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# High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance

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The frequency of phenotypic resistance to herbicides in previously untreated weed populations and the herbicide dose applied to these populations are key determinants of the dynamics of selection for resistance. In total, 31 *Lolium rigidum* populations were collected from sites with no previous history of exposure to herbicides and where there was little probability of gene flow from adjacent resistant populations. The mean survival frequency across all 31 populations following two applications of commercial rates (375 g ha$^{-1}$) of the acetyl-coenzyme A carboxylase (ACCase) inhibiting herbicide, diclofop-methyl was 0.43%. Survivors from five of these populations were grown to maturity and seed was collected. Dose–response experiments compared population level resistance to diclofop-methyl in these selected lines with their original parent populations. A single cycle of herbicide selection significantly increased resistance in all populations (LD$_{50}$ R:S ratios ranged from 2.8 to 23.2), confirming the inheritance and genetic basis of phenotypic resistance. *In vitro* assays of ACCase inhibition by diclofop acid indicated that resistance was due to a non-target-site mechanism. Following selection with diclofop-methyl, the five *L. rigidum* populations exhibited diverse patterns of cross-resistance to ACCase and ALS-inhibiting herbicides, suggesting that different genes or gene combinations were responsible for resistance. The relevance of these results to the management of herbicide resistance are discussed.
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## Introduction

Evolved herbicide resistance now compromises crop weed management throughout the world (reviewed in Powles and Shaner, 2001). The dynamics of herbicide resistance evolution are governed by the biology of weedy plant species, by the genetic determination of the resistance trait and by herbicide characteristics and use patterns. Genetic factors include the frequency, dominance and inheritance of major (large phenotypic effect) and minor (small phenotypic effect) resistance-endowing alleles and the fitness of resistant genotypes in the presence and absence of the selecting agent (reviewed by Georghiou and Taylor, 1986; Maxwell and Mortimer, 1994; Jasieniuk *et al*, 1996). Important herbicide-related factors include herbicidal mode of action, environmental persistence and frequency of use. The role of herbicide dose in determining the evolutionary dynamics of herbicide resistance has often not been fully considered. Where high herbicide doses are applied, only those individuals possessing resistance alleles conferring a high level of resistance (major alleles) will survive and frequencies of resistance to these high doses will likely be low. Conversely, as dose is reduced, other weaker

resistance mechanisms (minor alleles) will enable survival, and observed frequencies of putative herbicide resistance will be higher. Neve and Powles (2005) have demonstrated that under recurrent selection at low herbicide doses, *Lolium rigidum* is able to rapidly evolve high levels of resistance as multiple weaker mechanisms are selected and enriched.

Changes in herbicide target-site sensitivity conferred by point mutations at single major genes (target-site resistance) account for the majority of documented field-evolved herbicide resistance (reviewed by Darmency, 1994; Jasieniuk *et al*, 1996; Tranel and Wright, 2002). Rates of spontaneous mutation vary from organism to organism and from locus to locus, ranging from $10^{-7}$ mutations per base pair per replication in some bacteriophages to $10^{-11}$ per base pair per replication in humans (Drake *et al*, 1998). Relatively little is known of per locus rates of mutation *in planta*, although for herbicide resistance, Haughn and Somerville (1987) estimated the spontaneous rate of target-site acetolactate synthase (ALS) gene mutations in *Arabidopsis thaliana* to be of the order of $10^{-9}$. In unselected, herbicide susceptible weed populations, major resistance alleles will be at a mutation-selection equilibrium determined by the mutation rate and the selective disadvantage of the mutant alleles. Modelling studies that simulate herbicide resistance evolution in the field usually assume initial major resistance allele frequencies of between $10^{-7}$ and $10^{-5}$ (Gressel and Segel, 1978; Diggle *et al*, 2003). Preston and Powles (2002) measured frequencies

between $1 \times 10^{-5}$ and $1.2 \times 10^{-4}$ for target-site ALS herbicide resistant mutants in three unselected *L. rigidum* populations.

In addition to variation at major resistance genes, continuous and heritable variation in susceptibility to low herbicide rates has been documented in weed populations (see Holliday and Putwain 1980; Jasieniuk *et al*, 1996; Patzoldt *et al*, 2002). While the genetic basis for this variation is not reported, continuous variation usually indicates polygenic control at numerous minor loci. Rates of spontaneous mutation at minor loci influencing a quantitative trait are approximately $10^{-2}$ per gamete per generation (Lande, 1983) and in view of this, we may expect more variation in herbicide susceptibility at minor, compared with major loci in populations not previously exposed to herbicides, resulting in quantitative variation in herbicide susceptibility. Where pesticide selection pressure acts within the normal range of continuous phenotypic variation for pesticide sensitivity, variation at minor resistance-endowing loci may be enriched and responses to selection will be polygenic (McKenzie and Batterham 1994; McKenzie, 2000).

The annual grass species, *L. rigidum* is the most severe weed of Australian agriculture. Since the 1970s, *L. rigidum* control has been dominated by herbicides and many populations have evolved multiple- and cross-resistance (Burnet *et al*, 1994; Preston *et al*, 1996; Neve *et al*, 2004), in some cases after as few as 3–4 herbicide applications (Gill 1995). In a large random survey conducted in 1998, 46% of *L. rigidum* populations were resistant to field application rates of the acetyl-coenzyme A caboxylase (ACCase) inhibiting herbicide diclofop-methyl (Llewellyn and Powles, 2001). Reasons for the unprecedented scale and rapid evolution of resistance to ACCase and other herbicides in Australian *L. rigidum* populations are not fully understood. In Australia, large farm sizes, low yields and small per area profit margins mean that herbicide application rates are often lower than elsewhere in the world. We postulate that these relatively low herbicide use rates select for variation in diverse nontarget site resistance mechanisms, which are initially present at high frequencies in unselected *L. rigidum* populations. Under recurrent herbicide selection, these unspecified mechanisms will accumulate and result in the rapid evolution of herbicide resistance.

This study quantifies variation in the levels of susceptibility to field applied rates of diclofop-methyl in 31 *L. rigidum* populations with no history of herbicide treatment. The response to selection with diclofop-methyl and the mechanistic basis of resistance is described for five of these populations and is discussed in terms of observed patterns of herbicide resistance for this species in Australia.

## Materials and methods

### Plant material
Seeds of 31 *L. rigidum* populations were collected from sites in Western Australia (16), Victoria (12) and South Australia (3) in 2001. Collection sites (waste ground, roadsides, parkland and unimproved pasture) were selected to minimize the likelihood of previous exposure to ACCase herbicides and to ensure that there was very little chance of gene flow from herbicide treated areas. In Western Australia all sites were more than 50 km from agricultural areas where these herbicides were used. In South Australia and Victoria, farmers were consulted to ensure that adjacent agricultural fields had no history of herbicide application. At each site seed was harvested from at least 50 individual plants. After collection, seed was stored under hot, dry summer conditions with fluctuating day/night temperatures to facilitate dormancy release. During April 2002, seed was threshed and cleaned and was stored at constant 20°C until use.

### Screen for diclofop-methyl resistance
In May 2002, 400–500 seeds of each of the 31 populations (four replicates) were evenly spread onto the surface of seedling trays ($28 \times 33 \times 5 \, cm^3$) containing a standard potting mixture (50% peat:50% sand). Seeds were covered with a 2 mm layer of potting mixture. Trays were maintained in a cooled glasshouse (maximum daily temperature $20 \pm 5°C$) and were watered on a daily basis. At the 2–3 leaf stage, the number of emerged seedlings was counted and the commercial herbicide diclofop-methyl plus 0.25% of BS1000 nonionic surfactant was applied at the Australian use rate for *L. rigidum* control ($375 \, g \, ha^{-1}$). The herbicide was applied using a twin nozzle laboratory sprayer delivering herbicide in $100 \, l \, ha^{-1}$ of water at 210 kPa when travelling at $1 \, m \, s^{-1}$. Trays were returned to the glasshouse after herbicide application. Initial assessments of plant survival were made 21 days after herbicide treatment. The shoots of surviving plants were cut back to soil level and were allowed to regrow for 5 days. At this stage, diclofop-methyl was reapplied as described above. Plant survival was reassessed 21 days after the second herbicide application. Plants were scored as alive if they had resumed growth after cutting and continued to grow after the second herbicide application. The total number of treated and surviving plants was summed across the four replicates and percentage survival was calculated for each population.

Surviving plants from five populations (Busselton 1, Busselton 2, Gnangara, Kudardup, Victoria 9) were excavated from trays, individually repotted in 180 mm plastic pots and grown to maturity in the glasshouse. Prior to anthesis, each population was isolated to one bench in the glasshouse and plants were surrounded by a pollen-proof enclosure to ensure cross-pollination within populations only. At maturity, seed was harvested and stored over summer as described previously.

### Response to diclofop-methyl selection
In July 2004, dose–response experiments were conducted to compare levels of survival in the diclofop-methyl selected progeny and their original unselected parent populations. Seeds (12) of the original Busselton 1, Busselton 2, Gnangara, Kudardup and Victoria 9 populations and their diclofop-methyl selected progeny (for example, denoted as Busselton $1^+$ to indicate a selected line) were sown into 180 mm pots containing standard potting mixture and were maintained in the glasshouse as described previously. At the 2–3 leaf stage, emerged seedlings were sprayed with diclofop-methyl at 0, 47, 94, 188, 375, 750, 1500 or $3000 \, g \, ha^{-1}$ using the laboratory sprayer. There were three replicate pots per treatment.

Plant survival was assessed 21 days after herbicide treatment (plants were scored as alive if they had continued to grow following herbicide treatment) and percentage survival was calculated for each population by herbicide dose treatment. Survival data were analysed by probit analysis. Values for percentage survival were converted to probits and the function, probit = $a \log_{10}(\text{dose}) + b$ was fitted to the transformed data (Genstat version 6.1.0.200), where $a$ is the slope of the line and $b$ the intercept. The $LD_{50}$ values (diclofop-methyl dose causing 50% mortality), together with 95% confidence intervals, were calculated from probit analysis. The shoots of surviving and dead plants were harvested at the soil surface and fresh weight of all individuals was recorded. The mean shoot weight of treated individuals was calculated for each population by dose treatment and was expressed as a percentage of the mean fresh weight of untreated individuals for that population. These data were analysed by nonlinear regression (Genstat version 6.1.0.200) and fitted to the log-logistic model:

$$y = \frac{100}{1 + \exp[b(x - x_{50})]} \qquad (1)$$

where $y$ is the fresh biomass (% of mean untreated control), $x_{50}$ is the log of the dose required to reduce growth by 50% and $b$ is the slope around $x_{50}$. The diclofop-methyl dose required to reduce mean fresh above-ground biomass by 50% ($GR_{50}$) was calculated by back-transforming $x_{50}$.

## ACCase extraction and assay

*In vitro* assays of ACCase inhibition by diclofop acid were performed to determine if observed changes in resistance following selection with diclofop-methyl were the result of reduced sensitivity of the herbicide target enzyme. Seeds of the unselected *L. rigidum* populations and of their diclofop-methyl selected lines were sown into three replicate 180 mm pots per line (20 per pot) and were maintained in a glasshouse. At the 1.5–2 leaf stage, plants were harvested at the soil level and fresh shoot material was frozen in liquid nitrogen and stored at −20°C until use. Crude enzyme was extracted from a sample of shoot tissue (3 g) for each *L. rigidum* line and enzyme activity was assayed in duplicate over a range of diclofop acid concentrations.

Frozen tissue was homogenized in 10 ml extraction buffer containing 100 mM Tris (pH 8.0), 1 mM EDTA, 10% (v/v) glycerol, 2 mM isoascorbic acid, 1 mM PMSF, 0.5% PVP-40, 0.5% insoluble PVP, and 20 mM DTT. The homogenate was centrifuged at 27 000 g for 15 min. The supernatant was brought to 40% $(NH_4)_2SO_4$ saturation by dropwise addition of saturated $(NH_4)_2SO_4$ and stirred for 30 min. The solution was centrifuged at 27 000 g for 30 min. The pellet was resuspended in 2.5 ml elution buffer (50 mM Tricine, pH 8.0, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT) and desalted on a Sephadex G-25 (Pharmacia PD-10) column pre-equilibrated with elution buffer. The eluent was stored at −20°C until use (modified from Tardif *et al*, 1993).

ACCase activity was assayed by quantifying the incorporation of $NaH^{14}CO_3$ into an acid-stable product according to Seefeldt *et al* (1996) with modifications. The enzyme extract was incubated at 30°C in assay buffer

that contained 10 mM Tricine-KOH, pH 8.3, 5 mM ATP, 10 mM $MgCl_2$, 0.1% BSA, 2.5 mM DTT, 3.24 mM $NaHCO_3$ (including 18.5 kBq of $NaH^{14}CO_3$) and appropriate concentrations of technical grade diclofop acid. A diclofop acid concentration series (0.1, 1, 10, 50 and 100 μM) was prepared in 100 mM tricine buffer containing 10% acetone. The reaction was started by the addition of acetyl-CoA at a final concentration of 0.25 mM and was stopped after 10 min by the addition of concentrated HCl. A 50 μl aliquot of the reaction mixture was placed on glass filter paper and air-dried. After drying, the filter paper was placed in a scintillation vial containing 3 ml of liquid scintillant. Radioactivity was determined using a liquid scintillation counter. Enzyme activity (inhibition) was expressed as a percentage of the untreated control and results were analysed by log-logistic regression analysis (equation (1)) to determine and compare the $I_{50}$ values (diclofop acid concentration required to reduce enzyme activity by 50%) of the ACCase enzyme from diclofop-methyl selected and unselected lines. Pairwise comparisons of the $I_{50}$ values of unselected and selected lines were performed using *t*-test.

## Cross-resistance profiling

In August 2004, an experiment was established to determine levels of cross-resistance to four ACCase-inhibiting (fluazifop-P-butyl, haloxyfop-R-methyl, sethoxydim and tepraloxydim) and two ALS-inhibiting herbicides (chlorsulfuron and imazethapyr). Seeds (10) of the diclofop-methyl selected and unselected lines were sown in seedling trays containing a standard potting mixture and were placed on benches in an outdoor experimental garden at the University of Western Australia (three replicates per treatment). Trays were watered and fertilized as required. At the 2–3 leaf stage, emerged seedlings were counted and herbicides were applied at two rates (referred to as low and intermediate rates) using the laboratory sprayer. The herbicides applied and their application rates are in Table 1. Herbicide rates were selected on the basis of prior knowledge of normal responses of susceptible *L. rigidum* populations. Intermediate rates would usually result in 100% mortality of susceptible *L. rigidum* and low rates in low levels (<10%) of survival. Rates for the two ALS-inhibiting herbicides were higher than field recommended rates

**Table 1** Herbicides and rates applied to diclofop-methyl selected and unselected *L. rigidum* lines for cross-resistance profiling

| Herbicide active ingredient | Low rate (g ha⁻¹) | Intermediate rate (g ha⁻¹) | Recommended use rate (g ha⁻¹) |
|---|---|---|---|
| Fluazifop-P-butyl[a] | 27 | 53 | 53 |
| Haloxyfop-R-methyl[a] | 14 | 29 | 39 |
| Sethoxydim[b] | 33 | 70 | 93 |
| Tepraloxydim[b] | 18 | 38 | 50 |
| Chlorsulfuron[c] | 30 | 120 | 15 |
| Imazethapyr[d] | 70 | 175 | 70 |

[a]Aryloxyphenoxypropionate (ACCase inhibitor).
[b]Cyclohexanedione (ACCase inhibitor).
[c]Sulfonylurea (ALS inhibitor).
[d]Imidazolinone (ALS inhibitor).

based on relatively poor control of *L. rigidum* by these herbicides when applied to seedlings post-emergence in pot trials. Plant survival was assessed 21 days after herbicide treatment. Plants were assessed as previously described and mean percentage survival ($n = 3$) was calculated. The relative survival of the diclofop-methyl selected lines was calculated as the difference in survival of the selected and unselected lines for each herbicide rate.

## Results

### Frequency of phenotypic resistance to diclofop-methyl in unselected *L. rigidum*

Between 600 and 1500 individuals of each of 31 unselected *L. rigidum* populations were treated with the commercial rate (375 g ha$^{-1}$) of diclofop-methyl (differences in the total numbers of treated individuals reflected variations in seed germination and emergence of the populations). As expected, there was high mortality and the mean frequency of survivors was $4.3 \times 10^{-3}$ (0.43%) (Table 2). The highest frequency of survivors was observed for the Western Australian population, Augusta 2, in which 2.6% ($n = 24$) of sprayed individuals survived. There were no survivors in six of the populations screened. The significance of these

results for the evolution of herbicide resistance and its subsequent management will depend on the relative contribution of genetic and environmental factors to this putative phenotypic resistance. In order to confirm that plant survival was the result of heritable genetic variation, survivors from five of the previously unselected populations were grown to maturity, and each population was individually bulk-crossed to produce a diclofop-methyl selected line. The response to selection and heritability of resistance within the five populations was tested by detailed dose–response experiments.

### Dose–response of diclofop-methyl selected *L. rigidum* populations

Large differences in the dose–response characteristics of diclofop-methyl selected and unselected lines of five *L. rigidum* populations confirmed that high initial levels of survival to field recommended rates of diclofop-methyl were the result of heritable variations in herbicide sensitivity. The LD$_{50}$ values (Table 3) for the unselected lines ranged from 75 to 127 g ha$^{-1}$ diclofop-methyl and from 361 to 1924 g ha$^{-1}$ following diclofop-methyl selection. There was little correlation between the initial population LD$_{50}$ value and the response to selection. For example, Busselton 2, the population with the highest initial LD$_{50}$ showed the weakest response to selection (R:S resistance ratio of 2.8, see Table 3).

Log-logistic analysis of fresh biomass data has adequately described the response of selected and unselected lines to diclofop-methyl ($R^2 = 0.551$–0.975, Figure 1). GR$_{50}$ values are summarized in Table 4 and while these confirm the populations responses to selection, there are some noteworthy differences in LD$_{50}$ and GR$_{50}$ values. For instance, there is a large difference between the LD$_{50}$ (1924 g diclofop-methyl ha$^{-1}$) and GR$_{50}$ (751 g diclofop-methyl ha$^{-1}$) values for the Kudardup$^+$ line, indicating that the growth of individuals surviving herbicide treatments is significantly retarded. Conversely, there is little difference in the LD$_{50}$ and GR$_{50}$ values for the Busselton 1$^+$ line. While it is clear that the variations in herbicide sensitivity observed in the initial resistance screen (Table 2) are heritable, the actual magnitude of the response to selection varies significantly between

**Table 2** Frequencies of survival of previously unselected populations of *L. rigidum* from Western Australia (WA), Victoria and South Australia (SA) following application of diclofop-methyl

| Population | Plants treated | Plants survived | % survival |
|---|---|---|---|
| Augusta 2 (WA) | 914 | 24 | 2.6 |
| Bordertown (SA) | 1320 | 17 | 1.3 |
| Busselton 2 (WA) | 1170 | 13 | 1.1 |
| Nannup 3 (WA) | 1065 | 10 | 0.95 |
| Gnangara (WA) | 992 | 9 | 0.91 |
| M River 2 (WA) | 1459 | 10 | 0.69 |
| Busselton 1 (WA) | 1104 | 7 | 0.63 |
| Kudardup (WA) | 1227 | 6 | 0.49 |
| Augusta 1 (WA) | 1047 | 5 | 0.48 |
| Victoria 15 | 848 | 4 | 0.47 |
| M River 1 (WA) | 1284 | 6 | 0.47 |
| Calgardup (WA) | 609 | 2 | 0.33 |
| Swan Valley (WA) | 926 | 3 | 0.32 |
| Victoria 8 | 1255 | 4 | 0.32 |
| Victoria 9 | 1419 | 4 | 0.28 |
| Spring Gully (SA) | 1175 | 3 | 0.26 |
| Blessing (SA) | 1213 | 3 | 0.25 |
| Victoria 18 | 1183 | 3 | 0.25 |
| Victoria 2 | 1109 | 2 | 0.18 |
| Swan (WA) | 1300 | 2 | 0.15 |
| Victoria 21 | 948 | 1 | 0.11 |
| Victoria 5 | 1054 | 1 | 0.10 |
| Northcliffe (WA) | 1041 | 1 | 0.10 |
| Pemberton (WA) | 989 | 1 | 0.10 |
| Victoria 12 | 1117 | 1 | 0.09 |
| Denmark (WA) | 807 | 0 | 0 |
| Cowaramup (WA) | 1126 | 0 | 0 |
| Victoria 13 | 1128 | 0 | 0 |
| Victoria 6 | 1032 | 0 | 0 |
| Victoria 4 | 732 | 0 | 0 |
| Victoria 20 | 1188 | 0 | 0 |
| Total | 32 700 | 141 | 0.43 |

Survivors from underlined populations were grown to maturity to produce diclofop-methyl selected lines.

**Table 3** Diclofop-methyl dose required to cause 50% mortality (LD$_{50}$) for diclofop-methyl unselected and selected lines

| Population | LD$_{50}$ (g diclofop-methyl ha$^{-1}$) | | LD$_{50}$ R:S ratio[a] |
|---|---|---|---|
| | *Unselected* | *Selected* | |
| Busselton 1 | 95.6 (74.4–118) | 1279 (901–2048) | 13.4 |
| Busselton 2 | 127 (103–155) | 361 (276–472) | 2.8 |
| Gnangara | 74.9 (54.9–94.8) | 386 (293–510) | 5.2 |
| Kudardup | 82.9 (64.3–102) | 1924 (1230–3890) | 23.2 |
| Victoria 9 | 101 (76.0–128) | 623 (506–773) | 6.2 |

LD$_{50}$ estimates are back-transformed from probit analysis of diclofop-methyl dose-mortality response data. 95% confidence intervals are in parentheses.
[a]LD$_{50}$ R:S ratios are calculated as the ratio of the LD$_{50}$ of the selected and unselected lines.
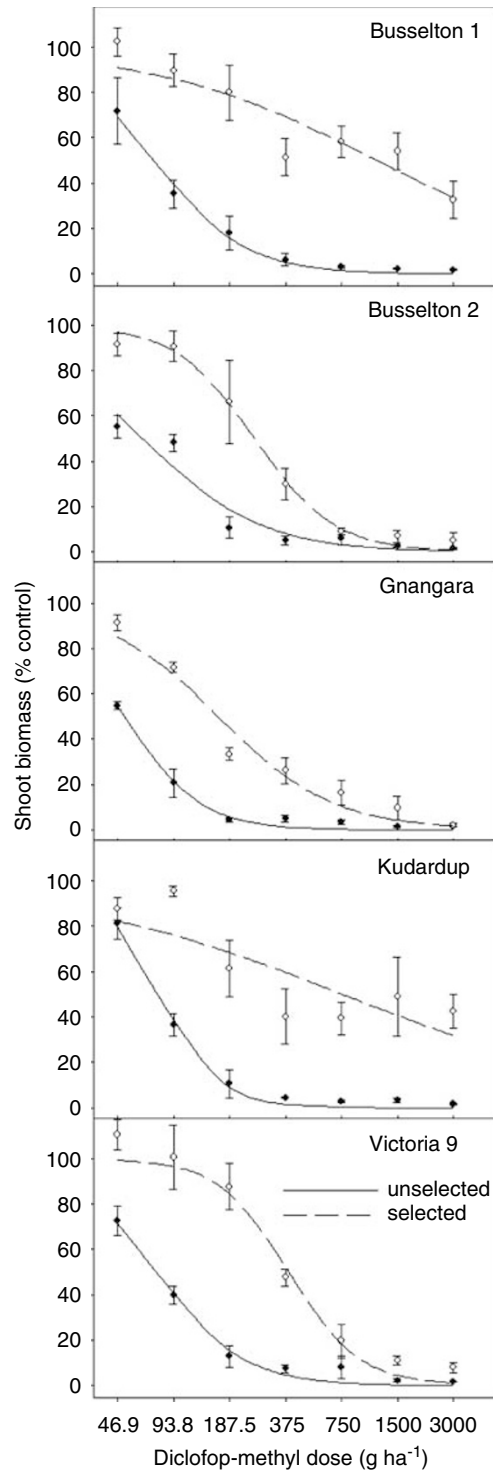
**Figure 1** Dose–response curves for above-ground fresh biomass production (% mean untreated control) 21 days after herbicide application for diclofop-methyl unselected and selected lines of five *L. rigidum* populations following application of diclofop-methyl at the 2–3 leaf stage. Symbols are mean observed % biomass production and error bars are ±one standard error of the mean. Predicted values for % biomass production (solid and broken lines) are derived from log-logistic regression analysis.

populations. These observations suggest that there may be physiological and genetic differences in the underlying mechanism(s) of these responses.

**Table 4** Diclofop-methyl dose required to reduce population fresh biomass by 50% ($GR_{50}$) for diclofop-methyl unselected and selected lines

| Population | $GR_{50}$ (g diclofop-methyl ha$^{-1}$) | | $GR_{50}$ R:S ratio[a] |
|---|---|---|---|
| | *Unselected* | *Selected* | |
| Busselton 1 | 74.8 (6.9) | 1151 (297) | 15.4 |
| Busselton 2 | 65.1 (6.1) | 256 (26.7) | 3.9 |
| Gnangara | 51.9 (2.7) | 163 (14.4) | 3.1 |
| Kudardup | 79.6 (5.8) | 751 (275) | 9.4 |
| Victoria 9 | 77.5 (6.8) | 392 (40.9) | 6.2 |

$GR_{50}$ estimates are back-transformed from log-logistic regression analysis. Standard errors of the estimates are in parentheses.
[a]$GR_{50}$ R:S ratios are calculated as the ratio of the $GR_{50}$ of the selected and unselected lines.

**Table 5** Diclofop acid concentration required to cause 50% inhibition of acetyl-coenzyme A carboxylase activity ($I_{50}$) for unselected and selected lines

| Population | $I_{50}$ ($\mu M$ diclofop acid) | |
|---|---|---|
| | *Unselected* | *Selected* |
| Busselton 1 | 4.30 (0.57) | 6.17 (0.91) |
| Busselton 2 | 5.51 (0.70) | 4.31 (0.58) |
| Gnangara | 7.13 (1.33) | 5.02 (0.86) |
| Kudardup | 5.26 (1.40) | 5.21 (0.56) |
| Victoria 9 | 4.61 (0.49) | 4.30 (0.77) |

$I_{50}$ estimates are back-transformed from log-logistic regression analysis. Standard errors of the estimates are in parentheses. $I_{50}$ values of unselected and selected lines were compared by *t*-test and were not statistically different for any population.

## *In vitro* inhibition of ACCase by diclofop acid

There were no significant differences in the *in vitro* diclofop acid inhibition of ACCase from unselected and diclofop-methyl selected lines (Table 5). These results established that observed increases in population level resistance were not target-site enzyme related due to any change in ACCase sensitivity, and must therefore be conferred by unknown nontarget site mechanism(s).

## Cross-resistance to other herbicides

The resistance status of selected and unselected lines to four ACCase and two ALS-inhibiting herbicides was tested to establish patterns of cross-resistance following a single selection with diclofop-methyl. Relative survival was calculated by subtracting the survival (%) of original untreated lines from observed survival of diclofop-methyl selected lines (Figure 2). It was immediately evident that selection with diclofop-methyl increased population level resistance to other ACCase and ALS herbicides. Even more notable was that populations exhibited very diverse patterns of cross-resistance (Figure 2). The Busselton 1[+] and Kudardup[+] lines exhibit cross-resistance to low and intermediate levels of the aryloxyphenoxypropionate (AOPP) herbicides, flua-zifop-P-butyl and haloxyfop-R-methyl, and the CHD herbicide sethoxydim, and very low-level cross-resistance to tepraloxydim. Interestingly, these two populations also exhibited the highest level of resistance to diclofop-methyl (Figure 1, Tables 3 and 4) suggesting that
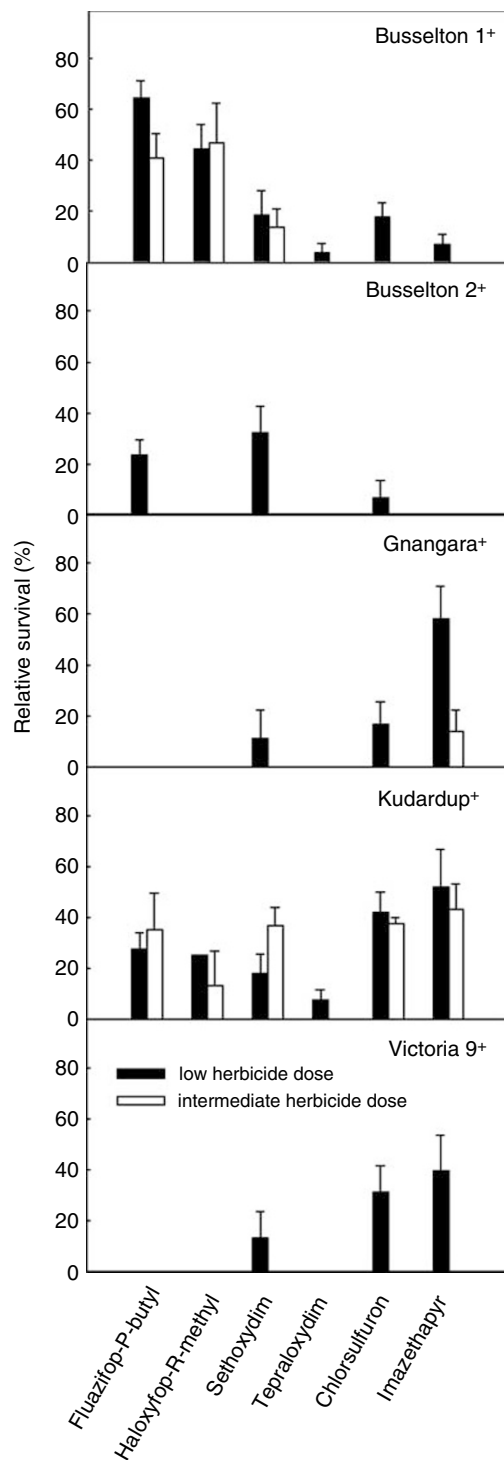
**Figure 2** Mean percentage relative survival of diclofop-methyl selected lines of five *L. rigidum* populations treated with low and intermediate doses of ACCase and ALS-inhibiting herbicides (see Table 1). Relative survival is calculated by subtracting the survival (%) of original untreated lines from observed survival of diclofop-methyl selected lines. Error bars are ±one standard error of the mean.

cross-resistance to chlorsulfuron and imazethapyr whereas Kudardup⁺ demonstrated significant levels of resistance to low and intermediate rates of these two herbicides (Figure 2). The Gnangara⁺ and Victoria 9⁺ lines had very low levels of cross-resistance to sethoxydim, but not to other ACCase inhibiting herbicides. They also had moderate resistance to chlorsulfuron and significant increases in survival at the low dose of imazethapyr. The Busselton 2⁺ line exhibited moderate cross-resistance at low doses of fluazifop-P-butyl and sethoxydim and no resistance to the ALS-inhibiting herbicides. These widely contrasting patterns of cross-resistance are intriguing as they further suggest that the precise mechanism and genetic basis of resistance in each selected line is different.

## Discussion

Australian populations of *L. rigidum* have demonstrated rapid and widespread evolution of resistance to selective herbicides (Gill, 1995; Nietschke *et al*, 1996; Llewellyn and Powles, 2001). The potential for, and rate of evolved responses to herbicides depends on the existence within plant populations of genetic variation for responses to herbicides (survival and growth). The results reported here have clearly demonstrated high survival frequencies (mean = 0.43%) in a number of pristine *L. rigidum* populations from across Australia following application of a commercial rate (375 g ha⁻¹) of the ACCase-inhibiting herbicide diclofop-methyl (Table 2). These results are similar to those reported by Matthews and Powles (1992), who found a mean diclofop-methyl survival frequency of 0.2% in a number of previously untreated *L. rigidum* populations from non-farm situations. For five of these populations we have demonstrated that this putative resistance was heritable (Tables 3 and 4 and Figure 1) and that resistance was not based on target-site insensitivity (Table 5). Additionally, a single cycle of selection with diclofop-methyl increased population-level resistance to a range of other herbicides (Figure 2), demonstrating the potential for rapid selection of diverse cross-resistance patterns. These observed phenotypic frequencies of resistance were orders of magnitude greater than rates of spontaneous target-site mutations, which give rise to target-site ALS resistance in *Arabidopsis thaliana* (Haughn and Somerville, 1987; Jander *et al*, 2003) and *Nicotiana tabacum* (Harms and DiMaio, 1991). These frequencies were also much greater than the initial frequency of target-site based resistance to ALS herbicides in three previously untreated *L. rigidum* populations (Preston and Powles, 2002).

The physiological and genetic basis of resistance has not been determined, although it is not due to single gene, target-site resistance (Table 5). Species-dependent variations in the rate and mechanism of detoxification of some herbicides are the basis of herbicide selectivity (Owen, 2000). Indeed, grass weed control in wheat by some ACCase herbicides is based on differential rates of herbicide metabolism mediated by the cytochrome *P*450 and glutathione-*S*-transferase enzyme families (see Cole and Edwards, 2000). The evolution of enhanced rates of herbicide detoxification, mediated by cytochrome *P*450 monooxygenases, has previously been documented as a mechanism of evolved resistance to ACCase herbicides in *L. rigidum* (Preston *et al*, 1996) and *Alopecurus*

their robust resistance to this herbicide confers diverse cross-resistance to other ACCase herbicides. The two populations did, however, differ in their cross-resistance to the ALS herbicides. Busselton 1⁺ exhibited slight

*myosuroides* (Letouze and Gasquez, 2003). Glutathione transferases have also been shown to contribute to evolved herbicide resistance in *A. myosuroides* (Cummins *et al*, 1999). These and other potentially unknown mechanisms are expected to be responsible for resistance in our unselected *L. rigidum* populations. Plant cytochrome *P*450s are the largest family of plant proteins and are responsible for synthesising a vast array of plant secondary metabolites, many of which are involved in plant defence against biotic and abiotic stress (Morant *et al*, 2003). Likewise, the glutathione-*S*-transferases have long been associated with stress tolerance in plants (Frova 2003). We speculate that *L. rigidum* populations in Australia maintain a high level of genetic variability for expression and substrate specificity within these multifunctional enzyme families and that exposure to low herbicide rates selects for individuals with initially low-level resistance to herbicides. In an outcrossing species such as *L. rigidum*, all minor resistance mechanisms will be selected and enriched, and will accumulate in subsequent generations leading to polygenically endowed herbicide resistance.

The diverse patterns of cross-resistance observed in the five selected *L. rigidum* populations further suggested the involvement of different genes or gene combinations. This observation has significant management implications as it indicates that selection with a single herbicide can result in complex and highly unpredictable patterns of cross-resistance. Given this, the availability and efficacy of alternative herbicides for *L. rigidum* control may often be compromised following selection for resistance to diclofop-methyl (or other) herbicides. Finally, it is worth reiterating that herbicide use rates in Australia are generally lower than elsewhere in the world and that this may contribute to the prevalence of evolved resistance as use rates are selecting within the normal range of phenotypic variation for herbicide sensitivity. Our results should be borne in mind as economic and environmental incentives to reduce herbicide application rates continue to increase in some parts of the world.

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