Weed Science

www.cambridge.org/wsc

Cross-resistance to acetyl-CoA carboxylase–inhibiting herbicides conferred by a target-site mutation in perennial ryegrass (*Lolium perenne*) from Argentina

Marcos Yanniccari[1] and Ramón Gigón[2]

[1]Researcher, Consejo Nacional de Investigaciones Científicas y Técnicas, Laboratory of Biotechnology and Plant Genetics, Chacra Experimental Integrada Barrow (MAA-INTA), Tres Arroyos, Argentina and [2]Private Consultant in Weed Control, Tres Arroyos, Argentina

Research Article

Cite this article: Yanniccari M and Gigón R (2020) Cross-resistance to acetyl-CoA carboxylase–inhibiting herbicides conferred by a target-site mutation in perennial ryegrass (*Lolium perenne*) from Argentina. Weed Sci. **68**: 116–124. doi: 10.1017/wsc.2020.1

Received: 12 September 2019
Revised: 18 November 2019
Accepted: 10 December 2019
First published online: 13 January 2020

Associate Editor:
Franck E. Dayan, Colorado State University

Keywords:
Asp-2078-Gly; clethodim; LOLPE; perennial ryegrass; pinoxaden; quizalofop; viable seed production

Author for correspondence:
Marcos Yanniccari, Consejo Nacional de Investigaciones Científicas y Técnicas, Chacra Experimental Integrada Barrow (MAA-INTA), RN 3 km 487, Tres Arroyos (7500), Argentina. (Email: marcosyanniccari@conicet.gov.ar)

Abstract

In Argentina, *Lolium* spp. occur in 40% of winter cereal crops from the Pampas. Several years ago, cases of glyphosate-resistant perennial ryegrass (*Lolium perenne* L.) were detected, and the use of acetyl-CoA carboxylase (ACCase)-inhibiting herbicides to eradicate these plants has been considered. The aim of this study was to evaluate the sensitivity of a putative pinoxaden-resistant *L. perenne* population to ACCase-inhibiting herbicides. Around 80% of plants from the putative resistant population survived at a recommended dose of pinoxaden, and they produced viable seeds. The resistance indices (RIs) to pinoxaden were 5.1 and 2.8 for plant survival and seed production, respectively. A single point mutation that conferred a Asp-2078-Gly substitution in ACCase was the source of the resistance. To match the plant control achieved in the susceptible population, the resistant population required 5.4- and 10.4-fold greater doses of clethodim and quizalofop, respectively. RIs for viable seed production when treated with clethodim and quizalofop were 3.3 and 6.6, respectively. The Asp-2078-Gly mutation endowed significant levels of resistance to pinoxaden, clethodim, and quizalofop. For three herbicides, the level of resistance of a pinoxaden-resistant *L. perenne* population to ACCase inhibitors was evaluated, based on an evaluation of dose response for plant survival and seed production. The RIs were higher for plant survival than for seed production. In Argentina, the selection pressure associated with clethodim and haloxifop preplant application and pinoxaden use on wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) crops, would have favored the propagation of the Asp-2078-Gly mutation with its associated resistance.

Introduction

The evolution of herbicide-resistant weeds has been highlighted as a major challenge for agriculture (Baucom 2019). Since the first case of herbicide-resistant rigid ryegrass (*Lolium rigidum* Gaudin) reported in 1982 (Heap and Knight 1982), *Lolium* species have evolved resistance to active compounds with more than 10 sites of action (Heap 2019).

Lolium spp. are a major weed problem in winter cereal crops from five continents, and they leads to wheat (*Triticum aestivum* L.) yield losses of up to 55% (Bararpour et al. 2018; De Prado et al. 2005; Lemerle et al. 1995; Mahmood et al. 2016; Sabet Zangeneh et al. 2018; Scursoni et al. 2012; Smit et al. 1999; Zhang et al. 2017). In Argentina, *L. perenne* and Italian ryegrass [*Lolium perenne* (L.) ssp. *multiflorum* (Lam.) Husnot] occur in 40% of wheat and barley (*Hordeum vulgare* L.) crops from the Pampas (Istilart and Yanniccari 2012; Scursoni et al. 2014). Chemical weed management has been based on glyphosate treatments during fallow periods before winter crop planting and acetyl-CoA carboxylase (ACCase)- or acetohydroxyacid synthase–inhibiting herbicides applied POST in wheat or barley crops.

In response to that management regime, cases of glyphosate-resistant *L. perenne* have been detected over large areas where glyphosate exerts a strong selection pressure on weeds (Yanniccari et al. 2012). ACCase-inhibiting herbicides have been used to control glyphosate-resistant plants; specifically, clethodim or haloxifop have been applied early preplant and pinoxaden or clodinafop later on wheat crops (Yanniccari et al. 2012). However, the evolution of ACCase herbicide–resistant *Lolium* spp. has been demonstrated in several countries (De Prado et al. 2005; Kaundun 2013a; Yu et al. 2007), and putative ACCase-resistant populations of *Lolium* spp. have been detected in Argentina (Gigón and Yanniccari 2018).

The biochemical basis of ACCase-herbicide resistance could mainly be either through enhanced rates of herbicide metabolism or molecular mutations in the *ACCase* gene, endowing target site–based herbicide resistance (Powles and Yu 2010; Yu et al. 2007). In the first case, herbicides are metabolized to products with reduced phytotoxicity by cytochrome P450

monooxygenases (Yu and Powles 2014). Since the first case of resistance to diclofop-methyl in *L. rigidum*, endowed by enhanced detoxification, several *Lolium* spp. populations have shown resistance to a large number of herbicides with several different modes of action (Preston 2004; Preston et al. 1996; Vila-Aiub et al. 2005).

Three classes of commercially available herbicides, aryloxyphenoxy propionates (FOPs), cyclohexanediones (DIMs), and phenylpyrazolines (DEN), target the carboxyltransferase (CT) activity of ACCase; however, 14 spontaneous mutations in the CT domain of this enzyme have been shown to confer herbicide resistance: Ile-1781-Leu, Ile-1781-Val, Ile-1781-Arg, Ile-1781-Thr, Trp-1999-Cys, Trp-1999-Leu, Trp-1999-Ser, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, Gly-2096-Ala, and Gly-2096-Ser (amino acid residues correspond to the native blackgrass [*Alopecurus myosuroides* Huds.] ACCase sequence) (Jang et al. 2013; Kaundun et al. 2013a, 2013b; Yu et al. 2007, 2010). Target site–based herbicide resistance can also be associated with major mutations underlying adaptive phenotypes (Baucom, 2019). In general terms, Asp-2078-Gly and Cys-2088-Arg mutations confer resistance to FOPs, DIMs, and DEN (Kukorelli et al. 2013). The remaining mutations confer different levels of resistance to these herbicides, depending on specific amino acid changes, the number of resistant alleles, weed species, plant growth stage, and recommended field rates for the herbicides (Kaundun 2014).

While a resistance gene increases its frequency depending on the herbicides used, operational and biological factors (Powles and Yu 2010) and knowledge of the mechanism of resistance can support an appropriate management strategy (Evans et al. 2016). That might mean an adequate rotation of herbicides with different sites of action (Scarabel et al. 2007) or that are detoxified by different metabolic pathways, for example, cytochrome P450 monooxygenases and glutathione *S*-transferase (Busi 2018); the correct mixture of specific herbicides (Beckie and Reboud 2009); the possibility of exploiting fitness costs(Keshtkar et al. 2019); and an assessment of the capacity for resistance spread within and among populations (Mithila and Godar 2013).

As indicated earlier, because of the importance of ACCase-inhibiting herbicides in controlling glyphosate-resistant *Lolium* spp. plants during both fallow and POST periods, a putative pinoxaden-resistant *L. perenne* population threatens to undermine the efficacy of these herbicides. As such, this study evaluated the sensitivity of a putative pinoxaden-resistant *L. perenne* population from Argentina to ACCase-inhibiting herbicides. In addition, the cause of the poor plant control achieved by pinoxaden in this population was determined.

Materials and Methods

Experimental Site and Plant Material

Experiments were carried out in Chacra Experimental Integrada Barrow (38.32°S, 60.23°W) from 2017 to 2019. Each experiment was repeated twice.

In December 2016, seeds were sampled from a putative pinoxaden-resistant *L. perenne* population that had arisen in a wheat field (38.32°S, 60.47°W), where the crop rotation involved wheat–soybean [*Glycine max* (L.) Merr.] and barley–soybean. In this system, weed control had been based on recurring applications of glyphosate and clethodim or haloxyfop during fallow; pinoxaden, dicamba, 2,4-D, and metsulfuron in wheat and barley crops; and glyphosate in soybean crop. In addition to the putative pinoxaden-resistant population, seeds were sampled from a neighboring population susceptible to ACCase-inhibiting herbicides established as weeds in a wheat field (38.43°S, 60.97°W) (without application of ACCase-inhibiting herbicides in the last 5 yr). Thirty plants of each population were chosen at random from wheat fields and were stored under laboratory conditions at 20 to 25 C before being used in the current study.

Dose–Response Assays

Herbicide sensitivities of progeny obtained from both populations were compared using the assessments outlined in the following sections.

Germination Test

Seed germination percentage and plumule growth were evaluated under different pinoxaden concentrations following the methodology described by Murray et al. (1996) with modifications. Fifty seeds were placed in petri dishes containing filter paper and 10 ml of the following: pinoxaden (Axial®, pinoxaden [50 g ai L^{-1}] plus cloquintocet-mexyl [12.5 g L^{-1}]; Syngenta Agro S.A., Av. Del Libertador 1855, Vicente López, Argentina) media (each pinoxaden concentration included cloquintocet-mexyl in a 4:1 ratio, respectively): 0, 0.01, 0.1, 1, 10, 100, and 1,000 µM. For each treatment, there were four replicate petri dishes in a completely randomized design. The seeds were incubated in a growth chamber with 75 mmol m^{-2} s^{-1} of photosynthetically active radiation in a 12-h light/12-h darkness regime and temperatures of 25 and 20 C for day and night, respectively. After 7 d, the number of germinated seeds for which the radicle protruded from the seed coat ≥ 0.5 mm was recorded. In addition, plumule length was measured from the point of attachment to the seed to the tip of the coleoptile.

Plant Survival and Seed Production

In August 2017 and 2018, seeds of each population were sown in 2-L pots filled with soil. Seedlings on emerging were thinned to four to five per pot. The plants were grown in a greenhouse at 21 C (average temperature) in a completely randomized design and were irrigated at least every 2 d. Pinoxaden, clethodim, or quizalofop-P-tefuryl was applied to plants with 1 to 2 tillers using a laboratory belt sprayer calibrated to deliver 200 l ha^{-1}. The herbicides were applied at the following doses: pinoxaden (Axial®, pinoxaden [50 g ai L^{-1}] plus cloquintocet-mexyl [12.5 g L^{-1}]; Syngenta Agro S.A.) at 0, 12.5, 25, 50, 100, and 200 g ai ha^{-1} (recommended dose: 30 to 40 g ai ha^{-1}); clethodim (Select®, clethodim 240 g ai L^{-1}; Bayer S.A., Ricardo Gutierrez 3652, Munro, Argentina) at 0, 32.5, 65, 130, 260, and 520 g ai ha^{-1} (recommended dose: 110 to 280 g ai ha^{-1}); and quizalofop (Rango® GR, quizalofop-P-tefuryl 120 g ai L^{-1}; Arysta Lifescience Argentina S.A., Enciso 1463, Tigre, Argentina) at 0, 21.8, 43.7, 87.5, 175, and 350 g ai ha^{-1} (recommended dose: 35 to 112 g ai ha^{-1}). Following the manufacturers' recommendations, 0.2% v/v ethoxylated alcohol was used as surfactant for clethodim and quizalofop spraying. For each treatment, there were five replicates in a completely randomized design, wherein each pot was a sampling unit.

At 45 d post herbicide application, plant survival was evaluated by recording the number of dead and live plants. Plants with severe visual injury (chlorosis of newly emerged leaves, general browning, and severe growth reduction) were recorded as "dead" plants, while "surviving" plants showed no differences in their growth or color of their leaves compared with control plants. These data were used to

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

calculate the percentage of surviving plants per sampling unit (the number of surviving plants was divided by the total number of plants per pot and multiplied by 100).

At the end of the plant life cycle, all plants were harvested and manually threshed. After seeds were separated from the residual inflorescences, the number of seeds produced per plant was determined using a seed counter (Pfeuffer GMBH, Kitzingen, Germany).

The seeds obtained were stored at room temperature for 3 mo (time required to terminate seed dormancy). The viability of the seeds obtained from surviving plants was evaluated by incubating seeds in petri dishes containing filter paper and 10 ml of water and determining the germination percentage. One hundred seeds per treatment were evaluated. This test was carried out in a growth chamber under the conditions previously described.

Peroxidase Activity under Pinoxaden Treatment

Plants obtained from seeds of the susceptible and putative pinoxaden-resistant populations were grown in a greenhouse following the methodology and using the materials described earlier. When plants had developed 1 to 2 tillers, four individuals from each population were treated with 0 or 50 g ha^{-1} pinoxaden. The last expanded leaf of each plant was sampled at 10 d after application of the herbicide. Immediately, 0.3 g fresh weight of these samples were ground in nitrogen liquid and placed in 1.5-ml tubes containing 1 ml 50 mM potassium phosphate buffer (pH 6.5) and 10% (v/v) glycerol at 4 C. The tubes were then centrifuged at 400 × *g* for 5 min at 4 C. Peroxidase activity was determined for an aliquot of each supernatant, based on the oxidation of pyrogallol to purpuro-galline ($\varepsilon = 2.47\ mM^{-1}\ cm^{-1}$) (Puntarulo et al. 1988). The reaction mixtures consisted of 50 mM potassium phosphate buffer (pH 6.5), 45 mM pyrogallol, and 8 mM H_2O_2 at 25 C. Absorbance was measured on a spectrophotometer at 430 nm, and peroxidase activity was expressed in terms of the amount of purpuro-galline produced (mmol min^{-1} g^{-1} fresh weight of leaf).

Test of the Inhibition of Pinoxaden Detoxification through Cytochrome P450 Monooxygenases

The inhibition of pinoxaden detoxification had been performed using malathion as a cytochrome P450 monooxygenase inhibitor (Keith et al. 2015; Matzrafi et al. 2014). Based on this, the interaction between pinoxaden and malathion was evaluated on plumule growth. The susceptible and putative pinoxaden-resistant populations were tested in this assessment; however, another pinoxaden-resistant *L. perenne* population was also included in the evaluation as a positive control, as this was known to undergo detoxification through cytochrome P450 monooxygenases (Yanniccari et al. 2018).

Three hundred seeds of each population were incubated in petri dishes containing a wet filter paper in a growth chamber, with 75 mmol m^{-2} s^{-1} of photosynthetically active radiation, in a 12-h light/12-h dark regime, at temperatures of 25 and 20 C for day and night, respectively. After 48 h, germinated seeds with a radicle length of ≥ 0.2 mm were transferred to 10-ml glass test tubes (4 seeds per tube) containing cotton and 1 ml of one of the following four treatments: 1 µM pinoxaden, 10 ppm malathion, 1 µM pinoxaden plus 10 ppm malathion, and deionized water (control). Ten tubes were used as replicates for each population and treatment. After incubation for 5 d in a growth chamber under the conditions described above, the plumule length was measured from the point of attachment to the seed to the tip of the coleoptile.

Partial Sequencing of ACCase

Total DNA was extracted from leaf tissue of six plants of the susceptible and resistant populations by the cetyltrimethylammonium bromide protocol of Doyle and Doyle (1990). DNA yield and quality were evaluated spectrophotometrically. The DNA was used as a template to amplify two regions of the *ACCase* gene sequence, using primer pairs, ACCase A (F 5′-GGCTCAGCTATGTTCCTGCT-3′ and R 5′-CAAGCCTACCCATGCATTCT-3′) and ACCase B (F 5′-GGCTCAGCTATGTTCCTGCT-3′ and R 5′-CAAGCCTACCCATGCATTCT-3′), according to Matzrafi et al. (2014). The amplified sequences encompassed the positions of all the known mutations described earlier. Polymerase chain reaction (PCR) products were purified by ethanol precipitation at −20 C. The samples were then centrifuged at 32,000 × *g* for 20 min at 4 C. The resulting pellets were washed three times with 70% ethanol and then resuspended in ultrapure water. Nucleotide sequences were obtained for the PCR fragments in these DNA preparations. The sequence data obtained were cleaned, aligned, translated, and compared at the Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, and Gly-2096 codons.

Statistical Analysis

Dose–response data were used to build dose–response curves with a nonlinear log-logistic regression model:

$$y = D/\left(1 + (x/I_{50})^b\right) \quad [1]$$

where y represents the response at herbicide dose x, D is the upper asymptote, I_{50} is the herbicide dose required to achieve 50% of the maximum response, and b is the slope of the line at I_{50}. To assess the accuracy of the model, *F*-tests for model significance, coefficient of determination (R^2), and residual variance analysis were calculated. Models obtained were compared with the extra sum-of-squares *F*-test using GraphPad Prism® v. 6.01 (GraphPad Software, San Diego, CA, USA). Based on this analysis, population data from replicated experiments were pooled when $P > 0.05$ and model parameters were recalculated using the combined evidence. Finally, the I_{50} values from susceptible and resistant populations were compared with the *F*-test ($P < 0.05$) (GraphPad Software). Resistance indices (RI) were calculated as the ratio of I_{50} of the pinoxaden-resistant population compared with the susceptible population.

Data from peroxidase activity assays and tests for inhibition of pinoxaden detoxification were analyzed by multifactorial analysis of variance (ANOVA). Data from replicated experiments were pooled when no significant differences between data sets were detected ($P > 0.05$). In all cases, residual plots indicated that variances were normally distributed and homogenous. When ANOVA indicated significant effects, means were compared using Fisher's protected least significant difference test ($P < 0.05$).

Results and Discussion

Pinoxaden Resistance

At a pinoxaden dose of 50 g ai ha^{-1} (25% higher than the recommended dose of 30 to 40 g ha^{-1}; Syngenta Agro S.A.) approximately 80% of plants from the putative resistant population survived, while only 7% of plants remained alive in the susceptible population (Figure 1A). However, only surviving plants from the pinoxaden-resistant population produced seeds at this dose (Figure 1B). When 200 g ai ha^{-1} of pinoxaden (the maximum dose

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

Table 1. Variables measured in dose–response assays and parameter estimates from the nonlinear log-logistic regression model ($y = D/(1 + (x/I_{50})^b)$) of resistant and susceptible *Lolium perenne* populations treated with pinoxaden (data from two replicated experiments).[a]

Variable	Population	*D*	*b*	I_{50}	R^2	P-value
		%		g ai ha^{-1}		
Plant survival	Susceptible	101.1	4.7	21.1	0.96	$P < 0.001$
	Resistant	99.6	1.0	108.1*	0.76	$P < 0.001$
		RI = 5.1				
		seeds $plant^{-1}$		g ai ha^{-1}		
Seed production	Susceptible	206.4	1.5	9.2	0.92	$P < 0.001$
	Resistant	205.0	0.8	26.2*	0.85	$P < 0.001$
		RI = 2.8				
		%		µM		
Germination	Susceptible	59.7	1.0	0.6	0.87	$P < 0.001$
	Resistant	67.4	1.0	16.2*	0.80	$P < 0.001$
		RI = 27.0				
		mm		µM		
Length of plumule	Susceptible	47.0	1.0	0.3	0.94	$P < 0.001$
	Resistant	53.9	1.0	8.8*	0.90	$P < 0.001$
		RI = 29.0				

[a]Asterisks indicate significant differences between herbicide doses required to achieve 50% of the maximum response calculated on susceptible and resistant populations. Resistance indices (RI) are shown.

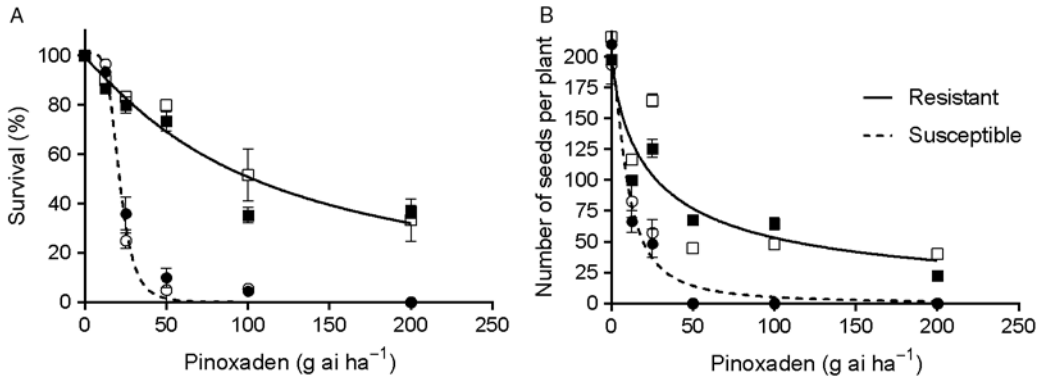

Figure 1. Effect of pinoxaden doses on plant survival (A) and seed production (B) in a resistant and a susceptible *Lolium perenne* population. Black and white symbols represent mean values from experiments conducted during 2017 and 2018, respectively, and bars indicate ±1 standard error of the mean. The predicted responses are shown by lines according to the adjusted models ($y = D/(1 + (x/I_{50})^b)$).

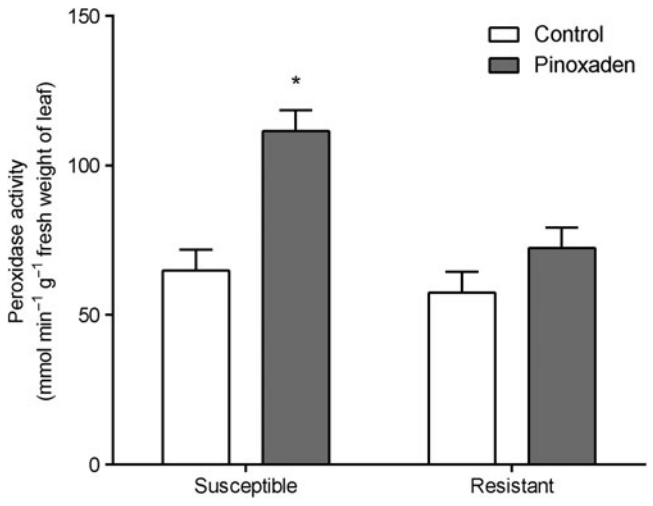

Figure 2. Peroxidase activity of susceptible and resistant *Lolium perenne* plants. Purpuro-galline produced by peroxidase at 10 d post application of pinoxaden compared with untreated control plants. Columns represent mean values, and vertical bars indicate the standard error of the mean (pooled data from replicated experiments). Asterisk indicates significant differences ($P < 0.05$) between a treatment and the control in the same population.

evaluated) was applied, pinoxaden-resistant plants produced around 15% of the seeds obtained from untreated control plants (Figure 1B).

Regression models of plant survival and seed production for both populations were compared, and I_{50} parameters differed significantly between susceptible and pinoxaden-resistant populations. RIs for plant survival and seed production were 5.1 and 2.8, respectively (Table 1).

When peroxidase activity was evaluated, ANOVA results showed a significant interaction between population (susceptible or resistant) and treatment (with or without pinoxaden) ($P = 0.04$), but differences between replicated experiments were not statistically significant ($P > 0.05$). After pinoxaden treatment, the susceptible plants showed indications of oxidative stress, with an increase in peroxidase activity of about 70% compared with controls not treated with herbicide (Figure 2). ACCase-inhibiting herbicides induce oxidative stress in grasses, resulting in enhanced lipid peroxidation and membrane damage (Lukatkin et al. 2013). Nevertheless, the putative pinoxaden-resistant plants showed no significant difference in peroxidase activity between the untreated control and those treated with pinoxaden (Figure 2). The putative pinoxaden-resistant plants also did not exhibit stress symptoms associated with the herbicide application and completed their life cycles, producing viable progeny,

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

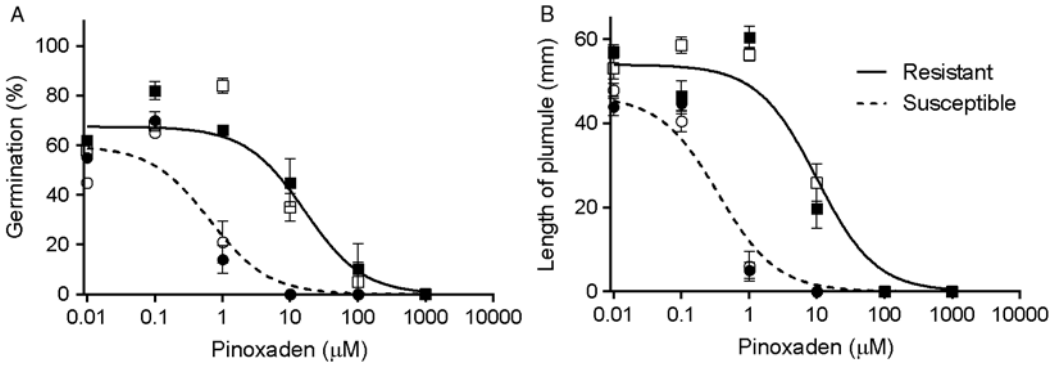

Figure 3. Effect of pinoxaden concentrations on germination (A) and plumule growth (B) in a resistant and a susceptible *Lolium perenne* population. Black and white symbols represent mean values from replicated experiments, and bars indicate ±1 standard error of the mean. The predicted responses are shown by lines according to the adjusted models ($y = D/(1 + (x/I_{50})^b)$).

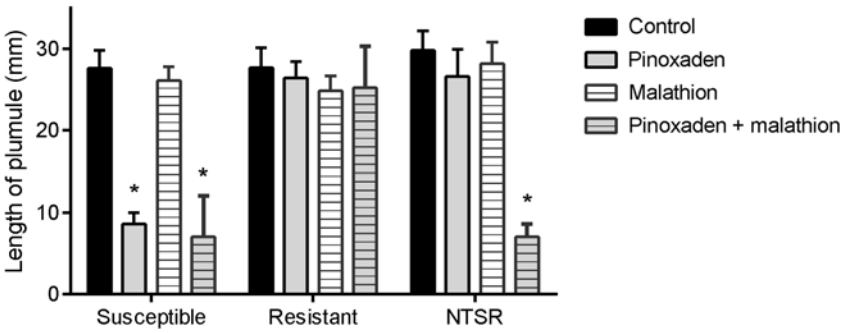

Figure 4. Effect of cytochrome P450 inhibition. Plumule growth of the susceptible and resistant *Lolium perenne* populations compared with an *L. perenne* population with cytochrome P450–mediated pinoxaden resistance (non–target site resistance [NTSR] as positive control), treated with 1 μM pinoxaden, 10 ppm malathion, 1 μM pinoxaden + 10 ppm malathion, and distilled water (control). Columns represent mean values, and vertical bars indicate the standard error of the mean (pooled data from replicated experiments). Asterisks indicate significant differences ($P < 0.05$) between a treatment and the control in the same population.

showing an average percentage germination of 77% (SD ±5%). As this result included seed from surviving plants of the susceptible population treated with pinoxaden, it illustrated the capacity of this species for perpetuation.

The results supported an inherited ability of plants from the putative resistant population to survive and reproduce after a normally lethal dose of pinoxaden. This population had a 5-fold lower sensitivity to pinoxaden than the susceptible population (Table 1). Interestingly, this level of resistance, relative to the susceptible population, seemed to change through the life cycle when RI values were compared. When the effects of pinoxaden on germination and plumule growth were evaluated, the resistant population showed RI values of 27.0 and 29.0, respectively (Table 1).

The low pinoxaden sensitivity of the resistant population was expressed from germination (Figure 3A and B). ACCase-inhibiting herbicides primarily cause the inhibition of fatty-acid biosynthesis, triggering reduction in growth (Cob and Reade 2010; Kukorelli et al. 2013). Fatty-acid synthesis is required for the early stages of cell growth, playing an important role during the processes of germination and seedling growth (Ghosh and Sen-Mandi 2018; Sasaki and Nagano 2004). The germination and plumule growth percentages for the susceptible population were reduced by half at pinoxaden concentrations of 0.6 and 0.3 μM, respectively, in comparison to controls without herbicide treatment (I_{50}; Table 1). However, those concentrations of pinoxaden did not affect the germination or plumule growth of the pinoxaden-resistant population (Figure 3A and B). These seedlings could adequately carry out fatty-acid biosynthesis at pinoxaden treatments ≤ 1 μM (Figure 3A and B).

As was discussed in a previous report, the contrasting behavior of susceptible and resistant plants on the germination test could not be explained by differential transport of the herbicide (Yanniccari et al. 2012). Imbibing embryos with pinoxaden ensured that all tissues were exposed to the herbicide, therefore, eliminating the consequences of any differential transport between populations.

Mechanism of Resistance

Frequently, the development of resistance to ACCase-inhibiting herbicides has been associated with elevated levels of cytochrome P450 monooxygenase activity, suggesting a mechanism of detoxification (Powles and Yu 2010; Siminszky 2006). In the current work, malathion was used as an inhibitor of cytochrome P450. Only the non–target site resistance (NTSR) population with its known pinoxaden detoxification responded to the inhibitor, neutralizing its mechanism of resistance. ANOVA results showed no differences between replicated experiments ($P > 0.05$), but a significant interaction between population (susceptible, resistant, and NTSR) and treatment (malathion, pinoxaden, pinoxaden + malathion, and control without herbicide) was detected ($P < 0.001$). The pinoxaden treatment did not significantly inhibit plumule growth of the NTSR population; however, the combination of pinoxaden + malathion reduced growth to the same level as the susceptible

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

```
R:  TATAATCAGCCTGCCTTTGTATATATCCCCAAGGCTGCAGAGCTACGTGGAGGGGCTTGG
    ·Y··N··Q··P··A··F··V··Y··I··P··K··A··A··E··L··R··G··G··A··W·
S:  TATAATCAGCCTGCCTTTGTATATATCCCCAAGGCTGCAGAGCTACGTGGAGGTGCTTGG
    ·Y··N··Q··P··A··F··V··Y··I··P··K··A··A··E··L··R··G··G··A··W·

                2078
R:  GTCGTGATTGGTAGCAAGATAAATCCAGATCGCATTGAGTGCTATGCTGAGACAACTGCA
    ·V··V··I··G··S··K··I··N··P··D··R··I··E··C··Y··A··E··T··T··A·
S:  GTCGTGATTGATAGCAAGATAAATCCAGATCGCATTGAGTGCTATGCTGAGACAACTGCA
    ·V··V··I··D··S··K··I··N··P··D··R··I··E··C··Y··A··E··T··T··A·
```

Figure 5. Sequence of the *ACCase* gene obtained from the pinoxaden-resistant (R) and pinoxaden-susceptible (S) plants and the conceptual translation of the amino acid sequence. The resistance-conferring codon is show in the box. Numbers refer to amino acid positions of full-length ACCase in *Alopecurus myosuroides*.

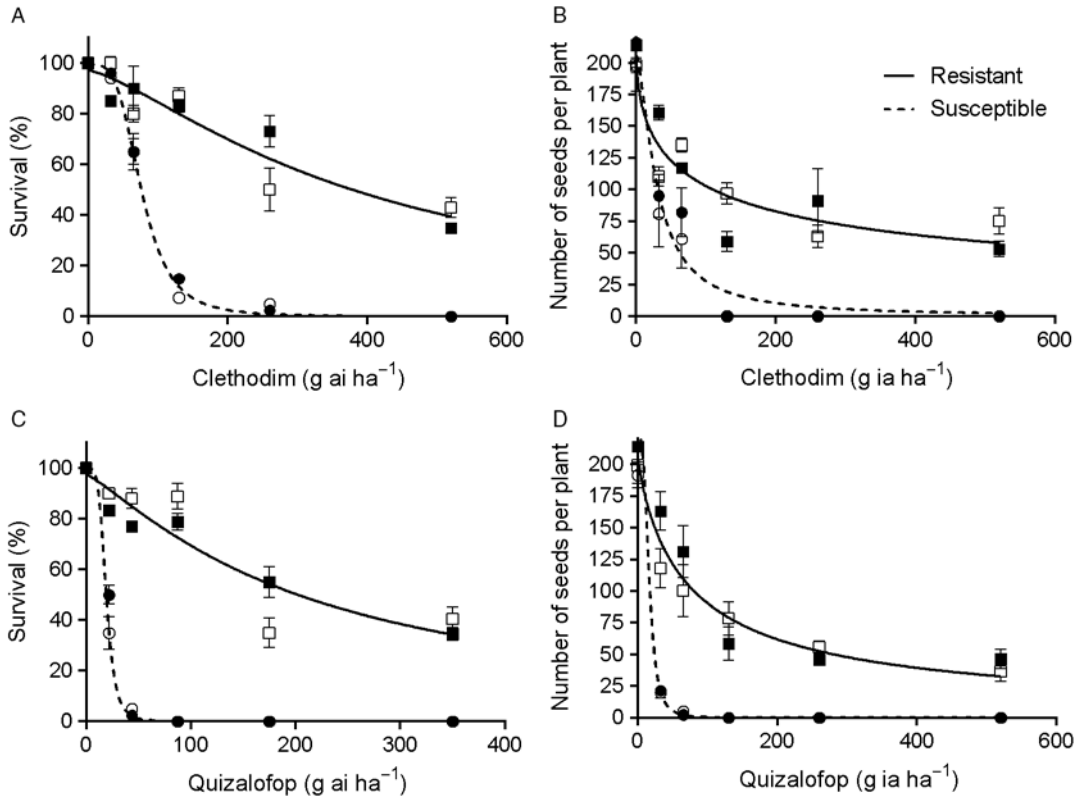

Figure 6. Dose response of a resistant and a susceptible population of *Lolium perenne* treated with clethodim, for plant survival (A) and seed production (B), and with quizalofop, for plant survival (C) and seed production (D). Black and white symbols represent mean values from experiments conducted during 2017 and 2018, respectively, and bars indicate ±1 standard error of the mean. The predicted responses are shown by lines according to the adjusted models ($y = D/(1 + (x/I_{50})^b)$).

population treated with the herbicide (Figure 4). By contrast, no significant differences were detected in plumule growth of the pinoxaden-resistant population treated with pinoxaden, pinoxaden + malathion, and the control without herbicide (Figure 4). As a result, there was no evidence of pinoxaden detoxification mediated by cytochrome P450 monooxygenase in this population.

When potential target-site mechanisms of resistance were explored, a single-point mutation from adenine to guanine was detected, indicating a Asp-2078-Gly substitution in ACCase as the source of the phenotype in the pinoxaden-resistant population (Figure 5). It is known that a Asp-2078-Gly mutation confers resistance to pinoxaden and/or other ACCase-inhibiting herbicides in *A. myosuroides*, wild oat (*Avena fatua* L.), sterile oat (*Avena sterilis* L.), goosegrass [*Eleusine indica* (L.) Gaertn.], *L. perenne* ssp. *multiflorum*, *L. rigidum*, littleseed canarygrass (*Phalaris minor* Retz.), and hood canarygrass (*Phalaris paradoxa* L.) (Beckie et al. 2012; Cruz-Hipolito et al. 2011, 2015; Délye 2005; Gherekhloo et al. 2012; Hochberg et al. 2009; Liu et al. 2007; Osuna et al. 2012; Petit et al. 2010; Yu et al. 2007). Although the D2078 residue is not part of either herbicide-binding site, a Asp-2078-Gly mutation would introduce large conformational changes in the binding site for the ACCase inhibitors, contributing to a low affinity for several herbicides (Jang et al. 2013; Yu et al. 2010).

Cross-resistance

The sensitivity of the populations to clethodim and quizalofop was also examined. While the recommended dose of clethodim ranged from 110 to 280 g ai ha^{-1} (Senseman 2007), there was 85% and 63% plant survival in the resistant population at 130 and 260 g ha^{-1}, respectively (Figure 6A). Moreover, around 40% of the plants survived at 520 g ha^{-1}, the highest dose of clethodim assayed

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

Table 2. Results of dose–response assays using clethodim and quizalofop showing variables measured and parameter estimates from the nonlinear log-logistic regression model ($y = D/(1 + (x/I_{50})^b)$) of resistant and susceptible *Lolium perenne* populations (data from two replicated experiments).[a]

Herbicide	Variable	Population	*D*	*b*	I_{50}	R^2	P-value
			%		g ai ha^{-1}		
Clethodim	Plant survival	Susceptible	99.5	3.7	76.6	0.98	$P < 0.001$
		Resistant	95.6	1.4	413.8*	0.81	$P < 0.001$
			RI = 5.4				
			seeds pl^{-1}		g ai ha^{-1}		
	Seed production	Susceptible	205.9	1.5	30.2	0.91	$P < 0.001$
		Resistant	207.2	0.5	99.8*	0.83	$P < 0.001$
			RI = 3.3				
			%		g ai ha^{-1}		
Quizalofop	Plant survival	Susceptible	100.0	4.2	20.3	0.96	$P < 0.001$
		Resistant	97.4	1.2	211.6*	0.78	$P < 0.001$
			RI = 10.4				
			seeds pl^{-1}		g ai ha^{-1}		
	Seed production	Susceptible	206.7	2.6	9.6	0.98	$P < 0.001$
		Resistant	207.7	1.0	63.4*	0.84	$P < 0.001$
			RI = 6.6				

[a]Asterisks indicate significant differences between herbicide doses required to achieve 50% of the maximum response calculated on susceptible and resistant populations. Resistance indices (RI) are shown.

(Figure 6A). At this dose, seed production was 45% that of control plants (without herbicide; Figure 6B). The seeds produced showed an average percentage of germination of 81% (SD ±7%), including all plants resistant to clethodim, indicating their capacity for perpetuation.

The I_{50} parameters calculated for plant survival and seed production were significantly different between the resistant and susceptible populations (Table 2). In the resistant population, a 5.4-fold greater dose of clethodim was necessary to match the level of plant death achieved in the susceptible population (RI; Table 2). The RI for seed production was 3.3 when comparing the effects of clethodim on the resistant and susceptible populations (Table 2). The Asp-2078-Gly mutation endowed a significant level of resistance to clethodim at the field rate of application, consistent with the findings of Yu et al. (2007). In addition, the RI values for plant survival and seed production on treatment with pinoxaden (5.1 and 2.8, respectively; Table 1) were similar to the RIs obtained for clethodim (5.4 and 3.3, respectively; Table 2). Therefore, the resistance conferred by the Asp-2078-Gly mutation resulted in the same level of insensitivity to both pinoxaden and clethodim.

Quizalofop application is recommended at doses of 35 to 112 g ha^{-1}, with the lowest dose sprayed on soybean crops and the highest dose applied to non-crop areas (Senseman 2007). However, at 43.7 and 87.5 g ha^{-1}, around 80% of the plants from the resistant population survived in the current study (Figure 6C), and they produced more than 50 seeds per plant (Figure 6D). At the highest dose sprayed (350 g ai ha^{-1}), plant survival was around 40% and every plant produced an average of 41 seeds (Figure 6D). The seeds produced showed an average percentage germination of 71% (SD ±6%) for all surviving plants treated with quizalofop. For plant survival and seed production, the resistant populations showed the highest RI values after quizalofop application compared with pinoxaden and clethodim treatments (Table 2). The Asp-2078-Gly mutation confers resistance to all three classes of herbicide. The effect of this mutation on the level of sensitivity of ACCase to several herbicides representing the three chemical classes (but not including clethodim) was studied by Jang et al. (2013), and they found that it provoked higher levels of resistance to quizalofop than pinoxaden.

The level of resistance to different ACCase-inhibiting herbicides depends on the specific mutation, weed species, plant growth stage, and recommended field rates of herbicides. In the case of Asp-2078-Gly and Cys-2088-Arg, both confer broad resistance to all ACCase-inhibiting herbicides currently tested (Kaundun 2014). However, the advantages provided to the plant by Asp-2078-Gly seem not be the general rule, as Vila-Aiub et al. (2015) found that this mutation was associated with constraints in ACCase activity. These constraints included significant reductions in the specific activity and the maximal reaction velocity of this enzyme, which may lead to a shortage of lipids available for rapid growth, correlated with detrimental effects in the growth of *L. rigidum* plants homozygous for this mutation. Similarly, *A. myosuroides* plants have displayed a reduction of around 40% in biomass and seed production associated with the Asp-2078-Gly mutation in the homozygous state (Menchari et al. 2008). Nevertheless, despite the fitness cost, Asp-2078-Gly has been the most frequently found *ACCase* mutation, being detected in nine of 54 *L. multiflorum* populations sampled from the United Kingdom (Alarcón-Reverte et al. 2013). The frequency of this mutation appeared to be governed by the herbicide selection pressure applied by FOPs and DIMs (Kaundun 2014). In Argentina, the selection pressure associated with the use of clethodim and haloxifop in preplant applications and pinoxaden on wheat and barley crops would also have favored the resistance mediated by the Asp-2078-Gly mutation in the *L. perenne* population studied.

The inherited ability of plants from the resistant population to survive and reproduce after a normally lethal dose of ACCase-inhibiting herbicides was demonstrated. Pinoxaden-, clethodim-, or quizalofop-treated *L. perenne* plants completed their life cycle, producing viable seeds.

The level of resistance to these ACCase inhibitors was assessed by dose–response evaluation of plant survival or seed production, and RIs were higher for plant survival than seed production. While growth or survival comparisons between susceptible and resistant plants gave an approximation about the relative sensitivity to an herbicide, considering these results, viable seed production should also be a variable taken into account in evaluating herbicide resistance.

The resistant *L. perenne* population would evolve under a high use of ACCase inhibitors in order to control glyphosate resistant plants. In this context, nonchemical methods of weed management (seedbank control, crop rotation, and competitive crops,

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press

among others) (Jabran and Chauhan 2018) and use of herbicide mixtures (Diggle et al. 2003) and herbicide rotations, taking into account the possible mechanisms of resistance (target-site resistance or detoxification, mainly), should be implemented to delay the evolution of herbicide resistance and prevent Asp-2078-Gly gene flow.

Acknowledgments. This research was partially supported by Consejo Nacional de Investigaciones Científicas y Técnicas and Instituto Nacional de Tecnología Agropecuaria (2019-PE-E4-I086-001). We sincerely thank four anonymous reviewers for their insightful comments that substantially improved the article. No conflicts of interest have been declared.

References

Alarcón-Reverte R, Shanley S, Kaundun S, Karp A, Moss S (2013) A SNaPshot assay for the rapid and simple detection of known point mutations conferring resistance to ACCase-inhibiting herbicides in *Lolium* spp. Weed Res 53:12–20

Bararpour T, Korres NE, Burgos NR, Hale RR, Tseng TP (2018) Performance of pinoxaden on the control of diclofop-resistant Italian ryegrass (*Lolium perenne* L. *ssp. multiflorum*) in winter wheat. Agriculture 8:114

Baucom RS (2019) Evolutionary and ecological insights from herbicide-resistant weeds: what have we learned about plant adaptation, and what is left to uncover? New Phytol 223:68–82

Beckie H, Warwick S, Sauder C (2012) Basis of resistance in Canadian populations of wild oat (*Avena fatua*). Weed Sci 60:10–18

Beckie HJ, Reboud X (2009) Selecting for weed resistance: herbicide rotation and mixture. Weed Technol 23:363–370

Busi R (2018) Herbicide resistance management: a 10-year case study and a look into the future. Pages 24–26 *in* Proceedings of the II Congreso Argentino de Malezas. Rosario, Argentina: Asociación Argentina de Ciencia de las Malezas

Cob AH, Reade JPH (2010) Inhibitors of lipid biosynthesis. Pages 157–175 *in* Cob AH, Reade JPH, eds. Herbicide and Plant Physiology. Chichester, UK: Wiley-Blackwell

Cruz-Hipolito H, Fernandez P, Alcantara P, Gherekhloo J, Osuna MD, De Prado R (2015) Ile-1781-Leu and Asp-2078-Gly mutations in ACCase gene, endow cross-resistance to APP, CHD, and PPZ in *Phalaris minor* from Mexico. Int J Mol Sci 16:21363–21377

Cruz-Hipolito H, Osuna MD, Domínguez-Valenzuela JA, Espinoza N, De Prado R (2011) Mechanism of resistance to ACCase-inhibiting herbicides in wild oat (*Avena fatua*) from Latin America. J Agric Food Chem 59: 7261–7267

Délye C (2005) Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. Weed Sci 53:728–746

De Prado JL, Osuna MD, Heredia A, De Prado R (2005) *Lolium rigidum*, a pool of resistance mechanisms to ACCase inhibitor herbicides. J Agric Food Chem 53:2185–2191

Diggle AJ, Neve PB, Smith FP (2003) Herbicides used in combination can reduce the probability of herbicide resistance in finite weed populations. Weed Res 43:371–382

Doyle J, Doyle L (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15

Evans JA, Tranel PJ, Hager AG, Schutte B, Wu C, Chatham LA, Davis AS (2016) Managing the evolution of herbicide resistance. Pest Manag Sci 72:74–80

Gherekhloo J, Osuna MD, De Prado R (2012) Biochemical and molecular basis of resistance to ACCase-inhibiting herbicides in Iranian *Phalaris minor* populations. Weed Res 52:367–372

Ghosh S, Sen-Mandi S (2018) Methylation status of ACCase promoter affects seed vigor-viability trait in *Oryza sativa* L. Trop Plant Res 5:1–7

Gigón R, Yanniccari M (2018) Evaluación de sensibilidad a diferentes herbicidas en poblaciones de *Lolium spp*. del centro sur de la provincia de Buenos Aires. Page 69 in Proceedings of II Congreso Argentino de Malezas. Rosario, Argentina: ASACIM

Heap I (2019) The International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed: February 7, 2019

Heap IM, Knight R (1982) A population of ryegrass tolerant to the herbicide diclofop-methyl. J Aust Inst Agric Sci 48:156–157

Hochberg O, Sibony M, Rubin B (2009) The response of ACCase-resistant *Phalaris paradoxa* populations involves two different target site mutations. Weed Res 49:37–46

Istilart C, Yanniccari M (2012) Análisis de la evolución de malezas en cereales de invierno durante 27 años en la zona sur de la pampa húmeda argentina. Revista Técnica Aapresid 2012(Malezas problema):47–50

Jabran K, Chauhan BS (2018) Overview and significance of non-chemical weed control. Pages 1–8 *in* Jabran K, Chauhan BS, eds. Non-chemical Weed Control. London: Elsevier

Jang S, Marjanovic J, Gornicki P (2013) Resistance to herbicides caused by single amino acid mutations in acetyl-CoA carboxylase in resistant populations of grassy weeds. New Phytol 197:1110–1116

Kaundun SS (2014) Resistance to acetyl-CoA carboxylase-inhibiting herbicides. Pest Manag Sci 70:1405–1417

Kaundun SS, Bailly GC, Dale RP, Hutchings S-J, McIndoe E (2013a) A novel W1999S mutation and non-target site resistance impact on acetyl-CoA carboxylase inhibiting herbicides to varying degrees in a UK *Lolium multiflorum* population. PLoS ONE 8:e58012

Kaundun SS, Hutchings S, Dale RP, McIndoe E (2013b) Role of a novel I1781T mutation and other mechanisms in conferring resistance to acetyl-CoA carboxylase inhibiting herbicides in a black grass population. PLoS ONE 8: e69568

Keith BK, Lehnhoff EA, Burns EE, Menalled FD, Dyer WE (2015) Characterisation of *Avena fatua* populations with resistance to multiple herbicides. Weed Res 55:621–630

Keshtkar E, Abdolshahi R, Sasanfar H, Zand E, Beffa R, Dayan FE, Kudsk P (2019) Assessing fitness costs from a herbicide-resistance management perspective: a review and insight. Weed Sci 67:137–148

Kukorelli G, Reisinger P, Pinke G (2013) ACCase inhibitor herbicide—selectivity, weed resistance and fitness cost: a review. Int J Pest Manag 59:165–173

Lemerle D, Verbeek B, Combes NE (1995) Losses in grain yield of winter crops from *Lolium rigidum* competition depend on crop species, cultivar and season. Weed Res 35:503–509

Liu W, Harrison DK, Chalupska D, Gornicki P, O'Donnell CC, Adkins SW, Haselkorn R, Williams RR (2007) Single-site mutations in the carboxyltransferase domain of plastid acetyl-CoA carboxylase confer resistance to grass-specific herbicides. Proc Natl Acad Sci USA 104:3627–3632

Lukatkin AS, Gar'kova AN, Bochkarjova AS, Nushtaeva OV, Teixeira da Silva JA (2013) Treatment with the herbicide TOPIK induces oxidative stress in cereal leaves. Pest Biochem Physiol 105:44–49

Mahmood K, Mathiassen SK, Kristensen M, Kudsk P (2016) Multiple herbicide resistance in *Lolium multiflorum* and Identification of conserved regulatory elements of herbicide resistance genes. Front Plant Sci 7:1160

Matzrafi M, Gadri Y, Frenkel E, Rubin B, Peleg Z (2014) Evolution of herbicide resistance mechanisms in grass weeds. Plant Sci 229:43–52

Menchari Y, Chauvel B, Darmency H, Délye C (2008) Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. J Appl Ecol 45:939–947

Mithila J, Godar AS (2013) Understanding genetics of herbicide resistance in weeds: implications for weed management. Adv Crop Sci Tech 1:1–4

Murray BG, Friesen LF, Beaulieu KJ, Morrison IN (1996) A seed bioassay to identify acetyl-CoA carboxylase inhibitor resistant wild oat (*Avena fatua*) populations. Weed Tech 10:85–89

Osuna MD, Goulart ICGR, Vidal RA, Kalsing A, Ruiz Santaella JP, De Prado R (2012) Resistance to ACCase inhibitors in *Eleusine indica* from Brazil involves a target site mutation. Planta Daninha 30:675–681

Petit C, Bay G, Pernin F, Délye C (2010) Prevalence of cross- or multiple resistance to the acetyl-coenzyme A carboxylase inhibitors fenoxaprop, clodinafop and pinoxaden in black-grass (*Alopecurus myosuroides* Huds.) in France. Pest Manag Sci 66:168–177

Powles S, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347

Preston C (2004) Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci 52:448–453

Preston C, Tardif EJ, Christopher JT, Powles SB (1996) Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to

enhanced activity of several herbicide degrading enzymes. Pestic Biochem Physiol 54:123–134

Puntarulo S, Sánchez R, Boveris A (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol 86:626–630

Sabet Zangeneh H, Mohammaddust Chamanabad HR, Zand E, Alcántara-de la Cruz R, Travlos IS, De Prado R, Alebrahim MT (2018) Clodinafop-propargyl resistance genes in *Lolium rigidum* Guad. populations are associated with fitness costs. Agronomy 8:106

Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. Biosci Biotechnol Biochem 68:1175–1184

Scarabel L, Varotto S, Sattin M (2007) A European biotype of *Amaranthus retroflexus* cross-resistant to ALS inhibitors and response to alternative herbicides. Weed Res 47:527–533

Scursoni J, Gigón R, Martin A, Vigna M, Leguizamón E, Istilart C, López R (2014) Changes in weed communities of spring wheat crops of Buenos Aires province of Argentina. Weed Sci 62:51–62

Scursoni JA, Palmano M, De Notta A, Delfino D (2012) Italian ryegrass (*Lolium multiflorum* Lam.) density and N fertilization on wheat (*Triticum aestivum* L.) yield in Argentina. Crop Prot 32:36–40

Senseman S, ed (2007) Herbicide Handbook. 9th ed. Lawrence, MA: Weed Science Society of America. 458 p

Siminszky B (2006) Plant cytochrome P450-mediated herbicide metabolism. Phytochem Rev 5:445–458

Smit JJ, Smit HA, de Villiers BL (1999) Differential efficacy of tralkoxydim and diclofop-methyl on a suspected resistant ryegrass (*Lolium rigidum* Gaud.) biotype. S Afr J Plant Soil 16:169–172

Vila-Aiub MM, Neve P, Powles SB (2005) Resistance cost of a cytochrome P450 herbicide metabolism mechanism but not an ACCase target site mutation in a multiple resistant *Lolium rigidum* population. New Phytol 167:787–796

Vila-Aiub MM, Yu Q, Han H, Powles SB (2015) Effect of herbicide resistance endowing Ile-1781-Leu and Asp-2078-Gly ACCase gene mutations on ACCase kinetics and growth traits in *Lolium rigidum*. J Exp Bot 66:4711–4718

Yanniccari M, Gigón R, Istilart C, Castro AM (2018) Mecanismos de resistencia a múltiples herbicidas en poblaciones de *Lolium spp*. del sur de la provincia de Buenos Aires. Page 242 in Proceedings of the II Congreso Argentino de Malezas. Rosario, Argentina: Asociación Argentina de Ciencia de las Malezas

Yanniccari M, Istilart C, Giménez DO, Castro AM (2012) Glyphosate resistance in perennial ryegrass (*Lolium perenne* L.) from Argentina. Crop Prot 32: 12–16

Yu LPC, Kim YS, Tong L (2010) Mechanism for the inhibition of the carboxyltransferase domain of acetyl-coenzyme A carboxylase by pinoxaden. Proc Natl Acad Sci USA 107:22072–22077

Yu Q, Collavo A, Zheng MQ, Owen M, Sattin M, Powles SB (2007) Diversity of acetyl-coenzyme A carboxylase mutations in resistant *Lolium* populations: evaluation using clethodim. Plant Physiol 145:547–548

Yu Q, Powles S (2014) Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. Plant Physiol 166:1106–1118

Zhang P, Wu H, Xu H, Gao Y, Zhang W (2017) Dong L, Mechanism of Fenoxaprop-P-ethyl Resistance in Italian Ryegrass (*Lolium perenne ssp. multiflorum*) from China. Weed Sci 65:1–8

https://doi.org/10.1017/wsc.2020.1 Published online by Cambridge University Press