

# Identification of resistance to either paraquat or ALS-inhibiting herbicides in two Western Australian *Hordeum leporinum* biotypes

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## Abstract

**BACKGROUND:** *Hordeum* populations are becoming increasingly difficult to control in cropping fields. Two herbicide-resistant *H. leporinum* populations were identified during a random crop survey after herbicides were applied. The study aimed to determine the herbicide resistance profile of these *H. leporinum* biotypes to a range of herbicides used for their control.

**RESULTS:** Based on dose–response studies, one *H. leporinum* population was very highly resistant to sulfosulfuron and sulfometuron (both sulfonylurea herbicides) and also displayed low-level resistance to imazamox (an imidazolinone herbicide). Reduced sensitivity of the ALS enzyme was identified with *in vitro* activity assays. Gene sequence analysis revealed a proline-to-threonine substitution at amino acid position 197 of ALS, which is likely to be the molecular basis for resistance in this population. Herbicide screening also revealed a different *H. leporinum* population with resistance to the bipyrindyl herbicide paraquat.

**CONCLUSION:** This study established the first cases of (1) sulfonylurea-to-imidazolinone cross-resistance and (2) field-evolved paraquat resistance in a *Hordeum* species in Western Australia.

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**Keywords:** resistance survey; resistance evolution; *Hordeum leporinum*; acetolactate synthase; paraquat

## 1 INTRODUCTION

*Hordeum* species including *H. glaucum* Steud. and *H. leporinum* Link (collectively known as barley grass) are important grass weeds in most parts of the world. These self-pollinating species are abundant in crops and pastures in southern Australia, with both species widespread across grain-growing regions.<sup>1</sup> *Hordeum* was introduced into Australia in the early days of European settlement, and is now ubiquitous in the annual pasture zone of southern Australia. It is considered undesirable for livestock because, although it can provide useful early feed and fodder during the year, at maturity its seeds can enter the skin and eyes of sheep as well as contaminating their wool, which may result in lost productivity.<sup>2</sup> In cereal-growing regions, *Hordeum* may also act as a host to cereal diseases.<sup>1,3</sup> *Hordeum* spp. are annual winter-growing plants and have become a problem in cropping fields in recent years as they are often difficult to control in wheat crops.<sup>4</sup> Two of the *Hordeum* species in Australia, *H. glaucum* and *H. leporinum*, have cases where herbicide resistance to non-selective (paraquat and diquat) and post-emergent selective (sulfosulfuron and sulfometuron) herbicides has been reported.<sup>5–7</sup>

Paraquat is a non-selective, broad-spectrum, rapid-action herbicide that has been used commercially since the 1970s. It is widely used in agricultural systems, generally as a burndown herbicide for grass weed control in minimum tillage and no-tillage cropping systems prior to planting. In contrast, the selective acetolactate synthase (ALS)- and acetyl-coenzyme A carboxylase (ACCCase)-inhibiting herbicides are used for post-planting control

of monocot weeds in a range of crops. ALS-inhibiting herbicides, including the sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl-thiobenzoates (PTBs) and sulfonylamino-carbonyl-triazolinones (SCTs), have been widely used because of their broad spectrum, low application rates, high efficacy in inhibiting the essential process of branched-chain amino acid synthesis and good crop safety.<sup>8,9,10</sup> Until recently, sulfosulfuron (Monza®), an SU-class herbicide, was the only product registered in Australia for selective post-emergent weed control of *Hordeum* spp. in wheat;<sup>4</sup> however, its efficacy can be poor on large plants and may only suppress, rather than kill, younger growing plants.<sup>4,11</sup> Herbicide persistence and rotational restrictions are two factors that may restrict its adoption.<sup>11</sup> The introduction of IMI-tolerant wheat (Clearfield™) to Australian growers provides the opportunity for effective control of *Hordeum* spp. with good crop safety.<sup>12</sup> IMI herbicides also have the advantage of controlling a number of other grass weeds.<sup>13</sup>

With continued and widespread use of both selective and non-selective herbicides, resistant populations have evolved. Worldwide, biotypes of 25 weed species from 13 countries have

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evolved paraquat resistance, while there are 113 species that are resistant to ALS-inhibiting herbicides,<sup>14</sup> either through mutations in the ALS protein (causing it to become insensitive to enzyme inhibitors) or through enhanced rates of metabolism (allowing the herbicide to be detoxified more rapidly). In eastern Australia, evolved paraquat resistance has been reported in a number of species, including *H. glaucum*<sup>7</sup> and *H. leporinum*,<sup>6</sup> as well as in *Arctotheca calendula*,<sup>15</sup> *Vulpia bromoides*<sup>16</sup> and, more recently, *Lolium rigidum*.<sup>14</sup> To date, there have been no reported cases of paraquat resistance in Western Australia, whereas resistance to the SU class of ALS-inhibiting herbicides is widespread in *L. rigidum*<sup>17,18</sup> and has also been observed in *Raphanus raphanistrum*<sup>19</sup> and *H. leporinum*.<sup>5</sup> The SU-resistant *H. leporinum* population was found to be susceptible to three IMI herbicides (imazamoz, imazapyr and imazapic + imazapyr) at field rates.<sup>5</sup> So far, there have been no reported cases of IMI-resistant *Hordeum* populations anywhere in the world.<sup>14</sup>

This study documents the first known case of resistance to both SU- and IMI-class ALS-inhibiting herbicides in an Australian population of *H. leporinum*, and the first case of field-evolved paraquat resistance in a Western Australian *H. leporinum* population. The herbicide resistance profile of these populations is also characterised.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

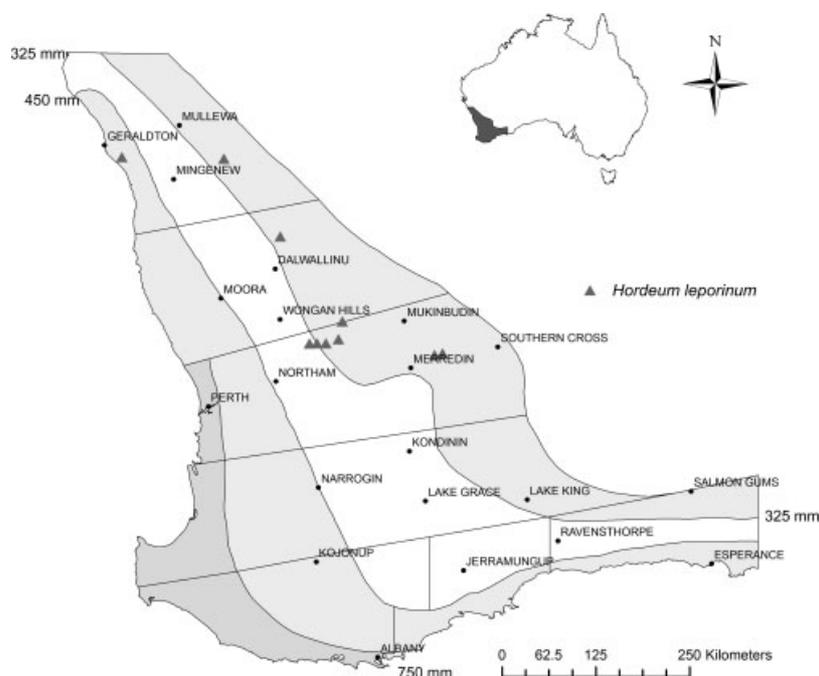
In 2005, weeds were sampled from crop fields during the months of October and November as part of a herbicide resistance survey, detailed in a previous paper (see Owen and Powles<sup>20</sup> for sampling details). The survey covered a 14 million ha region of the WA grain belt and sampled 677 cropping fields (Fig. 1). Mature *H. leporinum* seeds were collected from several plants in the sample area. Populations were only included in the study if there was enough seed to form a representative sample. To release

seed dormancy,<sup>21</sup> seed samples were stored in a glasshouse for 4 months with an average daily temperature of 26 °C over the summer months. Samples were then stored in the laboratory until screening. In total, nine *H. leporinum* populations were screened for resistance.

Starting in May 2008, *H. leporinum* seeds from each population were germinated for 5–7 days on 1% (w/v) agar at room temperature under normal room lighting. Fifty seedlings from each population were transplanted into potting mix (50% composted pine bark, 25% peat, 25% river sand) in plastic seedling trays and grown outdoors at the University of Western Australia during the May–September growing season. Plants were watered and fertilised as needed.

### 2.2 Herbicide resistance screening

The susceptibility of the *H. leporinum* biotypes to a range of herbicide chemistries was tested. At the 2–3-leaf stage of development, seedlings were treated with herbicide using a custom-built, dual-nozzle cabinet sprayer delivering herbicide in 100 L ha<sup>-1</sup> water at 210 kPa at a speed of 3.6 km h<sup>-1</sup>. The plants were sprayed at field rates known to control susceptible biotypes: 78 g ha<sup>-1</sup> fluazifop (Fusilade Forte 128 g L<sup>-1</sup> EC; Syngenta), 104 g ha<sup>-1</sup> haloxyfop (Verdict 520 g L<sup>-1</sup> EC; Dow Agroscience), 186 g ha<sup>-1</sup> sethoxydim (Sertin 186 g L<sup>-1</sup> EC; BayerCropscience), 60 g ha<sup>-1</sup> clethodim (Select 240 g L<sup>-1</sup> EC; Sumitomo), 37.5 g ha<sup>-1</sup> sulfosulfuron (Monza<sup>TM</sup> 750 g kg<sup>-1</sup> WG; Monsanto), 15 g ha<sup>-1</sup> sulfometuron (Oust<sup>®</sup> 750 g kg<sup>-1</sup> WG; Dupont), 21 g ha<sup>-1</sup> imazapic (75%) + 7 g ha<sup>-1</sup> imazapyr (25%) (Onduty<sup>®</sup> 525/175 g kg<sup>-1</sup> WG; BASF), 31.5 g ha<sup>-1</sup> imazamox (Raptor 700 g kg<sup>-1</sup> WG; BASF), 300 g ha<sup>-1</sup> paraquat (Gramoxone<sup>®</sup> 250 g L<sup>-1</sup> AC; Syngenta) or 540 g ha<sup>-1</sup> glyphosate (Roundup Power Max<sup>®</sup> 540 g L<sup>-1</sup> AC; Monsanto). Plants were visually assessed for survival 21 days after spraying, and were scored dead if the growing points of the plants were chlorotic and no new growth had formed. There were two replicates of



**Figure 1.** Map of south-western Western Australia showing the agronomic zones of the grain belt where *H. leporinum* samples were collected for herbicide resistance testing. Average annual rainfall isohyets are shown.

**Table 1.** Resistance status across differing herbicide chemistries for resistant *H. leporinum* biotypes WAHL8 and WAHL13. S denotes that the population was completely susceptible (all plants died) to the herbicide, and R denotes that the population was resistant (>95% survival for ALS herbicides and >30% for the bipyridyl herbicide). All other populations collected were susceptible to all herbicides tested

Herbicide chemical class	Herbicide mode of action	Active ingredient	Resistance status	
			WAHL8	WAHL13
Aryloxyphenoxypropionate	Inhibition of ACCase	Fluazifop	S	S
	Inhibition of ACCase	Haloxifop	S	S
Cyclohexanedione	Inhibition of ACCase	Sethoxydim	S	S
	Inhibition of ACCase	Clethodim	S	S
Sulfonylurea	Inhibition of ALS	Sulfometuron	S	R
	Inhibition of ALS	Sulfosulfuron	S	R
Imidazolinone	Inhibition of ALS	Imazapic + imazapyr	S	R
	Inhibition of ALS	Imazamox	S	R
Bipyridyl	Inhibition of photosystem I	Paraquat	R	S
Glycine	Inhibition of EPSPS	Glyphosate	S	S

50 plants each for all of the herbicide biotype treatments. For the SU herbicide, all plants in the resistant population survived, whereas for paraquat only 30% of the field population survived. Survivors were allowed to set seed, and this seed was used for further dose–response experiments.

Known resistant and susceptible populations<sup>5</sup> were used as controls for each herbicide treatment. In all experiments, with all herbicides, 100% mortality occurred in the known susceptible population, whereas with the known resistant populations there was always very high survival (>90%) with all herbicides used (data not shown).

### 2.3 Dose response to ALS herbicides

During the 2009 growing season (May to September), the level of resistance to ALS herbicides of each of the putative ALS-resistant *H. leporinum* populations was determined. Resistant (R) and susceptible (S) plants were sprayed with sulfometuron 750 g kg<sup>-1</sup> WG containing 0.25% (v/v) wetting agent (BS1000; Nufarm Australia) at 0, 3.75, 7.5, 15, 30, 60, 120 or 240 g ha<sup>-1</sup>, with imazamox 700 g kg<sup>-1</sup> WG together with 2% crop oil (Hasten; Victorian Chemicals Australia) at 0, 8, 16, 32, 64, 128, 256 or 512 g ha<sup>-1</sup> or with sulfosulfuron 750 g kg<sup>-1</sup> WG at 0, 37.5, 75, 150 or 300 g ha<sup>-1</sup>. Plants were maintained outdoors and the above-ground shoot material was harvested 28 days after spraying for determination of dry mass (material was dried for 72 h at 70 °C). The experiment contained 20 seedlings for each herbicide rate, was randomised with three replicates for each treatment and was conducted twice during the growing season.

### 2.4 Dose response to paraquat

During the 2009 growing season, dose–response studies were conducted to determine the level of resistance in the putative paraquat-resistant *H. leporinum* population. Paraquat 250 g kg<sup>-1</sup> AC containing 0.25% BS1000 was applied to R plants at 0, 62.5, 125, 250, 500, 1000, 2000 or 4000 g ha<sup>-1</sup>, and to S plants at 0, 16, 32, 62.5, 125, 250, 500 or 1000 g ha<sup>-1</sup>. Plants were maintained and assessed as for the ALS study above. The experiment was performed twice, contained four replicates of 40 seedlings for each treatment and was randomised.

### 2.5 In vitro ALS assay

Soluble leaf protein from S and R biotypes (four replicates of each, consisting of leaf blades collected from 4–8 individuals) was extracted using a modified method of Ray,<sup>22</sup> with all steps performed at 4 °C. Leaf blades were ground to powder in liquid nitrogen and extracted in 3 vol grinding buffer [0.1 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 1 mM sodium pyruvate, 0.5 mM MgCl<sub>2</sub>, 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]. The clarified extract was desalted into phosphate buffer (0.1 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 20 mM sodium pyruvate, 0.5 mM MgCl<sub>2</sub>, 1 mM DTT) on PD-10 columns (Pharmacia) and then assayed for ALS activity.

Enzyme and colour reactions were performed as in Ray,<sup>22</sup> except that enzyme reactions contained 185 μL desalted leaf extract in a final volume of 250 μL (rather than 500 μL), and so the volume of the colour reaction in Ray<sup>22</sup> was halved accordingly. To determine the sensitivity of ALS to inhibitors, technical-grade sulfometuron (Nufarm) or imazamox (Nufarm) at 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 or 100 μM was included in the enzyme reactions. Samples were incubated at 30 °C for 60 min (pilot studies showed that the enzyme reaction rate was linear over this time). Negative controls were inactivated with 0.55 N H<sub>2</sub>SO<sub>4</sub> prior to incubation at 30 °C, while the other reactions were stopped with H<sub>2</sub>SO<sub>4</sub> at the end of the incubation. Colour development after treatment with creatine and α-naphthol was measured spectrophotometrically at 530 nm, with acetoin (0–75 nmol) used as a standard. Total protein in leaf extracts was measured in duplicate according to Bradford<sup>23</sup> using BioRad dye reagent concentrate, with 0–25 μg bovine serum albumin used as a standard.

### 2.6 Detection of ALS gene mutations

Genomic DNA was extracted from leaf blades of S and R (one leaf from each) individuals using the phenol:chloroform extraction method of Guidet *et al.*,<sup>24</sup> scaled down to fit into a 1.5 mL microcentrifuge tube. DNA fragments corresponding to the regions surrounding the codons for Pro197 (fragment 1: 381 bp) and Trp574 (fragment 2: 1396 bp) were amplified by polymerase chain reaction using the primers from Yu *et al.*<sup>5</sup> for fragment 1 and Yu *et al.*<sup>25</sup> (primer pair ALSF197 and ALSR574) for fragment 2. Each 25 μL reaction contained 300 ng of genomic

DNA, 0.5  $\mu\text{M}$  of each primer and 12.5  $\mu\text{L}$  of KAPA Taq PCR 2 $\times$  ReadyMix (KAPA Biosystems), and was carried out in a Hybaid PCR Express thermocycler with the following reaction profile: 94 °C for 1 min, then 35 (fragment 1) or 40 (fragment 2) cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 (fragment 1) or 10 (fragment 2) min. PCR fragments were purified from agarose gels using a MinElute gel extraction kit (Qiagen) and then sequenced from the 3'-end with the reverse primer used in the fragment amplifications. Sequencing reactions were carried out using AB-BigDye Terminator with the following reaction profile: 96 °C for 30 s, then 30 cycles of 96 °C for 30 s, 50 °C for 5 s and 60 °C for 4 min. They were analysed at the Lotterywest State Biomedical Facility at Queen Elizabeth II hospital, Western Australia.

## 2.7 Statistical analysis

Datasets from repeated experiments were analysed by ANOVA (GenStat), with independent experiments included as a main factor (experiment). When the experiment factor between repeated experiments was not significant, pooled data were used for subsequent non-linear regression analysis.

The herbicide rate causing 50% mortality ( $\text{LD}_{50}$ ) or growth reduction ( $\text{GR}_{50}$ ) of plants and the herbicide concentration causing 50% inhibition ( $I_{50}$ ) of the enzyme activity were estimated by non-linear regression analysis using Sigma Plot software (v.11.0). The data were fitted to the log-logistic model<sup>26</sup>

$$y = C + \left\{ \frac{(D - C)}{[1 + (X/\text{ED}_{50})^b]} \right\}$$

where  $C$  is the lower limit,  $D$  is the upper limit,  $b$  is the slope and  $\text{ED}_{50}$  is the dose causing 50% reduction. The level of resistance was measured as the R : S (resistant : susceptible) ratio of estimated  $\text{LD}_{50}$  values. A  $t$ -test ( $P = 0.05$ ) was used to determine the level of significance. Mortality dose–response graphs are presented with untransformed data.

## 3 RESULTS

### 3.1 Herbicide resistance screening

In total, nine *Hordeum* populations were screened with a range of herbicide modes of action known to have activity on this grass weed. All *Hordeum* populations were susceptible to all herbicides tested except for one population (WAHL8) that exhibited resistance to paraquat, and another population (WAHL13) that displayed cross-resistance to ALS-inhibiting sulfonylurea and imidazolinone herbicides (Table 1).

### 3.2 Dose response to ALS herbicides

Dose–response studies confirmed *H. leporinum* population WAHL13 to be resistant to the ALS-inhibiting herbicides sulfometuron, sulfosulfuron and imazamox (Table 1). At the recommended field rate of 15 g ha<sup>-1</sup> for sulfometuron and 19 g ha<sup>-1</sup> for sulfosulfuron, the R biotype was unaffected, whereas the S biotype had significant mortality (Fig. 2). There was no reduction in R plant survival even at the highest rates of sulfometuron or sulfosulfuron; therefore,  $\text{LD}_{50}$  estimates could not be obtained but are higher than the dose required to control 50% of the susceptible population (Figs 2a and b; Table 2). There was no reduction in plant survival at the field rate of imazamox (32 g ha<sup>-1</sup>), but the population was, however, affected by higher rates of imazamox (Fig. 2b). The lethal dose required to control 50% of the population

**Table 2.**  $\text{LD}_{50}$  and  $\text{GR}_{50}$  values (with standard errors in parentheses) of WAHL13 population treated with sulfometuron and imazamox. R/S ratios were calculated as the ratio of  $\text{LD}_{50}$  and  $\text{GR}_{50}$  values of resistant and susceptible populations.<sup>a</sup> Data are means  $\pm$  SE of two experiments, each containing three replicates

Biotype	$\text{LD}_{50}$ (g ha <sup>-1</sup> )	R/S ratio of $\text{LD}_{50}$	$\text{GR}_{50}$ (g ha <sup>-1</sup> )	R/S ratio of $\text{GR}_{50}$
Sulfometuron:				
susceptible	10 (0.4)	n/a	15 (4.1)	n/a
resistant	>240	>24	>240	>16
Imazamox:				
susceptible	25 (0.3)	n/a	38 (6.1)	n/a
resistant	105 (3.2)	4.2	50 (8.1)	1.2

<sup>a</sup> Abbreviations:  $\text{LD}_{50}$ , the dose lethal to 50% of the population;  $\text{GR}_{50}$ , the dose required to reduce biomass by 50%; R, resistant; S, susceptible.

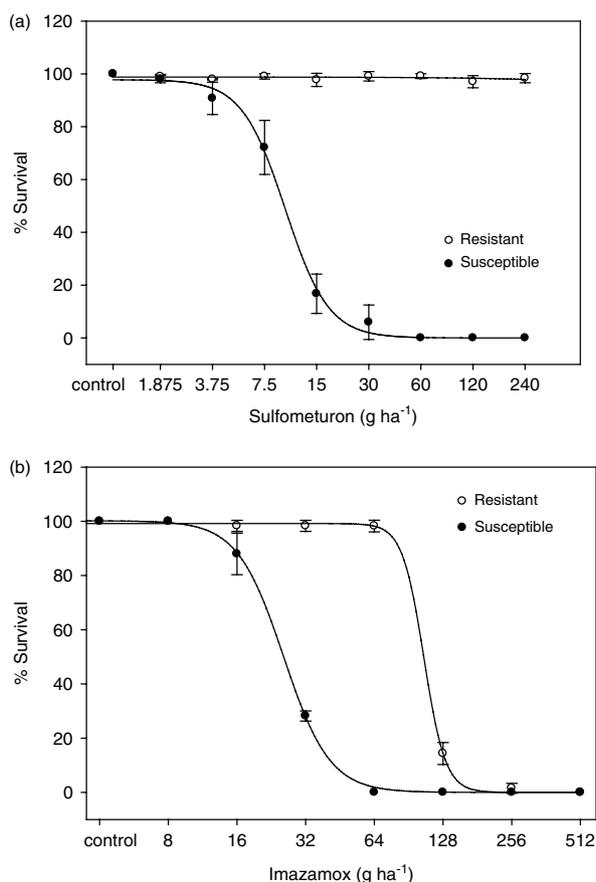
was 105 g ha<sup>-1</sup>, fourfold that to control the susceptible population (Fig. 2b; Table 2). However, biomass reduction was variable, and the dose required to reduce biomass to 50% ( $\text{GR}_{50}$ ) was 50 g ha<sup>-1</sup>, only 1.2-fold higher than that of the susceptible population (Table 2). The ALS-herbicide-resistant and -susceptible biotypes were susceptible to the ACCase-inhibiting herbicides fluazifop, haloxyfop, sethoxydim and clethodim, the bipyrindyl herbicide paraquat and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibiting herbicide glyphosate.

### 3.3 Dose response to paraquat

Dose response studies confirmed population WAHL8 to be resistant to paraquat. This population survived up to 16 times the recommended field rate (250 g ha<sup>-1</sup>) of paraquat (Fig. 3). While plant survival was unaffected in the R biotype, biomass production was reduced to 40% at the field rate of paraquat (data not shown). The rate causing 50% reduction in biomass for the R biotype was 28 g ha<sup>-1</sup>, which is fivefold higher than that of S, although this reduction was variable. In comparison, the lethal dose required to control R was 2583 g ha<sup>-1</sup>, more than 200-fold that of the susceptible (Table 3). There was no evidence of resistance to other herbicides with R and S biotypes equally susceptible to fluazifop, clethodim, sulfosulfuron, imazamox and glyphosate. When paraquat was applied during the warmer conditions of autumn (April), the R biotype showed a dramatic decrease in the level of resistance (35% survival) and a greater reduction in biomass (data not shown) at the recommended field rate. During the cooler winter months, a much higher level of resistance (close to 100% survival) was observed.

### 3.4 In vitro ALS activity and inhibition

In the absence of inhibitors, ALS activity was similar in the R and S biotypes. Inclusion of technical-grade sulfometuron in the enzyme reaction caused high variability between replicates, especially with the S biotype at lower concentrations (Fig. 4). However, there was a clear (threefold) difference between the S and R biotypes at 0.1, 1 and 10  $\mu\text{M}$  sulfometuron, before the activities of both biotypes decreased rapidly at 100  $\mu\text{M}$  (Fig. 4). The concentration of sulfometuron required to inhibit 50% ( $I_{50}$ ) enzyme activity of R was 11.31  $\mu\text{M}$ , while that required to inhibit 50% of S was 0.023  $\mu\text{M}$ . The effect of imazamox was similar to that of sulfometuron, with the activity of both R and S biotypes decreasing with increasing rates of herbicide (data not shown).



**Figure 2.** Dose–response curves for survival of an ALS-susceptible *H. leporinum* population (●) and an ALS-resistant *H. leporinum* population (Wahl13) (○) treated with a range of (a) sulfometuron doses and (b) imazamox doses. Each data point represents the mean percentage survival  $\pm$  SE of three replicate treatments.

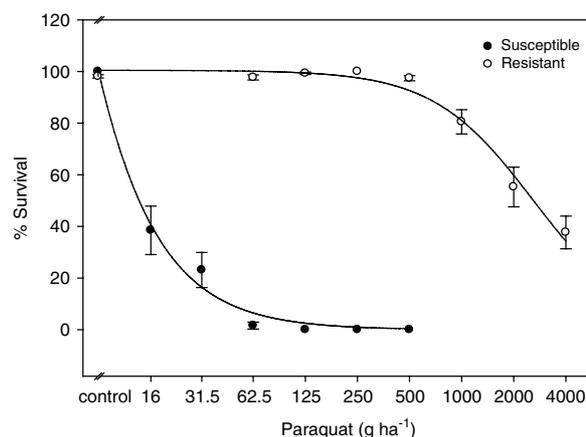
**Table 3.** LD<sub>50</sub> and GR<sub>50</sub> values (with standard errors in parentheses) of WAHL8 population treated with paraquat. R/S ratios were calculated as the ratio of LD<sub>50</sub> and GR<sub>50</sub> values of resistant and susceptible populations.<sup>a</sup> Data are means  $\pm$  SE of two experiments, each containing four replicates

Biotype	LD <sub>50</sub> (g ha <sup>-1</sup> )	R/S ratio of LD <sub>50</sub>	GR <sub>50</sub> (g ha <sup>-1</sup> )	R/S ratio of GR <sub>50</sub>
Susceptible	13 (1.8)	n/a	5 (1.1)	n/a
Resistant	2583 (201)	198.7	28 (18.4)	5.2

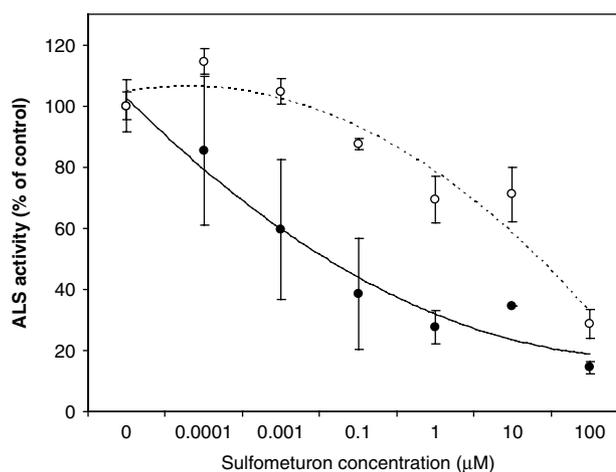
<sup>a</sup> Abbreviations: LD<sub>50</sub>, the dose lethal to 50% of the population; GR<sub>50</sub>, the dose required to reduce growth by 50%; R, resistant; S, susceptible.

### 3.5 Detection of ALS gene mutation

Previous studies have shown that most mutations of the ALS gene occur in the regions corresponding to positions Trp574 and Pro197 (reviewed in Powles and Yu<sup>27</sup>), and therefore sequencing focused on these regions. Both S and R biotypes had the wild-type (susceptible) codon TGG at the position corresponding to Trp574 (data not shown); however, sequencing of the region around the Pro197 codon confirmed a C-to-A point mutation in the R biotype, which changes proline (CCC) to threonine (ACC) (Fig. 5). The other two point mutations found (Fig. 5) were silent and did not change the amino acid sequence.



**Figure 3.** Dose–response curves for survival of a paraquat-susceptible *H. leporinum* population (●) and a paraquat-resistant *H. leporinum* population (WAHL8) (○) treated with a range of paraquat doses. Each data point represents the mean percentage survival of two experiments  $\pm$  SE each with four replicate treatments.

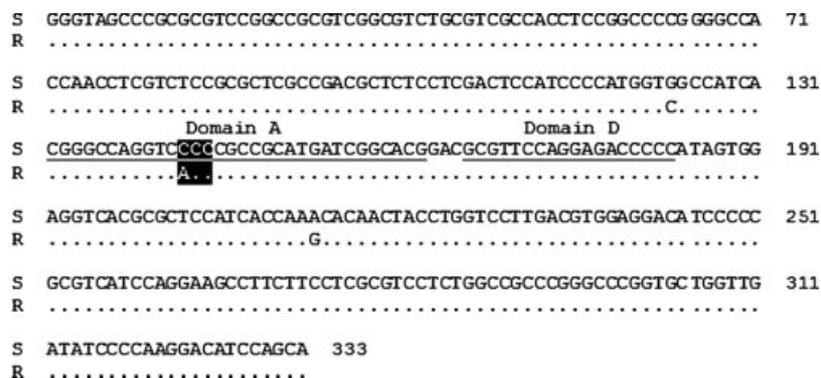


**Figure 4.** *In vitro* ALS enzyme activity for S (●) and R (○) biotypes in the presence of increasing concentrations of sulfometuron. ALS activity is expressed as the percentage of activity in the absence of herbicide (control activity). Each data point represents the mean activity  $\pm$  SE of three replicate treatments. The specific activity of the enzyme (nmol min<sup>-1</sup> mg<sup>-1</sup> protein) at 0  $\mu$ M sulfometuron (i.e. the control activity) was 1.42  $\pm$  0.063 for R (WAHL13) and 1.45  $\pm$  0.123 for S.

## 4 DISCUSSION

This study characterises the first known instance of resistance to both SU- and IMI-class ALS-inhibiting herbicides in a *H. leporinum* population. The first reported case of resistance to a SU herbicide (sulfosulfuron) in *H. leporinum* occurred in a population collected from the Western Australian wheat belt in 2004 and appeared without prior exposure to sulfosulfuron.<sup>5</sup> The repeated use of other SU herbicides for the control of other weed species in the area has thus provided the selection pressure for the development of resistance to sulfosulfuron.<sup>5</sup> The *H. leporinum* population used in the present study, WAHL13, was found to be not only highly resistant to the SU herbicides sulfosulfuron and sulfometuron (Fig. 2a; Table 2) but also moderately resistant to the IMI-class herbicides (Fig. 2b; Table 1).

*In vitro* enzyme activity assays confirmed that the ALS enzyme of WAHL13 was relatively insensitive to SU (Fig. 4) and IMI herbicides.



**Figure 5.** Alignment of the sequenced portion of the *H. leporinum* ALS gene from susceptible and resistant biotypes. The region around the codon corresponding to Pro197 was amplified and sequenced with ALS-specific primers; the highly-conserved A and D domains are underlined. Conserved bases between the S and R biotypes are represented by dots; the codon corresponding to Pro197 is in white type on a black background.

A proline-to-threonine substitution at amino acid position 197 of ALS, identified by sequencing of PCR fragments, is likely to be the molecular basis for resistance in this *H. leporinum* population. The cross-resistance pattern observed in WAHL13, i.e. high-level resistance to the SU herbicides and moderate-level resistance to the IMI herbicides (Fig. 2), is consistent with that of other resistant species possessing the Pro<sub>197</sub>-to-Thr substitution in their ALS enzyme, e.g. the dicots *Lactuca serriola*<sup>28</sup> and *Papaver rhoeas* L.<sup>29</sup> and the monocots *Alopecurus myosuroides*<sup>30</sup> and *Apera spica-venti*<sup>10</sup> (see also Powles and Yu<sup>27</sup> and Preston<sup>28</sup>). The proline-to-threonine mutation confers a different cross-resistance profile compared with the SU-specific resistance that was conferred by a proline-to-serine mutation at amino acid position 197.<sup>5</sup> The present authors believe that the different mutation is responsible for the differing resistance profiles, as the first population had no cross-resistance to the IMI herbicides when field rates were used.<sup>5</sup> This is generally consistent with previous studies on species with Pro-197-Ser and Pro-197-Thr mutations<sup>31</sup> (also reviewed in Powles and Yu<sup>27</sup>). Of the eight species with Pro-197-Ser mutations for which resistance to both SU and IMI was assessed, six were resistant to SU only; and of the six assessed species with Pro-197-Thr mutations, four were resistant to both SU and IMI, and two to SU only.<sup>31</sup>

The use of ACCase-inhibiting herbicides will initially allow the control of this SU- and IMI-resistant population; however, integrated weed management techniques should be used to minimise its risk of developing additional herbicide resistance, as there are reports of ACCase- and bipyridilium-resistant *Hordeum* populations from South Australia.<sup>6,7,14,32</sup> Other research has shown that biotypes of *Cyperus difformis* L. and *Amaranthus hybridus* L. have cross-resistance to five classes of ALS-inhibiting herbicides,<sup>33,34</sup> while many species have broad cross-resistance to ALS herbicides,<sup>31</sup> which means that the control of these biotypes with ALS-inhibiting herbicides is no longer possible.

This is the first report of paraquat resistance in an *H. leporinum* population from the Western Australian cropping region, although paraquat resistance in *Hordeum* spp. has been reported elsewhere in Australia.<sup>6,7,35</sup> Results from this study established that the WAHL8 population was highly resistant (> 195 fold) to paraquat (Fig. 3; Table 3) while remaining susceptible to other herbicide chemistries (Table 1). Therefore, the control of this population can be achieved using either glyphosate or ACCase- and ALS-inhibiting herbicides. As with other studies,<sup>35</sup> different levels of resistance were observed under different temperature conditions, with

higher temperatures causing a decrease in resistance levels (data not shown). The mechanism of paraquat resistance in a study by Purba<sup>35</sup> was found to be reduced translocation of paraquat to the growing tissue, and reduced penetration of herbicide to the active site of photosystem I. This and other studies<sup>36,37</sup> with paraquat-resistant *Hordeum* spp. have demonstrated that this mechanism of resistance is broken down at higher temperatures. Therefore, although paraquat resistance has developed in population WAHL8, an acceptable level of control with this herbicide may still be achieved under warm to hot temperatures.

To summarise, the first cases of (1) SU-to-IMI cross-resistance and (2) field-evolved paraquat resistance in *H. leporinum* in Western Australia have been identified in this study. An amino acid substitution (proline to threonine) at position 197 of the ALS enzyme, reducing its sensitivity to both SU and IMI herbicides, was the mechanism conferring resistance in the former case. With the continued use of ALS-inhibiting herbicides, it is likely that the selection of other resistant *Hordeum* populations will occur; therefore, management options that combine a number of weed control techniques are important to slow the rate of resistance evolution.

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