Research Article

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Loss of trifluralin metabolic resistance in *Lolium rigidum* plants exposed to prosulfocarb recurrent selection

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Abstract

BACKGROUND: Resistance to the dinitroaniline herbicide trifluralin in *Lolium rigidum* (annual ryegrass) often is mediated by the enhanced capacity to metabolize the herbicide to less toxic polar conjugates and/or by functionally recessive target-site mutations in α-tubulin.

RESULTS: In two *L. rigidum* populations possessing enhanced trifluralin metabolism, resistance was largely reversed by recurrent selection with the thiocarbamate herbicide prosulfocarb (i.e. plant survival was two- to >20-fold lower). Their ability to metabolize trifluralin was significantly decreased (by ≈2.3-fold) following recurrent prosulfocarb selection, to levels comparable to those observed in susceptible plants or when trifluralin metabolism was inhibited by treatment with the insecticide phorate.

CONCLUSIONS: This study provides evidence that trait(s) enabling efficient trifluralin metabolism in *L. rigidum* are purged from the population under prosulfocarb recurrent selection. The level of trifluralin metabolism *in vitro* and its inhibition caused by phorate action on trifluralin-metabolizing enzyme(s) is equivalent to the effect produced by prosulfocarb selection. The hypothetical link between the two phenomena is that the putative monooxygenase(s) conferring trifluralin metabolic resistance also mediate the activation of prosulfocarb to its toxic sulfoxide. Thus, we speculate that survival to prosulfocarb via a lack of metabolic herbicide activation, and survival to trifluralin conferred by enhanced herbicide metabolism, are mutually exclusive. These findings not only open up a new research direction in terms of the interaction between different herbicide resistance mechanisms in *L. rigidum*, but also offer strategies for immediate management of the population dynamics of metabolism-based resistance in the field.

Supporting information may be found in the online version of this article.

Keywords: annual ryegrass; cytochrome P450 monooxygenase; herbicide resistance; phorate; prosulfocarb; trifluralin

1 INTRODUCTION

Over-reliance on herbicides for weed control in global field crops over the past 60 years has resulted in the evolution of herbicide-resistant weed populations.[1] In Australia, the widespread adoption of conservation agriculture with reduced tillage, and the dominance of small-grain cereal crops (grown on >70% of arable land), has led to greater reliance on herbicides to control these weeds.[2] In cereal crops, the control of grass weeds is challenging owing to the difficulty in achieving effective weed kill while maintaining satisfactory crop safety. Nevertheless, several selective herbicides (i.e. metabolized by the cereal crop) from the acetyl CoA carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting groups, such as diclofop and chlorsulfuron have been used with great success from the late 1970s onwards. However, over-reliance on these selective post-emergence herbicides has led to widespread evolution of resistance in grass weed genera such as *Alopecurus*, *Avena* and *Lolium*.[3–5]

In Australian cropping systems, *Lolium rigidum* (annual ryegrass) is the most troublesome weed because it is widespread and extremely adaptable to single strategies deployed for its control. Recent surveys indicate that the incidence of multiple resistance to ACCase- and ALS-inhibiting herbicides in Western Australian *Lolium* populations is >95%.[6] In 70% of diclofop-resistant *Lolium* populations from Australia and France, resistance is endowed by

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a combination of two distinct mechanisms, namely enhanced metabolism (detoxification) of diclofop plus mutations of the target ACCase that minimize herbicide binding.[7] A similar situation is likely to exist for the ALS-inhibiting herbicides.[8] In response to escalating levels of multiple resistance to ACCase- and ALS-inhibiting herbicides, there has been an increasing adoption of soil-applied pre-emergence herbicides such as trifluralin and propyzamide (inhibitors of mitosis via disruption of microtubule assembly), pyroxasulfone (inhibitor of very long chain fatty acid biosynthesis), prosulfocarb and triallate (inhibitors of lipid synthesis).[8]

The dinitroaniline herbicide trifluralin has been almost universally used for pre-emergence weed control in Australian wheat crops for the past 25 years. Until recently, resistance has remained relatively uncommon, and in some cases dinitroaniline herbicides still provide satisfactory control of *L. rigidum* populations with resistance to multiple other herbicides.[6,9] However, in South Australia and Victoria, trifluralin resistance is now more common.[8] Only a few weed species are reported to have evolved prosulfocarb resistance in the field, namely *Alopecurus myosuroides* Huds. from Denmark[10] and *L. rigidum* from southern Australia.[11] Resistance to prosulfocarb was induced in a trifluralin-resistant *Lolium* population (SLR31) by recurrent selection with low doses of the isoxazoline herbicide pyroxasulfone.[12,13] Interestingly, in acquiring prosulfocarb resistance, the population seemed to have lost some of the pre-existing trifluralin resistance (see Supporting Information Table S2, from Busi and Powles[13]). Resistance to trifluralin, chlorsulfuron and pyroxasulfone in population SLR31 and its pyroxasulfone-selected progeny was partially reversed with the application of the organophosphate insecticide phorate.[14]

In theory, these responses can be explained by the fact that organophosphate insecticides are known to inhibit certain members of the cytochrome P450 monooxygenase (P450) superfamily.[15–17] P450s, along with flavin-containing monooxygenases (FMOs), are NADPH-requiring microsomal enzymes that catalyze the oxidative modification of hydrophobic molecules such as secondary metabolite precursors[18,19] and xenobiotics.[20] Whilst FMOs are known to detoxify xenobiotics in animals, they appear to be involved more in secondary metabolism than detoxification in plants.[19] For herbicides, the action of mixed-function oxygenases can result in detoxification via the conjugation of large, hydrophilic moieties to the activated group (e.g. the formation of phenolic glucosides of diclofop[21]), or conversely, in the activation of relatively inert herbicide molecules to their toxic form (e.g. conversion of prosulfocarb to its active sulfoxide[22,23] or of clomazone to a 5-hydroxylated derivative with higher toxicity[17]). The partial reversal of phenotypic trifluralin resistance by phorate suggests that trifluralin metabolism is mediated by one or more phorate-inhibitable enzymes (likely P450s),[14] but this has not yet been conclusively demonstrated.

In the current study, the potential role of phorate-inhibitable enzymes in mediating metabolic trifluralin resistance was investigated by quantifying the extent of trifluralin metabolism in the presence and absence of phorate in two populations with nontarget-site-based trifluralin resistance, and in their pyroxasulfone- and/or prosulfocarb-selected progeny. The levels of phenotypic resistance to trifluralin and prosulfocarb in these populations (plus or minus phorate) were confirmed in pot experiments at two doses of each herbicide, to further investigate the observation that selection with prosulfocarb may concurrently select for increased trifluralin sensitivity.[13]

2 MATERIALS AND METHODS

2.1 Plant material

A well-characterized *L. rigidum* population susceptible to all herbicides tested,[6] designated VLR1, was used as a susceptible control. Two herbicide-resistant field populations and their laboratory-selected progeny were used as resistant populations. Population SLR31, used as the parental population in previous studies[12,13] and with a known history of herbicide applications in the field,[24] is well-characterized as being resistant to the ACCase-inhibiting diclofop,[7] ALS-inhibiting chlorsulfuron,[3] cell division-inhibiting metolachlor[24] and microtubule-disrupting trifluralin.[25] Trifluralin resistance appears to be conferred solely by enhanced metabolism and not by mutations in the α-tubulin target site.[26,27] Following recurrent selection of the parental population (hereafter referred to as SLR31-P0) with pyroxasulfone at 60, 120, 120 and 240 g ha^{-1} and then prosulfocarb at 1000 g ha^{-1},[12,13] the resulting population (SLR31-P1) was further selected with 2000 g ha^{-1} prosulfocarb using the same methods as in Busi and Powles,[13] to yield population SLR31-P2. The populations SLR31-P0, SLR31-P1 and SLR31-P2 were used in the experiments described below.

The second herbicide-resistant population, designated as SELR68 (hereafter referred to as SELR68-P0), was collected in a random weed survey in 2012 near the town of Bordertown in the upper south-east region of South Australia and thus its history of herbicide selection in the field is unknown. It was confirmed as possessing high-level (>20% survival) resistance to trifluralin in a previous repeated pot trial (data not shown). This population, being initially susceptible to prosulfocarb, was selected twice with prosulfocarb at 500 g ha^{-1} to yield progeny populations SELR68-P1 and SELR68-P2. In each round of herbicide selection of SLR31 and SELR68 (using ≥100 individuals, four replicates of 25 seeds) there were at least ten survivors to generate seed for the next generation.

2.2 Herbicide treatments

Lolium rigidum seeds were sown on the surface of commercial potting mix in 2-L pots (25 seeds per pot, one pot per replicate). Thirty minutes before herbicide treatment, 10 kg ha^{-1} of the insecticide phorate (Thimet 100G; 100 g phorate kg^{-1}, Barmac Industries, Stapylton, QLD, Australia) was applied to some of the pots.[14] Pots then were sprayed with trifluralin (TriflurX, 480 g trifluralin L^{-1}; Nufarm, Laverton North, VIC, Australia) at 240 or 480 g ha^{-1} or with prosulfocarb (Arcade, 800 g prosulfocarb L^{-1}, Syngenta, North Ryde, NSW Australia) at 500 or 1000 g ha^{-1}. Control pots that were untreated, or treated with 10 kg ha^{-1} phorate only, also were included.

Herbicides were applied with a cabinet track sprayer travelling at 1 m s^{-1}, mounted with twin flat-fan nozzles (TeeJet 11 001, TeeJet Technologies, Springfield, IL, USA) and delivering a water volume of 240 L ha^{-1} per treatment at a pressure of 200 kPa. Following herbicide application, a 5 mm layer of potting mix was placed over the seeds and pots were returned to outdoor conditions at the University of Western Australia (mean maximum/minimum temperature, 20/11 °C; mean day length 12 h) and watered regularly. Survival, represented by plant emergence from the soil and active growth/establishment, was assessed 28 days post-treatment. At this time, the aboveground biomass was harvested, dried at 70 °C for three days, and the dry biomass was recorded. There were two replicates of each treatment, and the experiment was performed twice. Data from both experiments were pooled.

Phorate alone had no significant effect on plant emergence and survival or plant aboveground biomass (Fig. S1).

2.3 Analysis of trifluralin metabolism

Seeds of the VLR1, SLR31-P0, SLR31-P2, SELR68-P0 and SELR68-P2 populations were germinated in 90-mm diameter dishes containing 0.6% (w/v) agar plus 1 μM trifluralin (a concentration which discriminates between susceptible and resistant populations[28]) and incubated in the dark under a 12 h temperature cycle of 25/15 °C for five days. By this time, all populations had coleoptiles of 1–8 mm, depending on their trifluralin resistance level. Ten seedlings per population per replicate were used for treatment with radiolabelled trifluralin. In the trifluralin-susceptible populations, the ten seedlings with the longest coleoptiles (1–5 mm) were selected for treatment, whilst ten seedlings with a uniform coleoptile length of 8 mm were selected from the resistant populations. Radiolabelled trifluralin (ring-^{14}C[U]; 592 MBq mmol^{-1}; American Radiolabeled Chemicals, St Louis, MO, USA) was applied as a single 0.5-μL droplet of 1.5 kBq (corresponding to 5 mM trifluralin) in 0.2% (v/v) Tween 20 to the base of each coleoptile, and seedlings were incubated as above for a further 72 h. For the phorate treatments, 10 mg solid phorate was applied to the surface of the agar immediately after trifluralin application. Each agar plate of ten seedlings represented one experimental unit, and there were three replicates of each treatment.

At the end of the 72 h incubation period, seedlings were washed in a stream of 50% (v/v) methanol containing 0.1% (v/v) Tween 20 (1 mL per seedling), blotted dry, and the ten seedlings per treatment were homogenized in a total volume of 500 μL cold 80% (v/v) methanol[26] using a chilled mortar and pestle. To check for nonenzymatic degradation of [^{14}C]-trifluralin, untreated seedlings (ten per sample, two samples per experiment) were extracted in the presence of 15 kBq freshly-added [^{14}C]-trifluralin. Extracts were clarified by centrifugation at 12 000×*g* for 5 min, and the supernatant was collected and partitioned against an equal volume of 100% hexane[26] after removal of a small aliquot. Parent [^{14}C]-trifluralin (3 kBq per sample, three samples per experiment) was diluted into 400 μL of 80% methanol and also partitioned against hexane. The amount of ^{14}C in the seedling washes, crude clarified extract and the organic (hexane) and aqueous (80% methanol) phases was determined by liquid scintillation counting using Ultima Gold scintillation cocktail (Perkin-Elmer, Waltham, MA, USA) and a Packard TriCarb 1500 Liquid Scintillation Analyzer. On average, 87 ± 1% of the ^{14}C signal from hexane-partitioned parent trifluralin was recovered in the organic phase, and this value was used to correct the data from the seed extracts. Total recovery of applied ^{14}C from the [^{14}C]-trifluralin-treated seed extract plus the washes was 7.4 ± 0.4%, likely due to the volatility of trifluralin.[29] The relative volatility of parent trifluralin and its metabolite(s) was assessed by placing 20 μL of the organic and aqueous phases from extracts of ^{14}C-trifluralin-treated VLR1 and SLR31-P0 seedlings (three replicates per population) into scintillation vials and incubating at room temperature for 0, 1, 2, 4, 8, 24, 72, 120, 168 or 336 h before adding scintillation cocktail and immediately recording the amount of ^{14}C in the vial. Vials were left uncapped during incubation but were loosely covered with paper towel to prevent particulate matter from falling inside.

2.4 Statistical analysis

In order to compare across populations, data were expressed as a percentage of the untreated control, for each experiment, population, progeny and insecticide treatment. All analyses were performed using the R[30] and S-Plus[31] programming languages. The dataset was submitted to ANOVA, by considering the experiment, population, progeny, insecticide, herbicide, doses and all of their interactions as fixed factors. Graphical analyses of residuals showed that the dataset was clearly affected by heteroscedasticity, whereas normality was not a relevant issue. Therefore, generalized least squares were used for model-fitting, allowing the variance to be an exponential function of the fitted values.[32] The ANOVA showed that the effect of the experiment and its interactions with treatment factors were never significant and thus, those effects were removed from the model. Expected marginal means for the 'population × progeny × insecticide × herbicide × doses' combinations were obtained from model parameters, together with SEs; pairwise comparisons across means were performed by using Welch tests with Holm multiplicity correction.[33] Prism (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot the data.

3 RESULTS

3.1 Response to trifluralin in parental and selected *L. rigidum* populations

In the absence of phorate, survival of parental trifluralin-resistant populations SLR31-P0 and SELR68-P0 and the P1 progeny of SELR68 at 240 g trifluralin ha^{-1} was significantly (more than four-fold) higher than that of their P2 progeny and the susceptible population VLR1 [Fig. 1(a), (c)]. The same pattern was evident at 480 g trifluralin ha^{-1} for the SELR68 family [Fig. 1(d)], but at this rate of trifluralin, survival of SLR31-P1 was at the same low level as SLR31-P2 and VLR1 [Fig. 1(b)]. Phorate caused a three- to 11-fold decrease in the survival of the trifluralin-resistant P0 and P1 populations, but this was only statistically significant for the P1 populations, and only at 240 g trifluralin ha^{-1} (Fig. 1).

The biomass response of the populations in the presence and absence of phorate was statistically significant at 240 g ha^{-1} trifluralin for populations SLR31-P1 and SELR68-P1 and at 480 g ha^{-1} trifluralin for populations SELR68-P0 and SELR68-P1 (Fig. 2).

3.2 Response to prosulfocarb in parental and selected *L. rigidum* populations

Survival of prosulfocarb-resistant progeny SLR31-P2 was significantly higher than that of parental SLR31-P0 at both 500 and 1000 g prosulfocarb ha^{-1} in the absence of phorate [Fig. 3(a), (b)]. There were no statistically significant differences between SELR68 populations at either rate of prosulfocarb in the absence of phorate [Fig. 3(c), (d)]. However, addition of phorate caused a dramatic and significant increase in survival of SLR31-P0 and SELR68-P2 at 500 g prosulfocarb ha^{-1} (Fig. 3).

Significant differences in biomass were observed in population SLR31 in response to prosulfocarb and phorate treatments. SLR31-P2 had higher biomass than SLR31-P1 at 500 g prosulfocarb ha^{-1} and higher biomass than SLR31-P0 at both rates of prosulfocarb in the absence of phorate [Fig. 4(a), (b)]. Addition of phorate resulted in a significant increase in biomass of SLR31-P0. The effects of phorate on biomass of SELR68 treated with either rate of prosulfocarb were not significant [Fig. 4(c), (d)]. The profound visual effects of phorate on the survival and biomass of the parental populations are illustrated in Fig. S2.

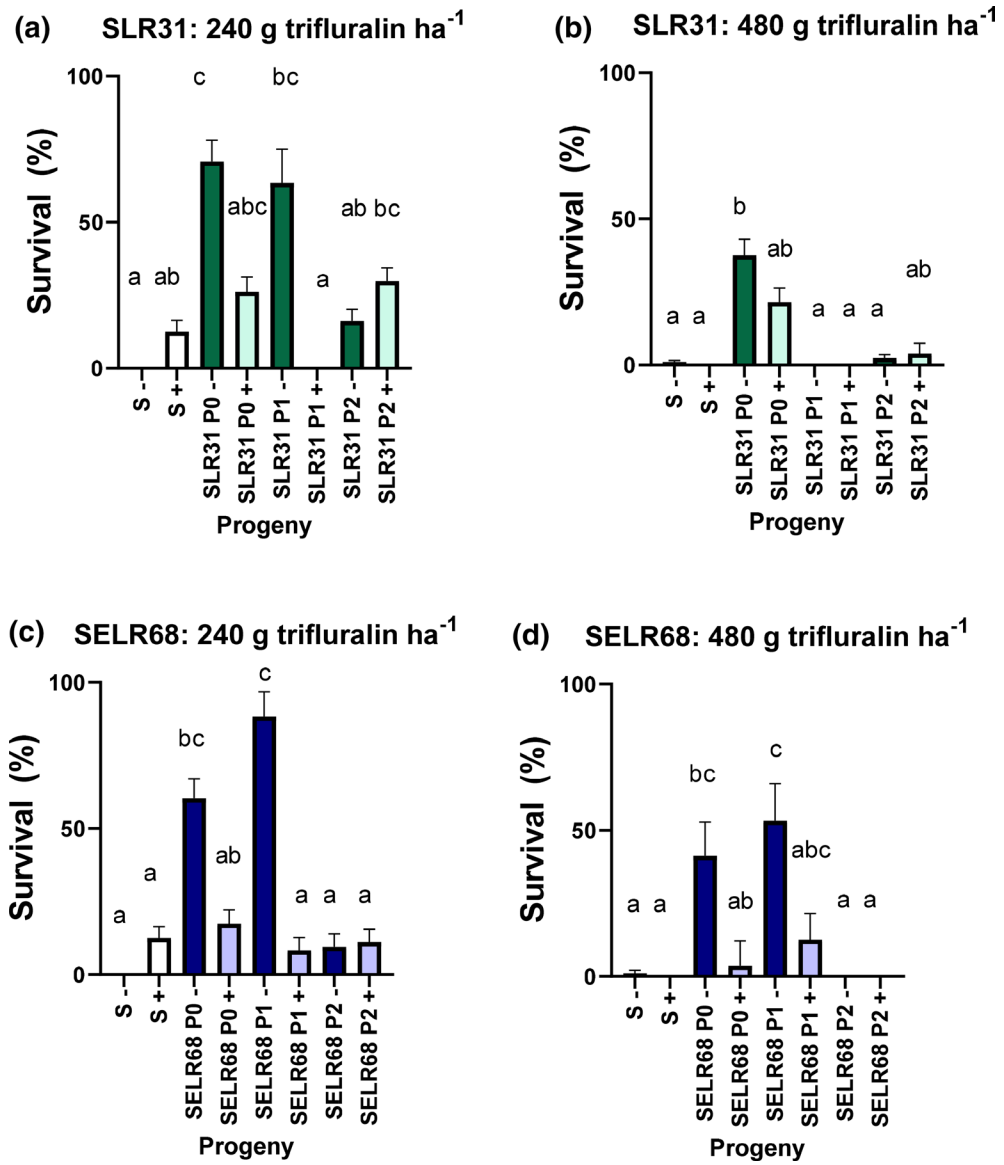

Figure 1 Survival of *L. rigidum* SLR31 (a, b) or SELR68 (c, d) -derived populations treated with 240 (a, c) or 480 (b, d) g ha^{-1} trifluralin in the presence (+) or absence (–) of the organophosphate insecticide phorate. S, susceptible population VLR1; P0, parental population; P1, P2, progeny following one or two rounds of prosulfocarb selection. Values are means ± SEs ($n = 4$). Different letters above columns in each panel denote significant ($P < 0.05$) differences between means.

3.3 Trifluralin metabolism and the effect of phorate

The capacity of each population to metabolize trifluralin was quantified as the proportion of ^{14}C recovered in the aqueous *versus* organic phase of hexane-partitioned extracts of [^{14}C]-trifluralin-treated seeds (with the aqueous ^{14}C representing polar metabolites of trifluralin). For the parental populations SLR31-P0 and SELR68-P0, 40–50% of the recovered ^{14}C was in the aqueous phase. By contrast, their prosulfocarb-selected progeny and the susceptible VLR1 population were not significantly different from the untreated, spiked controls, all showing ≤20% of ^{14}C in the aqueous phase (Fig. 5). However, incubation of the seedlings with phorate inhibited the conversion of [^{14}C]-trifluralin into polar metabolites, so that the extent of polar (trifluralin) metabolites in phorate-treated SLR31-P0 and SELR68-P0 seedlings was not significantly different to that in the untreated VLR1, SLR31-P2 and SELR68-P2 populations (Fig. 5). The phenotypes of the seedlings, photographed just before extraction, reflected the results of the pot study and the observed pattern of trifluralin metabolism in that only the SLR31-P0 and SELR68-P0 seedlings incubated without phorate were relatively free of the coleoptile swelling and stunting that are characteristic symptoms of trifluralin toxicity (Fig. S3).

Measurement of the loss of ^{14}C over time from the aqueous and organic phases incubated in open tubes showed that the ^{14}C in the latter, representing parent trifluralin, declined sharply in the first hour (to 70% of the starting value) and more gradually thereafter, until only 20% remained after 14 days incubation (Fig. S4). By contrast, the ^{14}C in the aqueous phase, representing polar trifluralin metabolites, remained at >90% of the starting value over the first 72 h, and was still >75% after 14 days (Fig. S4). There was no difference in the volatility of ^{14}C between the equivalent phases collected from VLR1 and SLR31-P0 extracts, indicating that

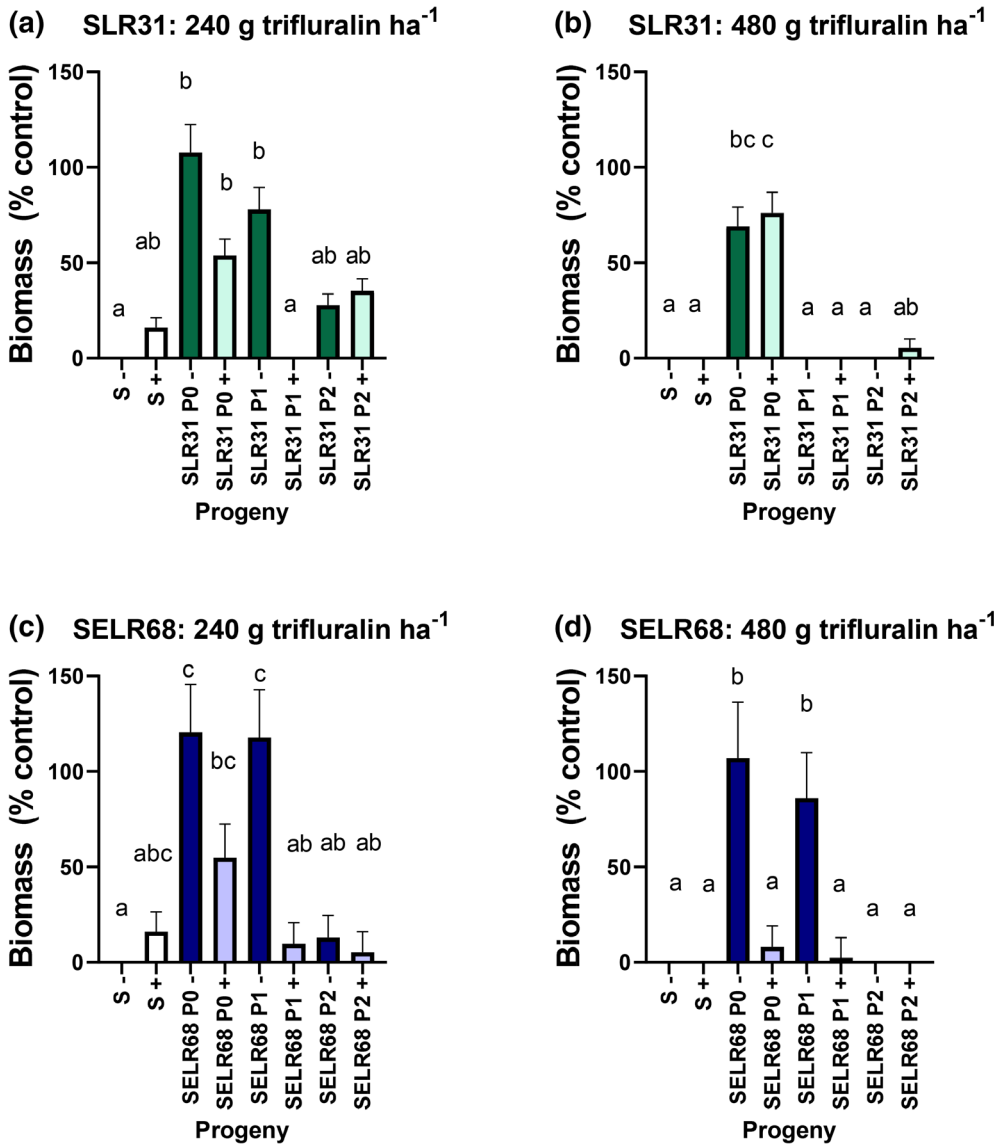

Figure 2 Survivor biomass of *L. rigidum* SLR31 (a, b) or SELR68 (c, d) -derived populations treated with 240 (a, c) or 480 (b, d) g ha^{-1} trifluralin in the presence (+) or absence (−) of the organophosphate insecticide phorate. S, susceptible population VLR1; P0, parental population; P1, P2, progeny following one or two rounds of prosulfocarb selection. Values are means ± SEs ($n = 4$). Different letters above columns in each panel denote significant ($P < 0.05$) differences between means.

comparisons of metabolite *versus* parent trifluralin ratios between different populations, in spite of the heavy losses of ^{14}C during seedling incubation and extraction, are valid.

4 DISCUSSION

4.1 Inhibition of trifluralin metabolism in resistant *L. rigidum*

In Australia, trifluralin is widely used in rotation in different crops to provide satisfactory control of multiple herbicide-resistant *L. rigidum* populations.[8] World wide, after nearly 60 years of use,[34] resistance to this herbicide has so far been reported in fewer than ten weed species,[1] and the spread of (target-site) resistance is slow in *L. rigidum* likely due to the functionally recessive nature of α-tubulin mutation inheritance.[35] Nevertheless, the incidence of trifluralin resistance in some regions of Australia is concerning.[8] The multiple-resistant population SLR31 was one of the first trifluralin-resistant biotypes confirmed,[25] with resistance mediated by enhanced metabolic activity leading to a decrease in the tissue concentration of the parent herbicide.[26]

Previous pot studies provided phenotypic evidence that the organophosphate insecticide phorate could reduce the level of chlorsulfuron, pyroxasulfone and trifluralin resistance in the pyroxasulfone-selected progeny of population SLR31, with the greatest synergistic effects observed when phorate was used in combination with trifluralin.[14] The current biochemical study confirms that phorate synergises trifluralin by inhibiting trifluralin metabolism *in vivo*, decreasing the formation of polar metabolites to levels similar to those observed in trifluralin-susceptible populations. Trifluralin metabolism in populations SLR31-P0 and SELR68-P0 is therefore likely mediated by a phorate-inhibitable enzyme(s) such as a P450(s). Additionally, this study provides evidence that recurrent prosulfocarb selection resulted in the loss of trifluralin metabolic resistance as evidenced by the inability of the

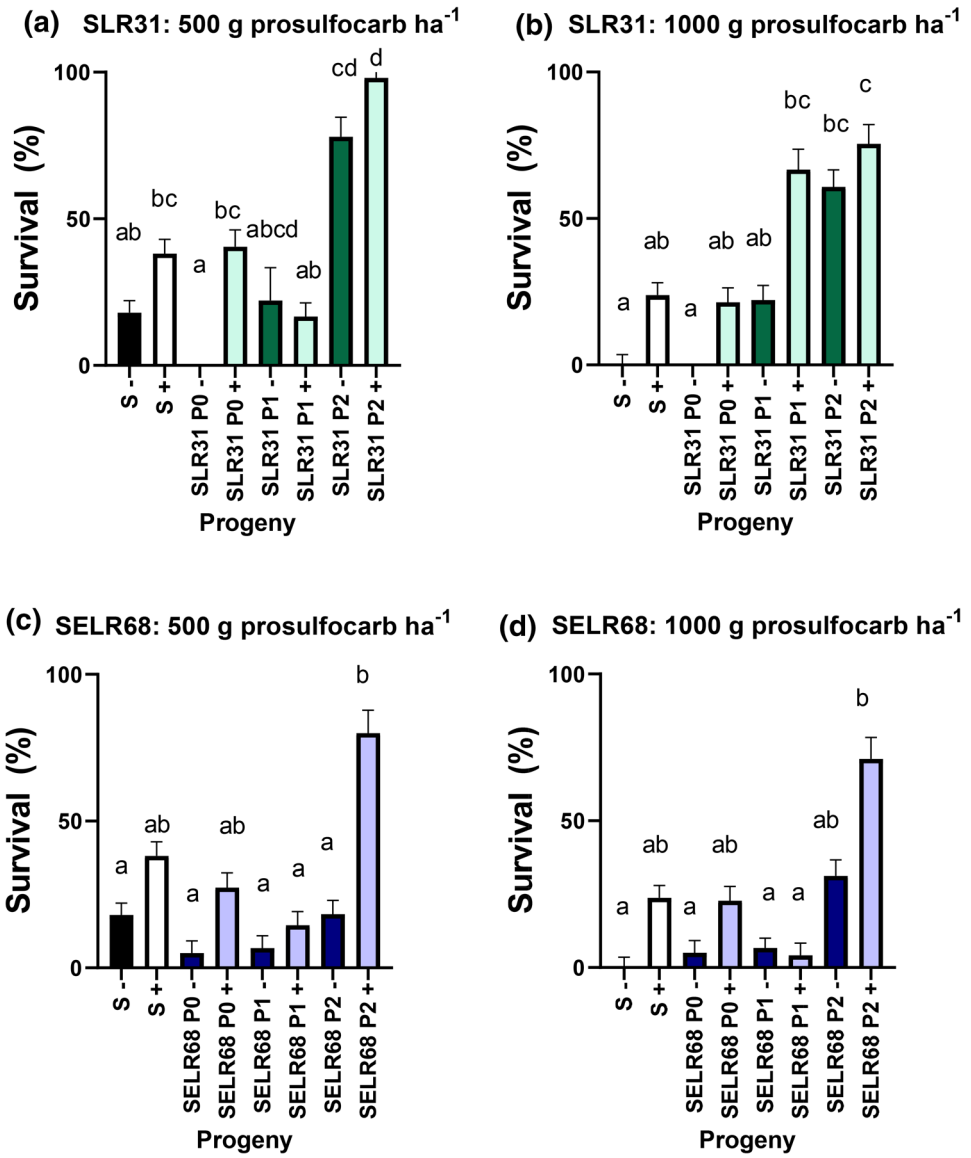

Figure 3 Survival of *L. rigidum* SLR31 (a, b) or SELR68 (c, d) -derived populations treated with 500 (a, c) or 1000 (b, d) g ha^{-1} prosulfocarb in the presence (+) or absence (–) of the organophosphate insecticide phorate. S, susceptible population VLR1; P0, parental population; P1, P2, progeny following one or two rounds of prosulfocarb selection. Values are means ± SEs (n = 4). Different letters above columns in each panel denote significant ($P < 0.05$) differences between means.

prosulfocarb-selected progeny to metabolize trifluralin. Thus, preliminary observations that sequential selection of SLR31-P0 with pyroxasulfone and prosulfocarb decreased the level of phenotypic trifluralin resistance[13] are confirmed.

4.2 A potential link between trifluralin and prosulfocarb responses

In this study we document that under two rounds of recurrent selection with prosulfocarb, the traits conferring efficient trifluralin detoxification via metabolism are purged from two field populations of *L. rigidum*. An equivalent decrease in trifluralin metabolism was observed by treating the trifluralin-resistant parent populations with phorate. Although phorate (and its oxidised metabolites) can inhibit or stimulate the activity of a range of mammalian and insect enzymes and is predominantly used as an insect cholinesterase inhibitor,[36,37] there is less information regarding its effect on plant enzymes, except that it is an inhibitor of several P450s[17] and that exposure to phorate causes moderate oxidative stress that leads to upregulation of antioxidant and pathogen defence genes.[37,38] Phorate has been used previously as a P450 inhibitor to investigate xenobiotic metabolism in plants.[14,17,39]

Likewise, little is known about the detoxification pathway of trifluralin in monocotyledonous plants, but dealkylation of the tertiary amine moiety, as observed in animals,[40,41] soil bacteria[42] and dicotyledonous crops[43,44] could be catalyzed by either P450s or FMOs,[45] and was shown to take place *in vitro* in a rat microsome system.[40] Oxidation of prosulfocarb to its active sulfoxide also is mediated by one or more of the microsomal oxidases.[22,23] Therefore, based on the reasonable speculation that phorate-inhibitable enzymes, probably P450s, are involved in the biotransformation of both trifluralin and prosulfocarb, we

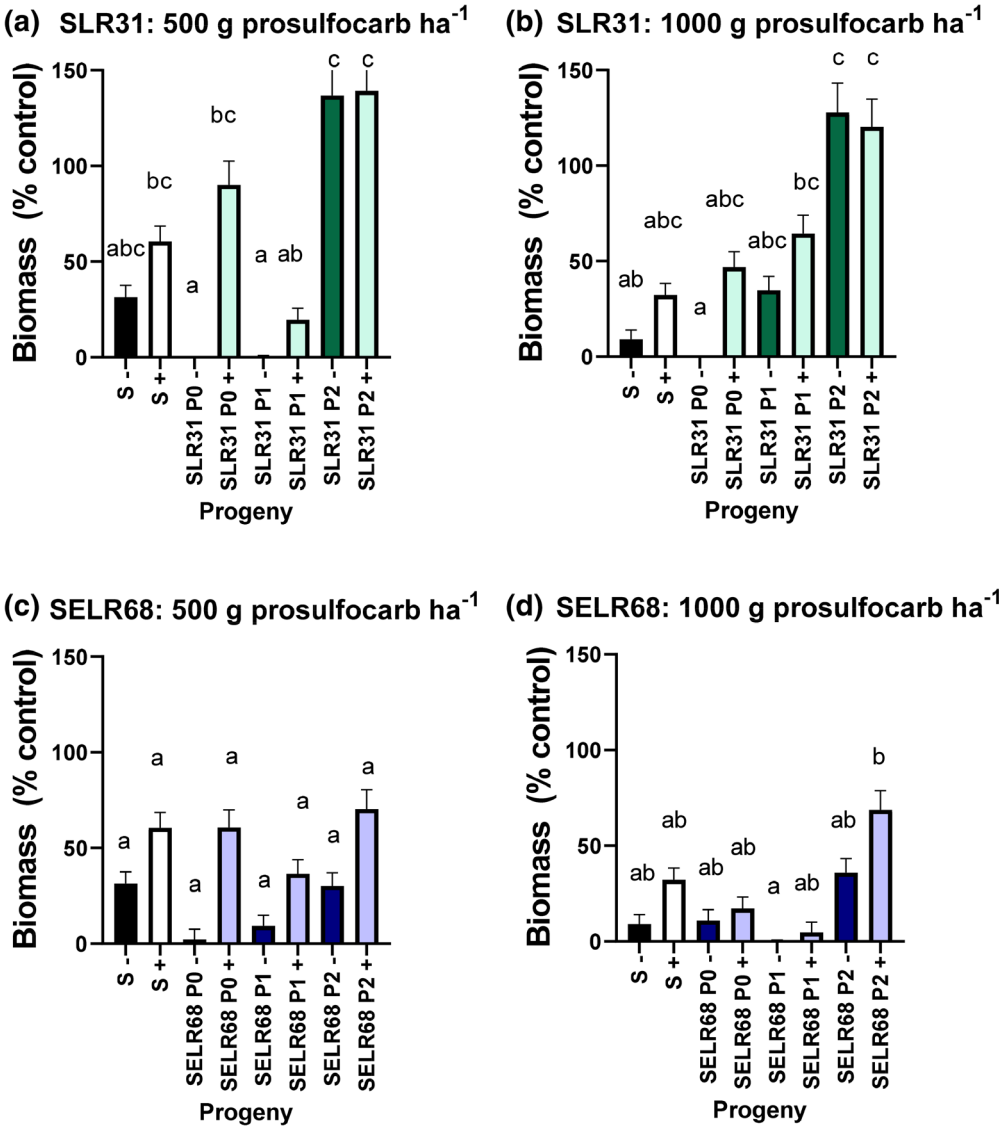

Figure 4 Survivor biomass of *L. rigidum* SLR31 (a, b) or SELR68 (c, d) -derived populations treated with 500 (a, c) or 1000 (B, D) g ha^{-1} prosulfocarb in the presence (+) or absence (–) of the organophosphate insecticide phorate. S, susceptible population VLR1; P0, parental population; P1, P2, progeny following one or two rounds of prosulfocarb selection. Values are means ± SEs ($n = 4$). Different letters above columns in each panel denote significant ($P < 0.05$) differences between means.

hypothesize that the expression of enzyme(s) conferring trifluralin metabolic resistance and those responsible for prosulfocarb activation are commonly controlled by an upstream regulator such as the ligand-activated transcription factors responsible for mediating expression of drug-metabolizing P450s.[46] This would account for the observation that metabolic resistance to trifluralin, and resistance to prosulfocarb via reduced activation, are mutually exclusive within a population.

For prosulfocarb, the hypothesis is supported by the fact that reduced activation has been documented to confer resistance to the thiocarbamate herbicide triallate in *Avena fatua*.[46] The same situation seems to be evolving in prosulfocarb-selected population SELR68-P2, and is mirrored by the enhanced survival of prosulfocarb-susceptible populations VLR1, SLR31-P0 and SELR68-P0 in the presence of P450-inhibiting phorate (Fig. 3). The hypothesis that survivors of recurrent selection with low doses of prosulfocarb may have purged the enzyme(s) responsible for activation of prosulfocarb,[47,48] and that these same enzymes contribute to trifluralin metabolism, can only be confirmed by identification of the trifluralin metabolites produced by resistant ryegrass, and of the putative P450s that catalyze biotransformation of trifluralin and prosulfocarb. Thus, a great deal more biochemical and genetic research is required.

4.3 Evolutionarily-instructed management of resistance

Recurrent selection studies allow the rapid assessment of the dynamics of herbicide selection and the subsequent risk of herbicide resistance evolution.[12,49] Likewise, this study aims to provide an 'evolutionarily-instructed' understanding of practical weed management.[50] Trifluralin resistance reversal via prosulfocarb field selection in *L. rigidum* is expected to be rapid when trifluralin resistance is only caused by enhanced metabolic capacity,[49,51] with the caveat that conditions in the field (seeds in different locations receiving different doses of prosulfocarb due to rainfall,

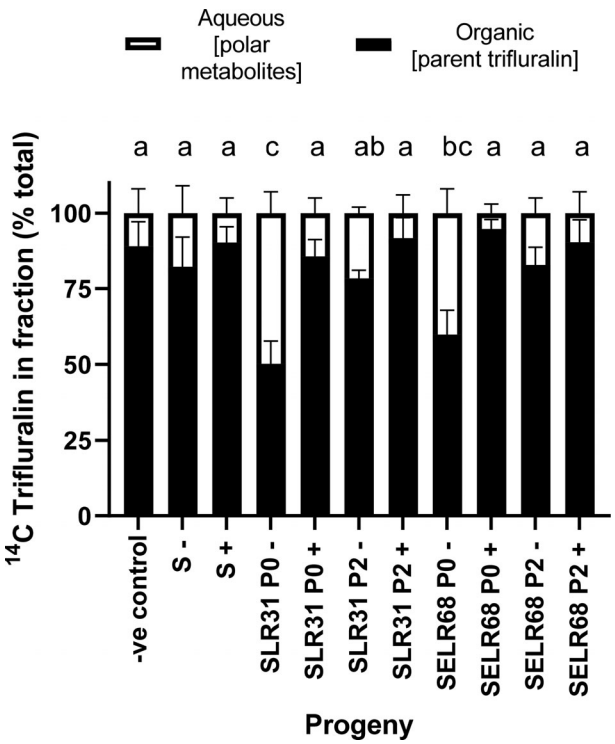

Figure 5 Metabolism of trifluralin in *L. rigidum* populations in the presence or absence of phorate. ^{14}C recovered from extracts of [^{14}C]-trifluralin-treated seedlings was partitioned into organic and aqueous phases, representing parent trifluralin and its polar metabolites, respectively, and quantified by liquid scintillation counting. To account for nonenzymatic modification of trifluralin, untreated seeds were extracted in the presence of ^{14}C-trifluralin (–ve control). Values are means ± SEs (n = 3). Different letters above columns denote significant ($P < 0.05$) differences between populations. S, susceptible population VLR1; SLR31 and SELR68, populations in the absence (–) or presence (+) of the organophosphate insecticide phorate; P0, parental population; P2, progeny following two rounds of prosulfocarb selection.

burial or uneven spray application; the confounding effects of seed dormancy) may slow the changes observed in pot studies. Conversely, the frequency of trifluralin resistance in *L. rigidum* populations exclusively mediated by a resistance-endowing target-site mutation would be minimally affected by selection with prosulfocarb, with some possible fluctuations due to genetic drift. In heterogeneous *L. rigidum* populations, multiple traits endowing herbicide resistance can co-exist. For example, target-site resistance mutations and traits conferring an enhanced capacity for herbicide metabolism are co-selected by use of ACCase herbicides.[7] A similar co-selection of multiple resistance mechanisms was shown to confer a greater level of phenotypic resistance to glyphosate,[52] and the co-evolution of target- and nontarget-site resistance traits for trifluralin resistance also has been reported.[27] It also is possible that trifluralin metabolism in *L. rigidum*, which is an obligate cross-pollinated species, is frequently co-selected in trifluralin resistant field populations to complement functionally recessive target-site trifluralin resistance mutations.[35] We would expect this to be the norm in heterogeneous field populations of *L. rigidum*. Thus, from a management perspective, the fundamental knowledge generated in the current study on the interaction between trifluralin metabolism, phorate and prosulfocarb selection provides insight into herbicide rotation use patterns. Indeed, a recent simulation modelling study found that a regime of rotating mixtures of trifluralin plus prosulfocarb+*S*-metolachlor and trifluralin plus pyroxasulfone could delay resistance evolution in a population with existing trifluralin resistance alleles.[53] If the effect of herbicide selection on resistance to other herbicides can be better understood, it could be exploited to diversify strategies for chemical intervention and management of the escalating problem of multiple herbicide resistance in *L. rigidum*.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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