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Inheritance of evolved resistance to a novel herbicide (pyroxasulfone)

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ABSTRACT

Agricultural weeds have rapidly adapted to intensive herbicide selection and resistance to herbicides has evolved within ecological timescales. Yet, the genetic basis of broad-spectrum generalist herbicide resistance is largely unknown. This study aims to determine the genetic control of non-target-site herbicide resistance trait(s) that rapidly evolved under recurrent selection of the novel lipid biosynthesis inhibitor pyroxasulfone in *Lolium rigidum*. The phenotypic segregation of pyroxasulfone resistance in parental, F_1 and back-cross (BC) families was assessed in plants exposed to a gradient of pyroxasulfone doses. The inheritance of resistance to chemically dissimilar herbicides (cross-resistance) was also evaluated. Evolved resistance to the novel selective agent (pyroxasulfone) is explained by Mendelian segregation of one semi-dominant allele incrementally herbicide-selected at higher frequency in the progeny. In BC families, cross-resistance is conferred by an incompletely dominant single major locus. This study confirms that herbicide resistance can rapidly evolve to any novel selective herbicide agents by continuous and repeated herbicide use. The results imply that the combination of herbicide options (rotation, mixtures or combinations) to exploit incomplete dominance can provide acceptable control of broad-spectrum generalist resistance-endowing monogenic traits. Herbicide diversity within a set of integrated management tactics can be one important component to reduce the herbicide selection intensity.

1. Introduction

Persistent herbicide use across agricultural landscapes has selected for herbicide-resistant plant populations. Since first predicted by Harper [1] many agricultural weed species have evolved adaptive traits in response to herbicide selection within ecological timescales [2]. Similarly to any other evolved plant trait, the adaptive response of plant populations to herbicide selection is determined by complex interactions between genotypes and environment [3]. Some broad-spectrum generalist physiological and biochemical traits endow herbicide resistance in plants by limiting the amount of herbicide reaching their site of action (non-target-site resistance). The inheritance of non-target-site resistance is often complex [4] and the molecular genetic identification of specific resistance-endowing enzymes conferring non-target-site resistance remains to be elucidated [5]. However, there is increasing (mostly indirect) evidence that non-target-site resistance is widespread in weedy plants and can be broad spectrum (i.e. across multiple modes of herbicide action) extending to herbicide ingredients that have never been used or even new herbicide discoveries [6,7].

Lolium rigidum (Gaud.) (annual ryegrass) is a prominent example of a global resistance-prone weed species [8,9]. Resistance evolution in *L. rigidum* in Australia has occurred at a dramatic rate [10] and to a lesser extent in other parts of the world [11]. *L. rigidum* has large genetic variability which is likely determined by its obligate out-breeding reproductive mode [12]. Populations possess heritable phenotypic variation in response to herbicides that enable rapid evolution of herbicide resistance [13]. Several directional selection studies have provided empirical evidence of rapid evolution to herbicide resistance in this species [14–18]. Interestingly, herbicide resistance in field populations of *L. rigidum* can equally occur through the evolved combination of target-site (e.g. resistance-endowing mutation(s) at the herbicide site of action) and non-target-site (e.g. enhanced capacity of herbicide metabolism) resistance mechanisms [9,19,20]. Major loci endowing resistance to anthropogenic toxins that often impose high intensity of selection have been well documented in plants [21,22]. The majority of field-evolved cases of herbicide resistant weeds are monogenic traits (reviewed by Darmency [23]). Inheritance studies improve the overall understanding of the rate and evolutionary dynamics leading to herbicide resistance by providing insights on the number and initial frequency of resistance alleles, genetic dominance and mode of inheritance [24,25].

Pyroxasulfone (chemical class isoxazoline) is a new herbicide safe to wheat and triticale crops but active on *L. rigidum* and other weedy grasses [26,27]. Pyroxasulfone acts on emerging seedlings

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by blocking lipid biosynthesis through inhibition of several very long chain fatty acid elongases (VLCFAE) [26,28]. Before its commercialisation, pyroxasulfone resistance evolved in a *L. rigidum* population (SLR31) after recurrent low-dose selection over three generations [16]. The aim of this study was to determine the inheritance of pyroxasulfone resistance trait that evolved under recurrent selection in the *L. rigidum* population SLR31 and the basis of cross-resistance by phenotypic segregation analysis of back-cross families.

2. Methods

2.1. Plant material

The *L. rigidum* population termed SLR31 has evolved herbicide resistance (hereinafter referred to as MR) in field conditions with most individuals displaying enhanced herbicide metabolism that endows resistance across several herbicide modes of action including ACCase- (diclofop), ALS- (chlorsulfuron), microtubule assembly- (trifluralin), and VLCFAE-inhibiting (*S*-metolachlor) herbicides [29,30,49]. This particular *L. rigidum* MR population was initially susceptible to pyroxasulfone but pyroxasulfone resistance evolved under recurrent pyroxasulfone selection. This occurred after three cycles of recurrent selection with 60 (first generation, MR1), 120 (second generation, MR2) and 120 (third generation, MR3) g pyroxasulfone ha^{-1}. Each generation was selected with a single herbicide application corresponding to 0.6*X*, 1.2*X* and 1.2*X*, respectively (*X* = recommended pyroxasulfone field rate = 100 g pyroxasulfone ha^{-1}). [16]. To continue to characterize and select the pyroxasulfone resistance, the MR3 population was further subjected to two consecutive cycles of selection at 240 (2.4*X*) g pyroxasulfone ha^{-1} (fourth generation, MR4) and 400 (4.0*X*) g pyroxasulfone ha^{-1} (fifth generation, MR5) (see below).

2.2. Generation of F_1 families

One hundred and fifty seeds of the pyroxasulfone-selected progeny MR3 (third generation) were germinated on 0.6% agar medium plates kept in a growth cabinet under 12 h artificial light at 20 °C (light)/12 °C (dark) temperatures, planted after seven days in 18 cm diameter pots containing a potting mix (50% peatmoss, 25% pine bark, 25% river sand), and covered with 0.5 cm of commercial potting soil. Pyroxasulfone treatment at a dose of 240 g ha^{-1} was applied at the soil surface one day after planting using a twin-nozzle laboratory sprayer calibrated to deliver 120 L of spray volume ha^{-1} at each pass at 210 kPa. Following pyroxasulfone treatment, pots were maintained outdoors during the normal winter growing season with 10 h sunlight at 15 °C (light)/11 °C (dark) temperatures. Plants that emerged and grew, surviving the high dose of 240 g pyroxasulfone ha^{-1}, were used as resistant parental plants and were kept for pair crossing. When those highly pyroxasulfone-resistant survivors reached the two-tiller vegetative stage each tiller was separated to generate two genetically identical one-tiller clones. Single pyroxasulfone-selected MR3 tillers were paired according to floral synchronicity to untreated plants of an herbicide susceptible population (S) and enclosed within a plastic-coated cylinder (1.5 m height), which excluded foreign pollen and ensured cross-pollination only between MR3 and S plants. The S plants were a *L. rigidum* population (VLR1) known to be susceptible to all herbicides (hereinafter referred to as S). Importantly, the herbicide-susceptible S plants did not contain major alleles for pyroxasulfone resistance [see 16] and *L. rigidum* is known as an obligate cross-pollinated species [12]. At maturity, seeds were collected and pooled within each of the five pair crosses between the MR3 and S parents (Fig. S1). Five separate F_1 families were thus generated. The remaining five pyroxasulfone-selected MR3 tillers were bulk crossed to produce the seed progeny MR4 (fourth generation) (Fig. S1).

2.3. Generation of backcross (BC) families

Fifty seeds from each F_1 family were treated as previously described with the recommended label pyroxasulfone dose (100 g ha^{-1}). Highly homogeneous response to this pyroxasulfone dose was observed and no significant heterogeneity in plant survival between the five F_1 families was established by a chi-square test ($\chi^2 = 6.88$; $P = 0.14$) (data not shown). Surviving F_1 individuals were cloned and one clone from each of ten plants was individually hybridized with parental S individuals to generate back-cross families. Ten backcross families (from five F_1 families, two individuals from each, hereinafter referred to as BC) were obtained after crossing individual F_1 plants back to S parental plants (Fig. S1). Seeds collected from both plants were pooled at harvest.

2.4. Phenotypic resistance to pyroxasulfone

Outdoor grown plants of parental S, MR, the pyroxasulfone-selected progenies MR1, MR2, MR3, MR4, MR5, five F_1 and 10 BC families were treated at 100 g pyroxasulfone ha^{-1}. There were three replications per parental population (S, MR and selected progenies), two replications for each of the 5 F_1 and each of the 10 BC families, and 30 seeds per replication. Plants were grown in 2 L plastic pots and kept well watered (>80% field capacity) and fertilized weekly (50 mg kg^{-1} of NO_3^-). After 21 days, emerged actively growing plants were counted and assessed for survival. Multiple comparisons among survival proportions were assessed by χ^2 heterogeneity test performed using the statistical software *R* (version 2.14.1) with the command *prop.test*. Confidence intervals were obtained for each single proportion by performing an exact binomial test with the command *binom.test*.

2.5. Pyroxasulfone resistance dominance level assessment

Plants from the five F_1 and 10 BC families together with the parental MR4 and S populations were grown outdoors under identical conditions as described earlier. Parental MR4 and S populations and F_1 families were treated with six pyroxasulfone doses including 0, 12.5, 25, 50, 100, 200 or 400 g ha^{-1}. Plant survival of parental and F_1 families in response to increasing herbicide doses was analyzed by fitting a three parameter log-logistic model (software *R* version 2.14.1). Survival values (survivors/pyroxasulfone treated seeds) ranged between 0 and 1 and a binomial distribution of errors was adopted in the non-linear regression analysis [31]. The herbicide doses causing 50% plant mortality (LD_{50}) in the selected and unselected populations at each generation were estimated by using the three-parameter logistic model (Eq. (1)):

$$Y = \frac{d}{1 + \exp[b(\log x - \log e)]} \quad (1)$$

where *Y* denotes the plant survival, *d* is the upper asymptotic value of *Y*, respectively, *b* is the slope of the curve, *e* is LD_{50}, and *x* is the herbicide dose. Chi-square (χ^2) analysis was used to test inheritance hypotheses for dominance level of genetic pyroxasulfone resistance by comparing survival rates of MR parental and F_1 lines at the recommended label rate and also across a gradient of pyroxasulfone doses by LD_{50} values analysis.

2.6. Pyroxasulfone resistance segregation

Back cross (BC) families were treated with three discriminating doses at 50, 100 or 400 g pyroxasulfone ha^{-1} (0.5×, 1× or

Table 1
Herbicide treatments to evaluate cross-resistance to different herbicide modes of action in BC families.

Active ingredient	Herbicide mode of action	Dose (g ha^{-1})	Recommended dose (g ha^{-1})
Pyroxasulfone	VLCFAE inhibition	100	100
S-metolachlor	VLCFAE inhibition	480	480–720
Prosulfocarb	Lipid synthesis inhibition	2000	2000
Triallate	Lipid synthesis inhibition	2000	800–1000
Trifluralin	Inhibition of microtubule assembly	960	720–960
Atrazine	Inhibition of PSII	2000	1000–2000

VLCFAE, very long chain fatty acid elongase.

4× the recommended label dose, respectively). The 0.5× dose allowed evaluation of pyroxasulfone resistance segregation at a below-label dose. The 1× dose was chosen to assess segregation at the recommended label dose at which plants are selected in the field. The higher dose (4×) was chosen to likely allow pyroxasulfone resistance segregation to occur with minimal interference of minor resistance loci. Two replications per dose and 30 seeds per replication were used. After 21 days, emerged alive plants were counted and aboveground oven-dried biomass was evaluated. The experimental null hypothesis was that pyroxasulfone resistance segregates as controlled by one locus with two alleles, R (resistant) and S (susceptible). The segregation analysis in BC families was based on the observed survival/mortality of resistant (alive) or susceptible (dead) plants compared to the expected survival/mortality assuming one resistance-endowing locus (Table S1). As described by Tabashnik [32], the expected number of surviving plants in BC families was calculated using the following equation referred to one gene locus model (Eq. (2)):

$$\text{Survivors (expected)} = 0.5\,(W_{RS} + W_{SS}) \quad (2)$$

where W_{RS} and W_{SS} are the observed surviving plants of the presumed RS (F_1) and SS (S parent) genotypes at a each herbicide dose tested (i.e. 50, 100 or 400 g pyroxasulfone ha^{-1}). Thus, for a particular dose, the expected number of survivors of each BC population was calculated with the total number of herbicide-treated plants multiplied by the theoretical one locus segregation ratio (e.g. for one locus model that ratio is 0.5F1:0.5S) multiplied by the observed survival ratios in F_1 and S populations (number of plants × segregation ratio × survival ratio; see example in Table S1). For each segregating BC family a goodness of fit chi-square (χ^2) test was used to compare the observed plant survival to the expected calculated values according to one-, two-, or three- resistance locus segregation models. *P*-values were obtained indicating the probability of type I error in rejecting the null hypothesis (H_0 = the BC family segregates as 0.5F_1:0.5S for one locus with two alleles) (see Table S1). The significance level was $\alpha = 0.05$ (two-sided).

2.7. Cross-resistance segregation

In a separate study, a sample of 26 seeds of each parental MR4 and S (two replicates), four F_1 and 10 BC families were also treated with the recommended label dose of various herbicides of dissimilar modes of action (Table 1). Plants were grown and maintained outdoors as described previously. After 21 days, emerged alive plants were counted. As before, differences in survival data of BC families in response to different herbicide modes of action were subjected to chi-square (χ^2) analysis. The null hypothesis H_0 was that the expected survival for any herbicide mode of action tested would have been the same across all BC families. Multiple comparisons among survival proportions of BC families were assessed by χ^2 heterogeneity test performed using the statistical software *R* with the command *prop.test*. Cross-resistance segregation in BC families was investigated as described above. For each segregating BC family a goodness of fit chi-square (χ^2) test was used to compare observed and expected plant survival according to one-, two-, or three- resistance locus segregation models (see also Table S1).

Table 2
Pyroxasulfone resistance evaluation in F_1 and BC *L. rigidum* families compared to parental pyroxasulfone-resistant (MR4) and – susceptible (S) populations. Estimated LD_{50} values after regression analysis are expressed as pyroxasulfone g ha^{-1} with standard errors in parentheses and resistance index (RI). Probability values (*P*) of difference between parental and selected populations in response to pyroxasulfone were assessed by the SI function in the *drc* package in the software programme R version 2.14.1.

Population	LD_{50} (g ha^{-1})	RI[a]	*P*
Pyroxasulfone			
S	16(1.5)	–	
MR4	226(24)	14	<0.01
F_1	76(4.3)	4.8	<0.01
BC	28(5.8)	1.8	0.05

[a] Resistance Index is the LD_{50} ratio between MR4, F_1 or BC population and the standard susceptible population S (VLR1).

3. Results

3.1. Phenotypic resistance to pyroxasulfone

Plant survival after pyroxasulfone treatments (at the label 100 g ha^{-1} pyroxasulfone dose) was assessed in the parental MR4 and S populations and contrasted with the F_1 and BC families (Fig. 1). As expected, the herbicide susceptible S plants were well controlled by 100 g pyroxasulfone ha^{-1}. Equally, the unselected parental MR plants were controlled. In contrast, high survival at this dose was equally observed in both MR4 parental plants and MR5 progeny ($\chi^2 = 0.001$, $P = 0.973$). The ability to survive pyroxasulfone significantly decreased in F_1 and BC families compared to the parental MR4 ($\chi^2 = 448.6$, $P < 0.001$). Pyroxasulfone survival in F_1 families was about 60% lower than MR4 parental plants. BC families exhibited the lowest level of pyroxasulfone survival (0.17) ($\chi^2 = 4.8$, $P = 0.028$) (Fig. 1).

3.2. Pyroxasulfone resistance dominance

Pyroxasulfone dose–response studies confirmed the highest level of pyroxasulfone resistance in the parental MR4 population, whereas the standard susceptible parental S population was confirmed to be pyroxasulfone-susceptible (Fig. 2). The overall pyroxasulfone resistance level in F_1 families ($n = 5$) was intermediate between the parental populations and significantly lower than parental MR4 ($P < 0.01$) (Table 2). As expected the dose–response study also confirmed that BC plants were less resistant than F_1 plants ($P < 0.01$) (Fig. 2 and Table 2).

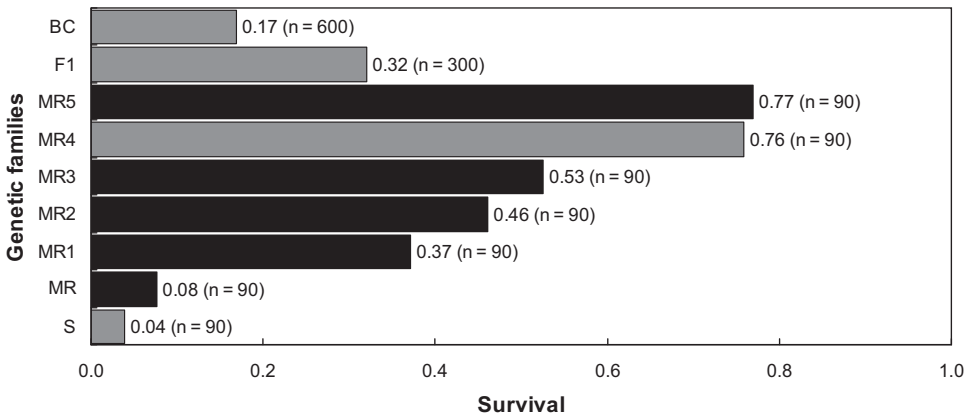

Fig. 1. Plant survival (n = sample size of treated plants) in response to 100 g ha^{-1} of pyroxasulfone in S (susceptible parent), F_1 and BC *L. rigidum* families. MR1, MR2, MR3, MR4 and MR5 were generated after one, two, three [16], four and five cycles of pyroxasulfone recurrent selection, respectively. Multiple comparisons among mean survival proportions of families were conducted and significant differences among the genetic families assessed by a χ^2 heterogeneity test. Grey bars indicate the genetic families investigated in following dose–response and segregation studies.

3.3. Segregation analysis of pyroxasulfone resistance

Overall, the responses obtained with back-cross (BC) families (three different pyroxasulfone doses) indicated that evolved pyroxasulfone resistance in the MR4 *L. rigidum* progeny is governed by one major locus. At pyroxasulfone doses lower than recommended (50 g ha^{-1}) the χ^2 test revealed that a one locus model best fitted the data. However, four of the ten BC poorly fitted the one locus model because of lower than expected survival (Table 3). Other genetic models, implying two or three segregating loci for pyroxasulfone resistance, resulted in highly significant deviations for all 10 BC families tested at that pyroxasulfone dose (P < 0.01; data not shown). At the pyroxasulfone recommended dose (100 g ha^{-1}), the one locus model best fitted eight BC families (Table 3). One BC family showed lower and the other greater than expected survival, respectively. Greater than expected survival could have been explained by a two-locus model (P = 0.48; data not shown). At pyroxasulfone rates higher than recommended (400 g ha^{-1}) the χ^2 test that confirmed that a one locus model best fitted the segregation data of all the BC families tested (Table 3).

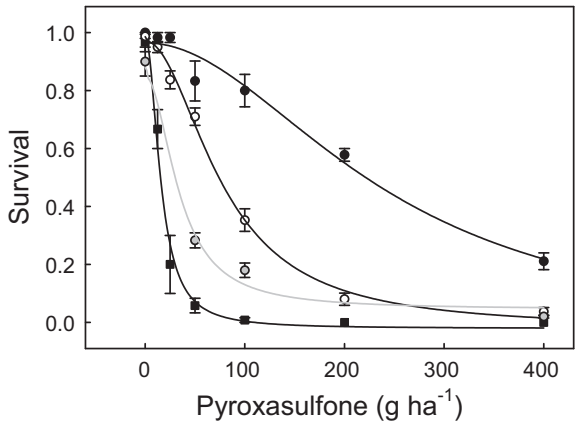

Fig. 2. Pyroxasulfone dose–response study of F_1 and BC *L. rigidum* families compared to parental multi-resistant MR4 and susceptible S populations. MR4 multi-resistant parent (filled circles and continuous black line), S, herbicide-susceptible parent (filled squares and black line); F_1 families (empty circles and continuous black line) and BC families (grey circles and continuous grey line). Symbols are mean of observed plant survival ratios ± SE. Each single pot was the experimental unit (n). MR4 n = 3, F_1 n = 10, BC n = 20 and S n = 3). Lines represent the fitted log-logistic model (Eq. (1)).

3.4. Dominance of cross-resistance

In addition to pyroxasulfone resistance, indications of cross-resistance to several other herbicide modes of action (at the recommended label rate) were assessed with MR4, S, F_1 and BC plants (Table 1). As expected, the parental S population was susceptible to all the herbicides tested (χ^2 = 8.65, P = 0.12) (Fig. 3). The parental MR4 population exhibited similar levels of resistance to prosulfocarb, pyroxasulfone, and triallate (χ^2 = 3.76, P = 0.15) (Table 4), whereas *S*-metolachlor resistance was significantly higher and trifluralin resistance was lower (χ^2 > 13.71, P < 0.003) (Fig. 3). The MR4 population was susceptible to the recommended dose of atrazine. Overall, there was significant heterogeneity in F_1 response to the different herbicides tested (χ^2 = 119, P < 0.001) and the resistance level was lower than that in MR4 parental plants (Fig. 3). As observed in the earlier experiment, pyroxasulfone resistance in F_1 families was significantly lower (P < 0.009) than in parental MR4 plants. The F_1 level of resistance to *S*-metolachlor was significantly greater than to the other herbicides (χ^2 > 37, P < 0.001) (Table 4). Pyroxasufone, triallate, and trifluralin F_1 resistance levels were similar (χ^2 = 1.1, P = 0.59), whereas resistance to prosulfocarb was lower (χ^2 > 8.2, P < 0.004) (Fig. 3, Table 4). Similarly, in BC, *S*-metolachlor resistance was significantly higher than resistance to all the other herbicide treatments (χ^2 = 258, P < 0.001). BC pyroxasulfone resistance was significantly (χ^2 = 48.9, P < 0.001)

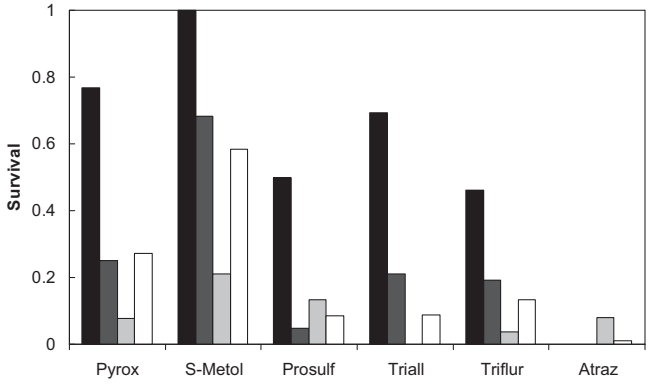

Fig. 3. Phenotypic resistance (survival) observed at the recommended rate of different herbicide modes of action (see Table 1). Significant differences of mean survival ratios among the genetic families were assessed by a χ^2 heterogeneity test. Sample size reported in parenthesis (n). Bar colours: black MR4 (n = 26), dark grey F_1 (n = 106), grey S (n = 52) and white BC (n = 260).

Table 3
Phenotypic resistance segregation observed in 10 back-cross (BC) families treated at three different pyroxasulfone doses. Chi-square (χ^2) analysis for expected plant survival assuming control of pyroxasulfone resistance by one major allele. Survivors were plants that emerged and grew.

Family	Pyroxasulfone dose (g ha^{-1})	Seeds treated	Survivors (observed)	Survival ratio	Survivors (expected)[a]	Segregation ratio	χ^2	P
MR4	50	60	53	0.88				
S	50	60	5	0.08				
F_1	50	300	213	0.71				
BC1	50	60	12	0.20	23.8	1F1:1S	9.7	0.00
BC2	50	60	18	0.30	23.8	1F1:1S	2.3	0.13
BC3	50	60	7	0.12	23.8	1F1:1S	19.7	0.00
BC4	50	60	18	0.30	23.8	1F1:1S	2.3	0.13
BC5	50	60	12	0.20	23.8	1F1:1S	9.7	0.00
BC6	50	60	12	0.20	23.8	1F1:1S	9.7	0.00
BC7	50	60	22	0.37	23.8	1F1:1S	0.2	0.63
BC8	50	60	24	0.40	23.8	1F1:1S	0.0	0.96
BC9	50	60	25	0.42	23.8	1F1:1S	0.1	0.75
BC10	50	60	20	0.33	23.8	1F1:1S	1.0	0.32
MR4	100	60	51	0.85				
S	100	60	0	0.00				
F_1	100	300	106	0.35				
BC1	100	60	5	0.08	10.6	1F1:1S	3.6	0.06
BC2	100	60	8	0.13	10.6	1F1:1S	0.8	0.38
BC3	100	60	4	0.07	10.6	1F1:1S	5.0	0.03
BC4	100	60	9	0.15	10.6	1F1:1S	0.3	0.59
BC5	100	60	10	0.17	10.6	1F1:1S	0.0	0.84
BC6	100	60	9	0.15	10.6	1F1:1S	0.3	0.59
BC7	100	60	11	0.18	10.6	1F1:1S	0.0	0.89
BC8	100	60	25	0.42	10.6	1F1:1S	23.8	0.00
BC9	100	60	16	0.28	10.6	1F1:1S	4.7	0.07
BC10	100	60	10	0.17	10.6	1F1:1S	0.0	0.84
MR4	400	60	14	0.85				
S	400	60	0	0.00				
F_1	400	300	11	0.04				
BC1	400	60	0	0.00	1.1	1F1:1S	1.1	0.29
BC2	400	60	0	0.00	1.1	1F1:1S	1.1	0.29
BC3	400	60	0	0.00	1.1	1F1:1S	1.1	0.29
BC4	400	60	1	0.02	1.1	1F1:1S	0.0	0.92
BC5	400	60	3	0.05	1.1	1F1:1S	3.3	0.07
BC6	400	60	1	0.02	1.1	1F1:1S	0.0	0.92
BC7	400	60	1	0.02	1.1	1F1:1S	0.0	0.92
BC8	400	60	2	0.03	1.1	1F1:1S	0.8	0.39
BC9	400	60	2	0.03	1.1	1F1:1S	0.8	0.39
BC10	400	60	2	0.03	1.1	1F1:1S	0.8	0.39

[a] Survivors expected in BC is the calculated number of seeds treated multiplied by the theoretical one locus segregation model ($0.5F_1$:0.5S) multiplied by the observed survival proportion in F_1 and S at that specific dose (see Eq. (2) in Section 2 and also Table S1).

higher than prosulfocarb, triallate, and trifluralin resistance that were all similarly low ($\chi^2 = 4.37$, $P = 0.112$).

3.5. Segregation analysis of cross-resistance

As previously determined, there was some degree of heterogeneity ($\chi^2 = 69.5$, $P < 0.001$) of BC families in response to pyroxasulfone (see also Table 3) and trifluralin ($\chi^2 = 35.1$, $P < 0.001$). No heterogeneity was found among BC families in response to *S*-metolachlor ($\chi^2 = 10.7$, $P = 0.296$), prosulfocarb ($\chi^2 = 10.7$, $P = 0.295$) and triallate ($\chi^2 = 16.3$, $P = 0.062$) treatments, respectively. Thus, we determined the genetic control of cross-resistance by segregation analysis of pooled BC. The segregation model of one resistance-endowing locus (Table 4) was confirmed to best fit pyroxasulfone, prosulfocarb, triallate and trifluralin. BC resistance to *S*-metolachlor was best explained by the segregation at two loci (Table 4), whereas one locus resulted in significant deviations from the expected plant survival/mortality ($\chi^2 = 19.9$, $P < 0.001$).

4. Discussion

4.1. Monogenic inheritance of pyroxasulfone resistance in Lolium rigidum

Exposure to pyroxasulfone selection over four generations resulted in rapid monogenic-based pyroxasulfone resistance evolution in a *L. rigidum* population (Fig. 1). We emphasize that this population was initially susceptible to the novel herbicide pyroxasulfone, although it had previously evolved resistance to other modes of herbicide action including trifluralin, *S*-metolachlor, diclofop, chlorsulfuron, etc. [16,49]. The present study, by analysis of back-cross (BC) families, confirmed that the evolved pyroxasulfone resistance is controlled by a semi-dominant allele that segregates at one major locus. We observed a deviation from one-locus segregation (lower than expected survival) in four BC families treated at the lowest pyroxasulfone dose (50 g ha^{-1}). Interference of minor resistance loci is possibly responsible for those deviations from monogenic segregation of pyroxasulfone resistance at a low dose of 50 g pyroxasulfone ha^{-1}. BC families were generated by highly pyroxasulfone-resistant parental plants hybridized with susceptible plants to generate resistant F_1 families that homogeneously survived the full recommended dose of 100 g pyroxasulfone ha^{-1}. This dose should have excluded sensitive plants or plants carrying only minor genes for pyroxasulfone resistance and selected only for pyroxasulfone-resistant phenotypes endowed by a major genetic trait. According to Tabashnik [32] greater than expected survival at relatively low doses (in this study 50 g pyroxasulfone ha^{-1}) would denote polygenic additive inheritance, whereas, as we recorded in this study, significant deviations from the expected survival/mortality segregation at doses near LD_{50} values may suggest non-additive interactions. Thus, it may be that non-additive (synergistic) allelic interactions,

Table 4
Herbicide cross-resistance segregation assessed in pooled data obtained from 10 BC families treated with the recommended rate of different herbicides. Pyroxasulfone or atrazine treatments were the control for phenotypic herbicide resistance and susceptibility in *L. rigidum* plants. Chi-square analysis was used to test the genetic control by assuming involvement of one or two resistance-endowing gene(s). Survivors were plants that emerged and grew.

Herbicide	Family	Seeds treated	Survivors (observed)	Survival ratio	Survivors (expected)[a]	Segregation ratio[a]	χ^2	*P*
Pyroxasulfone	MR4	26	16	0.62				
Pyroxasulfone	S	52	4	0.08				
Pyroxasulfone	F_1	104	26	0.25				
Pyroxasulfone	BC	260	71	0.27	59.4	1F1:1S	2.9	0.09
S-metolachlor	MR4	26	26	1.00				
S-metolachlor	S	52	11	0.21				
S-metolachlor	F_1	104	71	0.68				
S-metolachlor	BC	260	152	0.58	146.9	3F1:1S	0.4	0.52
Prosulfocarb	MR4	26	13	0.50				
Prosulfocarb	S	52	7	0.13				
Prosulfocarb	F_1	104	5	0.05				
Prosulfocarb	BC	260	22	0.00	23.8	1F1:1S	0.1	0.71
Triallate	MR4	26	18	0.69				
Triallate	S	52	0	0.00				
Triallate	F_1	104	22	0.21				
Triallate	BC	260	23	0.09	27.5	1F1:1S	0.8	0.36
Trifluralin	MR4	26	12	0.46				
Trifluralin	S	52	2	0.04				
Trifluralin	F_1	104	20	0.19				
Trifluralin	BC	260	35	0.13	30.0	1F1:1S	0.9	0.33
Atrazine	MR4	26	0	0.00				
Atrazine	S	52	4	0.08				
Atrazine	F_1	104	0	0.00				
Atrazine	BC	260	3	0.01	–	–	–	–

[a] Survivors expected in BC is the calculated number of seeds treated multiplied by the theoretical segregation model (0.5F1:0.5S; 1 locus with segregation ratio 1F1:1S) or (0.75F1:0.25S; 2 loci with segregation ratio 3F1:1S) multiplied by the observed survival proportion in F1 and S at that specific dose (see Eq. (2) and also Table S1). The model used (0.5F1:0.5S; 1 locus) or (0.75F1:0.25S; 2 loci) was found to be the best fit, based on evaluating both models, and results from the best fit are shown.

allowing high level pyroxasulfone resistance in pyroxasulfone-selected MR3 parental plants, could have been compromised in F_1 families produced by hybridization with herbicide susceptible S plants. Similar complexities have been shown in herbicide-resistant phenotypes of the cross-pollinated grass weed *Alopecurus myosuroides* [4]. Under the hypothesis of monogenic inheritance and F_1 families genetic structure based on a RR × SS pair-cross, the results seem to clearly indicate that the genetic trait(s) associated to pyroxasulfone resistance is semi-dominant [33]. It may be possible that F_1 families were not based on a RR × SS pair-cross but rather on a back-cross (RS × SS) structure. However, we observed homogenous response to pyroxasulfone between F_1 families suggesting homozygous resistant parental plants. In addition, the repeated directional selection with pyroxasulfone at incrementally higher doses should have selected for homozygous individuals for pyroxasulfone resistance and removed heterozygous individuals.

As advocated for other systems [34], management strategies should be designed to exploit the semi-dominant nature of pyroxasulfone resistance traits and delay pyroxasulfone resistance evolution by reducing the realized heritabilty. For example, crop and/or herbicide rotation practices that favour the crossing between pyroxasulfone-resistant (RR and RS) and – susceptible plants should be adopted. Long history of repeated herbicide field selection led the MR *L. rigidum* population to evolve cross-resistance to ACCase-, ALS-, microtubule assembly-, and VLCFAE-inhibiting herbicides [29,49]. Thus, the broad-spectrum cross-resistance observed in this MR population following pyroxasulfone recurrent selection is not conferred by modifications at multiple herbicide sites of action but is probably due to a mechanism other than resistant enzyme target sites (most likely enhanced herbicide metabolism) [30,35,36]. However, the exact molecular basis of such a mechanism endowing cross-resistance in this *L. rigidum* population remains unknown. It is emphasized that the observed cross-resistance patterns refer to the pyroxasulfone-selected MR *L. rigidum* population. The herbicides used in this study are only effective in the early phases of seed germination or coleoptiles elongation. Thus, it was not possible to investigate cross-resistance patterns on genetically identical clones (e.g. plant tillers).

Thus, pyroxasulfone resistance endowing trait(s) were likely enriched in this field population before pyroxasulfone discovery or use. Repeated field applications of unrelated herbicides maintained those trait(s) at low frequencies in parental MR plants. This can be explained by partial plant protection (cross-resistance) conferred by those trait(s) against herbicides historically used in the field [29]. Subsequently, recurrent pyroxasulfone selection rapidly favoured the selection of plants carrying a major trait (estimated initial phenotypic frequency of 0.08 in the original pyroxasulfone-unselected *L. rigidum* population MR, Fig. 1) more efficient in conferring survival to the specific selective agent pyroxasulfone, and thus causing rapid pyroxasulfone resistance evolution. Four generations of recurrent selection were necessary to increase the population frequency of resistance from 0.08 to 0.77 observed when plants were treated at the recommended pyroxasulfone rate. Modelling simulations with QU-GENE [38], under a range of simplified assumptions including initial gene frequency of 0.08 (defined in Fig. 1), mean selection proportion of 0.32 (mean selection proportion from MR to generate MR5), heritability of 0.2 and one semi-dominant gene, showed that the simulated shift of population mean was similar to the population-based pyroxasulfone resistance evolution reported here (Fig. 1 and Fig. S2). This may suggest some degree of stochasticity during the phenotypic selection of pyroxasulfone resistance under controlled conditions and therefore we suggest that low heritability may likely be associated the evolved genetic trait(s) conferring pyroxasulfone resistance [25,39]. Herbicides such as thiocarbamates and chloroacetamides are important components of herbicide programmes to manage weeds in glyphosate-resistant crops [40–42] as well as to limit the spread of multiple resistance evolution [43,44]. However, thus far field resistance to VLCFA inhibitor herbicides has evolved at a moderate rate [2].

4.2. The challenge posed by genes endowing cross-resistance

Pyroxasulfone resistance appears to be controlled by one major-effect resistance locus that likely contributes to cross-resistance. It is important to emphasize that in a previous study we have shown that under pyroxasulfone recurrent selection the population MR co-evolved cross-resistance to prosulfocarb and triallate [45]. In this study, as found for pyroxasulfone resistance, monogenic segregation has been shown to best fit the phenotypic cross-resistance observed in most of the BC families. Similarly to the results reported by Preston [46], likely, there may be different (distinct) loci endowing cross-resistance in *L. rigidum* with each single major locus conferring resistance to a specific herbicide. However, some degree of variation in response to each herbicide mode of action has been found. Variability in response to herbicide action has been often documented in weed populations of different species [47]. The level of broad-spectrum resistance observed in F_1 families suggests that the implementation of management strategies should be focused on the lowest levels of cross-resistance to minimize the likelihood of alleles endowing cross-resistance to be passed to the next generations [34]. Generally, this is achieved through crop rotation that allows full dose use of dissimilar herbicide modes of action in very different environmental settings [6,48]. The results of this study suggest that in this cross-resistant *L. rigidum* population some degree of control could be achieved by cycling pyroxasulfone (VLCFAE-inhibitor) with a different herbicide mode of action such as either trifluralin (microtubule inhibitor), to which survival of the pyroxasulfone resistant parental MR4 was low, or either prosulfocarb or triallate (lipid synthesis inhibitors), to which low levels of dominance in F_1 plants were observed (see Fig. 3). Thus, chemical control of *L. rigidum* plants by pre-emergence herbicide is best achieved by the combined use (alternations, combinations and/or mixtures) of novel and old herbicide alternatives used only at the recommended (label) doses that give high weed mortality with diversity in control practices. It is emphasized that this is best achieved by employing herbicides at their recommended (full) label rate which can make a heterozygous resistance trait functionally recessive (e.g. control of F_1 plants by prosulfocarb; Fig. 3).

In conclusion, this inheritance study has defined monogenic inheritance of evolved resistance to a novel herbicide and the associated cross-resistance to different herbicide modes of action. The generalist nature of the herbicide resistance trait(s) found in this population of *L. rigidum* suggests that best management tactics should be designed after careful assessment of (1) evolutionary dynamics from directional selection studies (2) inheritance, mechanism and genetic control of broad spectrum cross-resistance and (3) accurate predictions obtained by computational modelling. The evolutionary principles underlying the resistance to herbicides are analogous to those for antibiotic, anti-cancer agent, fungicide and insecticide resistance and we emphasize that more often scientific communities that study such agents should conduct evolutionary studies prior to commercialization of new products and share lessons learnt.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.12.005.

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