

Widespread occurrence of both metabolic and target-site herbicide resistance mechanisms in *Lolium rigidum* populations

Heping Han,^a Qin Yu,^{a*} Mechelle J Owen,^a Gregory R Cawthray^b and Stephen B Powles^a

Abstract:

BACKGROUND: *Lolium rigidum* populations in Australia and globally have demonstrated rapid and widespread evolution of resistance to acetyl coenzyme A carboxylase (ACCase)-inhibiting and acetolactate synthase (ALS)-inhibiting herbicides. Thirty-three resistant *L. rigidum* populations, randomly collected from crop fields in a most recent resistance survey, were analysed for non-target-site diclofop metabolism and all known target-site ACCase gene resistance-endowing mutations.

RESULTS: The HPLC profile of [¹⁴C]-diclofop-methyl *in vivo* metabolism revealed that 79% of these resistant *L. rigidum* populations showed enhanced capacity for diclofop acid metabolism (metabolic resistance). ACCase gene sequencing identified that 91% of the populations contain plants with ACCase resistance mutation(s). Importantly, 70% of the populations exhibit both non-target-site metabolic resistance and target-site ACCase mutations.

CONCLUSIONS: This work demonstrates that metabolic herbicide resistance is commonly occurring in *L. rigidum*, and coevolution of both metabolic resistance and target-site resistance is an evolutionary reality. Metabolic herbicide resistance can potentially endow resistance to many herbicides and poses a threat to herbicide sustainability and thus crop production, calling for major research and management efforts.

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Keywords: diclofop; herbicide metabolism; *Lolium rigidum*; metabolic resistance; target-site mutation; resistance evolution

1 INTRODUCTION

Lolium rigidum is a global crop weed and is by far the most widespread weed in Australian field crops. Excellent herbicidal control of *L. rigidum* infesting crops was initially achieved with the first used (in 1977) acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicide diclofop-methyl. However, diclofop-methyl resistance evolution in *L. rigidum* populations quickly occurred,¹ and, unexpectedly, the first *L. rigidum* population documented to be resistant to diclofop-methyl displayed cross-resistance to the dissimilar yet never used acetolactate synthase (ALS)-inhibiting herbicide chlorsulfuron.² With one of these first resistant *L. rigidum* populations to be identified (SLR31) it was established that resistance and cross-resistance were due to enhanced rates of herbicide metabolism (referred to as metabolic resistance).^{3–7} Such metabolic cross-resistance to diverse herbicides has been identified in many *L. rigidum* populations in Australia.^{8,9} Early work also established that for resistant Australian *L. rigidum* populations it is common for resistant individuals to exhibit both target-site and non-target-site resistance mechanisms.^{10,11} Target-site resistance due to target gene mutations has been widely reported.^{12–15} However, non-target-site resistance due to enhanced rates of herbicide metabolism (metabolic resistance) has been very much understudied. Yet, compared with target-site resistance, non-target-site herbicide metabolic resistance is economically more threatening

because it can confer wide-spectrum resistance to dissimilar herbicides and even herbicides not yet commercialised.^{9,15} Equally importantly, metabolic resistance has been shown to evolve rapidly in *L. rigidum* when herbicides are used at low rates.^{16–19} Therefore, metabolic resistance is a very important but underestimated threat to herbicide sustainability and thus crop production. Our several large-scale field herbicide resistance surveys have revealed ever-increasing and widespread resistance to ACCase- and ALS-inhibiting herbicides, and we have much, albeit indirect, evidence that *L. rigidum* across vast areas exhibits both non-target-site metabolic and target-site resistance to many herbicides.^{20–22} Here, to quantify the extent of coexistence of both metabolic and target-site resistance, we analysed diclofop-methyl *in vivo* metabolism (metabolic resistance) and all known ACCase target-site mutations (target-site resistance) in individuals of 33 resistant *L. rigidum* populations collected from our most recent random resistance survey.²² We provide direct evidence that both

* Correspondence to: Qin Yu, Australian Herbicide Resistance Initiative, School of Plant Biology, University of Western Australia, Crawley, WA, Australia. E-mail: qin.yu@uwa.edu.au

a Australian Herbicide Resistance Initiative, School of Plant Biology, University of Western Australia, Crawley, WA, Australia

b School of Plant Biology, University of Western Australia, Crawley, WA, Australia

metabolic and target-site herbicide resistance commonly co-occur in *L. rigidum* populations/individuals. Evolution of metabolic and target-site resistance to crop-selective herbicides is discussed.

2 MATERIALS AND METHODS

2.1 Origin of resistant populations

Seeds of multiple-resistant *L. rigidum* populations were collected from crop fields during our 2010 random herbicide resistance survey conducted across the Western Australian grain belt.²² From this survey, a subset of 33 resistant (R) populations were selected for mechanism studies, and these included 12 populations from high-rainfall (H, 450–470 mm), 18 populations from medium-rainfall (M, 325–450 mm) and three populations from low-rainfall (L, <325 mm) regions across very wide geographies (Table 1, Fig. 1). Fewer populations were selected from the low-rainfall regions mainly owing to relatively lower levels of resistance observed and limited seed availability. As controls, the three known herbicide-susceptible (S) *L. rigidum* populations, VLR1, SVLR1 and WALR1,^{18,19,23} were used, as well as three known metabolic-resistant *L. rigidum* populations.^{6,7,16,19,24}

2.2 Plant growth

For herbicide resistance testing, seeds were germinated on 0.6% agar-solidified water in a growth cabinet at day/night temperatures of 20/15 °C for 7 days. Germinating seedlings were transplanted to plastic trays (300 × 400 × 100 mm, 50 seedlings per tray) containing potting mix and grown outdoors at the University of Western Australia in the winter growing season (May to August), or in a controlled-environment room (CER) at 20/15 °C with a photon flux density of 300–400 μmol m⁻² s⁻¹, a photoperiod of 12 h and a relative humidity of 70–75%. For diclofop-methyl dose response studies, seedlings were transplanted into plastic pots (diameter 18 mm, 20 seedlings per pot) and grown in a glasshouse at 20–22 °C/10–15 °C. There were 3–4 replicates per treatment per population. For [¹⁴C]-diclofop-methyl uptake and metabolism experiments, individual seedlings were transplanted into small plastic cups (60 × 60 × 100 mm, one seedling per cup) and grown in the CER as described above.

2.3 Herbicide treatment

Diclofop-methyl (Hoegrass, 500 g Al L⁻¹; Bayer CropScience, Frankfurt, Germany) was applied to 2–3-leaf-stage plants in a 117 L ha⁻¹ spray volume delivered in two passes at 200 kPa with a cabinet sprayer equipped with two flat-fan nozzles. After spraying, plants were returned to the specific conditions as indicated above, and mortality was determined 3 weeks after treatment.

2.4 Herbicide uptake and metabolism analysis

[¹⁴C]-Diclofop-methyl (dichlorophenyl-UL-[¹⁴C]) with a specific activity of 6.6 MBq mg⁻¹ was obtained from Bayer CropScience. [¹⁴C]-Diclofop-methyl was dissolved in a small volume of acetonitrile and diluted in water containing 0.25% (v/v) nonionic surfactant BS1000. A treatment solution of approximately 3.76 kBq μL⁻¹ was prepared with a final diclofop-methyl concentration of about 1.6 mM (concentration equivalent to 63 g ha⁻¹). To mimic the field spray situation and induce metabolism activity, R and S seedlings at the 2–3-leaf stage were foliar sprayed with normal commercial (non-radiolabelled) diclofop-methyl at 25 g ha⁻¹, which is one-fifteenth of the Australian recommended field rate of diclofop-methyl (375 g ha⁻¹) and resulted in only 6% control of the

S populations under the above experimental conditions. After 3 h, 1 μL of the ¹⁴C-treatment solution was applied along the adaxial surface (close to the leaf base) of the second fully expanded leaf of each plant. Two replicate samples (each containing six plants as a bulk) of each population were harvested above ground 4 days after treatment (4 DAT). The treated leaf of each plant was rinsed with 20 mL of 20% (v/v) methanol plus 0.2% (v/v) Triton[®] X-100, blotted dry with paper tissue, snap frozen in liquid N₂ and stored at –80 °C until extraction and analysis. The rinse solution of 12 individual plants from each population was combined, and radioactivity present in the solution was quantified by liquid scintillation counter (LSC). Foliar uptake of [¹⁴C]-diclofop-methyl of each population was calculated as the difference between the radioactivity applied and the radioactivity in the rinse solution.

Extraction, separation and identification of the parent herbicide diclofop-methyl and its metabolites were done according to Yu *et al.*¹⁹ Briefly, above-ground plant tissue was ground in liquid N₂ with a prechilled mortar and pestle, and then homogenised with 5 mL of 80% (v/v) cold methanol. The crude homogenate was centrifuged at 8000 × *g* for 15 min at 4 °C. The supernatant was decanted, and the residue was re-extracted with 1.5 mL of 80% chill methanol, followed by a final extraction with 1.5 mL of 50% (v/v) cold methanol. The pooled supernatant was spin evaporated to dryness under vacuum, resuspended in 300 μL of 50% (v/v) methanol and centrifuged at 14 000 × *g* for 5 min. Radioactivity recovered in each sample extract was quantified by LSC (averaging 50–60% of total absorbed, with partial loss due to expected root translocation) and normalised for HPLC analysis. Diclofop-methyl and its metabolites were separated by gradient reverse-phase HPLC equipped with a 600E dual-head pump with a 717 Plus autosampler (Waters, Milford, MA, USA). Separation was conducted on a Waters Spherisorb 5 μm ODS2 column (250 mm long × 4.6 mm i.d.). Radioactivity was detected with an inline Beta-RAM model 2B detector (IN/US Systems Inc., Pine Brook, NJ). The HPLC conditions for sample separation were according to Yu *et al.*¹⁹ Injection volumes (30–90 μL) were adjusted to provide similar total radioactivity in each sample. The proportion of herbicide and metabolites was expressed as the percentage peak area of total radioactivity in the sample injection. The three S populations (SVLR1, VLR1 and WALR1) were used as controls, and the three known metabolic-resistant *L. rigidum* populations (PN, SLR31 and VLR69) were used as diclofop metabolism positive controls.

2.5 ACCase gene sequencing

Bulked leaf tissue of five diclofop-methyl survivors (at 375 g ha⁻¹) from each field-collected population, together with untreated plants from two S populations (VLR1, WALR1) and three metabolic-resistant populations (PN, SLR31 and VLR69), were harvested and DNA extracted.²⁵ A 1592 bp fragment covering nearly the entire CT domain and containing all known resistance-endowing ACCase mutations was amplified from each DNA sample using the *L. rigidum* specific primer pair 5'-ATGG TAGCCTGGATCTTGGACATG-3'/5'-GGAAGTGTCATGCAATTCAGCAA-3'.²⁶ The fragment was directly sequenced using two internal primers ACCF1 (5'-CACAGACCATGATGCGAGCTC-3') and ACCR6 (5'-TCCTGGATCAGCTGGGACG-3'), and PCR conditions were as described by Yu *et al.*²⁷

2.6 Statistical analysis

The LD₅₀ (herbicide rate causing 50% plant mortality) for herbicide dose response was estimated using the four-parameter

Table 1. Phenotypic resistance, diclofop metabolism and target-site ACCase gene mutations in plants of resistant field populations in comparison with three known herbicide-susceptible populations and three metabolic-resistant populations

Population	Survival (%) (diclofop-methyl 375 g ha ⁻¹)	Diclofop metabolism						Enhanced diclofop metabolism ^b (% increase)	Target-site ACCase mutations
		Diclofop acid (%)	<i>P</i> -value ^a	Metabolites (%)	<i>P</i> -value	Diclofop- methyl (%)	<i>P</i> -value		
Known susceptible populations									
SVLR1	0	65.22 ± 2.92		33.21 ± 2.8		1.58 ± 0.12			
VLR1	2	68.94 ± 2.95		30.48 ± 3.54		0.59 ± 0.59			
WALR1	8	67.09 ± 4.33		32.01 ± 5.24		0.91 ± 0.91			
Average		67.10 ± 1.69		31.90 ± 1.85		1.02 ± 0.34			
Field resistant populations									
L5/7	87	58.16 ± 1.50	*	36.90 ± 1.32	NS	4.95 ± 0.18	***	No	2027, 2041
M5/39	66	56.69 ± 0.05	*	36.70 ± 1.42	NS	6.61 ± 1.37	***	No	1781, 2078
M2/13	96	61.50 ± 4.88	NS	37.11 ± 4.50	NS	1.40 ± 0.38	NS	No	1781, 2041
H1/4	65	59.83 ± 1.42	NS	38.91 ± 0.54	NS	1.27 ± 0.89	NS	No	2041
M4/38	78	53.75 ± 0.68	**	39.45 ± 0.08	NS	6.81 ± 0.76	***	No	2041
M1/16	84	54.44 ± 3.43	*	40.75 ± 4.35	NS	4.81 ± 0.92	**	No	1781
H5/3	95	53.43 ± 0.29	**	40.00 ± 0.11	NS	6.58 ± 0.19	***	No	2041
M1/28	96	58.10 ± 0.41	*	40.90 ± 0.10	*	1.00 ± 0.31	NS	Yes (29%)	2041, 2078
H3/5	94	53.22 ± 1.03	**	42.06 ± 0.49	*	4.73 ± 0.54	**	Yes (32%)	2041
H1/1	81	54.93 ± 6.38	*	42.34 ± 4.43	*	2.73 ± 1.95	NS	Yes (33%)	2078
H2/3	72	53.12 ± 4.91	*	45.34 ± 4.80	*	1.54 ± 0.11	NS	Yes (42%)	Not detected
M4/26	94	48.90 ± 2.44	**	47.75 ± 2.59	**	3.36 ± 0.16	**	Yes (47%)	2078
M5/12	72	47.83 ± 4.13	**	48.88 ± 5.05	**	3.29 ± 0.92	*	Yes (53%)	2041, 2078
H5/19	90	45.02 ± 7.63	**	50.49 ± 7.81	**	4.50 ± 0.18	**	Yes (58%)	2041
H5/22	94	43.87 ± 3.14	***	50.59 ± 3.11	**	5.54 ± 0.03	***	Yes (59%)	1781, 2078, 2088
M4/10	91	43.64 ± 2.99	***	50.79 ± 1.23	**	5.58 ± 1.76	**	Yes (59%)	Not detected
M3/48	64	46.70 ± 2.56	***	51.26 ± 2.92	**	2.04 ± 0.36	NS	Yes (61%)	1781, 2041, 2088
M4/9	92	40.57 ± 8.53	***	53.27 ± 3.06	**	6.17 ± 2.37	**	Yes (67%)	2027
M5/34	100	42.81 ± 0.42	***	53.33 ± 0.17	***	3.87 ± 0.25	**	Yes (67%)	2041, 2078
M3/45	94	41.91 ± 15.4	*	54.69 ± 14.6	*	3.41 ± 0.74	*	Yes (71%)	2041, 2088
M3/31	86	41.78 ± 0.27	***	54.92 ± 0.20	***	3.31 ± 0.08	*	Yes (72%)	2041
H3/14	69	42.73 ± 10.77	**	55.35 ± 9.90	**	1.93 ± 0.87	NS	Yes (74%)	2041
L4/17	100	37.41 ± 1.49	***	58.39 ± 1.08	***	4.21 ± 0.41	**	Yes (83%)	1781, 2041, 2078
M1/13	100	38.59 ± 2.69	***	59.78 ± 1.80	***	1.64 ± 0.89	NS	Yes (87%)	1781
M2/23	92	34.83 ± 2.54	***	61.49 ± 2.91	***	3.69 ± 0.37	**	Yes (93%)	2041
H4/12	94	35.19 ± 2.87	***	62.69 ± 3.37	***	2.13 ± 0.50	NS	Yes (97%)	2041, 2078
H5/4	98	31.43 ± 0.26	***	64.26 ± 0.88	***	4.32 ± 1.14	**	Yes (101%)	1781, 2041, 2078, 2088
H4/13	92	32.90 ± 2.27	***	64.31 ± 3.59	***	2.80 ± 0.86	NS	Yes (102%)	1781
H4/1	92	30.70 ± 4.35	***	66.17 ± 4.54	***	3.14 ± 0.19	*	Yes (107%)	1781
M3/47	100	26.61 ± 0.08	***	68.05 ± 1.24	***	5.35 ± 1.32	**	Yes (113%)	2041
M3/35	90	27.47 ± 9.47	***	70.14 ± 9.68	***	2.40 ± 0.22	NS	Yes (120%)	2041
L3/14	96	25.38 ± 3.73	***	70.28 ± 2.34	***	4.35 ± 1.40	*	Yes (120%)	Not detected
M3/30	92	27.19 ± 4.33	***	70.36 ± 3.44	***	2.46 ± 0.89	NS	Yes (121%)	2041
Known metabolic-resistant populations									
SLR31	80	49.26 ± 0.63	**	49.16 ± 0.94	**	1.58 ± 0.31	NS	Yes (54%)	No mutations
PN	90	39.61 ± 1.50	***	58.62 ± 2.20	***	1.78 ± 0.70	NS	Yes (84%)	No mutations
VLR69	98	18.08 ± 3.71	***	75.15 ± 9.63	***	6.78 ± 5.92	NS	Yes (136%)	No mutations

^a Significance level (*P*-value) was indicated at 0.05 (*), 0.01 (**) or 0.001 (***). NS denotes not significant at the 0.05 level.

^b The percentage increase in diclofop metabolism is based on data for total metabolites relative to the average susceptible controls.

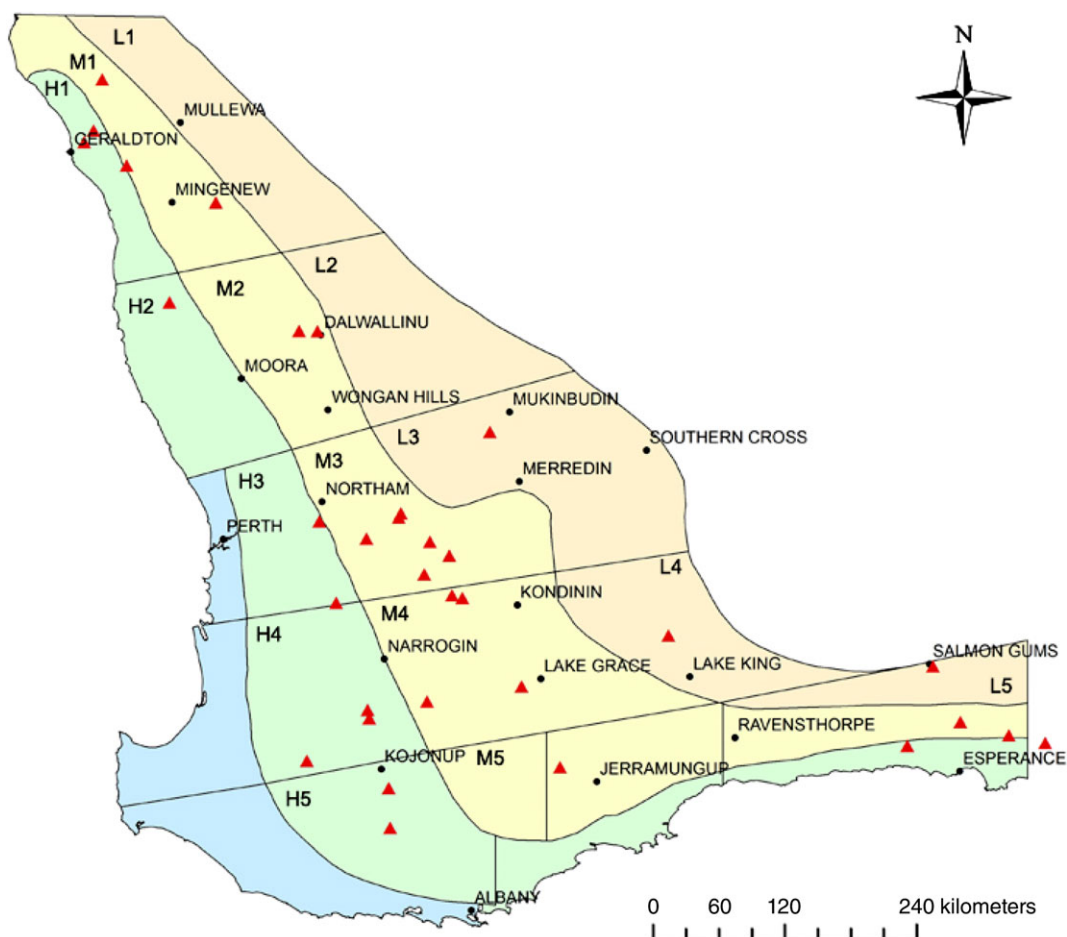


Figure 1. Map of south-western Western Australia, showing the agronomic zones of the grain belt where 33 *Lolium rigidum* populations were collected for herbicide resistance screening and herbicide metabolism analysis. Annual rainfall isohyets are shown. Rainfall regions are shown by H (high, 450–470 mm), M (medium, 325–450 mm) and L (low, <325 mm). Zones are indicated by 1 (north), 2 (north-central), 3 (central), 4 (south-central) and 5 (south).

logistic equation

$$y = C + (D - C) / [1 + (x/LD_{50})]^b$$

where *C* is the lower limit at indefinitely large doses, *D* is the upper limit close to untreated controls and *b* is the slope of the best-fitting curve through the LD₅₀. One-way analysis of variance (ANOVA) with an unpaired *t*-test was performed to compare differences in herbicide metabolism data between R and S populations (GraphPad Prism 5.0; GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA showed no significant differences in diclofop metabolism data among the three S control populations, and therefore data were pooled and the averaged S values were used for the *t*-test.

3 RESULTS

3.1 Herbicide resistance testing and confirmation of the selected resistant populations

A total of 33 resistant (R) *L. rigidum* populations were selected from our 2010 random herbicide resistance survey,²² and resistance to the ACCase-inhibiting herbicide diclofop-methyl was confirmed both with plants grown outdoors and in the CER (where diclofop-methyl metabolism analysis was also conducted). Under field conditions, 500 g diclofop-methyl ha⁻¹ gave full control of

three S populations, whereas the 33 R populations displayed a phenotypic resistance frequency ranging from 50 to 100% survival at this herbicide rate (data not shown). Under CER conditions (20/15 °C), a field rate of 375 g diclofop-methyl ha⁻¹ provided 90% control of the S populations, whereas the R populations had 64–100% survival at this rate (Table 1). Therefore, these tests confirmed resistance in these 33 R populations.

3.2 HPLC analysis of diclofop metabolism

In these experiments, the [¹⁴C]-diclofop-methyl dose was carefully determined so as to maximise the radioactive signal without causing visual damage to the S plants, and the plants were foliar pretreated with a very low dose of non-radioactive commercial formulation of diclofop-methyl. As *L. rigidum* is relatively slow in metabolising diclofop,^{19,23} the optimal time to show differences in diclofop metabolism between R and S plants was determined to be 4 DAT under our experimental conditions.

Foliar uptake of [¹⁴C]-diclofop-methyl was measured in 12 bulked individual plants of each of the 33 field R populations. About 91% of the total applied diclofop-methyl was absorbed by 4 DAT. No differences were found in diclofop-methyl leaf uptake among the 33 field R populations versus the six (3S and 3R) control populations (data not shown).

After foliar absorption, diclofop-methyl was converted by plant esterases to the herbicidally active diclofop acid, which was

further metabolised to non-toxic polar compounds (aryl hydroxylation followed by sugar conjugation).^{19,28} As shown in Fig. 2, under our HPLC conditions, diclofop-methyl, diclofop acid and its metabolites were resolved at about 31 min, 29 min and between 2 and 5 min (major metabolites) and 5 and 15 min (minor metabolites) respectively. The percentage of diclofop acid and its total metabolites and diclofop-methyl was quantified for each sample and compared with the S controls (Table 1). In S plants, by 4 DAT almost all diclofop-methyl had been converted to phytotoxic diclofop acid, which on average accounted for 67% of total radioactivity (Table 1). In contrast, in plants of the three known metabolic-resistant populations, while the diclofop-methyl level was the same as in S plants, significantly lower levels of diclofop acid (18–49%) and correspondingly higher levels of diclofop acid metabolites were observed (Table 1). There were differences in the capacity to metabolise diclofop acid among the 33 field R populations (Fig. 2, Table 1). Based on the statistical significance of difference in the levels of both diclofop acid and its metabolites, compared with the S populations, 79% of the field R populations (26 out of 33, in Table 1 starting from population M1/28 downwards) displayed enhanced capacity for diclofop acid metabolism. These field R populations metabolised 40–70% of the diclofop acid by 4 DAT, compared with 32% in the S populations (Table 1). The diclofop-methyl level in some field R samples was greater than that of the S samples (Table 1). However, diclofop-methyl accounted for less than 7% of the total radioactivity, and therefore this difference is considered to have a minor effect on resistance. In addition, incomplete removal of unabsorbed [¹⁴C]-diclofop-methyl from the leaf surface may result in variation. Based on the level of diclofop acid metabolites, field resistant populations displayed a 29–121% increase in diclofop acid metabolism capacity relative to the S populations (Table 1). The magnitude of the increase in diclofop acid metabolism in 81% of the field R populations (21 out of 26) (Table 1) is comparable with that displayed in the three known metabolic-resistant populations (SLR31, PN and VLR69). Therefore, metabolic resistance, evident as enhanced rates of diclofop acid metabolism, is common in these field-collected *L. rigidum* populations from the Western Australian grain belt.

3.3 ACCase sequencing

Nearly all known resistance-endowing target-site ACCase gene mutations were identified in 30 field resistant populations (Table 1). Half of the populations exhibited a single ACCase gene mutation (one of the ACCase gene mutations at 1781, 2027, 2041 or 2078), with the other 50% having multiple ACCase mutations. Among them, Ile-2041-Asn was the most commonly identified mutation (Table 1); however, the frequency of each ACCase mutation(s) within a population cannot be determined by the current approach (using bulked survivors). These mutations (single and/or in combinations) are known to confer resistance to diclofop-methyl and other ACCase-inhibiting herbicides (including clethodim) in *L. rigidum*.²⁷

Comparing the data for the presence of enhanced diclofop metabolism and ACCase mutations in Table 1, it is clear that 70% of the resistant *L. rigidum* populations possess both non-target-site metabolic resistance and target-site resistance due to ACCase gene mutations. Some 21% of the populations exhibited solely ACCase target-site mutation(s), and 9% of the populations displayed solely enhanced herbicide metabolism. These results show that both metabolic resistance and ACCase mutations co-occur in most resistant populations.

3.4 Diclofop-methyl dose response to determine the level of resistance conferred by metabolic resistance versus a target-site ACCase mutation

Two contrasting resistant populations were identified: H5/3, which has only the target-site ACCase 2041 mutation, and L3/14, which has only metabolic resistance (Table 1). Both populations are resistant at a field rate of 375 g diclofop-methyl ha⁻¹ (Table 1). The susceptible population (SVLR1) had a diclofop-methyl LD₅₀ value of 17 g ha⁻¹, whereas for the two resistant populations the LD₅₀ is >6000 g ha⁻¹ (Fig. 3). Based on the LD₅₀ R/S ratio, the two resistant populations are at least 350-fold more resistant to diclofop-methyl than the S population. It is therefore important to note that the mechanism based on enhanced herbicide metabolism in *L. rigidum* can endow high-level resistance, albeit lower than the target-site 2041 mutation (Fig. 3).

4 DISCUSSION

In studies with herbicide-resistant weeds to identify the biochemical basis of resistance, many researchers examine only for herbicide target-site mutations. This is understandable, as target-site resistance frequently occurs, can provide high level resistance (Table 1, Fig. 3) and is easy to determine. There can be many target-site mutations, and various molecular approaches/markers have been developed to monitor target-site resistance at the individual, population and geographic levels.^{15,29,30} In contrast, non-target-site metabolic resistance is difficult to measure and thus is often not determined. Indeed, when target-site resistance is identified, researchers often fail to investigate for the presence of other coexisting resistance mechanisms. This is unfortunate. Here, working with 33 randomly selected herbicide-resistant *L. rigidum* populations from different geographical zones of the Western Australian grain belt, we provide direct evidence that non-target-site metabolic resistance is present in most of these resistant populations (which also contain target-site ACCase mutations). Therefore, populations/individuals with both non-target site and target-site resistance to selective herbicides now represent the usual situation for *L. rigidum*, at least in Australia.

4.1 Evolution of metabolic herbicide resistance in *L. rigidum*

Rapid evolution of metabolic resistance to crop-selective herbicides has been demonstrated in Australian *L. rigidum* populations. For example, with the benefit of hindsight, metabolic resistance in *L. rigidum* was evident only 3 years after first use of diclofop-methyl.² This occurs because unselected herbicide-susceptible *L. rigidum* displays a low level of diclofop-methyl metabolism capacity, and individuals in a large population vary in their capacity to metabolise herbicides.^{9,19,23} Thus, when herbicides are at low rates, individuals with higher capacity for herbicide metabolism survive. Therefore, lower herbicide rates can rapidly select for metabolic resistance, and especially so in genetically diverse, obligate cross-pollinated species such as *L. rigidum*. This was early evident in the *L. rigidum* population WLR1, which was selected in wheat fields for seven successive years at a low chlorsulfuron dose.¹⁰ This rate-cutting practice resulted in the selection and accumulation of metabolic resistance to chlorsulfuron,¹⁰ as well as at least six different target-site ALS gene mutations.²⁵ High plant survival frequencies at relatively low Australian herbicide use rates have been revealed in many *L. rigidum* populations,¹⁷ and rapid selection of metabolic resistance by low herbicide rates has been demonstrated in several initially

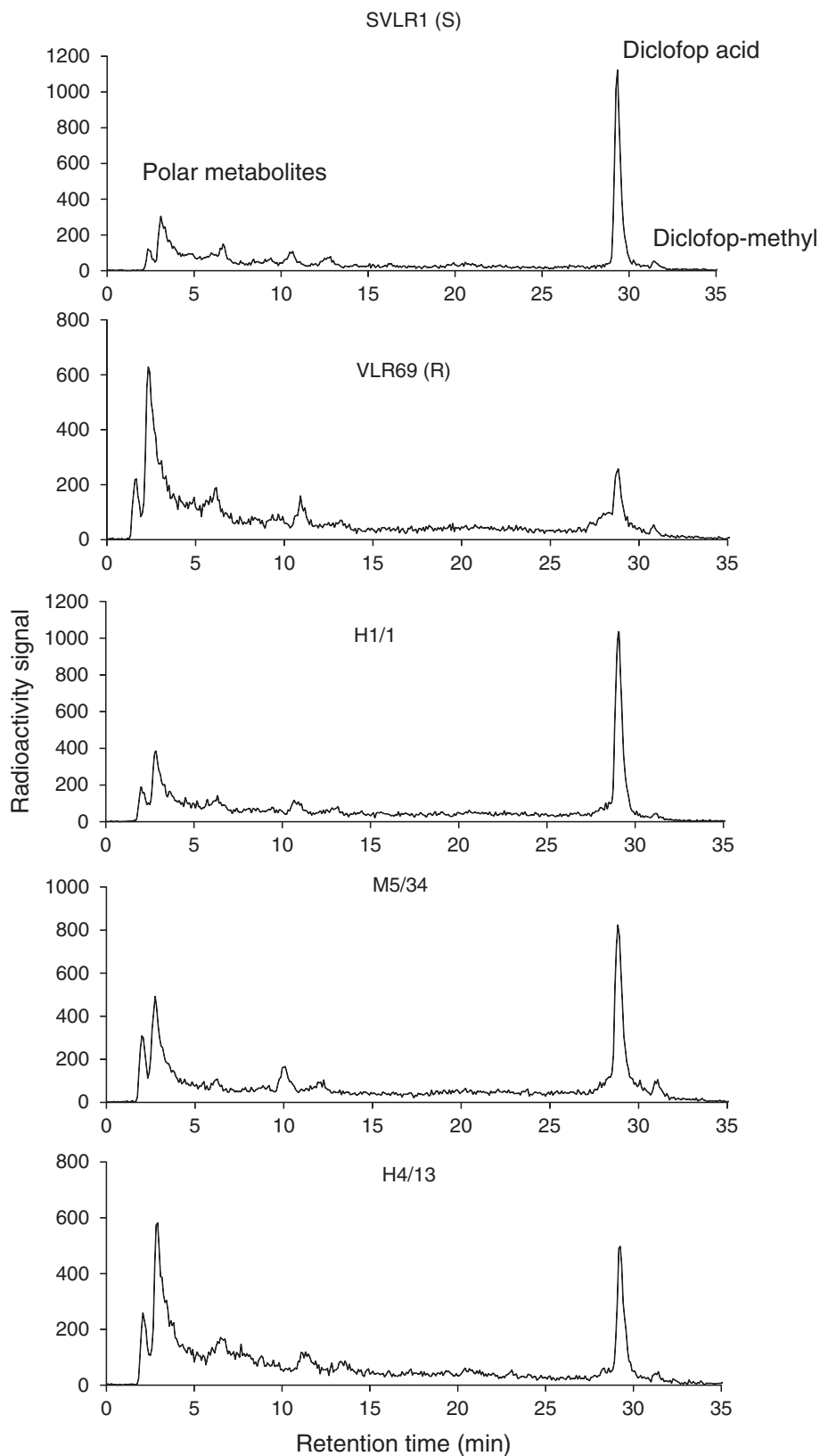


Figure 2. HPLC chromatograms comparing the elution profiles of diclofop-methyl, phytotoxic diclofop-acid and its metabolites (eluted at between 2 and 5 min and 10 and 15 min) in herbicide-susceptible (S) (SVLR1) and herbicide-resistant (R) (VLR69) populations and in three representative field-collected resistant *Lolium rigidum* populations (H1/1, M5/34 and H4/13) 96 h after treatment.

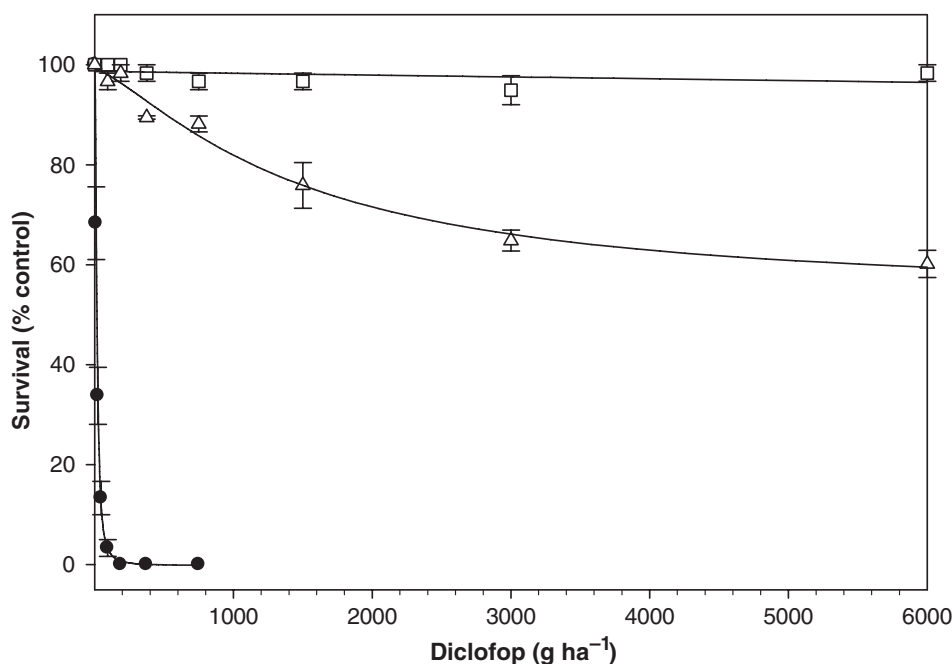


Figure 3. Diclofop dose response of *Lolium rigidum*, comparing non-target-site metabolic resistance conferred by enhanced herbicide metabolism capacity (Δ , population L3/14) and target-site resistance endowed by the ACCase Ile-2041-Asn mutation (\square , population H5/3) in relation to the herbicide-susceptible population (\bullet , SVLR1).

herbicide-susceptible *L. rigidum* populations.^{16,18,19} This is likely to have been occurring in Australian field *L. rigidum* populations, as herbicide rate cutting was initially widely practised. Given this, it is no surprise that metabolic herbicide resistance is found to be common in Australian *L. rigidum* (Table 1).

When metabolic resistance is initially selected from the pre-existing genetic variation in a *L. rigidum* population, it endows a level of resistance that enables some plant survival. If herbicide selection continues, then the resistance genes are recombined and additively enriched by obligate cross-pollination, resulting in a substantial level of resistance.^{16,31} This is evident in the present study in that populations varied in their herbicide metabolic capacity (Table 1, Fig. 2), with some exhibiting high-level metabolic resistance (Fig. 3). Nevertheless, even a slightly increased level of herbicide metabolic capacity (e.g. in population H2/3 in Table 1) is sufficient to endow resistance at the recommended diclofop field rate.

While here we analysed 33 field *L. rigidum* populations, direct evidence of metabolic resistance to diclofop-methyl in much larger numbers of resistant plants has recently been obtained using an automated ¹⁴C-herbicide metabolism screen at Bayer CropScience in Frankfurt. Analysis of >2000 individuals from 301 different resistant *Lolium* spp. populations from France revealed that 72% of the resistant populations displayed metabolic resistance to diclofop-methyl, with only 28% of the populations possessing solely target-site resistance (Beffa R, private communication). The Bayer resistance screening establishes, along with our study, that metabolic resistance in *Lolium* spp. is widespread. In addition to *L. rigidum*, metabolic resistance to diclofop-methyl was identified in resistant Australian *Avena* spp. populations.^{32,33} There is indirect evidence that metabolic resistance is common in other grass weed species. For example, resistance to ALS-inhibiting herbicides in six Australian *Bromus rigidus* populations was found to be non-target-site based.³⁴ In Canadian populations of *A. fatua*, resistance to ALS-inhibiting herbicides was found to be

prevalently metabolism based.³⁵ A large-scale survey of ACCase target-site resistance in *Alopecurus myosuroides* in France (over 10 000 seedlings in 243 populations) established that 75% of the resistant plants did not have target-site mutation(s) and thus must have had non-target-site resistance, although non-target-site mechanism studies were not conducted.²⁹ These results demonstrate that metabolic resistance to crop-selective herbicides is widespread and is a global issue.

The threat of metabolic resistance is that it not only can endow resistance to the selecting herbicide but also can confer cross-resistance to dissimilar and even never used herbicides.^{9,16,31} Indeed, cross-resistance patterns vary among populations,¹⁶ compromising resistance management strategies such as alternative herbicide options and herbicide mixture/rotations. In addition, populations that have developed metabolic resistance might be able to evolve resistance to new herbicides more rapidly and at a greater magnitude than herbicide-susceptible populations. Our recent work has demonstrated the differential capability of two *L. rigidum* populations, with different initial levels of susceptibility to a new selective herbicide pyroxasulfone, rapidly to evolve pyroxasulfone resistance under recurrent low-dose pyroxasulfone selection.³⁶ Highly pyroxasulfone-resistant progeny were selected in three generations in the multiple-resistant population (SLR31), and in contrast only a modest shift towards pyroxasulfone resistance was obtained in the S population (VLR1).³⁶ This is likely to be the case in fields where change from one selective herbicide to another occurs, and this poses resistance evolution risks on new crop-selective herbicides. Therefore, new selective herbicides must be used cautiously and at full rates, and resistance potential should be early evaluated with metabolic-resistant populations.

Our genetic studies have revealed that metabolic resistance in *L. rigidum* is inherited as polygenic gene traits.^{37–39} A few genes involved in metabolic resistance (including cytochrome P450s and glucosyl transferase) in *L. rigidum* have been recently identified,⁴⁰ and much will be revealed in the next few years on the

specific genes conferring metabolic resistance. This information is essential for better understanding and management of metabolic resistance.

4.2 Coevolution of metabolic and target-site resistance in *L. rigidum*

Over many years we have studied the biochemical and genetic bases of herbicide resistance and cross-resistance in *L. rigidum* and have established that resistant populations/individuals can exhibit from one to several coexisting resistance mechanisms, both non-target-site and target-site based.^{9,14,15,41,42} Especially for crop-selective herbicides, coevolution of non-target-site metabolic resistance and target-site resistance to a given herbicide within a single weed population was early revealed in many Australian *L. rigidum* populations.^{10,11,43} This is expected, as evolutionary reality teaches us that any and all gene traits that can endow plant survival to an herbicide will be selected.⁴¹ Nevertheless, selection of non-target-site metabolic resistance and/or target-site resistance in a given weed population is related to initial resistance gene frequencies, selecting herbicides (e.g. crop-selective versus non-selective) and selection history and herbicide rates used. For example, in herbicide-resistant *L. rigidum* populations (e.g. SLR31, VLR69) the majority of individuals displayed enhanced herbicide metabolism, while only a small percentage had target-site resistance mutations.^{11,43} The high frequency of individuals with metabolic resistance is probably related to the use of metabolisable herbicides (e.g. atrazine, chlorsulfuron, diclofop-methyl) that initially select plants with higher metabolic capacity for these herbicides. Furthermore, in large herbicide-treated, genetically diverse *L. rigidum* populations, individual plants can be subjected to different herbicide selection intensity owing to variations in plant developmental stages, herbicide metabolic capacity, etc., resulting in evolution of different resistance mechanisms. Therefore, various resistance mechanisms (e.g. enhanced herbicide metabolism, target-site mutations) can be initially selected in different individuals in a population and accumulated among resistant individuals by gene exchange due to obligate cross-pollination. Depending on the fitness of the resistance mechanisms in the presence and absence of herbicide selection under different environments, the frequency of the resistance mechanisms in a population may change dynamically over time.

In summary, the present *in vivo* herbicide metabolism study with 33 randomly selected resistant *L. rigidum* populations from across the Western Australian grain belt demonstrates the coexistence of metabolic resistance and target-site resistance. In particular, metabolic herbicide resistance is an important threat to herbicide efficacy and crop productivity, warranting considerable research and management efforts.

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REFERENCES

- Heap I and Knight R, A population of ryegrass tolerant to the herbicide diclofop-methyl. *J Aust Inst Agric Sci* **48**:156–157 (1982).
- Heap I and Knight R, The occurrence of herbicide cross-resistance in a population of annual ryegrass, *Lolium rigidum*, resistant to diclofop-methyl. *Aust J Agric Res* **37**:149–156 (1986).
- Matthews JM, Holtum JAM, Liljegren DR, Furness B and Powles SB, Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). I. Properties of the herbicide target enzymes acetyl-CoA carboxylase (ACC) and acetolactate synthase (ALS). *Plant Physiol* **94**:1180–1186 (1990).
- Christopher JT, Powles SB, Holtum JAM and Liljegren DR, Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). II. Chlorsulfuron resistance involves a wheat-like detoxification system. *Plant Physiol* **95**:1036–1045 (1991).
- Cotterman JC and Saari LL, Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in diclofop-methyl-resistant rigid ryegrass (*Lolium rigidum*) biotype SR4/84. *Pestic Biochem Physiol* **43**:182–192 (1992).
- Holtum JAM, Matthews JM, Liljegren DR and Powles SB, Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). III. On the mechanism of resistance to diclofop-methyl. *Plant Physiol* **97**:1026–1034 (1991).
- Preston C, Tardif FJ, Christopher JT and Powles SB, Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. *Pestic Biochem Physiol* **54**:123–134 (1996).
- Preston C, Herbicide resistance in weeds endowed by enhanced detoxification: complication for management. *Weed Sci* **52**:448–453 (2004).
- Yu Q and Powles SB, Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol* **166**:1106–1118 (2014).
- Christopher JT, Powles SB and Holtum JAM, Resistance to acetolactate synthase inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Physiol* **100**:1909–1913 (1992).
- Tardif FJ and Powles SB, Herbicide multiple-resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: isolation of a subset with resistant acetyl-CoA carboxylase. *Physiol Plantarum* **91**:488–494 (1994).
- Tranel PJ and Wright TR, Resistance of weeds to ALS inhibiting herbicides: what have we learned? *Weed Sci* **50**:700–712 (2002).
- Délye C, Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. *Weed Sci* **53**:728–746 (2005).
- Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317–347 (2010).
- Yu Q and Powles SB, Resistance to AHAS inhibitor herbicides: current understanding. *Pest Manag Sci* **70**:1340–1350 (2014).
- Neve P and Powles SB, Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*. *Theor Appl Genet* **110**:1154–1166 (2005).
- Neve P and Powles SB, High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. *Heredity* **95**:485–492 (2005).
- Manalil S, Busi R, Renton M and Powles SB, Rapid evolution of herbicide resistance by low herbicide dosages. *Weed Sci* **59**:210–217 (2011).
- Yu Q, Han HP, Cawthray GR, Wang SF and Powles SB, Enhanced rates of herbicide metabolism in low herbicide-dose selected resistant *Lolium rigidum*. *Plant Cell Environ* **36**:818–827 (2013).
- Llewellyn R and Powles SB, High levels of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across the Western Australian wheatbelt. *Weed Technol* **15**:242–248 (2001).
- Owen MJ, Walsh M, Llewellyn R and Powles SB, Widespread occurrence of multiple herbicide resistance in Western Australian annual ryegrass (*Lolium rigidum*) populations. *Aust J Agric Res* **58**:711–718 (2007).
- Owen MJ, Martinez NJ and Powles SB, Multiple herbicide-resistant *Lolium rigidum* (annual ryegrass) now dominates across the Western Australian grain belt. *Weed Res* **54**:314–324 (2014).
- Han HP, Yu Q, Cawthray G and Powles SB, Enhanced herbicide metabolism induced by 2,4-D in herbicide susceptible *Lolium rigidum* provides protection against herbicide injury. *Pest Manag Sci* **69**:996–1000 (2013).
- Vila-Aiub MM, Neve P, Steadman KJ and Powles SB, Ecological fitness of a multiple herbicide-resistant *Lolium rigidum* population: dynamics of seed germination and seedling emergence of resistant and susceptible phenotypes. *J Appl Ecol* **42**:288–298 (2005).

- 25 Yu Q, Han HP and Powles SB, Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Manag Sci* **64**:1229–1236 (2008).
- 26 Zhang XQ and Powles SB, Six amino acid substitutions in the carboxyl-transferase domain of the plastidic acetyl-CoA carboxylase gene are linked with resistance to herbicides in a *Lolium rigidum* population. *New Phytol* **172**:636–645 (2006).
- 27 Yu Q, Collavo A, Zheng MQ, Owen M, Sattin M and Powles SB, Diversity of acetyl-coenzyme A carboxylase mutations in resistant *Lolium* populations: evaluation using clethodim. *Plant Physiol* **145**:547–558 (2007).
- 28 Shimabukuro RH and Hoffer BL, Metabolism of diclofop-methyl in susceptible and resistant biotypes of *Lolium rigidum*. *Pestic Biochem Physiol* **39**:251–260 (1991).
- 29 Délye C, Menchari Y, Guillemin JP, Matějček A, Michel S, Camilleri C *et al.*, Status of black grass (*Alopecurus myosuroides*) resistance to acetyl-coenzyme A carboxylase inhibitors in France. *Weed Res* **47**:95–105 (2007).
- 30 Malone JM, Boutsalis P, Baker J and Preston C, Distribution of herbicide-resistant acetyl-coenzyme A carboxylase alleles in *Lolium rigidum* across rain cropping areas of South Australia. *Weed Res* **54**:78–86 (2014).
- 31 Busi R and Powles SB, Cross-resistance to prosulfocarb and triallate in pyroxasulfone-resistant *Lolium rigidum*. *Pest Manag Sci* **69**:1379–1384 (2013).
- 32 Manechote C, Preston C and Powles SB, A diclofop-methyl-resistant *Avena sterilis* biotype with a herbicide-resistant acetyl-coenzyme A carboxylase and enhanced metabolism of diclofop-methyl. *Pestic Sci* **49**:105–114 (1997).
- 33 Ahmad-Hamdani MS, Yu Q, Han HP, Cawthray GR, Wang SF and Powles SB, Herbicide resistance endowed by enhanced rates of herbicide metabolism in wild oat (*Avena* spp.) *Weed Sci* **61**:55–62 (2013).
- 34 Owen MJ, Goggin DE and Powles SB, Non-target-site-based resistance to ALS-inhibiting herbicides in six *Bromus rigidus* populations from Western Australian cropping fields. *Pest Manag Sci* **68**:1077–1082 (2012).
- 35 Beckie HJ, Warwich SI and Sauder CA, Basis for herbicide resistance in Canadian populations of wild oat (*Avena fatua*). *Weed Sci* **60**:10–18 (2012).
- 36 Busi R, Gaines T, Walsh MJ and Powles SB, Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: field selection at high doses versus recurrent selection at low doses. *Weed Res* **52**:489–499 (2012).
- 37 Busi R, Vila-Aiub MM and Powles SB, Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. *Heredity* **106**:817–824 (2011).
- 38 Busi R, Neve P and Powles SB, Evolved polygenic herbicide resistance in *Lolium rigidum* by low-dose herbicide selection within standing genetic variation. *Evol Appl* **6**:231–242 (2013).
- 39 Han HP, Yu Q, Vila-Aiub MM and Powles SB, Genetic inheritance of cytochrome P450-mediated metabolic resistance to chlorsulfuron in a multiple herbicide resistant *Lolium rigidum* population. *Crop Prot* **65**:57–63 (2014).
- 40 Gaines TA, Lorentz L, Figge A, Herrmann J, Maiwald F, Ott MC *et al.*, RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. *Plant J* **78**:865–876 (2014).
- 41 Powles SB and Matthews JM, Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*): a driving force for the adoption of integrated weed management strategies, in *Resistance 91: Achievements and Developments in Combating Pesticide Resistance*, ed. by Denholm I, Devonshire AL and Holloman DW. Elsevier Science Publishers, London, UK, pp. 75–87 (1992).
- 42 Hall LM, Holtum JAM and Powles SB, Mechanisms responsible for cross resistance and multiple resistance, in *Herbicide Resistance in Plants: Biology and Biochemistry*, ed. by Powles SB and Holtum JAM. Lewis Publishers, Boca Raton, FL, pp. 243–261 (1994).
- 43 Burnet MWM, Christopher JT, Holtum JAM and Powles SB, Identification of two mechanisms of resistance within one population of rigid ryegrass (*Lolium rigidum*) using a selective germination medium. *Weed Sci* **42**:468–473 (1994).