Varying responses of field-selected herbicide-resistant rigid ryegrass (*Lolium rigidum*) populations to combinations of phorate with PPI herbicides

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Abstract

Organophosphate insecticides, which have the capacity to inhibit specific herbicide-degrading (cytochrome P450) enzymes, have been used to explore metabolic herbicide-resistance mechanisms in weeds. This study investigates the response of seven field-selected rigid ryegrass (*Lolium rigidum* Gaudin) populations to herbicides from three different sites of action in the presence or absence of the P450 inhibitor phorate. Phorate antagonized the thiocarbamate herbicides triallate and prosulfocarb (8-fold increase in LD_{50}) in multiple resistant *L. rigidum* populations with resistance to three different site-of-action herbicides. In contrast, phorate synergized trifluralin and propyzamide in some populations, reducing the LD_{50} by 50%. Conversely, treatment with phorate had no significant effect on the LD_{50} for *S*-metolachlor or pyroxasulfone (inhibitors of very-long-chain fatty-acid synthesis). Phorate has diverse effects that are herbicide and population dependant in field-selected *L. rigidum*, suggesting P450 involvement in the metabolism of trifluralin and failure to activate thiocarbamate herbicides in these populations. This research highlights the need for implementation of diverse approaches other than herbicide alone as part of a long-term integrated strategy to reduce the likelihood of metabolism-based resistance to PPI herbicides in *L. rigidum*.

Introduction

Numerous weed species have evolved resistance to herbicides in many countries, which has made their management much more difficult and threatens crop production globally. Evolution of herbicide resistance is widely documented in southern Australian cropping systems in the genetically diverse, outcrossing species rigid ryegrass (*Lolium rigidum* Gaudin). Resistance in this species is conferred by mutations at the herbicide target site (Christopher et al. 1992; Malone et al. 2014) or non–target site mechanisms, such as enhanced herbicide metabolism, which can confer cross- and multiple resistance (Preston et al. 1996; Yu and Powles 2014). The role of different enzymatic processes involving cytochrome P450 enzymes have been identified as capable of herbicide metabolism in crop species, endowing crop selectivity to certain herbicides (Owen 1991; Van Eerd et al. 2003). Furthermore, the conjugation of some herbicides to the tripeptide glutathione occurs with glutathione *S*-transferases (GSTs), resulting in metabolic detoxification in crop species (Pan et al. 2012; Tanetani et al. 2013). Resistance mediated by P450 or GST enzymes via enhanced herbicide metabolism has led to resistance to multiple site-of-action herbicides in *L. rigidum* in Australia (Busi et al. 2018; Preston 2004), blackgrass (*Alopecurus myosuroides* Huds.) in England (Keshtkar et al. 2015), and wild oat (*Avena fatua* L.) in Canada (Mangin et al. 2016).

Organophosphate insecticides are highly reactive and chemically diverse molecules that possess a phosphorous atom with a covalent bond to either sulfur or oxygen (Durst et al. 1997). Certain organophosphate insecticides have inhibitory effects on some P450 enzymes through which some herbicides are detoxified (Durst et al. 1997; Kreuz et al. 1996). Organophosphate insecticides have been shown to significantly reduce phytotoxicity from clomazone (13$^{(F4)}$: inhibitors of carotenoid biosynthesis) when applied to cotton (*Gossypium hirsutum* L.) by inhibiting P450-mediated oxidation into toxic clomazone metabolites (Ferhatoglu et al. 2005). Similarly, the thiocarbamate herbicides require conversion into a toxic metabolite in order to exert their herbicidal effects. Activation involves the oxidation of the parent compound via P450 enzymes into the bioactive sulfoxide form (Casida et al. 1974; Fuerst 1987). The ability of organophosphate insecticides to act as a suicide substrate through

Table 1. Active ingredients, formulations, and manufacturers of herbicides and insecticide used in dose–response experiments.

Active ingredient	Trade name	Formulation	Manufacturer
Triallate	Avadex Xtra®	500 g L^{-1}	Nufarm Pty Ltd, Wingfield, South Australia
Prosulfocarb	Arcade®	800 g L^{-1}	Syngenta Crop Protection Pty Ltd, Eastwood, South Australia
S-metolachlor	Dual Gold®	960 g L^{-1}	Syngenta Crop Protection Pty Ltd, Eastwood, South Australia
Pyroxasulfone	Sakura®	850 g kg^{-1}	Bayer Crop Science Pty Ltd, Hawthorn East, Victoria, Australia
Trifluralin	Trifluralin	480 g L^{-1}	Imtrade Australia Pty Ltd, Kwinana Beach, Western Australia
Propyzamide	Kerb®	500 g L^{-1}	Corteva AgriScience Pty Ltd, Melbourne, Victoria, Australia
Phorate	Thimet®	100 g kg^{-1}	Barmac Industries Pty Ltd, Stapylton, Queensland, Australia

binding and catalysis results in a phosphorous atom of the insecticide substrate molecule being released and covalently bound to the P450, thereby inhibiting activation or metabolism of these herbicides by P450 enzymes that can result in either increased resistance (thiocarbamates) or susceptibility (dinitroaniline and benzamides) in *L. rigidum* (Busi et al. 2017; Tanetani et al. 2013; Tardif and Powles 1999).

The *L. rigidum* population SLR31 has been comprehensively studied because of its known metabolism-based multiple resistance to the herbicides diclofop-methyl (1$^{(A)}$: inhibitors of acetyl CoA carboxylase [ACCase]) and chlorsulfuron (2$^{(B)}$: inhibitors of acetolactate synthase [ALS]) (Heap and Knight 1986). Furthermore, it exhibits a moderate level of resistance to trifluralin (3$^{(K1)}$: inhibitors of microtubule polymerization) (McAlister et al. 1995), *S*-metolachlor (15$^{(K3)}$: inhibitors of very-long-chain fatty-acid [VLCFA] synthesis) (Burnet et al. 1994a), and triallate (8$^{(N)}$: inhibitors of fatty acid elongation) (Tardif and Powles 1999). Further studies with SLR31 have confirmed altered target-site (ACCase) and non–target site enhanced herbicide metabolism (ALS) resistance (Christopher et al. 1991, 1992, 1994). Evolutionary studies in the glasshouse using low herbicide dose recurrent selection of SLR31 have selected resistance to the sulfonylisoxazoline and thiocarbamate herbicides, pyroxasulfone and prosulfocarb (Busi and Powles 2013). Other than the studies by Busi et al. (2017) on the recurrently selected population, there is limited information on non–target site resistance to thiocarbamates and cross-resistance to other chemical classes in *L. rigidum*. Furthermore, non–target site resistance to these herbicides has not been investigated in any field-selected resistant populations of *L. rigidum*.

Previous studies have highlighted direct involvement of P450 enzymes as a mechanism of metabolic resistance to specific herbicides, including pyroxasulfone in the recurrently selected population (Busi and Powles 2016). In this study, we investigated the response of seven field-selected populations from across southern Australia to six different herbicides from three different sites of action. The objective was to understand whether the P450 inhibitor phorate could increase susceptibility to these herbicides and whether the response of these field-selected

L. rigidum populations would differ from those reported for SLR31 in previous studies (Busi et al. 2017).

Materials and Methods

Plant Material

The seed of *L. rigidum* populations 375-14 (R), 198-15 (R), 16.2 (R), RAC1 (R), A18 (R), and EP162 (R) used in this study was collected from cropping fields across southern Australia as described by Brunton et al. (2019). A well-characterized *L. rigidum* population, SLR4, was used as the susceptible (S) control (Boutsalis et al. 2012; Brunton et al. 2018). In addition, the extensively studied multiple resistant *L. rigidum* population, SLR31, was used as a known metabolic-resistant biotype (Burnet et al. 1994a, 1994b; McAlister et al. 1995). Seeds from SLR4, 375-14, 198-15, 16.2, EP162, A18, RAC1, and SLR31 were weighed (0.2 g = 50 to 60 seeds) and spread onto the surface of 9.5 cm by 8.5 cm by 9.5 cm punnet pots (Masrac Plastics, Dry Creek, South Australia) containing cocoa peat potting mix as described by Boutsalis et al. (2012).

Inhibitor and Herbicide Application

Before herbicide application (3 h), the insecticide phorate (Thimet®, Barmac Industries, Stapylton, QLD, Australia) was applied to the soil surface as described by Busi et al. (2017) at a dose of 0.076 g pot^{-1} corresponding to 10 kg ha^{-1} phorate (Table 1). Pots treated with the inhibitor only were also included. Herbicides were applied directly to seed and soil using a laboratory spray cabinet equipped with flat-fan nozzles (Hardi® ISO F-110-01, Hardi, Adelaide, SA, Australia) delivering 118 L ha^{-1} water at a pressure of 2.54 kPa. The control pots were not treated with any herbicide or inhibitor. The experiment was conducted twice outdoors under natural growing conditions in winter during the normal growing season (May to July 2018). Pots were watered as needed to maintain the potting mix near field capacity. There were three replicates for each herbicide dose, and pots were arranged in a randomized complete block design.

Dose–Response Experiment and Seedling Growth

The six PRE herbicides were applied (Table 1) following the methods described by Boutsalis et al. (2012). The herbicide triallate was applied to the S biotype at 50, 100, 200, 400, 800, and 1,600 g ha^{-1} and to the R biotypes at 400, 800, 1,600, 3,200, 6,400, and 12,800 g ha^{-1}. In Australia, the recommended label rate of triallate for *L. rigidum* control is 1,500 g ha^{-1}. Prosulfocarb was applied to the S biotype at 75, 150, 300, 600, 1,200, and 2,400 g ha^{-1} and to the R biotypes at 600, 1,200, 2,400, 4,800, 9,600, and 19,200 g ha^{-1}. The recommended label rate of prosulfocarb for *L. rigidum* control is 2,400 g ha^{-1}.

Pyroxasulfone was applied to the S biotype at 3.2, 6.4, 12.8, 25.5, 51, and 102 g ha^{-1} and to the R biotypes at 25.5, 51, 102, 204, and 408 g ha^{-1} with the recommended label rate for *L. rigidum* control being 100 g ha^{-1}. *S*-metolachlor was applied to the S biotype at 15, 30, 60, 120, 240, and 480 g ha^{-1} and to the R biotypes at 120, 240, 480, 960, 1,920, and 3,840 g ha^{-1} with the recommended label rate for *L. rigidum* control in Australia being 480 g ha^{-1}.

Trifluralin was applied to the S biotype at 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 g ha^{-1} and to the R biotypes at 125, 250, 500, 1,000, 2,000, and 4,000 g ha^{-1} with the recommended label

Table 2. Pooled dose–response data of thiocarbamate herbicides triallate and prosulfocarb with or without phorate required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations with 95% confidence intervals in parentheses and resistance index (RI).

Population	Inhibitor	LD_{50}	RI[a]	P[b]
		—g ai ha^{-1}—		
Triallate				
SLR4	+	1,595 (1,402, 1,827)	8.9	<0.01
SLR4	−	179 (159, 200)	1.0	
375-14	+	6,360 (5,654, 7,493)	35.5	<0.01
375-14	−	4,810 (4,372, 5,357)	26.9	
198-15	+	8,579 (7,970, 9,254)	47.9	0.34
198-15	−	8,089 (7,641, 8,576)	45.2	
16.2	+	2,738 (1,840, 3,254)	15.3	<0.01
16.2	−	1,648 (1,425, 1,819)	9.2	
RAC1	+	4,024 (3,752, 4,298)	22.5	<0.01
RAC1	−	3,124 (2,913, 3,352)	17.5	
EP162	+	4,147 (2,971, 4,975)	23.2	0.16
EP162	−	3,606 (2,405, 4,425)	20.1	
A18	+	5,630 (5,264, 6,243)	31.5	<0.01
A18	−	4,760 (4,369, 5,232)	26.6	
SLR31	+	3,263 (2,826, 3,799)	18.2	<0.01
SLR31	−	745 (691, 801)	4.2	
Prosulfocarb				
SLR4	+	2,006 (1,555, 2,860)	5.7	<0.01
SLR4	−	353 (270, 450)	1.0	
375-14	+	8,776 (6,382, 9,788)	24.9	0.44
375-14	−	6,949 (6,139, 8,061)	19.7	
198-15	+	18,329 (16,608, 25,491)	51.9	0.78
198-15	−	15,446 (12,287, 17,751)	43.8	
16.2	+	3,379 (2,881, 3,852)	9.6	<0.01
16.2	−	2,066 (1,508, 2,739)	5.9	
RAC1	+	4,383 (3,566, 5,310)	12.4	<0.01
RAC1	−	2,918 (2,783, 3,121)	8.3	
EP162	+	3,778 (3,279, 4,374)	10.7	0.24
EP162	−	3,382 (2,931, 3,900)	9.6	
A18	+	5,750 (4,840, 6,837)	16.3	<0.01
A18	−	4,317 (3,960, 4,705)	12.2	
SLR31	+	4,385 (3,734, 5,189)	12.4	<0.01
SLR31	−	998 (826, 1,157)	2.8	

[a] RI values calculated as the ratio between the LD50 of the resistant populations compared with the mean LD50 value of the susceptible population (SLR4) without phorate.
[b] Probability value (P) indicates the significance of differences in LD50 values between *L. rigidum* populations treated or not treated with phorate.

rate for *L. rigidum* control being 500 g ha^{-1}. Propyzamide was applied to the S biotype at 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 g ha^{-1} and to the R biotypes at 125, 250, 500, 1,000, and 2,000 g ha^{-1} with the recommended label rate for *L. rigidum* control in Australia being 500 g ha^{-1}. Pots were assessed at 28 d after herbicide treatment, and plants that had emerged and grown to the 2-leaf stage were counted. Percentage survival was assessed as the number of plants growing in the treated pots compared with the average number present in the untreated pots.

Statistical Analysis

Following an ANOVA, the data for both experimental runs were pooled, as there was no statistical difference between the runs. The data were analyzed using a log-logistic equation (GraphPad Prism v. 7.0, GraphPad Software, San Diego, CA, USA) fit to the percentage emergence data (Seefeldt et al. 1995). The normalized three-parameter logistic regression model was fit where y represents plant survival (%), x is the log-dose of the herbicide used, LD_{50} is the herbicide dose required to cause 50% reduction in plant emergence, and b denotes the slope of the curve. LD_{50} parameter estimates from the log-logistic analysis were used

to calculate the resistance index (RI), which is the resistant/susceptible ratio of the LD_{50}.

$$y = \frac{100}{1 + 10^{(\log\ LD_{50} - x) \times b}} \qquad [1]$$

To estimate no difference between estimated LD_{50} values for *L. rigidum* populations in the presence of the insecticide phorate, a *t*-test was conducted with the null hypothesis of no difference between resistant versus susceptible populations or their ratio was equal to 1 (Ritz et al. 2015).

Results and Discussion

Effect of Phorate on Thiocarbamates Herbicides

In the absence of herbicide, phorate at 10 kg ha^{-1} had no effect on the germination percentage of all *L. rigidum* populations when compared with nontreated controls (data not shown). The susceptible population SLR4 was completely controlled by both triallate and prosulfocarb applied at the recommended field rate. The LD_{50} value for SLR4 treated with triallate was 179 g ha^{-1} and ranged from 745 to 8,089 g ha^{-1} for the resistant populations (4.2- to 45.2-fold resistance compared with SLR4) (Table 2). The addition of phorate significantly (P < 0.01) increased the LD_{50} for triallate in SLR4, 375-14, 16.2, RAC1, A18, and SLR31 as compared with the herbicide-only treatment. In the presence of phorate, the LD_{50} for SLR4 was 1,595 g ha^{-1} and ranged from 2,738 to 8,579 g ha^{-1} for the resistant populations (15.3- to 47.9-fold resistance compared with SLR4) in the absence of phorate (Table 2). The *L. rigidum* populations SLR4, 16.2, RAC1, A18, and SLR31 treated with phorate and prosulfocarb displayed a significant (P < 0.01) antagonistic response similar to triallate. In the presence of phorate, the LD_{50} value for SLR4 was 2,006 g ha^{-1} (5.7-fold increase) and ranged from 3,379 to 18,329 g ha^{-1} (9.6- to 51.9-fold for the resistant biotypes compared with SLR4) in the absence of phorate (Table 2).

In this study, we report major differences between field-selected *L. rigidum* populations in response to herbicides when the P450 inhibitor phorate was present. Phorate was shown to antagonize the thiocarbamate herbicides triallate in SLR4, 375-14, 16.2, RAC1, A18, and SLR31 and prosulfocarb in SLR4, 16.2, RAC1, A18, and SLR31. As previously reported, P450 enzymes are involved in the activation of thiocarbamate herbicides (Fuerst 1987), and therefore, inhibition of P450s by phorate would reduce the biological activity of these herbicides. Reduced activation has been documented to elicit triallate resistance in *A. fatua* (Kern et al. 1996). This was also shown in the recurrently selected *L. rigidum* by Busi et al. (2017), who reported a herbicide-susceptible population treated with phorate exhibited an LD_{50} value similar to that seen in the resistant population by inhibiting the activation of either triallate or prosulfocarb.

The antagonism between triallate or prosulfocarb and phorate was also observed in the susceptible population SLR4, which indicates involvement of P450s in herbicide activation in this population as well. However, three of the resistant populations displayed no antagonism to phorate. If the P450 responsible for thiocarbamate activation is not active in these populations, then no antagonism with phorate would occur, suggesting a lack of activation as a possible resistance mechanism in these populations (Kern et al. 1996). Different P450s inhibited by phorate might bioactivate thiocarbamates in these populations or phorate might

Table 3. Pooled dose–response data of the very-long-chain fatty-acid–inhibiting herbicides *S*-metolachlor and pyroxasulfone with or without phorate required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations with 95% confidence intervals in parentheses and resistance index (RI).

Population	Inhibitor	LD_{50}	RI^a	P^b
		—g ai ha^{-1}—		
S-metolachlor				
SLR4	+	125 (91, 176)	0.8	0.16
SLR4	−	148 (116, 194)	1.0	
375-14	+	2,620 (2,135, 3,100)	17.7	0.49
375-14	−	2,830 (2,512, 3,281)	19.1	
198-15	+	3,620 (3,338, 4,157)	24.5	0.38
198-15	−	4,179 (3,857, 4,757)	28.2	
16.2	+	820 (527, 1,218)	5.5	0.55
16.2	−	1,105 (684, 1,496)	7.5	
RAC1	+	240 (214, 262)	1.6	0.19
RAC1	−	250 (249, 272)	1.7	
EP162	+	2,000 (1,750, 2,330)	13.5	0.76
EP162	−	2,303 (2,028, 2,673)	15.6	
A18	+	1,728 (1,513, 2,001)	11.7	0.27
A18	−	2,105 (1,852, 2,437)	14.2	
SLR31	+	571 (504, 643)	3.9	0.47
SLR31	−	697 (622, 779)	4.7	
Pyroxasulfone				
SLR4	+	9 (8, 10)	0.9	0.13
SLR4	−	10 (9, 11)	1.0	
375-14	+	128 (115, 142)	12.8	0.31
375-14	−	144 (124, 168)	14.4	
198-15	+	96 (85, 108)	9.6	0.54
198-15	−	114 (96, 135)	11.4	
16.2	+	26 (23, 30)	2.6	0.26
16.2	−	31 (26, 37)	3.1	
RAC1	+	29 (26, 32)	2.9	0.48
RAC1	−	32 (27, 38)	3.2	
EP162	+	76 (68, 83)	7.6	0.29
EP162	−	78 (73, 89)	7.8	
A18	+	70 (63, 78)	7.0	0.22
A18	−	82 (70, 95)	8.2	
SLR31	+	32 (28, 35)	3.2	0.29
SLR31	−	34 (29, 40)	3.4	

[a] RI values calculated as the ratio between the LD_{50} of the resistant populations compared with the mean LD_{50} value of the susceptible population (SLR4) without phorate.
[b] Probability value (P) indicates the significance of differences in LD_{50} values between *L. rigidum* populations treated or not treated with phorate.

phorate compared with herbicide-only treatment (Table 3). The *S*-metolachlor rate required to control 50% of the SLR4 population was 148 g ha^{-1}. In contrast, the LD_{50} for the resistant populations ranged from 250 to 4,179 g ha^{-1} (1.7- to 28.2-fold). In the presence of phorate, the LD_{50} was 125 g ha^{-1} for SLR4 and ranged from 240 to 3,620 g ha^{-1} (1.6- to 24.5-fold) in the resistant populations. As with *S*-metolachlor, phorate treatment had no significant effect on the LD_{50} response of the susceptible and resistant populations treated with pyroxasulfone compared with the absence of phorate (Table 3).

Our results suggest limited involvement of P450s in the metabolism of the VLCFA-inhibiting herbicides *S*-metolachlor and pyroxasulfone. More recently, studies have shown that rapid GST-mediated pyroxasulfone metabolism in wheat compared with slower metabolism in *L. rigidum* plants was responsible for herbicide selectivity (Tanetani et al. 2013). In addition, Busi et al. (2018) suggested that increased transcription of GST was the primary mechanism of resistance to the VLCFA-inhibiting herbicides and that the expression of five P450s did not substantially differ among individuals in the recurrently selected *L. rigidum* populations. They also showed a synergistic interaction between phorate and pyroxasulfone that substantially reduced (approximately 45%) the level of pyroxasulfone resistance. However, in this study, we found no significant synergistic effect between phorate and either VLCFA-inhibiting herbicide. Limited P450 response was observed with no change in LD_{50} either in the presence or absence of phorate in all populations. A possible explanation is that phorate does not inhibit specific P450s involved in the metabolism of *S*-metolachlor and pyroxasulfone. However, evidence of enhanced P450 activity conferring resistance to these herbicides in *L. rigidum* remains not fully understood, and it has been reported that selectivity of these herbicides in crops can be attributed to enhanced levels of GST activity (Lamoureux et al. 1991). The response of both resistant and susceptible *L. rigidum* populations to chloroacetamide and sulfonylisoxazoline herbicides suggests a possible pathway wherein herbicides are metabolized by GST conjugation similar to that reported in wheat and *L. rigidum* (Busi et al. 2018; Dücker et al. 2019; Tanetani et al. 2013).

Effect of Phorate on Microtubule Polymerization Inhibitors

The LD_{50} for *L. rigidum* population SLR4 treated with trifluralin in the absence of phorate was 52 g ha^{-1} compared with 73 g ha^{-1} when treated with phorate, indicating a significant (P < 0.01) antagonistic response (Table 4). However, there was a significant (P < 0.01) reduction in the rate of trifluralin required to control some *L. rigidum* populations when phorate was applied. The addition of phorate reduced the LD_{50} for trifluralin by about 50% in the resistant populations 16.2, RAC1, A18, and SLR31. The LD_{50} in the absence of phorate ranged from 146 to 1,471 g ha^{-1} (2.8- to 28.3-fold compared with SLR4), which reduced to 117 to 665 g ha^{-1} (2.3- to 12.8-fold resistance compared with SLR4) in the presence of phorate. However, three of the trifluralin-resistant populations (375-14, 198-15, and EP162) showed no change in LD_{50} to trifluralin with the addition of phorate. All *L. rigidum* populations were controlled by propyzamide at the recommended dose. Addition of phorate to propyzamide reduced LD_{50} (0.6- to 1.5-fold) in all *L. rigidum* populations; however, this was only significant (P < 0.01) for SLR4 (S), 16.2, RAC1, and SLR31 (Table 4).

There was significant synergism between trifluralin and phorate in some *L. rigidum* populations. Therefore, a possible P450-mediated herbicide detoxification mechanism appears to

not inhibit these P450s, leading to further detoxification of the sulfoxide activated by GSTs. The resistant populations 198-15 and EP162 treated with either triallate or prosulfocarb displayed an antagonistic response with phorate, suggesting that specific P450s involved in thiocarbamate conversion are still active and resistance is likely due to something other than the loss of a specific P450. The possible involvement of a target-site resistance mechanism is known to confer resistance to trifluralin, but not to other herbicides, including the thiocarbamates. This suggests a complex set of resistance mechanisms involving lack of herbicide activation, but also possible involvement at a later stage of glutathione conjugation of the herbicide following activation (Fuerst 1987). The involvement of GSTs mediating herbicide detoxification via glutathione conjugation is widely documented for chloroacetamide and thiocarbamate herbicides (Cummins et al. 1997; Siminszky 2006). Studies by Busi et al. (2017) suggested the presence of a metabolism-based mechanism in *L. rigidum* similar to that found in wheat (*Triticum aestivum* L.).

Effect of Phorate on VLCFA Inhibitors

All *L. rigidum* populations treated with *S*-metolachlor displayed no statistical difference (P > 0.05) in LD_{50} in the presence of

Table 4. Pooled dose–response data of the microtubule polymerization–inhibiting herbicides trifluralin and propyzamide with or without phorate required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations with 95% confidence intervals in parentheses and resistance index (RI).

Population	Inhibitor	LD_{50}	RI[a]	P[b]
		—g ai ha^{-1}—		
Trifluralin				
SLR4	+	73 (66, 81)	1.4	<0.01
SLR4	−	52 (47, 58)	1.0	
375-14	+	1,347 (1,204, 1,529)	26.0	0.48
375-14	−	1,438 (1,290, 1,606)	27.7	
198-15	+	752 (484, 827)	10.3	0.57
198-15	−	817 (730, 915)	15.7	
16.2	+	254 (218, 291)	4.9	<0.01
16.2	−	400 (361, 444)	7.7	
RAC1	+	117 (100, 131)	2.3	0.01
RAC1	−	146 (132, 164)	2.8	
EP162	+	356 (281, 447)	6.8	0.66
EP162	−	406 (361, 455)	7.8	
A18	+	665 (574, 777)	12.8	<0.01
A18	−	1,471 (1,315, 1,647)	28.3	
SLR31	+	525 (469, 588)	10.1	<0.01
SLR31	−	1,160 (1,047, 1,284)	22.3	
Propyzamide				
SLR4	+	17 (15, 19)	0.6	<0.01
SLR4	−	28 (26, 31)	1.0	
375-14	+	36 (20, 52)	1.3	0.17
375-14	−	54 (42, 69)	1.9	
198-15	+	39 (31, 51)	1.4	0.38
198-15	−	60 (46, 75)	2.1	
16.2	+	19 (18, 21)	0.7	<0.01
16.2	−	29 (27, 32)	1.0	
RAC1	+	18 (17, 19)	0.6	<0.01
RAC1	−	28 (25, 32)	1.0	
EP162	+	43 (34, 59)	1.5	0.73
EP162	−	69 (54, 82)	2.5	
A18	+	20 (17, 25)	0.7	0.56
A18	−	36 (23, 52)	1.3	
SLR31	+	18 (16, 21)	0.6	<0.01
SLR31	−	29 (28, 30)	1.0	

[a] RI values calculated as the ratio between the LD_{50} of the resistant populations compared with the mean LD_{50} value of the susceptible population (SLR4) without phorate.
[b] Probability value (P) indicates the significance of differences in LD_{50} values between *L. rigidum* populations treated or not treated with phorate.

be involved in microtubule polymerization inhibitor resistance in *L. rigidum*. In the susceptible population, phorate antagonized trifluralin. One possible explanation is that P450 inhibitors might induce P450 activity, resulting in herbicide metabolism (Hidayat and Preston 2001). Previous studies in *L. rigidum* and *A. myosuroides* have shown that the presence of resistance to the microtubule polymerization–inhibiting herbicide pendimethalin could be reversed with the P450 inhibitor malathion (James et al. 1995; Tardif and Powles 1999). Some of the populations of *L. rigidum* showed no synergism between phorate and trifluralin, which suggests the presence of a possible target-site resistance mechanism. The amino acid change from threonine to isoleucine at position 239 or valine to phenylalanine at position 2020 of the α-tubulin gene contributes greater resistance to trifluralin (>10-fold) compared with metabolic resistance (Fleet et al. 2018). All *L. rigidum* populations treated with propyzamide were susceptible and completely killed at the recommended dose. However, there was significant synergism between propyzamide and phorate for four out of the eight populations. Therefore, a metabolic P450 detoxification mechanism for propyzamide is likely in *L. rigidum*.

This study highlights an antagonistic response to the P450 inhibitor phorate in *L. rigidum* when treated with thiocarbamate

herbicides, no response when treated with VLCFA-inhibiting herbicides, and a synergistic response with microtubule polymerization–inhibiting herbicides. This suggests the possible involvement of a complex of P450-mediated and other mechanisms in resistant *L. rigidum* populations, similar to those conferring tolerance to thiocarbamate, chloroacetamide, and sulfonylisoxazoline herbicides in wheat (Busi et al. 2017; Tanetani et al. 2013). In addition, some populations showed no interaction between phorate and trifluralin, suggesting a possible target-site resistance mechanism. There were also differences among the resistant populations, with populations 198-15 and EP162 not responding to phorate in combination with any of the six herbicides used. The possibility that phorate may be detoxified before it is able to inhibit P450s in these populations is likely and would explain the limited response in these populations. The application of a GST inhibitor may clarify the possible involvement of GSTs in the studied field-selected *L. rigidum* populations. However, there remains a limited understanding about the possibility of a specific target-site mutation that confers resistance (>20-fold) to inhibitors of fatty-acid elongation and VLCFA inhibition in these populations.

Phorate enables a greater understanding of potential biochemical mechanisms involved in herbicide resistance, but it is unlikely that applications of this P450 inhibitor in broad-acre scale would be environmentally or economically sustainable. Furthermore, phorate was unable to completely overcome resistance and antagonized thiocarbamate-resistant and thiocarbamate-susceptible populations. The challenges of understanding metabolic resistance in *L. rigidum* reported in this study highlight the significant differences in response among populations, herbicides, and inhibitors. In the presence of phorate, populations 198-15 and EP162 showed no response when treated with either thiocarbamate or microtubule polymerization–inhibiting herbicides, suggesting the likelihood of a complex resistance mechanism. In addition to these populations showing no response to either herbicide, the response of the other populations varied significantly, highlighting a diversity among the resistant biotypes. *Lolium rigidum* populations 16.2, RAC1, and SLR31 responded to all site-of-action herbicides. In contrast, population 375-14 responded to triallate only, while A18 showed a response to both thiocarbamates and the microtubule polymerization inhibitor (trifluralin). Metabolic detoxification mechanisms in *L. rigidum* have the ability to confer resistance to herbicides that have never been applied and result in herbicides with different sites of action not controlling these populations in the field (Brunton et al. 2019; Mangin et al. 2016; Tardif and Powles 1999). Therefore, in addition to the use of herbicides, a diverse array of cultural and non-herbicidal weed control tactics should be implemented to manage *L. rigidum* populations to reduce the amount of viable seed returning to the weed seedbank (Beckie 2006).

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