

# Evolution of herbicide resistance in weeds: initial frequency of target site-based resistance to acetolactate synthase-inhibiting herbicides in *Lolium rigidum*

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The frequency of individuals resistant to two acetolactate synthase (ALS)-inhibiting herbicides in three previously untreated populations of *Lolium rigidum* was determined. The frequency of individuals resistant to the sulfonylurea herbicide sulfometuron-methyl varied from  $2.2 \times 10^{-5}$  to  $1.2 \times 10^{-4}$  and the frequency of individuals resistant to the imidazolinone herbicide imazapyr varied from  $1 \times 10^{-5}$  to  $5.8 \times 10^{-5}$  depending on the population. Application of sulfometu-

ron-methyl selected individuals with a herbicide-insensitive ALS, which was also cross-resistant to imazapyr. The high initial frequency of individuals resistant to ALS-inhibiting herbicides in *L. rigidum* populations never previously exposed to these herbicides helps explain the rapid evolution of herbicide resistance in this species once ALS-inhibiting herbicides were used.

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## Introduction

Herbicides are used extensively for weed control in crop and fibre production systems throughout the world. One consequence of the extensive use of herbicides has been the appearance of herbicide resistance in weed species. Currently herbicide resistance has appeared in more than 140 weed species and many thousands of crop fields worldwide (Heap, 1999). Herbicide resistance is known in all areas where herbicides are used intensively (Powles *et al*, 1997). Major factors influence the evolution of herbicide resistance, including the intensity of selection by herbicides and the initial frequency of herbicide resistant individuals in the population (reviewed by Jasieniuk *et al*, 1996). The first factor is relatively easy to determine, but there is little information pertaining to the initial frequency of herbicide resistant individuals in weed populations (Maxwell and Mortimer, 1994; Jasieniuk *et al*, 1996).

In Australia, *Lolium rigidum* is a major annual grass weed of cropping systems. This species was introduced into Australia in the 19<sup>th</sup> century and is now present at high densities across vast areas. *L. rigidum* reduces crop yields dramatically at high densities and is a major target of herbicides in this area. Herbicide resistance first

appeared in *L. rigidum* in 1980 (Heap and Knight, 1982) and is now widespread across the more intensively cropped regions of southern Australia (Preston *et al*, 1999). In some areas more than 40% of cropping fields are infested with herbicide-resistant *L. rigidum* populations (Nietschke *et al*, 1996). Multiple herbicide resistant populations of *L. rigidum* can be simultaneously resistant to herbicides from more than 12 herbicide chemistries representing seven modes of action (Hall *et al*, 1994; Preston *et al*, 1996). The majority of cases of herbicide resistance in *L. rigidum* populations are to herbicides that inhibit acetyl-coenzyme A carboxylase (ACCCase) or acetolactate synthase (ALS). One unusual aspect of herbicide resistance in southern Australia has been the rapid evolution of resistance. Resistance to both ACCCase- and ALS-inhibiting herbicides has occurred with as few as three applications of these herbicides (Tardif *et al*, 1993; Powles *et al*, 1997; Preston *et al*, 1999). Gill (1995) established that of the *L. rigidum* populations sent for testing for herbicide resistance, all receiving four or more applications of ALS-inhibiting herbicides, were resistant to these herbicides.

Two major mechanisms endowing resistance to ALS-inhibiting herbicides in *L. rigidum* have been established, a modified ALS or enhanced metabolism of herbicides (Christopher *et al*, 1992). The latter mechanism is correlated with cross-resistance selected by ACCCase-inhibiting herbicides (Christopher *et al*, 1991). Populations of *L. rigidum* with metabolism-based resistance to ALS-inhibiting herbicides are not resistant to sulfometuron-methyl or imazapyr (Christopher *et al*, 1991). Gill (1995) demonstrated that most populations of *L. rigidum* resistant to

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ALS-inhibiting herbicides were also resistant to sulfometuron-methyl and, therefore, probably contained a herbicide-resistant ALS.

The rate of spontaneous mutations to resistance to ALS-inhibiting herbicides has been estimated at  $1 \times 10^{-9}$  in *Arabidopsis thaliana* (Haughn and Somerville, 1987) and at about  $2.7 \times 10^{-8}$  in *Nicotiana tabacum* cell cultures (Harms and DiMaio, 1991). Depending on the relative fitness of the resistant individuals, the actual frequency of resistant individuals in a natural population is likely to be up to two orders of magnitude higher than the mutation frequency (Jasieniuk *et al*, 1996). The aim of this study was to determine the initial frequency of resistance to ALS-inhibiting herbicides in *L. rigidum* so as to understand the rapid evolution of resistance to these herbicides.

## Materials and methods

### Plant material

Three susceptible populations of *L. rigidum* were used in this study. Population 1 was from a pasture in western Victoria where no herbicides had been used. This population had never been exposed to ALS-inhibiting herbicides and has been in our collection since 1988, well before the first reports of widespread resistance to ALS-inhibiting herbicides in Australia. Before 1990, no examples of target site-based resistance were known in Australia (Christopher *et al*, 1992). The other two populations were obtained from the south-east of South Australia. Population 2 was obtained in 1990 from an organic field where herbicides had never been used. Population 3 was obtained in 1996 from a field where tillage was used to control weeds and the only herbicide exposure this population had received was occasional application of paraquat in pastures. Paraquat acts as a radical generating herbicide through accepting electrons at photosystem I and cross resistance between paraquat and ALS inhibitors is neither known nor expected (Preston, 1994). The original seed was collected by commercial harvesting equipment, either directly (population 1) or as screenings from harvesting of grain (populations 2 and 3). Each sample came from a single field with more than 50 kg of seed in each sample.

### Field selection experiment

A field experiment was conducted at Roseworthy, South Australia during the winter of 1994. An area of  $90 \times 40$  m was sown to *L. rigidum* population 1. A stand of about 350 plants/m<sup>2</sup> was established. The area was divided into four plots of  $20 \times 45$  m and each plot further subdivided into a smaller subplot of  $20 \times 13.75$  m and a larger subplot of  $20 \times 31.25$  m. Herbicide was applied to the plots 6 weeks after sowing, when plants were at the three-leaf to two-tiller stage. A commercial formulation of sulfometuron-methyl was applied at two rates of 4 or 16 g/ha. The high rate was applied once to the smaller subplot and four applications of the low rate were applied, about 3 weeks apart to the large subplots. Therefore, both treatments received a total of 16 g/ha sulfometuron-methyl. The herbicide was applied by motorbike fitted with a 6 m boom applying 67 l/ha of water. All plots were sprayed with two passes each at half of the stated rate. Thirteen weeks after application of the first herbicide

treatment, survivors were assessed. Plants with at least five fertile tillers were counted as survivors and transplanted into plots. These individuals were grouped by treatment and allowed to cross-pollinate. Some smaller plants present in some plots were assumed to be later germinating seedlings and were not collected.

### Laboratory selection experiment

Seedling germination on agar containing sulfometuron-methyl is an effective way of identifying individuals with a herbicide-resistant ALS (Burnet *et al*, 1994). Agar (100 ml) containing 30  $\mu$ M sulfometuron-methyl was set in 1 litre plastic containers with 50 containers per replicate and four replicates spaced in time. Exactly 4 g of *L. rigidum* seed was placed in each container, as well as in a control container with herbicide-free agar. The containers were placed in a germination cabinet set at 19°C with a 12 h, 30  $\mu$ E/m<sup>2</sup> s light period and a 12 h dark period for 6 days. The expected number of germinants from 4 g of seed was calculated from the container without herbicide. Four replicates of this experiment were conducted for each *L. rigidum* population. Imazapyr can be used to select for resistant individuals in the same way (Preston C, unpublished results). Therefore, a second selection experiment was conducted using 100  $\mu$ M imazapyr as the selecting herbicide.

Seedlings that had a green leaf extending through the coleoptile were transplanted to pots containing potting soil and placed in a growth room with a 19°C, 12 h, 300  $\mu$ E/m<sup>2</sup> s light period and a 15°C, 12 h dark period. In addition, for each replicate of each population, one pot containing 12 unselected seedlings was also transplanted. At the two- to three-leaf stage, plants were treated with 16 g/ha of a commercial formulation of sulfometuron-methyl containing 0.2% non-ionic surfactant or 25 g/ha of a commercial formulation of imazapyr. Herbicide was applied using a laboratory spray cabinet fitted with flat-fan nozzles and an output of 130 l/ha. Following herbicide application, plants were returned to the growth room and assessed for survival after 21 days. Surviving plants from the sulfometuron-methyl selection were grouped by population and just before flowering were placed outdoors, surrounded by plastic sheeting and allowed to cross pollinate.

### Heritability of resistance

Two separate measures were made to determine whether the individuals selected by the herbicide treatments carried a heritable resistance trait.

Seeds (1.5 or 0.3 g) obtained from plants selected in the field or the laboratory and allowed to cross within selections, were placed on agar containing 30  $\mu$ M sulfometuron-methyl. An equivalent weight of seed was placed on agar without herbicide as a control. The seed was placed in the germination cabinet. After 6 days, the numbers of seedlings with a green leaf protruding through the coleoptile were counted. As target site-based resistance to ALS-inhibiting herbicides is inherited as a single, largely dominant gene (Shaner, 1999), it is expected the progeny will segregate 3:1 for resistance. The results were compared with the expected segregation using a  $\chi^2$  test.

A second experiment examined whether resistance to sulfometuron-methyl and imazapyr were being selected together. For this experiment, progeny of sulfometuron-

methyl or imazapyr laboratory selections were germinated, planted into pots and grown outdoors. When plants had reached the four- to five-tiller stage, each plant was divided into two. The two clones were trimmed to 1 cm of root and 2 cm of shoot and planted in separate pots. When the clones had recovered and produced 5 to 7 cm of new leaf growth they were sprayed with herbicide. One clone of each plant was treated with 16 g/ha of a commercial formulation of sulfometuron-methyl containing 0.2% non-ionic surfactant and one plant with 25 g/ha of a commercial formulation of imazapyr. Herbicides were applied using the laboratory spray cabinet. Plants were returned outdoors and assessed for survival after 21 days. Plants were counted as alive if they had produced new green leaf tissue since spraying. Each plant was assessed for survival to sulfometuron-methyl and imazapyr.

#### ALS assay

Seeds obtained from the plants selected in the field or the laboratory were placed on agar containing 30  $\mu$ M sulfometuron-methyl. After 6 days, germinated seedlings were transplanted to soil and grown in a growth room set at 19°C, 12 h, 400  $\mu$ E/m<sup>2</sup>s light and 15°C, 12 h dark regime for 3 weeks. Leaves (3 g) were harvested and ground in a cold mortar in 3 ml of ice-cold Buffer A (100 mM potassium phosphate buffer (pH 7.5), 1 mM MgCl<sub>2</sub>, 0.5 mM thiamine pyrophosphate, 10  $\mu$ M flavin adenine dinucleotide, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium pyruvate) with 5 mg/ml insoluble polyvinylpyrrolidone, and a small amount of acid-washed sand. The slurry was transferred to a centrifuge tube and the mortar washed with a further 3 ml of Buffer A. The solution was centrifuged at 20 000 g for 10 min and the supernatant brought to 50% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The solution was stirred for 30 minutes on ice, centrifuged at 20 000 g for 30 min and the pellet resuspended in 0.5 ml of Buffer A without dithiothreitol or phenylmethylsulfonyl fluoride. This solution was desalted on a Sephadex G9 column previously equilibrated with Buffer B (100 mM potassium phosphate buffer (pH 7.0), 200 mM sodium pyruvate, 20 mM MgCl<sub>2</sub>, 2 mM thiamine pyrophosphate and 20  $\mu$ M flavin adenine dinucleotide) and the protein collected in 2 ml Buffer B. The partially purified protein was assayed for ALS activity in the presence of sulfometuron-methyl or imazapyr in 400  $\mu$ l well microtitre plates. To each well, 50  $\mu$ l of herbicide solution containing twice the desired final concentration was added. Wells 1 and 7 in each row contained water and 20  $\mu$ l of 6 N H<sub>2</sub>SO<sub>4</sub> was added to well 7 to inactivate the enzyme. The reaction was started by addition of 50  $\mu$ l of enzyme extract and incubated for 30 min at 37°C. The reaction was stopped by addition of 20  $\mu$ l of 6 N H<sub>2</sub>SO<sub>4</sub>, which also served to decarboxylate acetolactate to acetoin. The decarboxylation reaction was allowed to proceed for 15 min at 60°C. Colour development occurred following the sequential additions of 95  $\mu$ l 0.55% creatine and 95  $\mu$ l 5.5%  $\alpha$ -naphthol in 5 N NaOH and incubation at 60°C for 15 min. Duplicate samples were pooled and absorption measured at 530 nm. Absorption occurring in the samples where H<sub>2</sub>SO<sub>4</sub> was added before the assay was subtracted from the absorption of all other samples. ALS activity in the presence of herbicide was calculated as percentage of activity in the absence of herbicide.

## Results

### Field selection

About  $1.12 \times 10^6$  herbicide susceptible *L. rigidum* plants (population 1) were treated with four applications of 4 g/ha (total of 16 g/ha) of sulfometuron-methyl and  $3.7 \times 10^5$  plants with a single application of 16 g/ha of sulfometuron-methyl. The herbicide caused massive mortality; however, there were some survivors. A total of 139 plants survived the lower rates of sulfometuron-methyl and 46 survived the single application of the higher rate. The average proportion of survivors was similar for the two treatments at  $1.2 \times 10^{-4}$  (Table 1).

### Laboratory selection

The vast majority of seeds placed on agar containing sulfometuron-methyl or imazapyr failed to germinate. However, between 700 and 1000 seedlings were recovered from 4 g of seed placed on the herbicide-free agar. Between 10 and 70 seedlings with green shoots emerging from the coleoptiles were transplanted to soil for each population in each replicate of the experiment. However, most of these seedlings did not grow in soil, probably because the meristematic region had already been killed by herbicide. Those individuals that did grow were subsequently treated with 16 g/ha sulfometuron-methyl or 25 g/ha imazapyr when at the two-leaf stage and all survived, whereas no unselected seedlings survived this treatment. For sulfometuron-methyl, there were 12 survivors from an expected  $2 \times 10^5$  germinants from population 1, 16 survivors from an expected  $1.6 \times 10^5$  germinants from population 2, and three survivors from an expected  $1.5 \times 10^5$  germinants from population 3 over all replicates. The frequency of survivors was variable between populations in this experiment and ranged from  $2.2 \times 10^{-5}$  to  $1.1 \times 10^{-4}$  (Table 1). For imazapyr there were fewer survivors. Population 1 had three survivors from an expected  $2.2 \times 10^5$  germinants, population 2 had 11 survivors from an expected  $2 \times 10^5$  germinants, and population 3 had two survivors from an expected  $1.6 \times 10^5$  germinants. The frequency of survivors to imazapyr varied from  $1 \times 10^{-5}$  to  $5.8 \times 10^{-5}$  between populations (Table 1).

### Heritability of resistance

A considerable amount of seed was set by the survivors of the field selection. However, as a result of differences in flowering time of sulfometuron-methyl-resistant individuals, only a few seeds were set on survivors of populations 1 and 2 and none on survivors from population 3 from the laboratory selection. Likewise, only a few seeds were set on imazapyr survivors of populations 2 and 3 and none on population 1. Assessment of heritability of sulfometuron-methyl resistance occurred following germinating seed on agar containing 30  $\mu$ M sulfometuron-methyl.

In all cases, seed germination ranged from 65 to 80% of germination on agar without herbicide (Table 2). Survivorship of the laboratory selections fit a 3:1 ratio of resistant to susceptible, suggesting a single dominant gene encodes resistance. Survivorship of the field selections did not fit this ratio and was lower than for the survivors of the laboratory selection. This suggests that a few susceptible escapes or individuals with less effective resistance may have survived in the field selection, com-

**Table 1** Frequency of survivors of sulfometuron-methyl or imazapyr selection in *L. rigidum* populations

Population	Selection treatment	Frequency of survivors
<i>Field experiments</i>		
Population 1	4 × 4 g/ha sulfometuron-methyl	1.24 × 10 <sup>-4</sup> ± 7.8 × 10 <sup>-6</sup>
Population 1	16 g/ha sulfometuron-methyl	1.24 × 10 <sup>-4</sup> ± 1.4 × 10 <sup>-5</sup>
<i>Laboratory experiments</i>		
Population 1	30 μM sulfometuron-methyl	4.6 × 10 <sup>-5</sup> ± 3.1 × 10 <sup>-5</sup>
Population 1	100 μM imazapyr	1.0 × 10 <sup>-5</sup> ± 0.6 × 10 <sup>-6</sup>
Population 2	30 μM sulfometuron-methyl	1.08 × 10 <sup>-4</sup> ± 1.9 × 10 <sup>-5</sup>
Population 2	100 μM imazapyr	5.8 × 10 <sup>-5</sup> ± 2.3 × 10 <sup>-5</sup>
Population 3	30 μM sulfometuron-methyl	2.2 × 10 <sup>-5</sup> ± 1.2 × 10 <sup>-5</sup>
Population 3	100 μM imazapyr	1.4 × 10 <sup>-5</sup> ± 0.8 × 10 <sup>-6</sup>

Populations were selected in the field or the laboratory with discriminating rates of herbicides. Values are means ± s.e. of four plots.

**Table 2** Chi-square analysis of segregation for sulfometuron-methyl resistance in progeny of sulfometuron-methyl selections

Population and selection with sulfometuron-methyl	Germinants (control)	Germinants (30 μM sulfometuron-methyl)	χ <sup>2</sup> probability (3:1 ratio)
Population 1 16 g field selection	290	190	0.0002
Population 1 4 × 4 g field selection	211	142	0.01
Population 1 laboratory selection	48	37	0.74
Population 2 laboratory selection	68	54	0.40

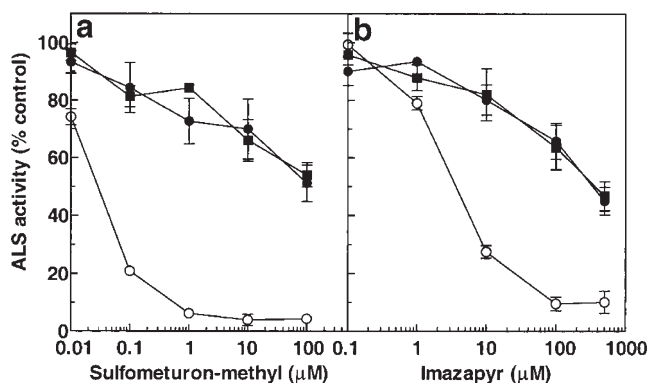
Seed (1.5 g or 0.3 g) was sown on agar or agar containing 30 μM sulfometuron-methyl.

pared with the laboratory selection. From the frequency of resistance in the progeny, it is estimated that as many as 8% of the plants collected from the field experiment may have been susceptible escapes.

When progeny of survivors of laboratory selections were examined for resistance to sulfometuron-methyl and imazapyr, only two phenotypes were observed. Plants were either resistant to sulfometuron-methyl and imazapyr or susceptible to both herbicides (Table 3). This result suggests that a single allele provides resistance to both herbicides in the selected populations.

#### ALS assay

As expected, the ALS enzyme extracted from the herbicide-susceptible *L. rigidum* population 1 was highly susceptible to the herbicides sulfometuron-methyl and imazapyr *in vitro* (Figure 1). ALS enzyme activity was greatly inhibited by 1 μM sulfometuron-methyl and 100 μM imazapyr. Susceptibility of ALS activity from the progeny of survivors of the field experiment was also measured. As the survivors of the herbicide treatment are expected to be heterozygous for the resistance gene, the progeny would be expected to segregate for resistance. Therefore,

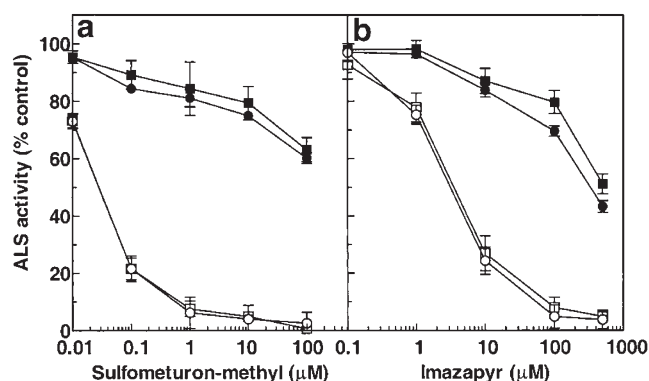


**Figure 1** Response to sulfometuron-methyl (a) and imazapyr (b) *in vitro* of ALS isolated from population 1 (○), and sulfometuron-methyl resistant progeny of the 4 × 4 g/ha (●) and 16 g/ha (■) selections from population 1. Each point is the mean ± s.e. of three determinations.

**Table 3** Phenotypes of individual plants from progeny of survivors of the laboratory selection regime

Population and selection	Plants susceptible to sulfometuron-methyl and imazapyr	Plants resistant to sulfometuron-methyl and imazapyr
Population 1 Sulfometuron-methyl	9	36
Population 2 Sulfometuron-methyl	8	32
Population 2 Imazapyr	9	26
Population 3 Imazapyr	6	17

Individual plants were cloned into two and clones treated with either 15 g/ha sulfometuron-methyl or 25 g/ha imazapyr. No individuals were resistant to only one herbicide.



**Figure 2** Response to sulfometuron-methyl (a) and imazapyr (b) *in vitro* of ALS isolated from population 1 (○), population 2 (□), and sulfometuron-methyl-resistant progeny of the selections from population 1 (●) and population 2 (■). Each point is the mean  $\pm$  s.e. of three determinations.

seeds were germinated on agar containing 30  $\mu\text{M}$  sulfometuron-methyl to remove susceptible individuals. Enzyme was extracted from leaves of survivors and assayed for susceptibility to herbicides. For both the 4  $\times$  4 g/ha and 16 g/ha sulfometuron-methyl selections, ALS activity was found to be highly resistant to sulfometuron-methyl and imazapyr (Figure 1). The level of resistance to sulfometuron-methyl and imazapyr was almost the same for both selections. Therefore, it is highly likely that the 4  $\times$  4 g/ha and 1  $\times$  16 g/ha sulfometuron-methyl selections provided identical selection pressures.

Sulfometuron-methyl- and imazapyr-inhibited ALS isolated from the susceptible populations 1 and 2 with similar inhibition kinetics (Figure 2). In contrast, ALS isolated from progeny of the laboratory selections from populations 1 and 2 were highly resistant to both sulfometuron-methyl and imazapyr. There was little difference in the level of resistance to sulfometuron-methyl between the resistant selections. However, ALS isolated from the population 2 selection was slightly more resistant to imazapyr (Figure 2).

## Discussion

This study has established that in natural populations of *L. rigidum* the frequency of individuals with an ALS enzyme resistant to the sulfonylurea herbicide sulfometuron-methyl ranges between  $4.6 \times 10^{-5}$  and  $1.2 \times 10^{-4}$ . In the same populations the frequency of individuals resistant to the imidazolinone herbicide imazapyr ranges between  $1 \times 10^{-5}$  and  $5.8 \times 10^{-5}$ . There were small differences in the frequency of sulfometuron-methyl resistant individuals observed in population 1 between the field experiment and the laboratory experiment (Table 1). This difference may be a result of different intensities of selection between the field and laboratory experiments. The sulfometuron-methyl selections from populations 1 and 2 also contained individuals resistant to imazapyr (Figures 1 and 2). As survivorship to sulfometuron and imazapyr within populations was not significantly different (*t* test), it seems likely that both herbicides selected mutations providing resistance to both herbicides. The linked inheritance of resistance to sulfometuron-methyl and imazapyr (Table 3) supports this conclusion.

The frequency of herbicide-resistant individuals in

weed populations is generally assumed to be about  $10^{-6}$  (Maxwell and Mortimer, 1994). However, there is no reason that this value should be uniform across herbicide modes of action. Indeed as demonstrated here for *L. rigidum*, resistance to ALS-inhibiting herbicides is up to 100 times more frequent. In a large population the equilibrium frequency of resistance is dependent on the mutation frequency and the fitness of the resistant mutation. The relationship for a partially or fully dominant gene is described by:

$$q_e = \mu/hs$$

where  $q_e$  is the equilibrium frequency of resistant alleles,  $\mu$  is the mutation frequency,  $h$  the degree of dominance, and  $s$  the fitness differential of the resistant allele in the absence of herbicide (Jasieniuk *et al.*, 1996). Assuming that the mutation frequency of  $10^{-8}$  to  $10^{-9}$ , calculated from selection experiments with *Arabidopsis thaliana* (Haugh and Somerville, 1987) and tobacco cell cultures (Harms and DiMaio, 1991), is similar in *L. rigidum*, then the resistant individuals should carry a fitness penalty of 0.01% compared with susceptible individuals. This is consistent with the lack of a measurable fitness penalty in plants containing mutations conferring resistance to ALS-inhibiting herbicides (Holt and Thill, 1994).

This study has exposed a surprisingly high frequency of individuals resistant to ALS-inhibiting herbicides in *L. rigidum* populations. It is possible that the high frequency of resistant individuals detected in the *L. rigidum* populations may have occurred by gene flow from outside the field. However, the fact that the frequencies of resistance in the three populations were similar, despite the populations being collected over 8 years during which time the incidence of resistance to these herbicides went from unknown to a major problem in Australia, argues against gene flow being important.

Mackenzie *et al.* (1997) performed a similar selection experiment on a commercial line of *L. perenne* using chlorsulfuron as the selecting agent and applying selection intensities to obtain 90 or 99.99% mortality of susceptible individuals. At the lower selection intensity the frequency of putative resistant individuals was  $4 \times 10^{-4}$  and at the higher selection intensity ranged from undetected to  $3 \times 10^{-5}$ . In the study of Mackenzie *et al.* (1997), the selected individuals did not contain a herbicide-insensitive ALS enzyme unlike the present study. Another study has determined high frequencies of individuals resistant to the Photosystem II-inhibiting triazine herbicides in *Chenopodium album* populations from private gardens in France (Darmency and Gasquez, 1990). However, in that case the relationship between the high initial frequency of resistance and the evolution of resistance in the field was not apparent.

Selection experiments using cell cultures in the absence of mutator chemicals have observed resistance cell lines at frequencies of  $10^{-7}$  in cotton (Rajasekaran *et al.*, 1996) and  $5 \times 10^{-8}$  in tobacco (Harms and DiMaio, 1991). As cell cultures are grown over a limited number of generations, the frequencies measured are likely to be closer to the mutation frequency than to the equilibrium frequency of resistant alleles.

This research may also have implications for selection in other systems. For example, selection for metal tolerance in plants occurs on old mine sites. Major genes contribute to some examples of metal tolerance and must be

present within populations for selection to occur (Macnair, 1993). Studies have demonstrated that some plant populations will evolve metal tolerance at some sites and not others (Al-Hiyaly *et al*, 1993). As rare genes will not be present in all small populations, large populations or a large number of populations will need to be treated to select for these rare genes.

A high initial gene frequency of resistant individuals within a population means that herbicide resistance will develop rapidly once herbicides are used. This has been the experience in *L. rigidum*, where all populations tested that had been exposed to four or more applications of sulfonylurea herbicides had more than 20% of individuals with resistance to triasulfuron (Gill, 1995). Had the initial frequency of resistant individuals been known before the introduction of the sulfonylurea herbicides, then the rapid evolution of resistance to these products could have been predicted and managed.

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