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ORIGINAL PAPER

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Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*

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Abstract There has been much debate regarding the potential for reduced rates of herbicide application to accelerate evolution of herbicide resistance. We report a series of experiments that demonstrate the potential for reduced rates of the acetyl-co enzyme A carboxylase (ACCase)-inhibiting herbicide diclofop-methyl to rapidly select for resistance in a susceptible biotype of *Lolium rigidum*. Thirty-six percent of individuals from the original VLR1 population survived application of 37.5 g diclofop-methyl ha^{-1} (10% of the recommended field application rate). These individuals were grown to maturity and bulk-crossed to produce the VLR1 low dose-selected line VLR1 (0.1). Subsequent comparisons of the dose-response characteristics of the original and low dose-selected VLR1 lines demonstrated increased tolerance of diclofop-methyl in the selected line. Two further rounds of selection produced VLR1 lines that were resistant to field-applied rates of diclofop-methyl. The LD_{50} (diclofop-methyl dose required to cause 50% mortality) of the most resistant line was 56-fold greater than that of the original unselected VLR1 population, indicating very large increases in mean population survival after three cycles of selection. In vitro ACCase inhibition by diclofop acid confirmed that resistance was not due to an insensitive herbicide target-site. Cross-resistance studies showed increases in resistance to four herbicides: fluazifop-P-butyl, haloxyfop-R-methyl, clethodim and imazethapyr. The potential genetic basis of the observed response and implications of reduced herbicide application rates for management of herbicide resistance are discussed.

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Introduction

Resistance to xenobiotics is a striking example of rapid evolution following a novel environmental challenge and is a recurring theme in agriculture (resistance to insecticides, fungicides and herbicides) and medicine (antimicrobial resistance). Herbicide resistance now compromises weed management practices in many agricultural situations worldwide (reviewed in Powles and Shaner 2001) and must be addressed from an evolutionary perspective (Via 1986). Georghiou and Taylor (1986) separated the factors influencing the evolution of pesticide resistance into genetic, biological/ecological and operational. Genetic factors include the frequency of resistance (*R*) alleles in populations prior to selection, the number of alleles or loci contributing to the resistance phenotype and the relative fitness of those alleles in the presence and absence of the herbicide (Maxwell and Mortimer 1994; Jasieniuk et al. 1996; Diggle and Neve 2001). Biological and ecological factors relate to individual species biology, and operational factors include the rate of herbicide applied (selection intensity) and herbicide use patterns (e.g. rotations and mixtures).

The relative contribution to adaptation of genetic variation at major (genes with a large phenotypic effect) and minor (genes with smaller additive effects) loci has been keenly discussed, and it is widely agreed that the majority of adaptation in natural populations occurs as a consequence of selection at many loci of small effect (Lande 1983; Orr and Coyne 1992). However, most of the documented cases of field-evolved resistance to xenobiotics result from the selection of single major genes (monogene resistance) (Roush and McKenzie 1987; Macnair 1991; Darmency 1994; Jasieniuk et al. 1996;

Shaw 2000; Daborn et al. 2002). Lande (1983) concluded that adaptation by single genes with large phenotypic effects (and potentially large pleiotropic fitness costs) would be favoured by a sudden and/or large change in the environment, such as that experienced following exposure to anthropogenic toxins (xenobiotics, heavy metals). Similarly, Macnair (1991) has argued that it is not the strength of selection, but the magnitude of phenotypic change required to achieve an adaptation that will determine whether monogenic or polygenic resistance occurs. In cases where the required magnitude of phenotypic change is large, this change may only be achieved by single genes of large effect.

Understanding the relationship between the selection intensity imposed by a xenobiotic and the genetic basis of resistance is crucial for the design and implementation of resistance management strategies. High dose rates have been advocated for resistance management for antimicrobials (Lipsitch and Levin 1997), insecticides (Tabashnik et al. 2004) and fungicides (Steva 1994), though in each case the theoretical basis for these recommendations has been somewhat different. Well known is the 'high-dose refuge' (HDR) strategy (see Tabashnik et al. 2004), developed to minimise insect adaptation to *Bacillus thuringiensis* (Bt) transgenic crops. This strategy relies on high toxin concentrations which make major gene resistance functionally recessive and on the maintenance of refuge areas of non-Bt crops that promote the survival of susceptible adults. These susceptible individuals mate with rare homozygote resistant individuals to produce heterozygotes. Lipsitch and Levin (1997) have shown that resistance evolves most rapidly following recurrent exposure to intermediate rates of antimicrobials. On this basis, they advocate the use of high doses applied infrequently.

It has been suggested that low or suboptimal herbicide rates result in the evolution of polygenic herbicide resistance (Gressel 1995, 2002). Empirical evidence for this phenomenon is largely anecdotal. However, many studies have found a high degree of continuous variation for tolerance to low herbicide rates within and between weed populations (Ellis and Kay 1975; Holliday and Putwain 1980; Price et al. 1983; DeGenarro and Weller 1984; Patzoldt et al. 2002). This continuous phenotypic variation is indicative of polygenic control, and McKenzie (2000) has argued that the genetic basis for evolved insecticide resistance (monogenic versus polygenic) depends on the way in which variation is channeled during selection. Where insecticide rates are high, selection acts outside of the normal range of distribution of susceptible phenotypes, meaning that only selection of rare monogenic mutations with large phenotypic effects occurs (McKenzie 2000). Conversely, low insecticide rates select for pre-existing polygenic variation (McKenzie and Batterham 1994; McKenzie 2000; Ffrench-Constant et al. 2004). These arguments related to pesticide resistance have a basis in the population genetic and evolutionary theory expounded by Lande (1983), Macnair (1991) and Orr and Coyne (1992). Where selection is intense and requires a large change in phenotype (high pesticide rates), adaptation will occur through the selection of major genes with large effect. Less intense selection (low or intermediate pesticide rates) may potentially favour adaptation by many genes of small effect.

In Australia, the annual grass *Lolium rigidum* Gaudin. has been introduced as a desirable pasture species. More recently, as wheat production has increased dramatically, *L. rigidum* has become the most widespread and troublesome weed of Australian grain production systems. Multiple introductions and the obligate outcrossing nature of *L. rigidum* mean that there is considerable genetic variation within and between populations. Since the 1970s herbicides have dominated *L. rigidum* control, and this species has demonstrated an unprecedented capacity to evolve resistance to multiple herbicides (Burnet et al. 1994; Preston et al. 1996). The acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicide, diclofop-methyl was introduced to Australia in 1978 and became widely used—primarily for *L. rigidum* control. By 1998, a random survey across 8 million ha of the Western Australian grain cropping region found that 46% of *L. rigidum* populations were resistant to field-applied rates of diclofop-methyl (Llewellyn and Powles 2001). In Australia, farm sizes and hence herbicide-treated areas are very large, and there is an economic incentive for growers to reduce herbicide rates where they believe these will achieve acceptable levels of control. It has been postulated that the use of relatively low herbicide rates over large and genetically variable *L. rigidum* populations has been responsible for the unprecedented incidence of herbicide resistance in Australian populations of this weed (Gressel 1995, 2002).

Our aim in this paper is to assess the potential for suboptimal rates of the ACCase-inhibiting herbicide diclofop-methyl to select for phenotypic variation in a known susceptible *L. rigidum* population and for this selection to result in the evolution of a herbicide-resistant population. These results will be discussed in terms of their relevance to field-evolved herbicide resistance and resistance management.

Materials and methods

Plant material

The *Lolium rigidum* biotype, VLR1, originates from a commercial seed source in Victoria, Australia and has previously been confirmed as susceptible to all herbicides commonly used for *L. rigidum* control. Seed stocks of VLR1 have been maintained and regularly multiplied without herbicide selection by S. Powles since 1985. During seed multiplication, ingress of pollen from surrounding *L. rigidum* populations has been prevented. Seed stocks used in the experiments described in this paper were collected in December 1998 following seed multiplication under field conditions at the University of

Western Australia experimental station. Following collection, threshed and cleaned seeds were stored in dry conditions at a constant 20°C until use.

VLR1 dose response to diclofop-methyl

Glasshouse experiments were conducted in August–September 2000 to establish the dose-mortality response of the VLR1 biotype treated with a range of diclofop-methyl rates up to the recommended Australian use rate for *L. rigidum* control ($x = 375$ g ha^{-1}). Seeds (50) were sown into plastic seedling trays (28×33×5 cm) containing a standard potting mixture (50% peat/50% sand). The trays were placed in a cooled glasshouse (maximum daily temperature of 20 ± 5°C) and were watered as required. When the majority of seedlings had reached the two-to three-leaf stage they were counted, and seedlings with more or fewer than two or three leaves were removed, resulting in a mean of 107 treated seedlings per herbicide rate. A commercial herbicide formulation was applied at rates of 18.75, 37.5, 75, 112.5, 187.5 and 375 g diclofop-methyl ha^{-1} (corresponding to 0.05*x*, 0.1*x*, 0.2*x*, 0.3*x*, 0.5*x* and 1.0*x*) with 0.25% BS1000 (non-ionic surfactant) using a twin-nozzle laboratory sprayer calibrated to deliver 100 l water ha^{-1} at 210 kPa when traveling at 1 m s^{-1}. An untreated control was sprayed with water, and there were three replicate trays per treatment. Following herbicide application, the trays were returned to the glasshouse. Plant survival was assessed 21 days after herbicide application. Plants were scored as alive if they had produced fresh green leaf material since the treatment and were actively growing. Plants that survived 37.5 g diclofop-methyl ha^{-1} (0.1×) ($n = 38$) were excavated from the trays, the roots were trimmed to approximately 10 mm in length and the shoots to 20 mm and the plants were individually repotted in 180-mm diameter pots containing potting mix. Prior to anthesis, all of the plants were placed in a pollen-proof enclosure to enable cross-pollination and to prevent the ingress of foreign pollen. Seeds were harvested at maturity and were stored as described previously. Seed collected was designated as the VLR1 (0.1) line, indicating that it had been selected at 10% of the diclofop-methyl recommended rate.

Recurrent selection of VLR1 with diclofop-methyl

In order to confirm a response in VLR1 to selection at low herbicide doses, we conducted experiments in 2001 to compare the dose-mortality response of the original VLR1 population with that of the VLR1 (0.1) line. Seeds of VLR1 or VLR1 (0.1) were sown in 180-mm plastic pots (12 per pot) containing a standard potting mix and maintained in a glasshouse as described previously. At the two- to three-leaf stage, seedlings were counted and diclofop-methyl was applied at rates of 37.5, 75, 187.5, 375 and 750 g ha^{-1} (0*x*, 0.1*x*, 0.2*x*, 0.5*x*, 1.0*x* and 2.0*x*) using the laboratory sprayer. There were four replicate pots per herbicide treatment. Plant survival was assessed 21 days after treatment. The experiment was repeated in April-May and June-July 2001 and, following appropriate statistical comparison, data sets were pooled for subsequent analysis to compare dose-mortality responses of the selected and unselected VLR1 lines.

Plants from the VLR1 (0.1) line that survived 75 g diclofop-methyl ha^{-1} ($n = 19$) and 187.5 g diclofop-methyl ha^{-1} ($n = 9$) in the second experiment (June–July) were repotted, maintained and separately crossed as previously described to produce VLR1 (0.1, 0.2) and VLR1 (0.1, 0.5) seed lines, respectively. In 2002, similar experiments were conducted to compare the dose-mortality responses of these two twice-selected lines and to assess the continued capacity of the VLR1 population to respond to selection with low and field-applied doses of diclofop-methyl (data not shown). Following scoring for survival, plants of the VLR1 (0.1, 0.2) line that had survived 187.5 g diclofop-methyl ha^{-1} ($n = 15$) and 375 g diclofop-methyl ha^{-1} ($n = 13$) and plants of the VLR1 (0.1, 0.5) line surviving 750 g ha^{-1} ($n = 23$) were repotted, grown to maturity and separately crossed as described previously to produce the VLR1 (0.1, 0.2, 0.5), VLR1 (0.1, 0.2, 1.0) and VLR1 (0.1, 0.5, 2.0) lines, respectively. Following collection and cleaning, seeds of all lines were stored in dry conditions at constant 20°C.

Diclofop-methyl dose-response profiles for low dose-selected VLR1 lines

In April-May 2004, experiments were conducted to compare the responses of all of the selected VLR1 lines to increasing doses of diclofop-methyl under identical conditions. Seeds of the original VLR1 population and of selected lines VLR (0.1), VLR1 (0.1, 0.2), VLR1 (0.1, 0.5), VLR1 (0.1, 0.2, 0.5), VLR1 (0.1, 0.2, 1.0) and VLR1 (0.1, 0.5, 2.0) were sown in 180-mm plastic pots (12 per pot) containing a standard potting mixture and maintained in the glasshouse as described previously. At the two- to three-leaf stage, pots were thinned to eight similarly sized seedlings. There were three replicate pots per herbicide rate × VLR1 line combination. Plants were sprayed with diclofop-methyl supplemented with 0.25% BS1000 at 0, 18.75 (0.05*x*), 37.5 (0.1*x*), 93.75 (0.25*x*), 187.5 (0.5*x*), 375(*x*), 750 (2*x*), 1,500 (4*x*), 3,000 (8*x*) and (16*x*) 6,000 g ha^{-1} using the laboratory sprayer and were returned to the glasshouse following the herbicide application. After 21 days, the plants were scored as dead or alive, and the above-ground fresh biomass of all plants (alive and dead) was determined as a percentage of the mean fresh biomass of the untreated control plants.

ACCase extraction and assay

In vitro assays of ACCase inhibition by diclofop acid were performed to determine if observed changes in

population level resistance following selection with diclofop-methyl were the result of a reduced sensitivity of the herbicide target enzyme. Seeds of the original VLR1 population and the VLR1 (0.1, 0.5, 2.0) line were sown in three replicate 180-mm pots (20 per pot) and maintained in a glasshouse. At the 1.5- to two-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.

VLR1 and VLR1 (0.1, 0.5, 2.0) frozen shoot tissue (3 g) was homogenized in 10 ml extraction buffer containing 100 m*M* Tris (pH 8.0), 1 m*M* EDTA, 10% (v/v) glycerol, 2 m*M* isoascorbic acid, 1 m*M* PMSF, 0.5% PVP-40, 0.5% insoluble PVP and 20 m*M* 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 m*M* Tricine, pH 8.0, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* 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 $Na[H^{14}]$ 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 m*M* Tricine-KOH, pH 8.3, 5 m*M* ATP, 10 m*M* $MgCl_2$, 0.1% bovine serum albumin (BSA), 2.5 m*M* DTT, 3.24 m*M* $NaHCO_3$ (including 18.5 kBq of $Na[H^{14}]$ 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 m*M* Tricine buffer containing 10% acetone. The reaction was started by the addition of acetyl-CoA at a final concentration of 0.25 m*M* 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 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.

Enzyme extraction was from two replicate VLR1 and VLR1 (0.1, 0.5, 2.0) samples. For each enzyme extraction, reaction assays were performed in duplicate. Enzyme activities from replicate extractions and duplicated assays were pooled for subsequent analysis.

Cross-resistance profiling of low dose-selected VLR1

A series of experiments were conducted to establish if low dose selection with diclofop-methyl had selected for changed sensitivity to other chemically similar and dissimilar herbicides commonly used for *L. rigidum* control. During June and July 2003, survival of the original VLR1 biotype and the selected line VLR1 (0.1, 0.5, 2.0) were compared following application of a rate close to the field-recommended rate of a range of herbicides. Herbicides and rates are shown in Table 1. Seeds were sown into 180-mm plastic pots (20 per pot) containing a standard potting mixture and maintained in a cooled glasshouse. At the two- to three-leaf stage, herbicides were applied and the plants returned to the glasshouse. There were three replicate pots per herbicide treatment. Plant survival was assessed 21 days after herbicide application.

For herbicides where the single rate screening described above indicated some level of cross-resistance, more detailed dose-mortality response experiments were conducted from August to October 2003. Three replicate pots with 20 seeds per pot for VLR1 and VLR1 (0.1, 0.5, 2.0) were established and maintained as previously described. At the two- to three-leaf stage, seedlings were sprayed with a series of doses of fluazifop-P-butyl (0, 10.6, 26.5, 38.75, 53, 106 g ha^{-1}), haloxyfop-R-methyl (0, 3.9, 7.8, 19.5, 39 g ha^{-1}), sethoxydim (0, 18.6, 37.2, 55.8, 74.4, 93 g ha^{-1}), clethodim (0, 2.4, 4.8, 12, 24 g ha^{-1}) and imazethapyr (0, 7, 14, 35, 70 g ha^{-1}). Plant survival was assessed 21 days after herbicide application.

Data analysis

Survivorship data from herbicide dose-response experiments were analysed by probit analysis. Values for

Table 1 Herbicides applied to VLR1 and VLR1 (0.1, 0.5, 2.0) seedlings to assess selection for cross-resistance following selection with low doses of diclofop-methyl

Herbicide active ingredient	Herbicide class	Herbicide target	Field rate (g ha^{-1})	Rate applied (g ha^{-1})
Fluazifop-P-butyl	Aryloxyphenoxypropionate	ACCase	53	32
Propaquizafop	Aryloxyphenoxypropionate	ACCase	30–45	25
Haloxyfop-R-methyl	Aryloxyphenoxypropionate	ACCase	39–52	39
Sethoxydim	Cyclohexanedione	ACCase	93	46.5
Clethodim	Cyclohexanedione	ACCase	36–60	30
Chlorsulfuron	Sulfonylurea	ALS	15	30
Sulfometuron-methyl	Sulfonylurea	ALS	7.5	7.5
Imazethapyr	Imidazolinone	ALS	70	70
Paraquat	Bypyridilium	Photosystem I	300–400	125
Glyphosate	Glycine	EPSP synthase	600–800	250

percentage survival were converted to probits, and the function probit = a $\log_{10}$(dose) + b was fitted to the transformed data (GENSTAT VER. 6.1.0.200), where a is the slope of the line and b the intercept. Probit analysis was used to determine LD_{50} and LD_{90} values (diclofop-methyl dose required to cause 50% and 90% population mortality, respectively).

Fresh biomass data from dose-response experiments and ACCase activity from enzyme inhibition studies were expressed as a percentage of the mean untreated control. These data were analysed using the non-linear regression procedure in GENSTAT and fitted to the log-logistic model in Eq. 1,

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

where y = fresh biomass or ACCase enzyme activity (% of mean untreated control), x_{50} is the log of the dose required to obtain a 50% reduction in growth or 50% enzyme inhibition and b is the slope around x_{50}.

Results

VLR1 dose response to diclofop-methyl

The herbicide-susceptible VLR1 *L. rigidum* biotype was well controlled at field-recommended rates of diclofop-methyl (375 g ha^1), and there was little difference in the level of control when this rate was halved (Fig. 1). As rates of diclofop-methyl were reduced further, a dose response became apparent with considerable increases in the number of survivors (Fig. 1), indicating phenotypic variation for herbicide susceptibility at low-applied doses. The LD_{50} for this biotype was 30.3 g ha^{-1} (25.8–34.8) and the LD_{99} was 300 g ha^{-1} (228.5–430.9), indicating that more than 99% of the population is controlled by the recommended field application rate.

Selection was imposed at an intensity (S) of 0.64 (36% survival) by allowing the 28 individuals that survived 37.5 g diclofop-methyl ha^{-1} to grow to maturity and bulk-cross (*L. rigidum* is obligate cross-pollinated) to produce the once-selected VLR1 (0.1) line. Selection at these relatively low intensities enabled us to test the hypothesis that selection within the range of phenotypic variation for herbicide response in susceptible populations can ultimately result in resistance to field-applied rates.

Confirmation of inheritance of putative resistance in the VLR1 (0.1) line.

Experiments were conducted in 2001 to compare the dose-mortality response of the original VLR1 biotype with the once-selected VLR1 (0.1) line. Probit analyses identified significant differences in the dose-response characteristics of the selected and unselected lines (Fig. 2), indicating a phenotypic response to low-dose selection. The dose-response curve for the unselected VLR1 biotype (Fig. 2) differs from that obtained in 2000 (Fig. 1), with the LD_{50} value in 2001 (66.6 g diclofop-methyl ha^{-1}) being over twice that observed in 2000. This result illustrates the potential for year-to-year environmental differences to effect dose-response characteristics and highlights the need for selected and unselected lines to be compared under identical conditions if responses to selection are to be confirmed.

The diclofop-methyl LD_{50} value for the VLR1 (0.1) line was 87.2 g ha^{-1}, representing a small, but significant increase in mean population level resistance (*R*:*S* LD_{50}

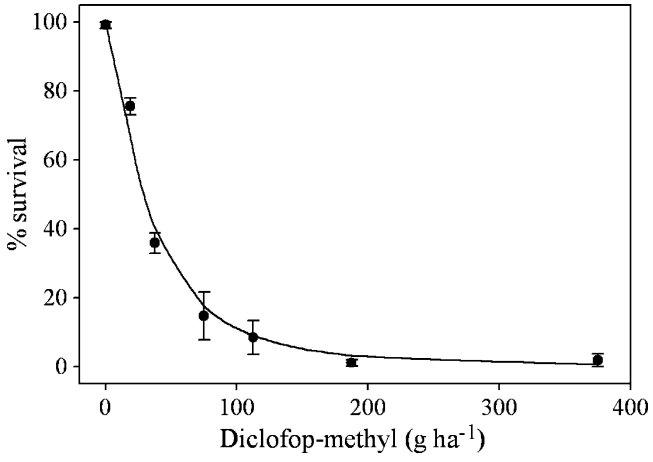

Fig. 1 Dose-mortality response curve for *Lolium rigidum* biotype VLR1 following application of a range of doses of diclofop-methyl at the two- to three-leaf stage in 2000. The *symbols* are the mean observed percentage survival; *error bars* are ± one standard error of the mean (n = 3). Predicted values for percentage survival (*solid line*) are back-transformed from probit analysis

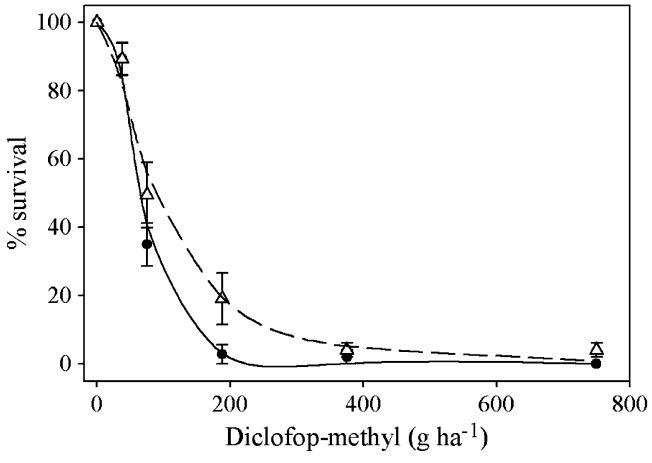

Fig. 2 Dose-mortality response curves for *L. rigidum* biotype VLR1 (*solid line*, *black circle*) and selected line VLR1 (0.1) (*broken line*, *open triangle*) following application of a range of doses of diclofop-methyl at the two- to three-leaf stage in 2001. *Symbols* are the mean observed percentage survival; *error bars* are ± one standard error of the mean (n = 8). Predicted values for percentage survival (*solid and broken lines*) are back-transformed from probit analysis

ratio = 1.31). There was a greater difference in diclofop-methyl LD_{99} values, which were estimated to be 244.1 g ha^{-1} and 706.7 g ha^{-1}, for VLR1 and VLR1 (0.1), respectively (*R*:*S* LD_{99} ratio = 2.90). The results in Fig. 2 confirm that a single selection with a low dose of diclofop-methyl has increased population level resistance in the VLR1 biotype.

VLR1 (0.1) individuals that survived 75 (*S* = 0.34) and 187.5 g diclofop-methyl ha^{-1} (*S* = 0.77) were grown to maturity and separately bulk-crossed to produce the twice-selected lines, VLR1 (0.1, 0.2) and VLR1 (0.1, 0.5), respectively. In 2002, dose-response experiments were performed to confirm the continued response to low-dose selection of the VLR1 biotype. Twice-selected lines showed highly significant increases in population level resistance (data not shown). VLR1 (0.1, 0.2) individuals that survived 187.5 (*S* = 0.44) and 375 g diclofop-methyl ha^{-1} (*S* = 0.58) were separately bulk-crossed to produce the three-times selected VLR1 (0.1, 0.2, 0.5) and VLR1 (0.1, 0.2, 1.0) lines. VLR1 (0.1, 0.5) individuals that survived 750 g diclofop-methyl ha^{-1} (*S* = 0.56) were bulk-crossed to produce a VLR1 (0.1, 0.5, 2.0) line.

Dose-mortality responses of VLR1 low dose-selected lines

Dose-response experiments performed in 2004 compared the dose-mortality characteristics of VLR1 with all of the twice- and three-times selected lines under identical conditions (Fig. 3, Table 2). In this experiment, the LD_{50} of VLR1 was estimated to be 61.4 g diclofop-methyl ha^{-1} . The LD_{50} of all of the selected lines was substantially higher than that of VLR1 (Table 2). A second cycle of low-dose selection resulted in large increases in the mean population level resistance of VLR1 lines. The LD_{50} values of the VLR1 (0.1, 0.2) and VLR1 (0.1, 0.5) lines were estimated to be 453 g diclofop-methyl ha^{-1} and 670 g diclofop-methyl ha^{-1}, respectively, corresponding to LD_{50} *R*:*S* ratios of 7.4 and 10.9. Observed mean survival percentages at the field application rate for diclofop-methyl were 54% and 83%, respectively, indicating that the population had rapidly achieved economic levels of resistance. A third cycle of selection, this time with diclofop-methyl doses up to and including the field-recommended rate, resulted in further increases in the resistance status of the VLR1 lines. Estimated diclofop-methyl LD_{50} values were 722, 3429 and 2462 g ha^{-1} for VLR1 (0.1, 0.2, 0.5), VLR1 (0.1, 0.2, 1.0) and VLR1 (0.1, 0.5, 2.0), respectively, corresponding to LD_{50} *R*:*S* ratios of 11.8, 55.8 and 40.1.

The rate of herbicide resistance evolution in the field will depend on overall population fitness, which is a function not only of the number of individuals that survive, but also of the subsequent ability of those survivors to grow and set seed despite intense competition from crop plants (a small number of vigorous survivors will likely result in a more rapid evolution of resistance

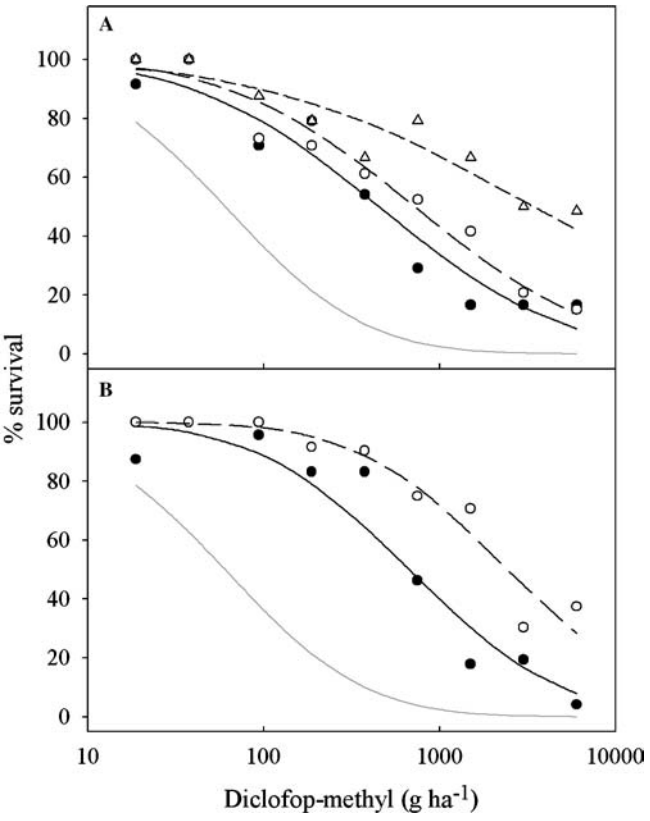

Fig. 3 Dose-mortality response curves for *L. rigidum* biotypes—**A** VLR1 (0.1, 0.2) (*solid line*, *black circle*), VLR1 (0.1, 0.2, 0.5) (*broken line*, *open circle*), VLR1 (0.1, 0.2, 1.0) (*stippled line*, *open triangle*); **B** VLR1 (0.1, 0.5) (*solid line*, *black circle*), VLR1 (0.1, 0.5, 2.0) (*broken line*, *open circle*)—following application of a range of doses of diclofop-methyl at the two- to three-leaf stage in 2004. *Symbols* are the mean observed percentage survival. Predicted values for percentage survival (*solid and broken lines*) are back-transformed from probit analysis. *Solid gray line* represents probit back-transformed dose-mortality response for unselected VLR1 biotype in the 2004 experiment

than a large number of severely retarded and uncompetitive individuals which set very little seed). Though no attempt was made to measure seed production, a truer reflection of relative population fitness under herbicide selection is given by population and individual biomass

Table 2 Estimates and standard errors of probit transformed LD_{50} and *b* (slope) values from dose-mortality response data for the VLR1 biotype and low dose-selected VLR1 lines treated with a range of doses of diclofop-methyl in 2004

Line	LD_{50}[a]	*b*[a]	LD_{50} *R*:*S* ratio[b]
VLR1	1.79 (0.084)	−1.62 (0.211)	
VLR1 (0.1, 0.2)	2.66 (0.086)	−1.22 (0.143)	7.4
VLR1 (0.1, 0.2, 0.5)	2.86 (0.092)	−1.201 (0.151)	11.8
VLR1 (0.1, 0.2, 1.0)	3.54 (0.210)	−0.83 (0.145)	55.8
VLR1 (0.1, 0.5)	2.83 (0.078)	−1.47 (0.172)	10.9
VLR1 (0.1, 0.5, 2.0)	3.39 (0.100)	−1.48 (0.219)	40.1

[a]Standard errors shown in parenthesis
[b]LD_{50} *R*:*S* ratios calculated as back-transformed LD_{50} for selected line/back-transformed LD_{50} for unselected VLR1 biotype

production following herbicide treatment. As expected, trends for population level growth responses are similar to those observed for survival, though in all cases GR_{50} values (diclofop-methyl rates required to reduce growth of the population by 50%) are lower than LD_{50} values (Fig. 4, Table 3).

Log-logistic analyses which considered only the biomass production of surviving individuals were performed to assess how individual growth is affected by recurrent selection with herbicides. These analyses clearly indicate that the mean biomass production of the surviving individuals continues to increase with recurrent herbicide selection. A resistance score has been calculated for each line as the product of the proportion of individuals that survive the recommended Australian use rate for diclofop-methyl (375 g ha^{-1}) and the relative fitness of those surviving individuals (mean percentage biomass production of untreated control (Table 4)). The resistance index (RI) is calculated by dividing the resistance score for each selected line with that of the unselected VLR1 biotype (Table 4). The results summarised in Table 4 show that the higher relative fitness of the VLR1 lines under recurrent herbicide selection is the consequence of increases in the number of individuals surviving herbicide application and increases in biomass production of the surviving individuals.

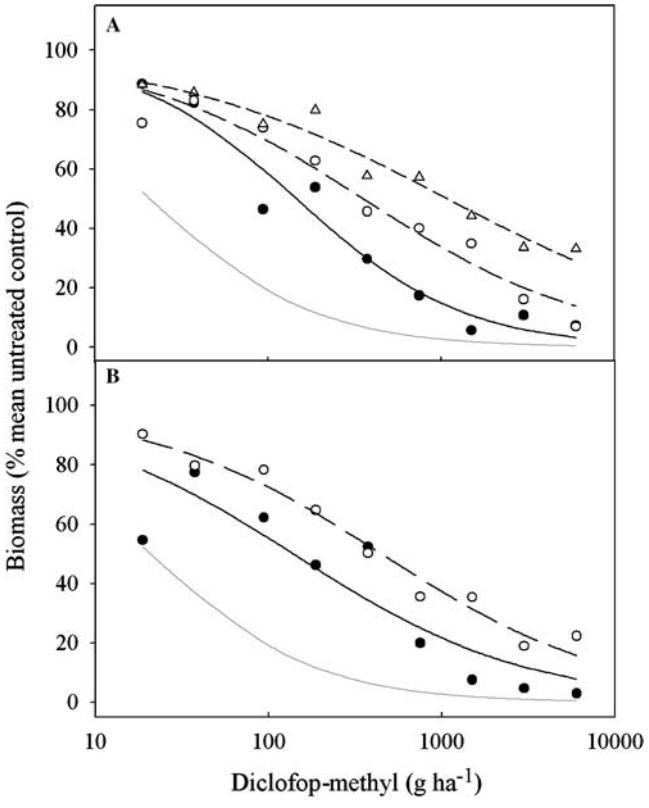

Fig. 4 Dose-response curves for per plant (dead and alive) fresh above-ground biomass production (percentage mean untreated control) 21 days after herbicide application of *L. rigidum* biotypes—**A** VLR1 (0.1, 0.2) (*solid line*, *black circle*), VLR1 (0.1, 0.2, 0.5) (*broken line*, *open circle*), VLR1 (0.1, 0.2, 1.0) (*stippled line*, *open triangle*); **B** VLR1 (0.1, 0.5) (*solid line*, *black circle*), VLR1 (0.1, 0.5, 2.0) (*broken line*, *open circle*)—following the application of a range of doses of diclofop-methyl at the two- to three-leaf stage in 2004. *Symbols* are mean observed percentage biomass production. Predicted values for percentage biomass production (*solid and broken lines*) are derived from log-logistic regression analysis. The *solid gray line* represents log-logistic dose-response curves for the original VLR1 biotype in the 2004 experiment

Table 3 Estimates and standard errors of GR_{50} and *b* (slope) parameters from log-logistic analysis of growth-response data for the VLR1 biotype and low dose-selected VLR1 lines treated with a range of doses of diclofop-methyl in 2004

Line	GR_{50}[a]	*b*[a]	GR_{50} *R*:*S* ratio[b]
VLR1	21.9 (3.0)	−2.16 (0.26)	
VLR1 (0.1, 0.2)	147.1 (24.6)	−2.10 (0.31)	6.7
VLR1 (0.1, 0.2, 0.5)	356.6 (77.0)	−1.50 (0.23)	16.3
VLR1 (0.1, 0.2, 1.0)	1089 (381)	−1.21 (0.27)	49.3
VLR1 (0.1, 0.5)	139.7 (31.7)	−1.50 (0.23)	6.4
VLR1 (0.1, 0.5, 2.0)	447.7 (93.0)	−1.49 (0.22)	20.4

[a]Standard errors are shown in parenthesis
[b]GR_{50} *R*:*S* ratios calculated as GR_{50} for selected line/GR_{50} for unselected VLR1 biotype

ACCase inhibition by diclofop acid

A comparison of in vitro inhibition of ACCase from VLR1 and VLR1 (0.1, 0.5, 2.0) lines by diclofop acid indicates that differences in whole-plant resistance are not due to a decreased sensitivity of the herbicide target-site (Fig. 5). The I_{50} values (diclofop acid concentration required to reduce ACCase activity by 50%) were 4.88 μM and 5.28 μM for VLR1 and VLR1 (0.1, 0.5, 2.0), respectively and were not significantly different. These results confirm that the mechanism of resistance selected with recurrent low rates of diclofop-methyl is not ACCase target-site based.

Cross-resistance profile of VLR1 (0.1, 0.5, 2.0)

An initial cross-resistance screen compared the survival of individuals of the VLR1 biotype and the

Table 4 Values for survival frequency, percentage above-ground biomass production and derived resistance scores and resistance indices for VLR1 and herbicide-selected VLR1 lines at the recommended field use rate for diclofop-methyl (375 g ha^{-1})

Line	Survival frequency (375 g ha^{-1})	Biomass (% mean control) (375 g ha^{-1})	*R* score	*R* index
VLR1	0.10	22.8	2.3	1.0
VLR1 (0.1, 0.2)	0.54	57.5	31.1	13.6
VLR1 (0.1, 0.2, 0.5)	0.63	78.8	49.6	21.8
VLR1 (0.1, 0.2, 1.0)	0.79	80.9	63.9	28.0
VLR1 (0.1, 0.5)	0.65	40.3	26.2	11.5
VLR1 (0.1, 0.5, 2.0)	0.89	64.4	57.3	25.1

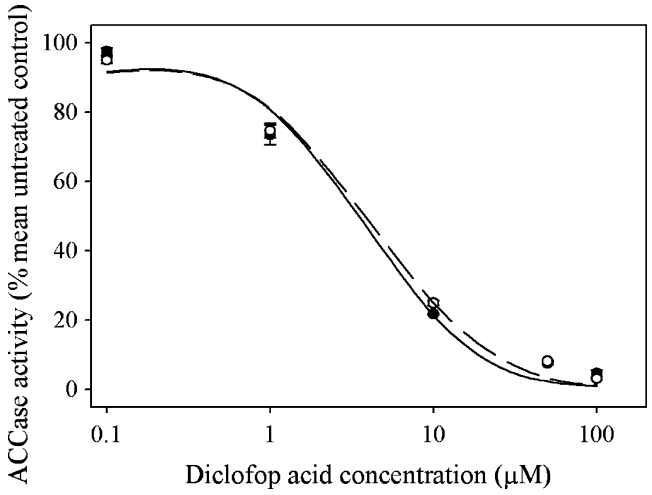

Fig. 5 In vitro enzyme inhibition curves for ACCase extracted from the unselected VLR1 biotype (*solid line*, *black circle*) and the VLR1 (0.1, 0.5, 2.0) (*broken line*, *open circle*) line and exposed to a range of diclofop acid concentrations. *Symbols* represent mean percentage ACCase inhibition (% of untreated control); *error bars* are ± one standard error of the mean. Predicted values (*solid and broken lines*) are derived from log-logistic regression analysis

diclofop-methyl-selected line, VLR1 (0.1, 0.5, 2.0) following application of a single rate of ten herbicides that usually control *L. rigidum* (Table 1). There were no differences in susceptibility to propaquizafop, an ACCase-inhibiting herbicide from the same chemical class as diclofop-methyl (AOPP) or to the chemically dissimilar herbicides glyphosate, paraquat, chlorsulfuron or sulfometuron-methyl (data not shown). For the five remaining herbicides [four ACCase-inhibitors and one acetolactate-synthase (ALS) inhibitor], there was increased survival of the VLR1 (0.1, 0.5, 2.0) selected line compared with the original biotype and, consequently, more detailed dose-response experiments were conducted (Fig. 6, Table 5).

Increased survival of the diclofop-methyl selected VLR1 line was observed for four of the five herbicides (Fig. 6), indicating some degree of cross-resistance. In no cases, however, did population level resistance (see *R*:*S* LD_{50} ratios in Table 5) approach that observed for diclofop-methyl, indicating that the mechanism(s) of resistance selected in VLR1 were reasonably specific to diclofop-methyl and endowed relatively low levels of resistance to other herbicides. The greatest increase in resistance was to the ALS-inhibiting imidazolinone herbicide, imazethapyr which has a different target-site and is chemically unrelated to diclofop-methyl. This result reaffirms that resistance is not target-site based and more likely arises from changes in the expression and/or substrate specificity of one or a suite of enzymes with the ability to metabolise chemically dissimilar herbicides. Only slight increases in resistance to the chemically related AOPP herbicides, fluazifop-P-butyl and haloxyfop-R-methyl were apparent (Fig. 6, Table 5), though in both cases these increases would have resulted in a less effective control at field-recommended rates, which could presumably result in the rapid evolution of economic levels of resistance. A very slight increase in resistance to the CHD herbicide clethodim was recorded (Fig. 6), though control obtained at recommended field rates was identical for selected and unselected lines. There were no differences in susceptibility to sethoxydim (Fig. 6).

Discussion

L. rigidum response to recurrent selection with low doses of diclofop-methyl.

The existence of phenotypic variation for diclofop-methyl susceptibility at low herbicide application rates has been demonstrated in a herbicide-susceptible *L. rigidum* biotype (Fig. 1). Continuous or quantitative variation in responses to treatment with low rates of herbicide have previously been reported within and between weed populations for a number of herbicides, both prior to and following herbicide selection (Faulkner 1974; Holliday and Putwain 1980; Price et al. 1983, 1985; DeGennaro and Weller 1984; Jasieniuk et al. 1996; Patzoldt et al. 2002). Inheritance studies have confirmed a genetic basis for this variation (reviewed by Jasieniuk et al. 1996). For insect populations treated with insecticides, it has been argued that resistance will evolve as a polygenic trait if selection acts within the normal range of phenotypic variation for 'susceptible' populations and as a monogenic trait when it acts outside of this distribution (McKenzie and Batterham 1994; McKenzie 2000; Ffrench-Constant et al. 2004). Our results demonstrate the potential for a low herbicide dose to select for genetic variation which segregates and recombines under recurrent selection to produce phenotypes in fewer than three generations that are resistant to field-applied herbicide rates. We have not determined the genetic basis of diclofop-methyl resistance in selected VLR1 lines, though the theoretical considerations above and a close examination of the dose-response characteristics of the selected lines suggest it to be polygenic. The following discussions speculate on the genetic and mechanistic basis of this low dose-selected resistance and will consider management implications for the use of reduced herbicide rates.

The mechanistic and genetic basis of responses to low diclofop-methyl doses in *L. rigidum*.

Resistance to ACCase-inhibiting herbicides is often enzyme target-site based as a result of single amino acid substitutions that render the ACCase enzyme insensitive to the herbicide (Zagnitko et al. 2001; Brown et al. 2002; Delye et al. 2002, 2003). Such mutations are rare, would not likely be found in the small number of plants treated in our study and would produce a resistant phenotype

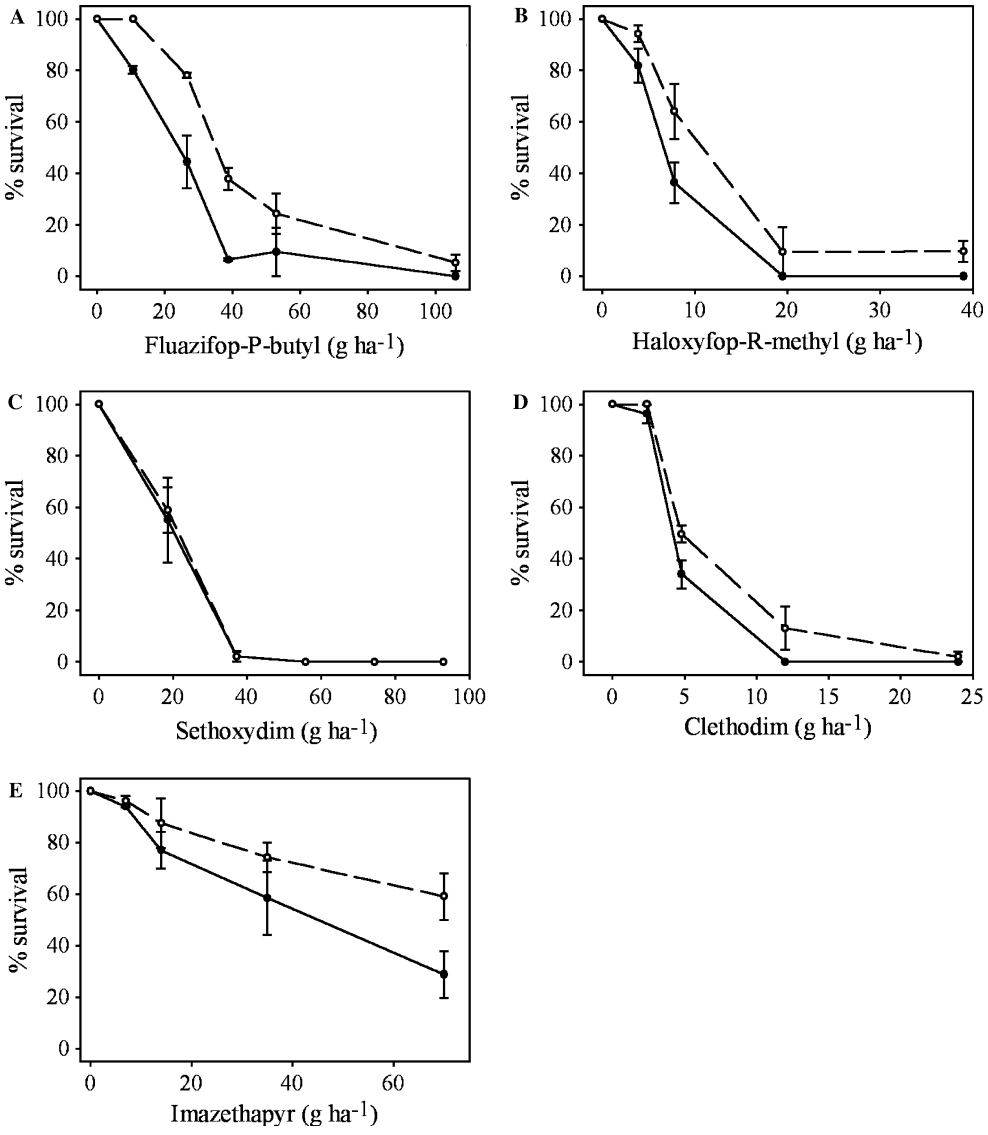

Fig. 6 Dose-mortality response curves for *L. rigidum* biotype VLR1 (*solid line*, *black circle*) and VLR1 (0.1, 0.5, 2.0) (*broken line*, *open circle*) treated with a range of doses of fluazifop-butyl (**A**), haloxyfop-R-methyl (**B**), sethoxydim (**C**), clethodim (**D**) and imazethapyr (**E**). *Symbols* represent mean percentage survival; *error bars* are ± one standard error of the mean

outside of the normal range of phenotypic variation for herbicide response. Indeed, as expected, we have shown that resistance to diclofop-methyl in the selected VLR1 lines is not ACCase-based (Fig. 5). Enhanced herbicide metabolism mediated by herbicide-degrading members of the cytochrome P450 monooxygenase (P450) enzyme family is also capable of endowing non-target-site resistance to ACCase herbicides in *L. rigidum* (Christopher et al. 1991; Preston et al. 1996) and *A. myosuroides* (Hall et al. 1997; Cocker et al. 1999; Letouze and Gasquez 2003). It is possible, though not confirmed, that resistance in the selected VLR1 lines is due to enhanced herbicide metabolism mediated by P450 herbicide-degrading enzymes.

Table 5 Estimates and standard errors of probit-transformed LD_{50} values from dose-mortality response data for the VLR1 biotype and the diclofop-methyl selected VLR1 (0.1, 0.5, 2.0) line treated with a range of doses of selected ACCase and ALS-inhibiting herbicides

Herbicide	LD_{50}VLR1[a]	LD_{50} VLR1 (0.1, 0.5, 2.0)[a]	LD_{50} *R*:*S* ratio[b]
Fluazifop-butyl	1.325 (0.034)	1.583 (0.023)	1.80
Haloxyfop-R-methyl	0.865 (0.026)	1.066 (0.035)	1.59
Sethoxydim	1.307 (0.028)	1.320 (0.024)	1.03
Clethodim	0.720 (0.019)	0.840 (0.028)	1.32
Imazethapyr	1.593 (0.055)	1.963 (0.140)	2.35

[a]Standard errors are shown in parenthesis
[b]LD_{50} *R*:*S* ratios calculated as back-transformed LD_{50} for VLR1 (0.1, 0.5, 2.0)/back-transformed LD_{50} for unselected VLR1

Typically, the frequency of major single gene resistance alleles will rapidly increase in populations under selection with high herbicide rates, resulting in the sudden and dramatic appearance of a high level resistance. Evidence obtained with *L. rigidum* from field selections (Tardif et al. 1993) together with predictions from simulation models of resistance evolution (Gressel and Segel 1978; Maxwell et al. 1990; Diggle and Neve 2001) certainly support this expectation. Contrary to this, incremental increases in the LD_{50} and GR_{50}values for the low

dose-selected VLR1 biotype, the shallow gradient of dose-response curves (see Via 1986) and the continued increases in fitness of surviving individuals under selection support our assertion that resistance is polygenic.

Global gene expression profiling of yeast cells treated with the ALS-inhibiting herbicide sulfometuron-methyl resulted in the up-regulation of 241 genes and the repression of 121 genes (Jia et al. 2000). Similarly, inoculation of *Arabidopsis thaliana* with the fungal pathogen *Alternaria brassicicola* resulted in changes in the abundance of 705 mRNAs (Schenk et al. 2000). These results demonstrate the vast array of molecular genetic responses elicited upon exposure to novel chemical or pathogenic challenges. Recent transcription profiling of DDT-resistant and susceptible lines of *Drosophila melanogaster* has shown significant overexpression of multiple cytochrome P450 and GST genes with potential insecticide-degrading capacities (Pedra et al. 2004). It has been argued that natural variation within populations for levels of gene expression may be a more potent force for evolution than structural and functional differences in enzymes (Oleksiak et al. 2002). Given the multitude of molecular responses to xenobiotics and pathogens outlined above, we can envisage how exposure to sublethal doses of herbicides could select for differences in expression at many of these loci and result in polygenic resistance traits. Alternatively, selection may act on genetic variation in expression levels at a single gene coding for an enzyme with herbicide-degrading activity. This enzyme will probably have a role in normal metabolism, and diversion from this role may incur significant pleiotropic fitness costs. Subsequent selection at modifier loci may compensate for these costs and contribute to increased fitness of the resistance phenotype (Uyenoyama 1986). In effect, resistance evolution will proceed by selecting the genetic backgrounds in which this one (or a few) gene(s) is (are) preferentially expressed and incurs the lowest fitness cost, and resistance will appear as a continuous polygenic character.

Evolution of herbicide resistance in the field

Our results present a compelling case for the potential of low herbicide rates to select for polygenically based resistance. They do not, however, completely mimic selection in the field where very large populations are exposed to herbicides, and empirical evidence suggests that monogenic responses predominate (reviewed by Darmency 1994; Jasieniuk et al. 1996). The same is true for insecticide resistance (Roush and McKenzie 1987), though laboratory selection for insecticide resistance has often resulted in a polygenic response (McKenzie and Batterham 1994). The reasons for this discrepancy are well understood; small laboratory populations are unlikely to contain rare single-gene mutations (McKenzie and Batterham 1994). Clearly, the same qualifications apply to our results, though we consider that the preponderance of reports of monogenic resistance in the literature may in part reflect the comparative ease with which this can be confirmed compared to more complex metabolism-based traits whose genetic determination in unknown.

The rate of herbicide applied to large, genetically diverse weed populations will influence which mechanisms of resistance are selected. At high doses, only those individuals outside of the normal range of susceptible phenotypes and possessing rare (and probably single gene) mutations will survive. At low or intermediate doses, a wider range of weaker resistance mechanisms may favour survival, and all of these mechanisms will be selected. The subsequent evolutionary dynamics of these mechanisms will depend on numerous factors, including their frequency in the population, relative fitness and subsequent herbicide use patterns. Given assumptions about the initial frequency and relative fitness of major and minor insecticide resistance genes, Groeters and Tabashnik (2000) showed that responses to selection were dominated by major genes regardless of selection intensity. However, whereas at high selection intensities (1% survival), minor genes did not contribute to resistance, low and intermediate selection resulted in increases in the minor genes also. Lande (1983) has shown that polygenic responses will be favoured when the initial frequencies of major genes are much lower than minor genes, selection intensities are low and major genes incur a large fitness penalty. The relatively high frequency of phenotypic resistance at low doses of diclofop-methyl evident in *L. rigidum* may predispose this species to polygenic resistance in the field.

The rapid evolution of polygenically based resistance relies on outcrossing between neighbouring plants and the accumulation in their progeny of minor genes with additive and/or multiplicative effects. This process has been facilitated in our study by the application of herbicides to even-aged individuals under highly controlled laboratory conditions and their subsequent crossing in the absence of fully susceptible survivors. However, in a field treated at a low herbicide dose, a number of highly susceptible individuals may survive as a result of emergence after herbicide application or because of other factors which enable them to escape herbicide application. In a cross-pollinated species such as *L. rigidum* these susceptible individuals will considerably reduce the frequency of minor resistance genes in the population and hence the potential for rapid evolution of polygenic resistance.

Implications for management and future research priorities

Our results demonstrate, for the first time, the potential for recurrent selection with low herbicide rates to rapidly select for herbicide resistance. We believe these results have important implications for directing future research towards more informed management to reduce

the impact of herbicide resistance. This will be particularly true as environmental and economic incentives to reduce herbicide application rates continue to increase.

The predominance of monogenic responses to herbicide selection is a central tenet of herbicide resistance management and is supported by empirical evidence (Darmency 1994; Jasieniuk et al. 1996). On this basis, almost all models which simulate herbicide resistance evolution assume monogenic control of resistance traits (Gressel and Segel 1978; Maxwell et al. 1990; Diggle et al. 2003). Under the assumption that initially low herbicide doses will select for both major and minor gene resistance, subsequent evolution towards greater fitness under recurrent herbicide selection will depend on the genetic and biological factors discussed previously and on herbicide-use patterns. Where a weed population is repeatedly selected with the same herbicide, low doses may result in the selection of monogenic or polygenic responses, depending on the initial genetic variability for herbicide response in the population. High doses will most probably favour major gene resistance. Where herbicide rotation is practiced, whether specifically for resistance management or because of crop rotation, low herbicide dose regimens may have some unforeseen consequences. In the VLR1 lines selected with low doses of diclofop-methyl, some interesting patterns of cross-resistance were observed. In particular, a threefold increase in resistance to the chemically unrelated imidazolinone herbicide, imazethapyr was noted. Subsequent rotation to this herbicide in the field would have resulted in the rapid evolution of economic levels of resistance. We postulate that herbicide rotation strategies based on low rates of herbicides select for 'generalist' resistance mechanisms and lead to the loss of a range of herbicide options. Indeed, the widespread existence of cross-resistance in Australian populations of *L. rigidum* may be, in part, a result of the frequent use of low or sub-optimal herbicide rates.

Large-scale and long-term field-based trials to compare selection for herbicide resistance at low, intermediate and high herbicide use rates are plagued with difficulties. They require experimentation on large populations of weeds which usually only produce a single generation per year. We propose two approaches that may overcome some of these difficulties and limitations. The first involves modifications to existing two-locus stochastic herbicide resistance models (Diggle et al. 2003; Neve et al. 2003) to incorporate polygenically inherited resistance traits. These models may be used to simulate major and minor gene herbicide resistance under a range of herbicide use patterns with various assumptions about the initial allele frequencies and their fitness in the presence and absence of herbicide selection. A second more empirical approach would create artificial populations of model plants such as *Arabidopsis thaliana* with known frequencies of major and minor gene resistance traits. These populations could then be subjected to a range of herbicide use regimens to assess evolutionary outcomes. *A. thaliana* has the benefit of a short generation time, enabling multiple generations to be produced over a relatively short period. In addition, the availability of well-defined, rapid and relatively cheap molecular tools would enable the effective screening of changes in allele frequencies and resistance mechanisms under selection.

In conclusion, our results have demonstrated the existence of quantitative phenotypic variation for herbicide response in a *L. rigidum* population with no previous history of exposure to herbicides. Our observations suggest that this variation is polygenically based and that selection with low herbicide rates results in the accumulation of minor genes with additive or multiplicative effects. We propose that too little attention has been given to the potential contribution of polygenic traits to herbicide resistance evolution in the field. Often research has documented the involvement of single major resistance genes but has not considered the fact that other loci with minor effects may also have been selected and contribute to the resistance phenotype. Studies of the inheritance of herbicide resistance have often used a single herbicide dose to characterise the resistance phenotype of progeny from pair crosses and in doing so have assumed, a priori, that resistance is a qualitative trait. Variation at minor genes may be particularly important during the early stages of resistance evolution, and the importance of this variation may be underestimated in studies that concentrate on highly evolved herbicide-resistant weed populations. Increasing environmental pressure for reductions in herbicide application rates together with the continuing development and adoption of herbicide-resistant transgenic crops means that it is crucial to continue to increase our understanding of the evolution and population genetics of herbicide resistance.

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