Non-target-site-based resistance to ALS-inhibiting herbicides in six *Bromus rigidus* populations from Western Australian cropping fields

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**Abstract**

**BACKGROUND:** *Bromus rigidus* is a common weed species that has increased in cropping fields owing to limited control options. During a random field survey in Western Australia, six *B. rigidus* populations that had survived in-crop weed control programmes were collected. The study aimed to determine the resistance profile of these six populations.

**RESULTS:** Based on dose–response studies, all six *B. rigidus* populations had a low-level resistance to sulfosulfuron and sulfometuron (both sulfonylurea herbicides) while remaining susceptible to herbicides with other modes of action. ALS *in vitro* activity assays revealed no differences in enzyme sensitivity between susceptible and resistant populations, while the use of malathion (a cytochrome P450 inhibitor) in combination with sulfosulfuron caused the resistant populations to behave like the susceptible population.

**CONCLUSION:** This study established that these six *B. rigidus* populations have a low-level resistance to the ALS-inhibiting sulfonylurea herbicides, but are able to be controlled by other herbicide modes of action. The low-level, malathion-reversible resistance, together with a sensitive ALS, strongly suggest that a non-target-site enhanced metabolism is the mechanism of resistance.

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**Keywords:** resistance survey; resistance evolution; *Bromus* spp.; ALS resistance

1 INTRODUCTION

Rigid brome (*Bromus rigidus* Roth) is a self-pollinated winter annual grass of Mediterranean origin. *Bromus* species are commonly found on lighter sandy soil across the wheat belt of southern Australia and are well adapted to the hot dry summers and mild wet winters of this climate.1–4 In recent years, *Bromus* spp. have become more abundant in cereal-growing regions, largely owing to increased cereal cropping frequency and the widespread adoption of conservation tillage. This technique often relies heavily on herbicide use, with limited in-crop herbicide options for *Bromus* control.2,4,6 *Bromus* spp. are competitive in both crops and pastures, resulting in reduced crop growth and yield;5 furthermore, its seeds can contaminate grain.7 On account of seed dormancy, germination of *Bromus* spp. can extend over the crop-growing season,8,8 with cohorts emerging in crop that require selective herbicide treatments.

Many herbicides target the plastidic enzyme acetolactate synthase (ALS), and these ALS herbicides are used to control a wide range of weed species in a variety of field crops. The acetyl-coenzyme A carboxylase (ACCase)– and ALS-inhibiting herbicides are the most important for *Bromus* control in Australia, and the newer ALS-inhibiting herbicides sulfosulfuron (Monza®) and mesosulfuron-methyl (Atlantis®) provide selective control of *Bromus* spp. in wheat.10 However, these soil-residual herbicides can have persistence issues11 and must be applied no later than the three-leaf growth stage to achieve good control. The continued use of ALS herbicides has led to the widespread evolution of ALS-herbicide-resistant weed populations (reviewed in Saari et al.,12 Tranel and Wright13 and Powles and Yu14). Worldwide, there are now 113 species with resistance to the ALS herbicides.15 While to date there are no published cases of ALS-herbicide-resistant *Bromus* populations in Australia, ACCase herbicide resistance in *Bromus* species is known.15,16 *Bromus tectorum* (downy brome), a widespread and competitive weed in crops in the Pacific Northwest of the United States, has evolved sulfonylurea (SU)-class ALS herbicide resistance.15,17,18 A recent survey in Western Australia found evidence of *B. rigidus* biotypes that were difficult to control during the cropping phase.19 The focus of the present study was to assess resistance to ALS-inhibiting SU herbicides in *B. rigidus* populations from Australia. The study characterises six *B. rigidus* populations resistant to ALS herbicides and demonstrates that resistance is likely, and

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unexpectedly, due to non-target-site-based enhanced rates of herbicide metabolism.

2 MATERIALS AND METHODS

2.1 Seed collection and germination

In 2005, 677 cropping fields were randomly visited across the Western Australian grain belt (Fig. 1) (see Owen and Powles for detailed methodology). A small number of fields were found to have high numbers of *B. rigidus* plants, and seeds from six field populations were collected for resistance screening. All populations tested came from the northern agricultural region (Fig. 1), as plants in the southern region had immature seed at the time of collection.

*B. rigidus* seeds (after-ripened and thus non-dormant) were germinated on water solidified with agar (1%) in 500 mL plastic containers for 7–10 days under ambient conditions in the laboratory. For each population and herbicide treatment, 50 seedlings were transplanted into plastic trays containing a standard potting mix (50% composted pine bark, 25% peat and 25% river sand). Seedlings were grown at the University of Western Australia in an outdoor plot during the normal growing season (May to September) and were watered and fertilised regularly.

2.2 Single-rate herbicide resistance testing

A range of herbicides inhibiting ACCase, ALS, photosystem I or 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) were tested at recommended field rates (Table 1) to determine the susceptibility of the *B. rigidus* biotypes to several different herbicide chemistries. When seedlings reached the 2–3-leaf stage, they were treated with herbicide (see Owen and Powles for details of the spraying equipment used). Twenty-one days later, survival of plants was assessed by inspecting the growing points: if these were chlorotic or necrotic and there were no new tillers forming, and leaf tissue was easily fragmented, the plants were considered to be dead. There were 50 plants per herbicide treatment, with two replicates for each biotype. A previously verified susceptible population (from South Australia) was used as a control for each herbicide treatment. For all herbicides, 100% mortality occurred in the known susceptible population (data not shown).

2.3 Dose response to ALS-inhibiting herbicides

Seedlings from each of the putative resistant populations were tested during May–September 2009 to quantify the level of resistance to ALS herbicides. Plants (two-leaf stage) were sprayed with sulfometuron 750 g kg$^{-1}$ WG together with 0.25% (v/v) wetting agent BS1000 at 0, 3.75, 7.5, 15, 30, 60, 120 and 240 g ha$^{-1}$, with imazamox 700 g kg$^{-1}$ WG together with 2% crop oil (Hasten; Victorian Chemicals Australia) at 0, 8, 16, 32 and 64 g ha$^{-1}$ or with sulfosulfuron 750 g kg$^{-1}$ WG at 0, 37.5, 75, 150, 300 and 600 g ha$^{-1}$.

Assessment of survival was conducted 28 days after spraying, and the above-ground tissue was harvested, dried at $70$ °C for 72 h and weighed. The experiment, performed twice during the growing season, was randomised with 20 seedlings per pot, and had three replicates per treatment.
2.4 Effect of malathion on sulfosulfuron resistance
Malathion is an organophosphate insecticide known to be an inhibitor of cytochrome P450 monoxygenases. Malathion is a synergist for the herbicide chlorosulfuron in *Lolium rigidum* and the herbicide primisulfuron in *Zea mays* (maize). Preliminary studies showed that there was no adverse effect on the seedling growth of *B. rigidus* when malathion was applied alone at 1000 g ha⁻¹; therefore, this rate was used in this study. During May–July 2011, seeds of each of the resistant *B. rigidus* populations were sprayed with sulfosulfuron 750 g kg⁻¹ WG at 0, 18.75, 37.5, 75, 150, 300 and 600 g ha⁻¹, with or without malathion. Malathion was applied 30 min prior to the application of sulfosulfuron using the herbicide sprayer. Plants were assessed 21 days after treatment, and the above-ground tissue was harvested, dried and weighed as above. The experiment was randomised, with 20 seedlings per pot, with or without malathion, with two replicates per treatment. A second susceptible population collected from the south-west of Western Australia was also used as a control in these experiments.

2.5 Statistical analyses for herbicide treatment studies
Non-linear regression was used to estimate the herbicide rate causing 50% mortality (LD₅₀) or growth reduction (GR₅₀) of plants using Sigma Plot software (v.11.0). The R:S (resistant:susceptible) ratio of estimated LD₅₀ values was used as the measure of resistance. A t-test (*P = 0.05*) was used to determine the level of significance. Mortality dose–response graphs are presented with untransformed data. The data were fitted to the log-logistic model:

\[ y = C + \frac{D - C}{1 + (X/ED_{50})^b} \]

where *C* is the lower limit, *D* is the upper slope, *b* is the slope and *ED₅₀* is the dose causing 50% reduction. When it was not possible to fit a log-logistic model to the biomass data, an exponential decay model was used. Datasets were analysed by ANOVA, and LSD (*P = 0.05*) was used to determine significant differences between populations.

2.6 In vitro ALS enzyme activity
ALS enzyme activity in leaf blades (1 g of young fully expanded tissue collected from 4–8 individuals for each replicate) was measured colorimetrically using a method modified from Ray. Leaf blades were snap frozen and pulverised in liquid nitrogen, then extracted in 3 mL of cold grinding buffer [0.1 M K₂HPO₄, pH 7.5, 1 mM sodium pyruvate, 0.5 mM MgCl₂, 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavine adenine dinucleotide (FAD), 1% (w/v) polyvinylpyrrolidone, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride and clarified by centrifugation at 12 000 × g at 4 °C. Using PD-10 (Pharmacia) columns, the supernatant was desalted into phosphate buffer (0.1 M K₂HPO₄, pH 7.5, 20 mM sodium pyruvate, 0.5 mM MgCl₂, 1 mM DTT) and then assayed for ALS activity in a 250 μL reaction containing 185 μL of desalted extract. The sensitivity of ALS to inhibitors was assessed by adding technical-grade sulfometuron (Nufarm) at 0, 0.0001, 0.001, 0.01, 1, 10 or 100 μM to the reaction. Reactions were incubated at 30 °C for 60 min (during which time the reaction rate was linear) and terminated with the addition of H₂SO₄ to 0.55 N (H₂SO₄ was added to negative controls at 0 min). Production of acetoin was monitored at 530 nm following treatment of the reaction mixture with creatine and α-naphthol, with 0–75 nmol pure acetoin being used as a standard. The protein concentration in the leaf extracts was measured as in Bradford using 0–25 μg of bovine serum albumin as a standard and colour development with BioRad dye reagent concentrate. Each enzyme assay was performed with four independent biological replicates, and protein concentration was measured in duplicate in each sample.

### Table 1. Resistance status across differing herbicide chemistries for all six resistant biotypes of *B. rigidus*. S denotes that the populations were completely susceptible (i.e. all plants died) to the herbicide, and R denotes that the populations were resistant (>95% of plants survived)

<table>
<thead>
<tr>
<th>Herbicide chemical class</th>
<th>Active ingredient</th>
<th>Herbicide mode of action</th>
<th>Rate (g ha⁻¹)</th>
<th>Resistance status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryloxyphenoxypropionate</td>
<td>Fluazifop</td>
<td>Inhibition of ACCase</td>
<td>78</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Haloxyfop</td>
<td>Inhibition of ACCase</td>
<td>104</td>
<td>S</td>
</tr>
<tr>
<td>Cyclohexanediene</td>
<td>Sethoxydim</td>
<td>Inhibition of ACCase</td>
<td>186</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Clethodim</td>
<td>Inhibition of ACCase</td>
<td>60</td>
<td>S</td>
</tr>
<tr>
<td>Sulfonyleurea</td>
<td>Sulfometuron</td>
<td>Inhibition of ALS</td>
<td>15</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Sulfsulfuron</td>
<td>Inhibition of ALS</td>
<td>37.5</td>
<td>R</td>
</tr>
<tr>
<td>Imidazolinone</td>
<td>Imazapic + imazapy</td>
<td>Inhibition of ALS</td>
<td>7 + 21</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Imazamax</td>
<td>Inhibition of ALS</td>
<td>31.5</td>
<td>S</td>
</tr>
<tr>
<td>Bipyridyl</td>
<td>Paraquat</td>
<td>Inhibition of photosystem I</td>
<td>300</td>
<td>S</td>
</tr>
<tr>
<td>Glycine</td>
<td>Glyphosate</td>
<td>Inhibition of EPSPS</td>
<td>540</td>
<td>S</td>
</tr>
</tbody>
</table>

3 RESULTS

3.1 Single-rate herbicide resistance testing
Six *B. rigidus* populations were screened with a range of herbicides with known activity on this species. All six populations responded similarly, being resistant to the ALS-inhibiting SU-class herbicides sulfometuron and sulfosulfuron but susceptible to the imidazoline (IMI)-class ALS herbicides and the inhibitors of ACCase, EPSPS and photosystem I (Table 1).

3.2 Dose response to ALS-inhibiting herbicides
Detailed dose–response studies confirmed that all six *B. rigidus* populations tested were resistant to the ALS-inhibiting herbicides sulfometuron and sulfosulfuron (Tables 2 and 3). The susceptible (S) biotype was controlled at field rates, in contrast to the six resistant (R) biotypes (Figs 2 and 3a). The lethal dose of SU herbicides required to kill 50% of the R biotypes was 3–4 times greater than that required to kill the S biotype for sulfometuron (Table 2), and 6–11 times greater for sulfosulfuron (Table 3). However, biomass data revealed that the growth of the R populations was clearly affected by sulfosulfuron, with high herbicide rates significantly reducing the biomass (Fig 3b).
Table 2. LD$_{50}$ values (standard errors in parentheses; $n = 3$) of B. rigidus populations treated with sulfometuron. R/S ratios were calculated as the ratio of LD$_{50}$ values of resistant and susceptible populations.

<table>
<thead>
<tr>
<th>Biotype</th>
<th>LD$_{50}$ (g ha$^{-1}$)</th>
<th>R/S ratio of LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>10 (0.8)</td>
<td>n/a</td>
</tr>
<tr>
<td>R1</td>
<td>30 (2.4)</td>
<td>3</td>
</tr>
<tr>
<td>R2</td>
<td>33 (3.7)</td>
<td>3.3</td>
</tr>
<tr>
<td>R3</td>
<td>31 (2.2)</td>
<td>3.1</td>
</tr>
<tr>
<td>R4</td>
<td>30 (2.9)</td>
<td>3</td>
</tr>
<tr>
<td>R5</td>
<td>38 (1.1)</td>
<td>3.8</td>
</tr>
<tr>
<td>R6</td>
<td>41 (2.0)</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^a$ LD$_{50}$: the dose lethal to 50% of the population; R: resistant; S: susceptible.

Table 3. LD$_{50}$ values (standard errors in parentheses; $n = 2$) of B. rigidus populations treated with sulfosulfuron ± 1000 g ha$^{-1}$ malathion. R/S ratios were calculated as the ratio of LD$_{50}$ values of resistant and susceptible populations.

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Sulfoxuron (no malathion)</th>
<th>R/S ratio of LD$_{50}$</th>
<th>Sulfoxuron (plus malathion)</th>
<th>R/S ratio of LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>21 (1.3)</td>
<td>n/a</td>
<td>6 (0.1)</td>
<td>n/a</td>
</tr>
<tr>
<td>R1</td>
<td>183 (21.0)</td>
<td>8.7</td>
<td>16 (0.38)</td>
<td>2.6</td>
</tr>
<tr>
<td>R2</td>
<td>166 (18.6)</td>
<td>7.9</td>
<td>10 (0.01)</td>
<td>1.6</td>
</tr>
<tr>
<td>R3</td>
<td>173 (11.6)</td>
<td>8.2</td>
<td>16 (0.02)</td>
<td>2.6</td>
</tr>
<tr>
<td>R4</td>
<td>239 (27.7)</td>
<td>11.3</td>
<td>13 (0.26)</td>
<td>2.1</td>
</tr>
<tr>
<td>R5</td>
<td>140 (12.9)</td>
<td>6.6</td>
<td>12 (0.01)</td>
<td>2.0</td>
</tr>
<tr>
<td>R6</td>
<td>127 (8.2)</td>
<td>6.0</td>
<td>11 (0.16)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

3.3 Effect of malathion on sulfoxuron resistance

When malathion was applied alone at 1000 g ha$^{-1}$, there was no effect on survival (Fig. 3a) or biomass (data not shown) of either the S or R biotypes. In the presence of malathion, the R biotypes became susceptible to sulfoxuron and the mortality of the already susceptible biotypes was increased further (Fig. 3a, Table 3). Similar results were obtained for the above-ground biomass (data not shown).

3.4 In vitro ALS activity and inhibition

All R biotypes showed similar ALS activity, and therefore one population was selected as a representative sample for testing of the sensitivity of the enzyme to technical-grade sulfoxuron. In the absence of the herbicide, ALS activity was the same in leaf extracts from both the R and S biotypes ($P = 0.16$), with an overall average activity of 1.3 ± 0.1 mol acetoin formed min$^{-1}$ mg$^{-1}$ protein. Sulfoxuron, when added to the reactions, almost completely inhibited ALS activity from both the S and R biotypes at concentrations of 0.1 µM and above (Fig. 4). The $I_{50}$ for the S biotype was 0.00314 ± 0.0017 µM and for the R biotype 0.0019 ± 0.0008 µM, which were not significantly different ($P = 0.878$). Therefore, the whole-plant resistance evident in the R biotypes is not due to target-site ALS enzyme insensitivity.

Figure 2. Dose–response curves for survival of an ALS-susceptible B. rigidus population and the six ALS-resistant B. rigidus populations treated with a range of sulfoxuron doses. Each data point represents the mean percentage survival ± SE of three replicate treatments.

4 DISCUSSION

This study is the first report to characterise ALS herbicide resistance in Australian populations of B. rigidus. Six biotypes were found to have relatively low-level resistance to the SU herbicides sulfoxuron and sulfofuron. Enhanced metabolism catalysed via enhanced rates of metabolism of the herbicide molecule at the herbicide-binding site of the ALS enzyme, resulting in reduced sensitivity to the herbicide. In other cases, non-target-site resistance can be the result of herbicide detoxification mechanism found across six different herbicide groups, which has evolved in at least nine weed species including Bromus species. This mechanism of resistance has been observed in several populations of L. rigidum from Australia and Alopecurus myosuroides from Europe, where these biotypes have displayed non-target-site cross-resistance across several herbicide modes of action, including herbicide groups never used (reviewed in Tranel and Wright 2013 and Powles and Yu 2014).

Synergistic interactions between organophosphate insecticides, which inhibit cytochrome P450 activity, and SU herbicides have been well documented, and previous studies have shown that malathion is an effective inhibitor of P450-mediated herbicide resistance in weed species such as L. rigidum. In the present study there are three lines of evidence suggesting that SU resistance in these B. rigidus populations is due to P450-mediated enhanced metabolism of the herbicides rather than a target-site mutation: (1) the relatively low-level resistance to sulfoxuron and sulfofuron, further illustrated by the decrease in biomass...
be excluded because sulfometuron equally inhibited ALS in R and S plants (Fig. 4). The I$_{50}$ in this study (average 0.0025 $\mu$M) was similar for other monocot species, with the $H. leporinum$ ALS I$_{50}$ for sulfluramifuron being 0.004 $\mu$M (Yu et al.$^{29}$). Although other mechanisms of resistance (e.g. reduced herbicide uptake or translocation) cannot yet be discounted, the present results are consistent with previous studies identifying potential P450-based resistance.$^{30,33}$ Several studies with $L. rigidum$ have shown that resistance in certain populations is likely to be metabolism based, involving cytochrome P450, although different P450 enzymes are probably involved for different herbicides.$^{21,26,34}$ A study using Sinapis arvensis L.$^{35}$ resistant to ethametsulfuron revealed that there were no differences in the resistant and susceptible biotypes with regard to herbicide absorption and translocation, and both biotypes had a sensitive ALS; however, the herbicide was metabolised more rapidly in the resistant biotype. Applying piperonyl butoxide, an inhibitor of cytochrome P450, decreased the rate of metabolism of ethametsulfuron in the resistant biotype, indicating that enhanced herbicide metabolism was the prominent mechanism of resistance.

The control of these ALS-resistant $B. rigidus$ biotypes may initially be achieved by the use of the ALS IMI herbicides, as well as by other herbicide modes of action (Table 1). The development of IMI-tolerant wheat (Clearfield$^{36}$) provides growers with the option to use IMI herbicides, which also have the advantage of controlling a number of other grass weed species.$^{19}$ Metribuzin can also be used selectively to control Bromus spp. in barley crops, although high rates may cause crop damage on some soil types.$^{36}$ Most importantly, weed numbers should be kept at a minimum through the use of integrated weed management options such as competitive crops and tools that deplete the soil weed seed bank, as resistance to some of the alternative herbicide control options has already been reported.$^{16–18}$

**ACKNOWLEDGEMENTS**

Thanks to AHRI staff and in particular Roslyn Owen, who provided invaluable technical assistance in many areas of the research that contributed to this paper. Valuable comments and advice from Dr Qin Yu both while conducting this research and in preparing this manuscript are greatly appreciated. The authors are grateful to the GRDC for providing funding for this research.

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**Figure 3.** Dose–response curves for (a) survival of an ALS-susceptible $B. rigidus$ population and the ALS-resistant $B. rigidus$ populations treated with a range of sulfosulfuron doses plus or minus 1000 g ha$^{-1}$ malathion (M) and (b) plant biomass of an ALS-susceptible $B. rigidus$ population and the ALS-resistant $B. rigidus$ populations treated with a range of sulfosulfuron doses. Each data point represents the mean percentage survival $\pm$ SE of two replicate treatments. As the six resistant populations all gave a similar response, data have been averaged across these populations and presented as one curve for clarity.

**Figure 4.** *In vitro* ALS enzyme activity in the presence of increasing concentrations of sulfometuron. Each data point represents the mean activity $\pm$ SE of three replicate treatments.
REFERENCES


