Plant Science 217–218 (2014) 127–134

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Inheritance of evolved resistance to a novel herbicide (pyroxasulfone)

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ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 6 December 2013
Accepted 8 December 2013
Available online 14 December 2013

Keywords:
Adaptation
Agriculture
Experimental evolution
Herbicide resistance
Plant science
Selection intensity

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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0168-9452/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2013.12.005

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