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Resistance to bixlozone and clomazone in cross-resistant rigid ryegrass (*Lolium rigidum*) populations from southern Australia

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Research Article

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Abstract

Three resistant (R) rigid ryegrass (*Lolium rigidum* Gaudin) populations from southern Australia (EP162, 375-14, and 198-15) with cross-resistance to thiocarbamate, chloroacetamide, and sulfonylisoxazoline herbicides displayed reduced sensitivity to the isoxazolidinone herbicides bixlozone and clomazone. Each of these R populations was exposed to two cycles of recurrent selection (RS) in which plants were treated with the field rate of bixlozone, survivors were bulk crossed, and seed was collected. After the first cycle of recurrent selection (RS1), the LD_{50} to bixlozone in population 198-15 increased to 17.5-fold compared with the S population and increased further to 26.9-fold after a second cycle of recurrent selection (RS2). The recurrent selection process also increased the level of resistance to bixlozone in populations EP162 and 375-14 (7.8- to 18.4-fold) compared with the S population. Phorate antagonized bixlozone and clomazone in SLR4 (34.6- and 28.1-fold increase in LD_{50}) and both herbicides in populations EP162 (36.5- to 46.6-fold), 375-14 (71.4- to 73.9-fold), and 198-15 (86.4- to 91.5-fold) compared with the absence of phorate. The increase in LD_{50} of all *L. rigidum* RS populations when treated with phorate suggests a lack of herbicide activation is not the likely resistance mechanism to these herbicides. This research highlights the elevated risk of thiocarbamate-resistant *L. rigidum* populations to rapidly evolve resistance to the isoxazolidinone herbicides bixlozone and clomazone.

Introduction

The continued reliance on herbicides for weed control has resulted in an increased number of weed species evolving resistance to herbicides. Of great concern are non–target site resistance mechanisms, which can confer resistance to a range of different site-of-action (SOA) herbicides (Dücker et al. 2016; Keshtkar et al. 2015; Yu and Powles 2014; Yuan et al. 2007). Rigid ryegrass (*Lolium rigidum* Gaudin), a genetically diverse, obligate-outcrossing species has been extensively studied and reported as having the capacity to evolve resistance to different SOA herbicides, including populations with cross-resistance to the thiocarbamate and sulfonylisoxazoline herbicides prosulfocarb and pyroxasulfone ($15^{(K3)}$: inhibitors of very-long-chain fatty-acid synthesis) (Brunton et al. 2018; Busi and Powles 2016). Research into the possibility of *L. rigidum* evolving resistance to more diverse SOA herbicides has provided some understanding of the likelihood of resistance evolution to herbicides not yet discovered (Busi et al. 2012). The current practice of managing *L. rigidum* in Australia in wheat (*Triticum aestivum* L.) relies heavily on the use of PRE herbicides, including trifluralin ($3^{(K1)}$: inhibitors of microtubule assembly), prosulfocarb, and pyroxasulfone (Boutsalis et al. 2014; Brunton et al. 2020a). A greater reliance has been placed on PRE herbicides due to significant and widespread resistance to the herbicides diclofop-methyl ($1^{(A)}$: inhibitors of acetyl CoA carboxylase) and chlorsulfuron ($2^{(B)}$: inhibitors of acetolactate synthase) (Broster and Pratley 2006).

Clomazone ($13^{(F4)}$: inhibitors of deoxy-D-xylulose phosphate [DXP] synthase) is registered in Australia for the control of broadleaf weeds in opium poppy (*Papaver somniferum* L.), cotton (*Gossypium hirsutum* L.), and vegetable crops. Clomazone has most commonly been used for the control of broadleaf and grass weeds in rice (*Oryza sativa* L.) (Ferhatoglu and Barrett 2006). Clomazone is not registered for *L. rigidum* control in Australia, and as a result, resistance to clomazone remains rare. Resistance to clomazone was reported by Tardif and Powles (1999) in the population SLR31, despite the fact that the population had never before been treated with this herbicide. In contrast to clomazone, the isoxazolidinone herbicide bixlozone ($13^{(F4)}$) will provide an additional SOA for the control of *L. rigidum* populations in wheat in Australia.

Several herbicides require in vitro biochemical conversion to exert herbicidal effects in plants. This process involves the metabolic activation of the herbicide into an active metabolite

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(Kern et al. 1996) and the oxidation of the parent compound through specific cytochrome P450s (CYP450s) (Casida et al. 1974; Fuerst 1987). Herbicides that require activation through this process include the thiocarbamates and isoxazolidinones (Casida et al. 1974; Ferhatoglu and Barrett 2006; Weimer et al. 1991). The competition in P450-mediated reactions between herbicides and insecticides as well as oxidative desulfuration can confer protection against herbicides such as clomazone (Durst et al. 1997; Ferhatoglu et al. 2005; Fuerst 1987). Organophosphate insecticides are highly diverse and reactive molecules that possess a phosphorous atom with a covalent bond to either sulfur or oxygen. To be active, both clomazone and bixlozone require metabolic conversion to the active keto-variant (5-ketoclomazone), which inhibits DXP synthase, leading to the biosynthesis of isopentenyl pyrophosphate in plastids (Ferhatoglu and Barrett 2006). In cotton, phorate and disulfoton enhanced protection through inhibiting P450-mediated bioactivation and preventing the production of 5-ketoclomazone (Ferhatoglu et al. 2005).

Metabolic resistance to herbicides is frequently associated with increased levels of CYP450 activity leading to enhanced detoxification of these primary metabolites (Siminszky 2006). Both clomazone and bixlozone are at risk of P450-mediated metabolism of the primary keto variant 5-OH clomazone (Ferhatoglu et al. 2005), a precursor of the active metabolite 5-ketoclomazone (Ferhatoglu and Barrett 2006). Studies in corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] have linked the degradation of major clomazone metabolites and hydroxylated derivatives through specific P450s (ElNaggar et al. 1992; Ferhatoglu et al. 2005). Resistance to clomazone has been reported in late watergrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] and has been linked to cytochrome P450-mediated metabolism that confers resistance to other SOA herbicides (Yasuor et al. 2008, 2010). In contrast, no cases of resistance to clomazone have been reported in blackgrass (*Alopecurus myosuroides* Huds.), which has evolved diverse metabolic resistance mechanisms (Keshtkar et al. 2015). The evolution of metabolic resistance is of significant concern and has been reported to endow cross-resistance to a diverse range of herbicides (Beckie and Tardif 2012; Brunton et al. 2018). Metabolic resistance has also been shown to evolve resistance to chemically unrelated herbicides (Brunton et al. 2019; Busi and Powles 2016).

To date, there are no reports of resistance to bixlozone, but there is a risk of cross-resistance evolution from prosulfocarb for other herbicides that require activation through P450s. A previous study by Busi et al. (2012) highlight the importance of research investigating the likelihood and potential of resistance evolution to diverse SOA herbicides in *L. rigidum*. The current research investigates possible resistance mechanisms to bixlozone in three recurrently selected *L. rigidum* populations from southern Australia with known resistance to thiocarbamate herbicides and multiple other SOA herbicides.

Materials and Methods

Plant Material

Lolium rigidum populations 375-14 (R), 198-15 (R), and EP162 (R) used in this study were collected from cropping fields across southern Australia as described by Brunton et al. (2018). A well-characterized *L. rigidum* population, SLR4, was used as the susceptible (S) control (Boutsalis et al. 2012). Seeds of each population 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, SA, Australia) containing cocoa peat potting mix, as described by Boutsalis et al. (2012).

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

Active ingredient	Trade name	Formulation	Manufacturer
Bixlozone	Overwatch®	400 g L^{-1}	FMC Australia Pty Ltd, North Ryde, NSW, Australia
Clomazone	Command®	480 g L^{-1}	FMC Australia Pty Ltd, North Ryde, NSW, Australia
Phorate	Thimet®	100 g kg^{-1}	Barmac Industries, Stapylton, QLD, Australia

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 on 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. Control pots were not treated with any herbicide or inhibitor. The experiment was conducted outdoors under natural growing conditions in winter during the normal growing season (May to July). Pots were watered as needed to maintain potting mix near field capacity. There were three replicates for each herbicide dose, and pots were arranged in a randomized complete block design, and the experiment was repeated.

Generation of RS1 and RS2 Families

Seed from the resistant populations (375-14, 198-15, and EP162) were treated with the recommended field rate of bixlozone (500 g ha^{-1}) in 2018, and survivors were collected. Four surviving plants of each R population were grown in a single 30-cm-diameter pot containing standard potting mix. Plants were grown outdoors under natural growing conditions during the months May to November 2018 and watered as required. Before plants flowered, pots were encased in a 1.2-m-long polypropylene sleeve supported by a wire mesh cage that was open at the top. This pollen sleeve was used to minimize the risk of cross-pollination from other plants present in the area. Seeds were harvested separately from each population and labeled RS1 for each population. To generate the RS2 population, the seeds of progeny of RS1 were further treated with the recommended field rate of bixlozone in 2019, and survivors were grown in a single 30-cm pot as described earlier during the months May to November 2019. At maturity, seeds from all plants within each pot were pooled to create the RS2 families. RS2 populations were then selected for inclusion in dose–response experiments in 2020.

Dose–Response Experiment and Seedling Growth

Dose–response experiments were conducted on S, R, RS1, and RS2 populations and repeated. Two PRE herbicides were applied (Table 1) directly onto the seed following the method described by Boutsalis et al. (2012). The herbicide bixlozone was applied to the S biotype at 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, 4,000, and

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Table 2. Pooled dose–response data of bixlozone and clomazone dose required for 50% mortality (LD_{50}) of the resistant parent, RS1, RS2 crosses, and susceptible *Lolium rigidum* populations (95% confidence intervals in parentheses) and resistance index (RI).[a]

Population	LD_{50}	RI[b]	P[c]	Population	LD_{50}	RI[b]	P[c]
	—g ai ha^{-1}—				—g ai ha^{-1}—		
Bixlozone				Clomazone			
SLR4	43 (32, 56)	—	—	SLR4	56 (44, 71)	—	—
375-14	253 (231, 277)	5.9	<0.01	375-14	319 (291, 350)	5.7	<0.01
375-14 RS1	379 (345, 417)	8.8	0.01	375-14 RS1	444 (412, 477)	7.9	0.01
375-14 RS2	793 (640, 932)	18.4	<0.01	375-14 RS2	690 (623, 747)	12.3	0.01
198-15	433 (368, 515)	10.1	<0.01	198-15	551 (452, 671)	9.8	<0.01
198-15 RS1	753 (681, 836)	17.5	<0.01	198-15 RS1	635 (577, 698)	11.3	<0.01
198-15 RS2	1,157 (1063, 1259)	26.9	<0.01	198-15 RS2	879 (690, 1093)	15.7	<0.01
EP162	108 (93, 124)	2.5	<0.01	EP162	214 (197, 234)	3.8	<0.01
EP162 RS1	336 (282, 389)	7.8	0.01	EP162 RS1	355 (308, 394)	6.3	0.01
EP162 RS2	579 (474, 701)	13.5	0.01	EP162 RS2	544 (498, 592)	9.7	0.01

[a]RS1, first-generation recurrent selection; RS2, second-generation recurrent selection.
[b]RI values calculated as the ratio between the LD_{50} of the resistant populations compared with the mean LD_{50} of the susceptible population (SLR4).
[c]P-value indicates significant difference in LD_{50} values between *L. rigidum* populations treated with a particular herbicide compared with SLR4.

8,000 g ha^{-1}, with the recommended label rate for *L. rigidum* control in Australia at 500 g ha^{-1}. Clomazone 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, 3,840, and 7,680 g ha^{-1}, with the recommended label rate being 240 to 480 g ha^{-1}. The resistant parent and recurrently selected RS1 and RS2 populations were tested during the normal growing season (May to July) 2020. 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 control pots.

Statistical Analysis

Following 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. 8.0, 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}.

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

To compare the 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

Herbicide Response of Recurrent Selection in Lolium rigidum Populations

The susceptible *L. rigidum* population SLR4 was completely controlled by bixlozone at the recommended field rate of 500 g ha^{-1} with an LD_{50} of 43 g ha^{-1} (Table 2; Figure 1). Furthermore, SLR4 was completely controlled by clomazone at the recommended field

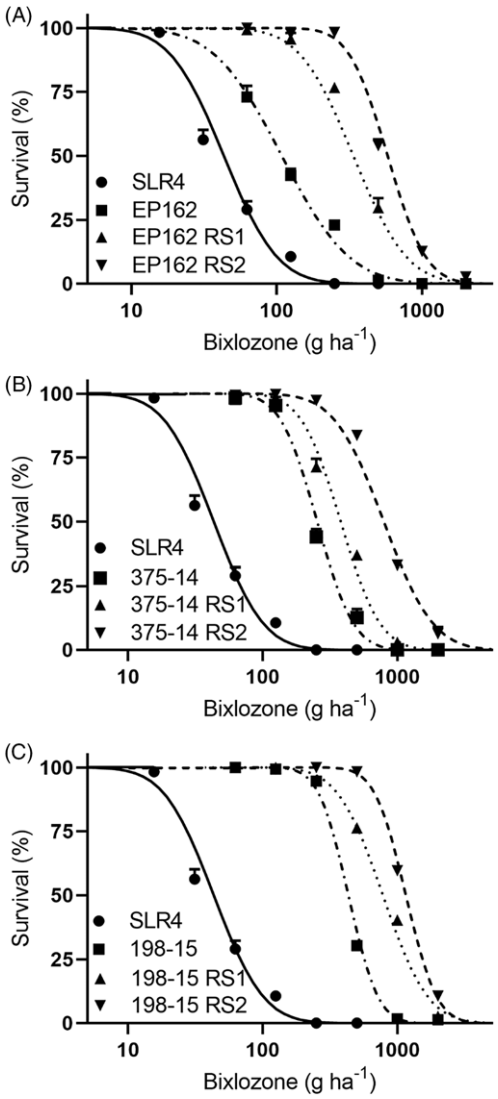

Figure 1. Response of SLR4 (solid line, ●), resistant parent (dotted and dashed line, •), recurrent progeny RS1 (dotted line, ▲), and RS2 (dashed line, ▼) of *Lolium rigidum* populations EP162 (A), 375-14 (B), and 198-15 (C) treated with varying rates of bixlozone. Each data point is the mean of six replicates, and bars represent the standard error of the mean.

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rate of 480 g ha^{-1} with an LD_{50} of 56 g ha^{-1} (Table 2). The bixlozone rates required for 50% mortality (LD_{50}) for the resistant populations EP162, 375-14, and 198-15 were 108, 253, and 433 g ha^{-1}, respectively (Table 2). *Lolium rigidum* population EP162 had a lower LD_{50} for bixlozone compared with the two other resistant populations. The RI for bixlozone was 2.5- to 10.1-fold greater than that of the S population SLR4 (Table 2). The LD_{50} values for populations EP162, 375-14, and 198-15 treated with clomazone were higher than that of the S population at 214, 319, and 551 g ha^{-1}, respectively giving an RI of 3.8 to 9.8 compared with the S population.

The response of the RS1 survivors showed a significant ($P < 0.01$) increase in LD_{50} as compared with the parent populations. The RS1 populations EP162 RS1 and 375-14 RS1 showed 29% and 37% survival to bixlozone at 500 g ha^{-1}, respectively, while 198-15 RS1 showed 75% survival at this rate (Figure 1). The populations EP162 RS1, 375-15 RS1, and 198-15 RS1 recorded RIs for bixlozone that were 7.8-, 8.8-, and 17.5-fold greater than that of the S population, respectively (Table 2). The RS1 populations also showed a significant ($P < 0.01$) increase in LD_{50} for clomazone (Table 2), with RIs to clomazone for populations EP162 RS1, 375-14 RS1, and 198-15 RS1 being 6.3-, 7.9-, and 11.3-fold compared with SLR4, respectively.

Further selection with bixlozone in the second generation (RS2) resulted in a significant increase ($P < 0.01$) in LD_{50} for bixlozone in all populations. All RS2 *L. rigidum* populations had LD_{50} values greater than the recommended field rate of 500 g ha^{-1} and were 579, 793, and 1,157 g ha^{-1} for EP162 RS2, 375-14 RS2, and 198-15 RS2, respectively. This increase in LD_{50} in the RS2 populations was reflected in 54%, 84%, and 98% survival at the recommended rate of bixlozone (Figure 1). The RIs for the RS2 populations were 13.5-, 18.4-, and 26.9-fold greater than that of the S population for EP162 RS2, 375-14 RS2, and 198-15 RS2, respectively (Table 2). Furthermore, all RS2 populations treated with clomazone had LD_{50} values greater than the recommended field rate 480g ha^{-1} and were 544, 690, and 879 g ha^{-1} for EP162 RS2, 375-14 RS2, and 198-15 RS2, respectively, giving RIs of 9.7-, 12.3-, and 15.7-fold compared with the S population.

The parental populations EP162, 375-14, and 198-15 with known cross-resistance to thiocarbamate, chloroacetamide, and sulfonylisoxazoline herbicides (Brunton et al. 2019) displayed reduced sensitivity to the isoxazolidinone herbicides bixlozone and clomazone. Cross-resistance to the isoxazolidinone herbicide clomazone was previously reported by Tardif and Powles (1999) in *L. rigidum* population SLR31, which had a long and complex history of exposure to herbicides. In this study, we show a similar level of resistance to bixlozone and clomazone in parent populations EP162, 375-14, and 198-15, as previously reported in *E. phyllopogon* by Guo et al. (2019) (Table 2). Furthermore, the response of the RS1 and RS2 populations demonstrated that recurrent selection of multiple-resistant *L. rigidum* populations with a full herbicide dose significantly increased the level of resistance to bixlozone and clomazone (Table 2).

The *L. rigidum* parental populations in this study had not been exposed to bixlozone in the field; their only exposure to bixlozone was in pots during generations RS1 and RS2. These findings highlight the capacity for *L. rigidum* to evolve resistance to herbicides in the absence of direct selection (Busi and Powles 2013; Keshtkar et al. 2015). Furthermore, observed resistance to clomazone suggests the possibility of a similar P450 mechanism conferring resistance to both herbicides.

Table 3. Pooled dose–response data of isoxazolidinone herbicides bixlozone and clomazone with or without phorate required for 50% mortality (LD_{50}) of resistant and susceptible *Lolium rigidum* populations (95% confidence intervals in parentheses) and resistance index (RI).

Population	Inhibitor	LD_{50} —g ai ha^{-1}—	RI[a]	P[b]
Bixlozone				
SLR4	+	1,627 (1,487, 1,833)	34.6	<0.01
SLR4	–	47 (38, 56)	1.0	
375-14	+	3,576 (3,229, 3,923)	73.9	<0.01
375-14	–	273 (230, 316)	5.8	
198-15	+	4,061 (3,838, 4,284)	86.4	<0.01
198-15	–	445 (382, 508)	9.5	
EP162	+	2,188 (1,966, 2,410)	46.6	<0.01
EP162	–	122 (103, 141)	2.6	
Clomazone				
SLR4	+	1,575 (1,348, 1,802)	28.1	<0.01
SLR4	–	56 (44, 71)	1.0	
375-14	+	3,997 (3,659, 4,335)	71.4	<0.01
375-14	–	319 (291, 350)	5.7	
198-15	+	5,127 (4,766, 5,488)	91.5	<0.01
198-15	–	551 (452, 671)	9.8	
EP162	+	2,044 (1,892, 2,196)	36.5	<0.01
EP162	–	214 (197, 234)	3.8	

[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]P-value indicates the significance of differences in LD_{50} values between *L. rigidum* populations treated or not treated with phorate.

Effect of Phorate on Isoxazolidinone Herbicides

The susceptible population SLR4 was completely controlled by both bixlozone and clomazone applied at the recommended field rate. 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 control (data not shown). The LD_{50} for *L. rigidum* population SLR4 treated with bixlozone was 47 g ha^{-1} and ranged from 122 to 455 g ha^{-1} for the resistant parent populations (2.6- to 9.5-fold resistance compared with SLR4) (Table 3). The addition of phorate significantly ($P < 0.01$) increased the LD_{50} for bixlozone in all populations compared with the herbicide-only treatment. In the presence of phorate, the LD_{50} for SLR4 was 1,627 g ha^{-1} (Table 3) and ranged from 2,188 to 4,061 g ha^{-1} for the resistant populations, giving RIs of 46.6- to 86.4-fold compared with SLR4 in the absence of phorate (Table 3). The *L. rigidum* populations SLR4, EP162, 375-14, and 198-15 treated with phorate and clomazone displayed a significant ($P < 0.01$) antagonistic response similar to the response to bixlozone. In the presence of phorate, the LD_{50} value for SLR4 was 1,575 g ha^{-1} (28.1-fold increase) and ranged from 2,044 to 5,127 g ha^{-1} with RIs of 36.5- to 91.5-fold compared with SLR4 in the absence of phorate (Table 3).

In this study, we report major difference between *L. rigidum* populations in response to bixlozone and clomazone in the presence of the P450 inhibitor phorate. As reported previously, P450 enzymes are involved in the activation (thiocarbamates) or hydroxylation (isoxazolidinones) of the parent compound into the bioactive form of clomazone, 5-ketoclomazone (Ferhatoglu and Barrett 2006; Fuerst 1987; Norman et al. 1990), and inhibition of P450s by phorate would therefore reduce the activity of these herbicides. Reduced activation has been reported as a possible mechanism conferring resistance to the thiocarbamate herbicide triallate (Kern et al. 1996). The antagonism between phorate and bixlozone or clomazone observed in the susceptible population SLR4 and the three resistant populations (Table 3) indicates involvement of P450s in herbicide activation (Brunton et al. 2020b; Busi

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et al. 2017), but lack of herbicide activation is not the mechanism of resistance to these herbicides. Whether resistance to bixlozone in *L. rigidum* is based on a similar mechanism involved in resistance to thiocarbamates remains to be determined.

In a previous study, this resistant population (EP162) displayed no antagonism between phorate and the thiocarbamate herbicide prosulfocarb (Brunton et al. 2020b), which suggested loss of activation as the mechanism of resistance in this population. The response of these populations is consistent with recent studies in which clomazone injury in cotton was reduced with phorate (Culpepper et al. 2001). Research with clomazone-resistant arabidopsis (*Arabidopsis thaliana* L.) revealed that upregulation of two P450 genes *CYP81A15* and *CYP81A21* was correlated with (>10-fold) resistance to clomazone (Guo et al. 2019), suggesting P450-mediated metabolism of clomazone as a possible mechanism. It is possible that a similar mechanism could be selected in *L. rigidum*.

Understanding the risk of resistance evolution to new herbicide discoveries requires early examination of the activity of herbicides and identifying the likelihood of metabolic resistance in weed populations so that resistance can be managed proactively (Busi et al. 2012; Yu and Powles 2014). The evolution of metabolic resistance and unexpected failures of key herbicides has been well reported in weed species globally (Hall et al. 1994; Mangin et al. 2016; Preston et al. 1996; Tardif and Powles 1999). Resistance evolution to bixlozone and clomazone in *L. rigidum* highlights the significant challenges in managing this species effectively while maintaining the sustainability of diverse SOA herbicides into the future. Furthermore, the resistance shift observed in both herbicides following two generations of recurrent selection at full field rates demonstrates the capacity of *L. rigidum* to rapidly evolve resistance without any prior exposure. The mechanism of resistance to clomazone and bixlozone in *L. rigidum* remains unknown; however, it is clear there are existing resistance mechanisms present in multiple-resistant populations. In a field context, use of this herbicide could rapidly select for resistant individuals, unless a diverse array of herbicide, non-herbicide, and cultural weed control tactics are being implemented (Beckie 2006).

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