	PRE (stand-alone)	375	0 (0)	a
Pyroxasulfone	15	PRE (stand-alone)	100	3.8 (2)	c
Trifluralin	3	PRE (stand-alone)	960	6.4 (1)	d

Summer and winter data were analysed separately; within each season, herbicides followed by different letters are significantly different ($P < 0.05$). The compact-letter-display (CLD) refers to *post hoc* pairwise comparisons based on generalised linear model fitting and the multivariate *t* distribution to account for multiplicity.[29]

pyroxasulfone, prosulfocarb, trifluralin), simulating field conditions in an outdoors setting. We hypothesized that at lower temperature, the efficacy of volatile herbicides such as cinmethylin would be greater and the subsequent resistance frequency lower. Plant survival was assessed 4 weeks after herbicide treatments when herbicide injury symptoms were maximal and there was a clear discrimination between plants dead or alive. Survivors were defined as plants that emerged, established and/or grew after herbicide treatments. Herbicide-susceptible weed populations were used as controls and >99.5% mortality of the susceptible control populations was always achieved at the full herbicide rates applied. More detailed methodology is described elsewhere.[12,26]

2.2 Herbicide resistance test, detection and categorization

Herbicide resistance was confirmed based on specific plant survival thresholds and categorized in three arbitrary categories as previously described by Busi and Beckie[26]: survival ≤5% indicated a herbicide 'susceptible' population, survival values within the range of 6–19% identified as field populations with a 'developing resistance' status and survival ≥20% interpreted as fully herbicide 'resistant' field populations. These three categories to define the level of herbicide resistance are often used by farmers who visually recognise resistance in their field when at a level of approximately 20% survival (80% herbicide efficacy). Thus, weed survival ≥20% would result in commercial failure of the herbicide, at which point farmers may stop using the herbicide or consider alternative management options. Alternatively, 6–19% survival indicates that a sufficiently high number of resistant individuals are present in the population that would likely result in commercial failure the next time the herbicide is applied. Conversely, survival ≤5% would likely reflect a field situation in which herbicide efficacy remains high (≥95%). As this level of weed control would be deemed acceptable, the field population was categorized as susceptible.

2.3 Data analysis

The probability density function for survival data (proportion of surviving plants out of the total number of treated plants) was non-normal and highly skewed, with a high proportion of susceptible samples and long tails to the right due to a relatively low number of populations characterised by survival values ranging from 20% to 80%. Therefore, this data were described by using box-whisker plots, with the median (as a horizontal black line), the 25th and 75th percentiles (as boxes), and maximum and minimum values (as whiskers) (Fig. 1). The survival data were analysed using a generalised linear model (GLM) with binomial error and logit link; a scale parameter was added to account for overdispersion. Categorical data (number of samples in the three resistance classes) were analysed by a multinomial GLM for nominal data. With both types of models, back-transformed proportions were derived and compared using a multiple comparison procedure based on the multivariate *t* distribution and multiplicity correction[29,30] The correlation of herbicide efficacy across samples was assessed using the Spearman rank correlation coefficient.

3 RESULTS

A total of 579 *L. rigidum* field populations from 298 farms were tested for resistance in response to 21 herbicide treatments.

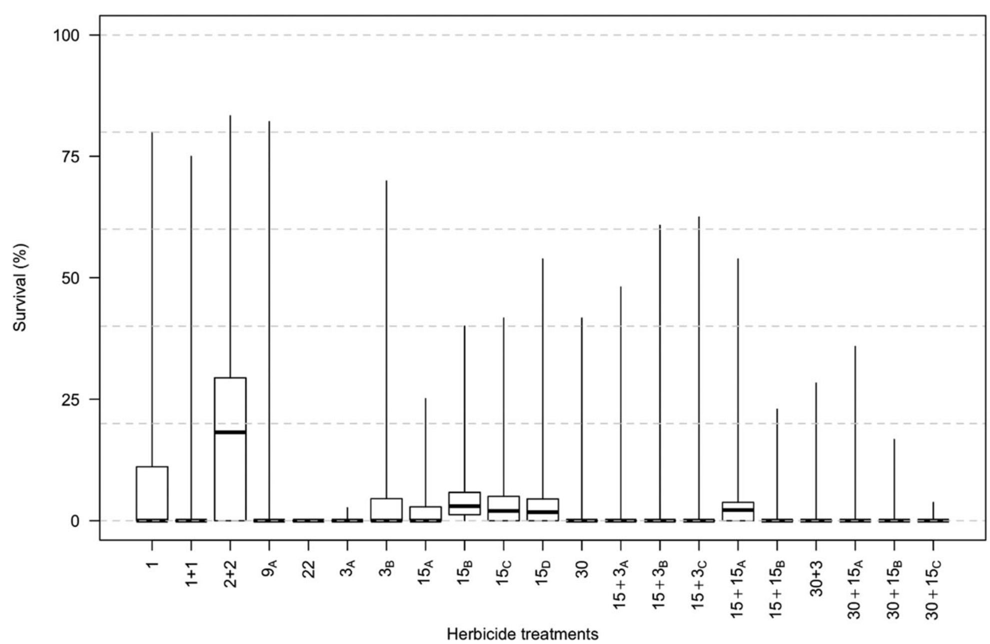

Figure 1. Box-whisker plots of the survival (%) for 579 field populations of *Lolium rigidum* collected in Australia in 2019–2020 when tested for herbicide resistance at the recommended label dose of 21 different herbicide options. The horizontal line is the median, the box highlights the 25th and 75th percentiles while the whiskers indicate maximum values. Herbicide codes reflect the Herbicide Resistance Action Committee (HRAC) herbicide classification codes: 1, clethodim 120 g ha^{-1}; 1 + 1, clethodim 120 g ha^{-1} + butroxydim 45 g ha^{-1}; 2 + 2, imazamox 25 g ha^{-1} + imazapyr 11 g ha^{-1}; 9_A, glyphosate 1080 g ha^{-1} (+ ammonium sulfate 1%); 22, paraquat 250 g ha^{-1}; 3_A, propyzamide 500 g ha^{-1}; 3_B, trifluralin 960 g ha^{-1}; 15_A, metazachlor 900 g ha^{-1}; 15_B, prosulfocarb 2400 g ha^{-1}; 15_C, pyroxasulfone 100 g ha^{-1}; 15_D, triallate 1500 g ha^{-1}; 30, cinmethylin 375 g ha^{-1}; 15 + 3_A, prosulfocarb 2400 g ha^{-1} + trifluralin 960 g ha^{-1}; 15 + 3_B, pyroxasulfone 100 g ha^{-1} + trifluralin 960 g ha^{-1}; 15 + 3_C, triallate 1500 g ha^{-1} + trifluralin 960 g ha^{-1}; 15 + 15_A, prosulfocarb 2000 g ha^{-1} + *S*-metolachlor 300 g ha^{-1}; 15 + 15_B, pyroxasulfone 100 g ha^{-1} + triallate 1500 g ha^{-1}; 30 + 3, cinmethylin 375 g ha^{-1} + trifluralin 960 g ha^{-1}; 30 + 15_A, cinmethylin 375 g ha^{-1} + prosulfocarb 2400 g ha^{-1}; 30 + 15_B, cinmethylin 375 g ha^{-1} + pyroxasulfone 100 g ha^{-1}; 30 + 15_C, cinmethylin 375 g ha^{-1} + triallate 1500 g ha^{-1}.

Table 3. Herbicide resistance frequencies of 579 populations of *Lolium rigidum* collected in Australia in 2019–2020 and tested for herbicide resistance to 21 herbicides and including nine binary mixtures

Herbicide	Developing resistant samples (%) 6–19% survival	GROUP	Resistant samples (%) ≥20% survival	GROUP
Summer–autumn testing (POST)				
Clethodim	15.1	de	16.2	f
Clethodim + butroxydim	7.4	bc	6.2	e
Imazamox + imazapyr	22.4	ef	48.7	g
Glyphosate	4.3	bc	1.7	abcd
Paraquat	0	a	0	ab
Summer–autumn testing (PRE)				
Prosulfocarb	31.2	f	2.7	cde
Prosulfocarb + trifluralin	4.6	bc	1.5	abcd
Triallate	20.4	e	1.0	abc
Prosulfocarb + *S*-metolachlor	14.7	de	0.86	ab
Metazachlor	9.5	cd	0.68	abc
Propyzamide	0	a	0	b
Cinmethylin	19.5	e	1.5	abcd
Cinmethylin + pyroxasulfone	4.1	bc	0	a
Cinmethylin + prosulfocarb	4.3	bc	0.51	abc
Cinmethylin + triallate	0	a	0	a
Cinmethylin + trifluralin	3.3	b	0.68	ab
Pyroxasulfone	21.9	ef	3.3	cde
Pyroxasulfone + triallate	5.1	de	0.34	abc
Pyroxasulfone + trifluralin	3.7	bc	1.2	abcd
Trifluralin	14.8	de	5.0	de
Triallate + trifluralin	3.4	b	1.0	abc
Winter testing (POST)				
Glyphosate + 1% ammonium sulfate	3.6	b	2.9	bc
Paraquat	0	a	0	a
Winter testing (PRE)				
Prosulfocarb	19.3	d	1.3	ab
Cinmethylin	0	a	0	a
Pyroxasulfone	18.1	d	2.5	bc
Trifluralin	15.3	cd	12.8	c

Summer and winter data were analysed separately; within each season, herbicides followed by different letters are significantly different ($P < 0.05$). Standard errors are in parentheses.Samples were divided into three categories according to the percentage survival observed at the recommended label dose. Herbicide 'resistance' was diagnosed with ≥20% survival, 'developing' resistance with survival ranging between >5–19% and 'Susceptible' samples with survival ≤5%. Within each column different letters indicate significantly different resistance frequencies (as proportions of samples resistant, developing or susceptible to each respective herbicide). The compact-letter-display (CLD) refers to *post hoc* pairwise comparisons based on multinomial generalised linear model fitting and the multivariate t distribution to account for multiplicity.[29]

Approximately two million seeds were screened in two experiments conducted as part of the field survey study. A total of 15 876 resistance tests were conducted overall, resulting in a total of 10 855 records (population by herbicide) of 0% survival (68%). Overall, only 10% of the tested populations were found to be susceptible to all herbicide treatments (Table 1). Conversely, a significantly greater proportion (approximately 62% of the populations) was susceptible to each of the nine binary (tank) herbicide mixtures tested (Table 1). A proportion of >50% of the populations tested was resistant to at least three different herbicides. Two populations originating in SA (populations 138- and 291-2020) and one population from WA (population 50-2020) were found to be resistant to 11 herbicidal options of which five were binary mixtures with five different MOA, ACCase, ALS, microtubule assembly, lipid synthesis and very long chain fatty acid elongase inhibitors, but not glyphosate (Table 1). Most resistant populations were not resistant to the mixture cinmethylin + triallate (MoA 30 + 15) (Table 1).

3.1 Post-emergence herbicides

The mean survival in response to POST herbicides was 4.9% compared to 2.1% survival observed in response to PRE herbicides (Table 2).

3.1.1 *ACCase carboxylase inhibitors*

The overall survival to clethodim was 7.4% (92.6% reciprocal control efficacy), which graded this herbicide option as the second least effective treatment (Table 2). Overall, the frequency of

Table 4. Resistance frequencies to key herbicides and binary mixtures observed in *Lolium rigidum* in four Australian states including New South Wales (NSW), South Australia (SA), Victoria (VIC) and Western Australia (WA)

When	Herbicide	NSW	SA	VIC	WA
Summer–autumn testing					
Summer	Clethodim	12 a	23 a	25 a	37 b
Summer	Clethodim + butroxydim	0 a	9.0 b	5.9 b	18 c
Summer	Propyzamide	0 a	0 a	0.01 a	0 a
Summer	Cinmethylin + triallate	0 a	0 a	0.01 a	0 a
Summer	Cinmethylin + pyroxasulfone	0 a	3.1 ab	0 a	5.9 b
Summer	Cinmethylin + prosulfocarb	0 a	0 a	0 a	7.9 b
Summer	Cinmethylin + trifluralin	0 a	3.1 ab	4.9 b	4.2 ab
Summer	Prosulfocarb + trifluralin	0 a	15 c	2.9 ab	5.1 b
Summer	Triallate + trifluralin	0 a	11 c	5.8 bc	2.5 b
Summer	Pyroxasulfone + triallate	0 a	9.2 b	1.0 a	6.2 b
Summer	Pyroxasulfone + trifluralin	0 a	9.2 b	6.8 b	3.7 b
Winter testing					
Winter	Glyphosate + % ammonium sulfate	42 b	8.8 a	8.8 a	2.7 a
Winter	Paraquat	0 a	0 a	0.01 a	0 a
Winter	Prosulfocarb	0 a	28 b	23 b	18 b
Winter	Cinmethylin	0 a	0 a	0 a	0 a
Winter	Pyroxasulfone	0 a	46 c	40 c	8.7 b
Winter	Trifluralin	0 a	49 c	57 c	16 b

Within each herbicide, regions followed by different letters are significantly different ($P < 0.05$).Herbicide resistance frequencies reported are the sum of 'developing resistant' and 'resistant' field-sampled populations with comparisons across each row to show current trends and cross-resistance profiles. The compact-letter-display refers to *post hoc* pairwise comparisons based on multinomial generalised linear model fitting and the multivariate t distribution to account for multiplicity.[29]

resistance was approximately 31%, with 15% developing resistant and 16% resistant field populations (Table 3). A significantly higher frequency of resistance to clethodim was found in WA, with a total of 40% of samples affected by herbicide resistance (Table 4). The calculated Spearman coefficient for correlation between clethodim and clethodim + butroxydim survival was approximately 0.6. Conversely, Spearman coefficient was <0.2 for all other herbicides. A negligible proportion of samples resistant to glyphosate were also clethodim resistant (data not shown). The binary mixture clethodim + butroxydim was significantly more effective than clethodim as a stand-alone treatment (Table 2). The overall efficacy of clethodim + butroxydim was greater than prosulfocarb and pyroxasulfone, but not significantly different to trifluralin and triallate (Table 2). The overall resistance frequency to the mixture clethodim + butroxydim was 13%. Incidence was considerably greater in WA, with a 2-fold greater frequency of clethodim resistance than the other states (Tables 3 and 4).

3.1.2 ALS inhibitors

The preformulated mixture of imazamox + imazapyr was the least effective herbicide treatment investigated in the study due to a resistance frequency of approximately 60% (Tables 2 and 3). With some variation, there was a similar incidence of imazamox + imazapyr resistance across NSW, SA and VIC (data not shown), whereas there was a greater incidence of imidazolinone resistance in WA.

3.1.3 5-enolpyruvylshikimate-3-phosphate synthase inhibitor

In the two experiments (repeated in summer and winter), the overall efficacy of glyphosate was >98.4% with a similar level of resistance detected in both studies (Table 2 and Fig. 1). The incidence of glyphosate resistance in *L. rigidum* across Australia was found to be relatively low at a frequency of 6% (Table 3). However, the incidence of glyphosate resistance in *L. rigidum* populations collected from NSW was significantly (5-fold) greater than in other states (Table 4). Thus, NSW appears to be a current pocket for high-level glyphosate resistance with an overall frequency >40% (Table 4).

3.1.4 photosystem I inhibitor

Paraquat was effective, with no resistance detected in repeated experiments across the 583 populations of *L. rigidum* treated at the recommended label rate (Tables 2–4).

3.2 Pre-emergence herbicides

3.2.1 Inhibitors of microtubule assembly

Propyzamide was fully effective, with no plant emergence, survival and resistance observed across all populations tested (Tables 2–4).

The overall survival to trifluralin across two experiments ranged between 3.7% in summer and 6.4% in winter (Table 2). Thus, trifluralin appeared to be somewhat more effective in summer than in winter. In summer, trifluralin efficacy was similar to prosulfocarb or pyroxasulfone, but in winter it was significantly less effective than those two stand-alone herbicides (Table 2 and Fig. 1). Despite a substantial proportion (19.8%) of annual ryegrass populations displaying a level of resistance in summer, the overall frequency of resistance observed was significantly greater in winter (28.1%) (Table 3). In both studies, there was a greater frequency of trifluralin resistance found in SA than WA or NSW (Table 4).

3.2.2 Inhibitors of very long chain fatty acid synthesis

The overall plant survival to prosulfocarb in summer (4.4%) was very similar to winter survival (4.1%) (Table 2 and Fig. 1). However, the summer testing detected a greater proportion of populations categorized as 'developing resistant' (Table 3). There were no major differences among the frequencies of resistance to prosulfocarb found in populations from NSW, SA, VIC or WA (Table 4).

Overall, the preformulated mixture of prosulfocarb + *S*-metolachlor and the other thiocarbamate herbicide, triallate, were slightly more effective than prosulfocarb with approximately a 3% survival response (Table 2 and Fig. 1). The frequency of 'developing resistant' populations found in response to prosulfocarb + *S*-metolachlor and triallate was significantly lower than for prosulfocarb (Table 3). In general, there was good correlation among the population responses to those three different herbicides with a calculated Spearman coefficient ranging from 0.36 to 0.41 (data not shown), suggesting cross-resistance can extend across these herbicides.

Survival responses to pyroxasulfone were not significantly different in the summer and winter studies (Table 2 and Fig. 1). Thus, the observed frequency of resistance to pyroxasulfone was consistently found to be approximately 25% in summer and 21% in winter (Table 3). It was observed that the overall efficacy of pyroxasulfone was no different to that of prosulfocarb, prosulfocarb + *S*-metolachlor or triallate (Tables 2 and 3). However, there was a significantly (4-fold) greater level of pyroxasulfone resistance in populations collected from SA and VIC (≥40% as the overall frequency of resistant samples) than in WA or NSW (Table 4). A correlation coefficient up to 0.31 was determined for survival responses to prosulfocarb and pyroxasulfone.

The efficacy of the herbicide metazachlor at its maximum recommended rate was significantly greater than several other PRE herbicides, including prosulfocarb, pyroxasulfone and trifluralin (Table 2). The frequency of resistance to metazachlor (approx. 10% nationally) was lower than pyroxasulfone (Table 3). There was a similar correlation between survival to pyroxasulfone and metazachlor and also between pyroxasulfone and prosulfocarb.

3.2.3 Mixtures of inhibitors of very long chain fatty acid synthesis and inhibitors of microtubule assembly

There was a similar efficacy of the three different binary mixtures (pyroxasulfone + trifluralin, prosulfocarb + trifluralin and triallate + trifluralin), with resistance ranging from 4.5% for trifluralin + triallate up to 6.2% for trifluralin + prosulfocarb (Tables 2 and 3, and Fig. 1). In SA, the level of resistance (approximately 15%) to the mixture trifluralin + prosulfocarb was found to be significantly greater than in other states (≤5%) (Table 4). In WA, the frequency of resistance to trifluralin + triallate was significantly (4-fold) lower than in SA (Table 4).

3.2.4 Mixture of inhibitors of very long chain fatty acid synthesis

The overall survival to the mixture pyroxasulfone + triallate was <1%, with a total frequency of resistance of approximately 5% (Tables 2 and 3). Resistance to pyroxasulfone + triallate was significantly greater in populations collected from SA and WA (Table 4).

3.2.5 Inhibitor of fatty acid thio-esterases

The overall efficacy of cinmethylin was 97.3% in summer *versus* 100% observed during the winter herbicide screening (Table 2 and Fig. 1). The overall frequency of populations affected by resistance during the summer test was 21%. However, the results from the winter test clearly indicated no emergence, survival and resistance to cinmethylin (Tables 3 and 4).

3.2.6 Mixtures of inhibitor of fatty acid thio-esterases and mitosis inhibitors

The overall efficacy of cinmethylin + trifluralin (99.3%) was significantly greater than cinmethylin stand-alone (Table 2), with an overall frequency of 'developing resistant' populations of <4% with no variations found across WA, VIC and SA (Tables 3 and 4).

3.2.7 Mixtures of inhibitor of fatty acid thio-esterases and inhibitors of very long chain fatty acid synthesis

Similar efficacy (>99%) was observed for cinmethylin + prosulfocarb and cinmethylin + pyroxasulfone (Table 2). There was a similar level of resistance to cinmethylin + pyroxasulfone (<6%) across WA, VIC and SA, whereas there was 8% developing resistant samples to cinmethylin + prosulfocarb in WA (Table 4). Conversely, cinmethylin + triallate was the most effective wheat-selective treatment of the study with a significantly greater efficacy (99.96%) than any other treatment, with the exception of paraquat and propyzamide (Table 2). Thus, no resistance was observed in response to this highly effective mixture of cinmethylin + triallate (Tables 3 and 4).

4 DISCUSSION

This is the first study to report the frequency and geographical distribution of resistance in *L. rigidum* in a national-scale study involving approximately 600 populations collected from nearly 300 farms located in the southern Australian wheat-belt and treated with several stand-alone herbicides or binary herbicide mixtures. In this study, we aimed to provide a comprehensive update of frequencies of resistance to herbicides most commonly used in the southern Australian cropping system (extending for approximately 25 million ha) and to document the level of evolving resistance to new stand-alone herbicides (e.g. cinmethylin in 2020) of recent introduction (e.g. pyroxasulfone in 2012) and old herbicides (e.g. trifluralin, triallate in the 1960s) that are often used in a mixture to increase and complement the efficacy of a newer herbicide.[26] The most frequent result obtained, as the median of survival response to many herbicides tested, was 0% survival (100% efficacy). Thus, the study highlights the importance of resistance testing to identify herbicide treatments for maximal weed control efficacy and minimal risk of weed resistance selection.

4.1 Frequency of herbicide resistance in Australia

The frequency of herbicide resistance found in weed populations in individual fields is continuously fluctuating due to the interplay of weed genotypes and recurrent selection caused by persistent herbicide use.[9,31] In the last five decades at a global scale, the selection and evolution of herbicide-resistant weed species and biotypes have shown a steadily increasing trend.[2] The southern Australian cropping system, given its high reliance on minimum tillage and reliance on herbicide technology for weed control, has been one of the major epicenters of herbicide resistance evolution.[32,33] The ability of *L. rigidum*, the most damaging weed in Australia, to rapidly adapt to herbicide selection has elevated it to the most herbicide resistance-prone model weed ever documented.[34] In the dryland Australian cropping systems, the use of herbicides in every growing season comes at a substantial cost,[10] but it is often seen as a necessary measure to preserve soil moisture, maintain the benefits of the minimum tillage system and ensure highly effective control against a range of dominant and competitive grass weeds such as *L. rigidum*, *Avena* spp., *Bromus* spp., *Hordeum* spp. or dicot weeds including *Raphanus raphanistrum* and *Conyza* spp.[35]

As documented in other geographical surveys in Australia,[19,36] this study reports and confirms the absence of evolved resistance to paraquat and propyzamide in a broadacre field crop. Thus, resistance to these two herbicides in *L. rigidum* remains almost as an anomaly, being confined to two individual populations resistant to paraquat in a vineyard setting[37,38] or a 2-fold level resistance to propyzamide, with no documented survival at the labelled rate.[39,40] No resistance was found to the mixture of cinmethylin + triallate, thereby identified as the most effective wheat-selective treatment of the study. The efficacy of cinmethylin + triallate was significantly higher than the other three binary mixtures with cinmethylin, and research is warranted to elucidate and better understand this interaction between cinmethylin and triallate that appears to be greater than simply additive. As recently reviewed[26,41,42] and shown by empirical field studies[43] and modelling simulations,[44] this study reaffirms the considerable efficacy of herbicide mixtures and their resilient nature to resistance selection. The ability of *L. rigidum* to evolve resistance to herbicide mixtures appears constrained: >60% of *L. rigidum* field populations remains fully susceptible to herbicide mixtures and the resistance frequency to the binary mixtures is at least 2-fold lower than stand-alone treatments for the tested POST herbicides (i.e. clethodim) and 3- to 7-fold lower for the PRE herbicides.

Among the several options for herbicide rotation tested here, the most effective and least affected by resistance appears to be the herbicide cinmethylin newly introduced to Australia in 2020. The herbicide did not perform as expected during the summer testing, but its efficacy was 100% with plants grown under colder winter conditions. As temperatures were higher in summer (25 °C *versus* 16 °C in winter), it is likely that a reduction in cinmethylin efficacy in summer is caused by the intrinsic high volatility of cinmethylin leading to evaporative losses at higher temperatures.[45] It was observed from the winter testing that no *L. rigidum* population was affected by resistance to cinmethylin, most likely due mainly to the recent introduction of cinmethylin. Moreover, no cross-resistance caused by the field use of other herbicide MOA was detected. In contrast, there was a relatively high frequency of evolved resistance affecting other widely adopted PRE herbicides such as pyroxasulfone and trifluralin.

4.2 Distribution of herbicide resistance in Australia

In Australia, herbicide resistance is prevalent, with a heterogeneous geographical distribution.[46] Several survey studies have documented the incidence of resistance in *L. rigidum* at a local level in NSW,[13] SA and VIC,[14] Tasmania[15] and WA.[12,16–18] In this unique comparative study, the frequency of resistance observed in response to a large number of herbicide treatments indicate that populations in the state of SA are the most affected by resistance, with an overall frequency of 17%, while VIC and WA are significantly less affected, with 14% and 13% resistance, respectively (Table S1).

Glyphosate resistance in *L rigidum* was documented in 1996,[47,48] but has progressed slowly across the entire southern Australian cropping region.[19,49] The documented levels of glyphosate resistance in SA, VIC and WA are consistent with frequencies reported previously, whereas the significantly greater frequency found in *L. rigidum* populations in NSW is clearly an outlier (>10-fold greater than previously reported). Most populations originated from the south-west part of the state. It is likely that anomalies in rainfall patterns (drought conditions) in the last few growing seasons led to overreliance on glyphosate for knock-down treatments in the absence of a growing crop. Double-knock intervention strategies (i.e. sequential application of glyphosate followed by paraquat) could offer an immediate solution to reduce the risk of further selection and spread of glyphosate resistance in those regions.[50] However, research is warranted *via* random surveys in NSW to confirm if the observed frequency of glyphosate resistance found in populations tested in this study accurately reflects the mean frequency in populations across the state.

The analysis of resistance distribution clearly indicates that there is a much greater frequency of clethodim resistance in *L. rigidum* populations collected in WA. Very high levels of ACCase resistance (>95% diclofop resistance) were reported a decade ago in WA and the frequency of clethodim resistance remains substantially unchanged.[12,14,36,51] As the frequency of resistance to the mixture of clethodim + butroxydim was often significantly reduced across a large number of *L. rigidum* populations, it appears logical to recommend resistance testing and adoption of mixtures of ACCase herbicides (dims and fops) permitted by labels that can deliver cost-effective solutions for the control of *L. rigidum* in break (nonwheat) crops.[52,53]

In Australia, the rapid increase of resistance to wheat-selective POST herbicides in *L. rigidum* has resulted in greater adoption of PRE herbicides such as prosulfocarb, pyroxasulfone, triallate and trifluralin.[22] The recent introduction of new active ingredients (e.g. cinmethylin, bixlozone) now provide additional weed control options to farmers.[28,54] Trifluralin has been widely used for almost 60 years to control *L. rigidum*, with a historically slow selection of resistance.[14] Resistance to very long chain fatty acid synthesis inhibitors including pyroxasulfone and thiocarbamate herbicides such as triallate and prosulfocarb has been reported in recurrent selection studies[22,55–57] and a few field populations by historical[58,59] and more recent characterization.[39,40,60] A recent study indicates resistance to bixlozone in a few *L. rigidum* field populations from SA even before introduction into Australia.[61]

This study shows that resistance to pyroxasulfone and trifluralin has reached concerning levels in SA and VIC, but remains at relatively low levels in WA and NSW. As resistance evolution in weed populations is driven by selection through recurrent use of the same practice, these results may lead to a reduction in the adoption of these two herbicides as a stand-alone use. As SA has been most affected by trifluralin resistance[14] due to trifluralin historical use on SA alkaline soils to control *L. rigidum*, the selection of pyroxasulfone resistance could be directly related to overreliance on this herbicide for the selective control of trifluralin-resistant *L. rigidum* in wheat.[21] The result is only partly surprising for three reasons: (i) evolved resistance to *S*-metolachlor and other chloroacetamides (with same MOA of pyroxasulfone) was documented about 30 years ago in *L. rigidum*[22,58]; (ii) recurrent selection studies showed that resistance to pyroxasulfone (and cross-resistance to prosulfocarb and triallate) was selected in pots in just three generations; and (iii) recent modelling simulations indicated that resistance could have been realistically selected in <10 years in field situations when little or no diversity in herbicide use is prevalent.[44]

Overall, it appeared that the mixture of pyroxasulfone + triallate provides an effective control option for *L. rigidum*. In samples collected from SA where resistance to thiocarbamate herbicides such as prosulfocarb and triallate has been previously documented[23,60] and appears more prevalent than in other states (Table 4), the frequency of resistance to the mixture of pyroxasulfone and triallate was <10%. Thus, binary mixtures with triallate appear to remain effective despite resistance to either stand-alone component. Therefore, triallate should be considered as an important mixing partner capable of minimizing the impact of PRE herbicide resistance. The future management of PRE herbicide resistance in these regions (SA and VIC) will require a careful integration of new and old herbicides guided by regular and systematic resistance testing and even greater adoption of harvest weed seed control and other nonchemical tactics to further reduce weed infestation densities.[62]

4.3 Herbicide resistance testing research and extension

This aim of this study was to investigate and rank the frequencies of resistance in *L. rigidum*, and its distribution across different states of the Australian continent. We also profiled cross- and multiple-resistance patterns in *L. rigidum* in response to a large number of herbicide options that included herbicide binary mixtures.

The study emphasizes the utility of herbicide mixtures for chemical intervention to mitigate resistance and to exploit the current susceptibility response found in large proportions of *L. rigidum* field populations. The prolonged drought of introduction of new MOA herbicides into Australia has finally ended. The newly introduced herbicide cinmethylin has the potential to restore the effective control of multiple-resistant *L. rigidum* populations in Australia, especially when used in a mixture with triallate. Furthermore, new herbicides will be available from 2021 in Australia (e.g. bixlozone, carbetamide, tiafenacil, trifludimoxazin). We aim to continue resistance evaluation of key agricultural weed species through systematic herbicide screening of field populations to characterize newly emerging patterns of resistance evolution.

This study also reflects the collective views of several influential agronomist consultants operating in different cropping regions of Australia who provided input for the herbicide treatments to be tested and contributed to the discussion of the results and their implications. We call for a robust effort for extension of these results via multiple stakeholders' involvement to raise awareness of rapidly emerging patterns of herbicide resistance, with end-users encouraged to adopt of a range of cost-effective, diverse and integrated strategies for the long-term mitigation of weed resistance evolution.

A common view shared among the authors is that this research should continue to inform the Australian grains industry in a timely manner on the most efficient and effective use of the available technology for weed control, facilitate early detection of the evolution of putative resistant *L. rigidum* and other weed populations to new (and old) herbicides, and help extend herbicide longevity by maximizing the heterogeneity of herbicide use on-farm.

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The service of herbicide resistance testing was offered at the University of Western Australia for the first time in 2020. The aim is to provide a yearly update on herbicide resistance frequency and distribution in Australia with direct and indirect input from influential Australian consultant agronomists and champion growers. Thus, we thank the support from more than 300 Australian farmers and consultant agronomists who collected and submitted annual ryegrass seed samples for comprehensive testing of herbicide resistance: a special mention to Andy Bates, Ryan Pearce, the Nutrien team, Dan Taylor DKT, Glenn Bryant Mullewa Farm Supplies and Nick Zordan for their support and feed-back. We are grateful for the skilled research assistance from Paula Reeve, Ci Sun, Bowen Zhang, Geide Figueiredo Junior and Martina Badano. The grant funding from BASF SE Germany (Agreement RecID 87592212 'Herbicide resistance risk of LuximoTM in *Lolium rigidum*') is acknowledged.

CONFLICT OF INTEREST

We acknowledge BASF to have partially funded the research. No other conflict of interest is declared.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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