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A "Stressed" Alfalfa-Based Cropping System Leads to the Selection of Quizalofop-Resistant Italian Ryegrass (*Lolium perenne* ssp. *multiflorum*)

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Italian ryegrass populations investigated in this study were harvested in an alfalfa-based cropping system. In that system, the agronomic practices and chemical weed management, based on the use of aryloxyphenoxy-propionates herbicides (i.e., quizalofop ethyl ester), were optimized to obtain a dual seed–forage production. Five of seven populations tested were confirmed resistant to quizalofop ethyl ester with resistance indexes ranging from 4.5 to >209. Both target- and nontarget-site resistance mechanisms were most likely involved. Three allelic variants were detected (Ile-1781–Leu, Trp-2027–Cys, and Ile-2041–Asn) in four resistant populations, whereas no known mutations were found in one resistant population. The herbicide treatment on Italian ryegrass plants at different phenological stages suggested that to control regrowth, it is necessary to use two to fives times the herbicide dose suitable for younger plants. This situation is encountered in fields when Italian ryegrass plants need to be controlled to maximize the alfalfa seed production, and it is comparable to using a sublethal herbicide dose, leading to the selection of herbicide-resistant biotypes. In such a situation, the cropping system is not sustainable, and integrated weed management should be implemented to deplete the soil weed seed bank and prevent new weed seed production.
Nomenclature: Quizalofop ethyl ester; Italian ryegrass, *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot, LOLMU; alfalfa, *Medicago sativa* L.
Key words: ACCase inhibitors, cross-resistance, herbicide efficacy, herbicide resistance, phenological stage, target-site resistance mechanism.

In intensive agriculture, the decline of "mixed" farming systems, based on suitable crop rotations, possibly including perennial forage crops, has reduced diversity in space and time within cropping systems and in agroecosystems (Sattin et al. 1995). Diversity drives the impact and evolution of weed communities (Busi et al. 2013; Shaner and Beckie 2014) and is the key underlying element of integrated weed management, which is a well-recognized pillar of sustainable agroecosystems management (Barzman et al. 2015; Mortensen et al. 2012; Vasileiadis et al. 2015).

The most rapid and dramatic changes in weed communities are brought about by the selection of herbicide-resistant biotypes because of recurrent treatments with herbicides having the same site of action (SoA) or metabolic-degradation pathway (Collavo et al. 2013; Délye et al. 2013; Powles and Yu 2010). The more frequent and widespread the use of the same SoA, the higher the risk is of quickly selecting resistant weed populations. Even the implementation of crop rotation per se does not significantly decrease the herbicide selection pressure if a rotation of herbicide SoA is not adopted (Powles and Yu 2010) and the reliance on chemical weed control reduced (Barzman et al. 2014). In fact, herbicide resistance has been steadily increasing in the past decade (GIRE 2016; Heap 2016), and the most standardized cropping systems are more quickly and intensely affected (Dauer et al. 2009; GIRE 2016). However, perennial legume and grass forage crops are rarely involved (Heap 2016), likely because the herbicide selection pressure is usually rather low.

Ryegrass species (*Lolium* spp.) are troublesome weeds, spread in many crops worldwide, with various populations that have evolved resistance to 11 different SoAs (Heap 2016). *Lolium* spp. are principally weeds of cereals but also occur in perennials (e.g., orchards and alfalfa) and, in Italy, have already evolved resistance in standardized cropping systems, such as wheat (*Triticum aestivum* L.), olive (*Olea europaea* L. ssp. *europaea*) groves, and vineyards (Collavo and Sattin 2012, 2014; Collavo et. al. 2013; Panozzo et al. 2015). The self-

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incompatible *Lolium* spp. are prone to rapidly evolve resistance under herbicide selection pressure because of the combination of three characteristics: high genetic variability, adaptability, and fecundity (Gill et al. 1996; Holt et al. 2013). Moreover, they produce dense and highly competitive infestations (Lemerle et al. 2001), have a weak dormancy (Goggin et al. 2012), and seed production can be as high as a few thousand seeds per plant (Pedersen et al. 2007), with up to 45,000 seeds m^{-2} (Rerkasem et al. 1980). However, the seed longevity of *Lolium* spp. is rather short compared with many other invasive species (Ellery et al. 2003), which is favorable for weed management as the viable seed bank can be depleted relatively rapidly (Goggin et al. 2012).

Both target- and nontarget-site mediated resistance mechanisms have been found in *Lolium* spp. populations (Han et al. 2015). The former involves the selection of an altered, but still active, target enzyme, whereas the latter includes a few mechanisms: exclusion of the herbicide molecules from the target site because of differential uptake and/or translocation (Powles and Yu 2010), sequestration or increased metabolic detoxification (Gaines et al. 2014; Preston 2004) and gene amplification (Salas et al. 2012).

The populations of Italian ryegrass investigated in this study were selected from an alfalfa-based cropping system in Ravenna province (northern Italy). Dual seed–forage production is frequent in that area, with the main crop lasting 4 to 6 yr and then rotated with wheat for 1 to 2 yr. Starting the second year, alfalfa is cut in May and then allowed to set seeds, which are harvested in July/August. A final forage cut is attempted at the end of summer. This cropping system led to higher profits for farmers in areas where limited water availability in summer restricted forage production. In most years, throughout the rotation cycle, a treatment with an acetyl-coenzyme A carboxylase (ACCase) inhibitor is applied, and there is no rotation of herbicide SoA.

Agronomic practices and chemical weed management are finely adjusted to optimize the dual production. In the first year, imazamox (an acetolactate synthase [ALS] inhibitor) is usually sprayed to control weeds during the crop establishment phase. From the second year onward, quizalofop ethyl ester (an ACCase inhibitor, hereafter called *quizalofop*) is applied once after the first cut. This is because the presence of grasses is desirable before the first cut to increase biomass production as well as reduce alfalfa plant density, which will favor the later crop seed production.

However, after the first cut, grasses and particularly Italian ryegrass, have to be controlled to avoid negative effects on crop seed production and quality; therefore, the regrowth is treated with quizalofop. This implies that *Lolium* spp. plants are treated when they are much older than the recommended growth stage/age for herbicide treatments (i.e., three- to four-leaf stage, BBCH [Biologische Bundesanstalt, Bundessortenamt, and CHemical industry] scales 13 to 14). Although there are very few hard data available in the literature on the effect of plant age on herbicide efficacy (Eure et al. 2013; Wauchope et al. 1997), it is commonly assumed that herbicide efficacy is lower when sprayed on the regrowth of adult plants, and so, the less susceptible individuals of the population may survive and reproduce. This represents a major risk for selecting resistance. Therefore, the overall selection pressure exerted by the above-described cropping system is rather high.

The objectives of this research were to (1) elucidate the resistance profile of putative ACCase-resistant populations of Italian ryegrass, (2) quantify the effect of plant age on the efficacy of quizalofop on Italian ryegrass, (3) investigate the presence of target-site–mediated resistance mechanisms, and (4) discuss management strategies for aryloxyphenoxy-propionate (FOP)-resistant Italian ryegrass selected in alfalfa.

Materials and Methods

Plant Material and Plants Preparation. Seed samples of Italian ryegrass suspected of being resistant to quizalofop were collected in 2010 from alfalfa fields after farmers' complaints to local extension services about poor herbicide efficacy. Seeds were collected from at least 10 to 15 plants (Panozzo et al. 2015) that had survived a quizalofop treatment at seven sites located in Ravenna province (Emilia-Romagna region, Italy) at least 2 km apart from each other. All alfalfa fields were cultivated for dual forage and seed production and treated with quizalofop every year after the first forage cut. Two susceptible populations were included in the experiments: the standard commonly used in our laboratory (Rigid ryegrass [*Lolium rigidum* Gaudin] S-204L) and another collected in the same area as the putative resistant ones (*Lolium multiflorum* S-389). After collection, seeds were cleaned, stored in double paper envelopes, and exposed to the natural

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indoor fluctuations of temperature and humidity. Experiments were conducted on the experimental farm of Padova University at Legnaro, Italy (45.35°N, 11.95557°E).

Plants used in all experiments were grown from seeds vernalized for 7 d at 4 C to break dormancy and to obtain simultaneous germination. Vernalized seeds were put in petri dishes containing 0.6% (wt/vol) agar and placed in a germination cabinet at 25/15 C (day/night temperature) and 12-h photoperiod with neon tubes providing a photosynthetic photon flux density (PPFD) of 15 to 30 μmol m^{-2} s^{-1}. Germinated seedlings at a similar growth stage were transplanted into plastic trays (32.5 by 26.5 by 9.5 cm) for the greenhouse experiments or into pots for the outdoor dose–response experiment (15 by 15 by 20 cm). Plastic trays and pots were filled with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite, 10% peat). Plants were watered as needed to keep the substrate at or near field capacity.

Whole-Plant Resistance Testing. *Preliminary Greenhouse Screenings*. Thirty seedlings at a similar growth stage were transplanted into plastic trays and placed in a heated greenhouse in which the average daily minimum and maximum temperatures were 16.5 and 27.7 C, respectively. The experimental layout was a completely randomized design with two replicates of 30 plants each. Herbicides were applied at the three- to four-leaf stage (BBCH 13 to 14; Hess et al. 1997) as commercial formulations plus adjuvants (when required) using a precision bench sprayer with a boom equipped with three flat-fan hydraulic nozzles (TeeJet XR11002-VK; Teejet Technologies, Springfield, IL), 0.5 m apart, delivering a spray volume of 300 L ha^{-1} at a pressure of 215 kPa and speed of 0.75 m s^{-1}. A spray volume of 200 L ha^{-1} and TeeJet TP11001-VH hydraulic nozzles were used for glyphosate treatments. An untreated control for each population was included in all experiments.

Italian ryegrass populations were screened twice, once during winter 2011 and once during spring 2012. Three ACCase inhibitors were tested at the recommended field dose (1×) and at three times that rate (3×). However, for glyphosate, the recommended field dose (1×) and double that rate (2×) had previously been identified as suitable for glyphosate-resistance screenings (Collavo and Sattin 2012). The other four herbicides with different SoAs were tested at the recommended field dose only (Table 1).

In the greenhouse, light was supplemented during a 14-h photoperiod using 400 W metal-halide lamps, which provided a PPFD of about 150 μmol m^{-2} s^{-1}. At 4 wk after treatment (WAT), plant survival was recorded. Plants were assessed as being dead if they showed no active growth, regardless of color or other appearance (Panozzo et al. 2015). Populations were classified as resistant when more than 20% of plants survived the herbicide dose at 1× and highly resistant when survivors were more than 20% at a herbicide dose of 1× and more than 10% at herbicide dose of 3× (Panozzo et al. 2015).

Outdoor Dose–Response Bioassay. Quizalofop efficacy on the seven populations of Italian ryegrass plus a susceptible population (S-204L) was also tested in an outdoor dose–response experiment during April to May 2012. Plants were treated at BBCH stages 13 to 14, and the experimental layout was a completely randomized design with three replicates of nine plants each. Herbicide doses ranged from 2.3 to 2,400 g ai ha^{-1}. Each population was sprayed with eight doses plus an untreated check, and the dose range varied based on the results obtained from the greenhouse experiments. Spraying equipment was as described in the previous section. Average daily minimum and maximum temperatures during the experiment were 8.7 and 18.4 C, respectively.

Plant survival and fresh weight per pot were recorded at 4 WAT. Plants were assessed as being dead if they showed no active growth, regardless of color or other appearance (Panozzo et al. 2015). Survival and fresh weight were expressed as a percentage of the untreated control. The 50% lethal dose (LD_{50}) and LD_{80} (based on plant survival), 50% growth reduction (GR_{50}), and GR_{80} (based on fresh weight), and their relative standard errors, were calculated using a nonlinear regression analysis performed with the macro BIOASSAY97 (version 2.651, Onofri A, Borgo XX Giugno 74, University of Perugia, Italy) running in Windows (Microsoft, Redmond, WA) Excel. The macro is based on a log–logistic equation to fit the data:

$$Y = C + \{(D - C)/[1 + (x/I_{50})b]\} \quad [1]$$

where Y is the fresh weight or survival; C and D are the lower and upper asymptotes at higher and zero doses, respectively; I_{50} is the herbicide dose resulting in a 50% inhibition in plant survival (LD_{50}) or biomass (GR_{50}); and b is the slope. To calculate the 80% inhibition in survival or growth, I_{80} was been used in the log–logistic equation.

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Table 1. Herbicide common and trade names, herbicide rate in active ingredients (ai) or acid equivalents (ae), name of respective herbicide manufacturer, and manufacturer's city, state, Web site, and recommended field dose (1×).

Common name	Trade name	Herbicide rate (ai or ae concentration)	Herbicide manufacturer	Herbicide manufacturer details	Herbicide dose[a]
Quizalofop ethyl ester	Leopard 5 EC	50 g ai L^{-1}	Adama Agan	Ashdod (Israel) http://www.adama.com	75 g ai ha^{-1}*
Pinoxaden	Axial Pronto	50 g ai L^{-1}	Syngenta Crop Protection	Monthey (Switzerland) http://www.syngenta.com	45 g ai ha^{-1}*
Cycloxydim	Stratos Ultra	100 g ai L^{-1}	BASF	Ludwigshafen am Rhein (Germany) http://www.basf.com	160 g ai ha^{-1}*
Mesosulfuron + iodosulfuron	Atlantis WG	30 + 6 g ai kg^{-1}	Bayer CropScience	Monheim am Rhein (Germany) http://www.cropscience.bayer.com	15 + 3 g ai ha^{-1}*
Imazamox	Tuareg	40 g ai L^{-1}	BASF	Ludwigshafen am Rhein (Germany) http://www.basf.com	40 g ai ha^{-1}*
Chlortoluron	Dicuran 700 FW	700 g ai L^{-1}	Syngenta Crop Protection	Monthey (Switzerland) http://www.syngenta.com	1,250 g ai ha^{-1}*
Propyzamide	Kerb Flo	400 g ai L^{-1}	Dow Agro Sciences	Midland, MO (USA) http://www.dowagro.com	1,600 g ai ha^{-1}*
Glyphosate	Roundup Platinum	480 g ae L^{-1}	Monsanto	St. Louis, MO (USA) http://www.monsanto.com	360 g ae ha^{-1}**

[a] *, recommended field dose in Italy (1×); **, minimum label dose recommended at the time the populations were sampled for annual weed species.

The procedure estimates the standard error of the parameters and performs the Box–Cox power transformation family. Data were analyzed as described in Seefeldt et al. (1995) by regressing all the considered curves together using independent parameters. The complex model with independent parameters for each population was then compared with relatively simplified models having common parameters among curves. The lack-of-fit *F* test was performed at each step, and the simplification stopped when a significant lack of fit occurred. For biological reasons, and to improve the estimates of other parameters, the upper asymptote was forced to 100, whereas the lower asymptote was not constrained to allow an appropriate adjustment of the regression curves with the observed data of resistant or susceptible populations. The resistance indexes—$RI = LD_{50}$ (or LD_{80}, GR_{50}, GR_{80}) of the resistant population (*R*)/LD_{50} (or LD_{80}, GR_{50}, GR_{80}) of the susceptible population (*S*)—for each putative resistant population were calculated.

ACCase Gene Sequencing. Plant material for molecular analyses was collected from the second greenhouse screening. Fresh leaf tissue from 10 plants per population that survived a quizalofop treatment at the dose 1× was analyzed. Amino acid substitutions in the carboxyl transferase (CT) domain of the ACCase gene responsible for target-site resistance were investigated. A bulk of leaves of the untreated susceptible S-204L and S-389 were used as wild-type controls.

The leaf samples were ground to powder using an electric drill (Skil, Breda, The Netherlands) equipped with a plastic pestle (Sigma-Aldrich, Saint Louis, MO). Genomic DNA was extracted following the CTAB method (Doyle and Doyle 1987) and then quantified through spectrophotometer analysis using a NanoDrop 2000C (Thermo Scientific, Wilmington, DE). The polymerase chain reaction (PCR) analysis was performed using a T1 Thermocycler (Biometra, Göttingen, Germany). An 1,100-base pair region of the ACCase CT domain was amplified using the primers LOL_FOR (5′-CTGTCTGAAGAAGACTATGGCCG-3′) and LOL_REV_CT (5′-ATGCATGGGTAGGCTT GATCCAG-3′). The reaction volume was 50 µL including 200 ng of genomic DNA, 0.6 µM of each ACCase primer, 0.2 mM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 10 µm of 5× Colorless GoTaq Flexi buffer (Promega Corporation, Fitchburg, WI), and 1.25 µm of GoTaq G2 Hot Start polymerase (Promega). Amplification was performed using the following program: denatur-

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ation step for 2 min at 95 C, 35 cycles of 30 s at 95 C, 30 s at 58 C, and 75 s at 72 C, followed by a final extension step of 5 min at 72 C. The PCR product was directly purified with the NucleoSpin Gel and PCR Clean UP kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and sequenced using the primers LOL_FOR and LOL_FOR_SEQ (5′-GAGGTGGCTCAGCTATGTTCCTG-3′) with an ABI 3730 XL sequencer (Applied Biosystem, Foster City, CA) by BMR Genomics (Padova, Italy). Sequences were edited with FinchTV 1.4.0 software (Geospiza Inc., Seattle, WA) and nucleotide sequences were manipulated using MEGA 5.05 software (Tamura et al. 2007). The amino acid positions in the CT domain of the ACCase gene, known to endow herbicide resistance, were investigated (Kaundun 2014).

Effect of Growth Stage on Quizalofop Efficacy. To quantify the effect of weed age/phenological stage on quizalofop efficacy, a dose–response experiment was performed in the greenhouse during February to April 2012 on two quizalofop susceptible *Lolium* populations (S-204L and S-389) treated at three different phenological stages: three to four leaves (BBCH scale 13 to 14), hereafter, called *A*; tillering (BBCH scale 14 to 21), hereafter, called *B*; and regrowth (shoots were cut about 3 cm above the soil surface, and the regrowth was treated 12 d later), hereafter, called *C*. The experimental layout was a completely randomized design with three replicates of 15 plants (plants/tray/treatment). The herbicide was applied at eight doses calculated using a geometric progression ranging from 4.7 to 150 g ai ha^{-1}. An untreated check was also included. Spraying equipment was as described above. The average daily minimum and maximum temperatures during the experimental period were 16.5 and 27.7 C, respectively. Plant survival and fresh weight were recorded at 4 WAT. Plants were assessed as being dead if they showed no active growth, regardless of color or other appearance (Panozzo et al. 2015).

Data were recorded and analyzed as reported above and LD_{50}, LD_{90}, GR_{50}, GR_{90}, and their relative standard errors were calculated. For biological reasons, and to improve the estimates of other parameters, the upper and lower asymptotes were forced to 100 and 0, respectively. A tolerance index (TI; de Mol et al. 2015) was calculated to express variable susceptibility at different phenological stages. It was defined as the ratio between the LD_{50} (or LD_{90}, GR_{50}, GR_{90}) of a specific phenological stage (i.e., tillering or regrowth) and the LD_{50} (or LD_{90}, GR_{50}, GR_{90}) of the "optimum" phenological stage treatment (i.e., three to four leaves).

Field Experiments. The field experiments were repeated twice, once in 2011 and once in 2012, to evaluate the efficacy of quizalofop on a resistant population of Italian ryegrass. The two alternative herbicides authorized in Italy for alfalfa crops (propyzamide and imazamox) were also tested, as well as herbicides reported to be used in the field history. The experiment was set up at a commercial farm near Ravenna, Italy (44.516667°N, 12.85°E; 8 m above sea level) where population 375 was originally sampled. The alfalfa-based cropping system was the same as described above. The experiment was repeated in two adjacent fields for 2 consecutive yr (the fourth and fifth years of continuous alfalfa cultivation). Field records showed that the use of ACCase inhibitors had been continuous for at least 5 yr, and glyphosate was occasionally sprayed on not actively growing alfalfa, that is, at the end of winter. The experimental layout was a randomized block design with four replicates; the size of each plot was 20 m^2 (2.5 by 8 m).

Quizalofop, propyzamide, and imazamox (Table 1) were sprayed at the recommended field doses. Untreated plots were also included in the experiments. Herbicides were applied as commercial formulations (plus adjuvants when required) using a backpack sprayer with a 2.5 m boom equipped with five flat-fan hydraulic nozzles (Teejet 110-03 VK-XR), spaced 0.5 m apart, delivering a spray volume of 300 L ha^{-1} at a pressure of 250 kPa, and sprayed at a speed of 1.1 m s^{-1}. Herbicides were applied in early February when alfalfa was not actively growing or in May, 10 d after the first forage cut.

Herbicide efficacy, in relation to the untreated control, was calculated in terms of both number of ears per square meter and the biomass of the Italian ryegrass. The number of ears per square meter was determined by counting the number of ears in four randomly placed quadrates of 0.5 m by 0.5 m, for a total sampled area of 1 m^2 $plot^{-1}$. Biomass was estimated using the visual estimated biomass (VEB) scale in the central portion of each plot. A visual comparison of plant biomass between treated and untreated plots was done giving a score from 10 (plants not affected by the herbicide) to 0 (plants clearly dead) to each treated plot (Panozzo et al. 2015). Weed assessments were done in April and

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June for treatments applied in February, whereas only in June for treatments applied in May.

The effects of control strategies on the infestation level were calculated using Abbott's formula:

$$\text{Efficacy}(\%) = (1 - \text{Nta}/\text{Nca}) \times 100 \qquad [2]$$

where Nta was the number of ears in treated plots, and Nca was the number of ears in the untreated control plots.

Statistica 7 (version 7.0, StatSoft, Tulsa, OK) software was used to analyze the data. Preliminary analysis was done to normalize the data using Bartlett's and Levene's tests, but because of their variability, normalization was not possible, even by applying the most common data transformations {e.g., arcsen(P), arcsen[$\sqrt{(P)}$], $\sqrt{(P+1)}$, etc.}.

Results and Discussion

Characterization of Resistant Populations. *Preliminary Greenhouse Screenings.* The effect of screening replication was tested using ANOVA, which indicated that data from the two experiments were not significantly different ($P < 0.05$); therefore, data for each treatment were pooled across experiments. Five of seven populations were resistant to quizalofop (Table 2), whereas the other two (376 and 380) had less than 20% of survivors at the recommended field dose (Table 1). Resistant populations were selected on different farms through several quizalofop treatments to the regrowth of Italian ryegrass using a dose recommended for three- to four-leaf stage. It should be stressed that all populations came from alfalfa crops grown for dual production, and several thousand hectares were affected.

The pattern of cross-resistance to other ACCase inhibitors differed among populations (Table 2). The results indicated that three of five populations resistant to the FOP herbicide were controlled by pinoxaden, a herbicide belonging to a different chemical family of ACCase inhibitors, the phenylpyrazolins (DEN). Populations 375 and 377 were cross-resistant to quizalofop and pinoxaden, whereas a few plants of population 388 survived the treatment with pinoxaden. Populations 378 and 379 were highly resistant only to quizalofop (Table 2). This is valuable information because pinoxaden is one of the most used graminicides in wheat, which is the crop usually rotated with alfalfa. A few individuals of population 375 and population 388 survived cycloxydim treatments (Table 2), suggesting that a resistance mechanism related to an altered

Table 2. Plant survival (percentage ± SE) of *Lolium* spp. populations calculated based on two greenhouse screenings for each herbicide and dose tested.

	Quizalofop		Pinoxaden		Cycloxydim		Mesosulfuron + iodosulfuron	Imazamox	Chlortoluron	Glyphosate	
Population	75	225	45	135	160	480	15 + 3	40	1,250	360	720
					g ai (or ae) ha^{-1}						
S-204L	0	0	0	0	0	0	0	0	6.8 ± 3.01	0	0
S-389	0	0	0	0	0	0	0	0	8.9 ± 2.45	0	0
375	96.6 ± 1.96	94.7 ± 1.07	43.9 ± 4.57	15 ± 5.18	9.1 ± 1.05	6.8 ± 1.34	0	1.7 ± 0.96	5.1 ± 1.71	9.4 ± 6.51	0
376	10.4 ± 5.11	9.3 ± 1.58	0	0	0	0	1.7 ± 0.96	0	5.9 ± 3.69	13.5 ± 11.11	0
377	98.3 ± 0.96	80.8 ± 2.5	21.7 ± 2.15	1.7 ± 0.96	0	0	0	0.8 ± 0.83	5 ± 3.19	4.3 ± 2.57	0
378	95.8 ± 1.63	76.7 ± 5.27	0	0	0	0	0	0.8 ± 0.83	6.7 ± 3.02	5 ± 3.19	0
379	41.2 ± 3.43	32.5 ± 0.83	0	1.7 ± 0.96	3.5 ± 2.53	0.9 ± 0.86	0	1.7 ± 0.96	8.5 ± 2.23	4.2 ± 2.50	0
380	9.2 ± 2.10	7.7 ± 2.08	0	0	1.7 ± 0.96	0	0	0	0.8 ± 0.83	17.4 ± 11.12	0
388	73.1 ± 4.49	70.8 ± 1.60	13.4 ± 1.29	4.2 ± 0.82	12.6 ± 1.72	11.8 ± 1.03	0	0	7.6 ± 3.15	0	0

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Table 3. Plant survival: lethal dose, the herbicide dose that reduce 50% of plant survival (LD_{50} and LD_{80}) ± SE and slope (*b*) calculated through nonlinear regression analysis of quizalofop dose–response experiment. Resistant indexes (RIs) calculated based on susceptible check S-204L LD_{50} or LD_{80}. Upper asymptote was fixed at 100.

Population	LD_{50}	LD_{80}	*b*	RI_{LD50}	RI_{LD80}
S-204L	9.6 ± 0.29	11.5 ± 0.67	7.9 ± 2.51	—	—
375	> 2,400	> 2,400	6.4 ± 2.18	> 250	> 209
376	9.2 ± 1.23	28.8 ± 10.25	2.3 ± 1.01	1.0	2.5
377	464 ± 39.8	869 ± 233.7	2.9 ± 0.88	48	76
378	511 ±3 8.71	> 2,400	4.4 ± 1.50	53	> 209
379	42.8 ± 6.39	314 ± 72.3	0.7 ± 0.17	4.5	27
380	14.6 ± 0.82	21.7 ± 2.40	4.7 ± 1.09	1.5	1.9
388	456 ± 90.54	1426 ± 604.8	0.8 ± 0.38	48	124

ACCase enzyme is involved because cycloxydim is not metabolizable (Keshtkar et al. 2015). Chlortoluron controlled Italian ryegrass more than 90% and equal to susceptible checks (Table 2). The mix of two ALS inhibitors tested (mesosulfuron + iodosulfuron) adequately controlled all populations, and the few survivors were heavily injured and most likely unable to produce seeds. At dose 720 g ae ha^{-1} (2×), glyphosate controlled *Lolium* species 100% (Table 2). This dose proved to be suitable for controlling *Lolium* spp. populations in Italy (Collavo and Sattin 2012). Summarizing, all quizalofop-resistant populations were controlled by herbicides having SoA different from ACCase inhibitors. However, the few survivors detected after the treatment with 360 g ae ha^{-1} of glyphosate, representing the low dose commonly used to control *Lolium* spp., confirm that the efficacy of this important herbicide needs careful monitoring to maintain its control even in arable crops that are not genetically modified (Collavo and Sattin 2014). In this situation, it is fundamental to reduce Italian ryegrass seed set and to keep efficacy close to 100% to limit the selection of resistant alleles (Manalil et al. 2011; Neve and Powles 2005a).

Dose–Response Bioassays. The response of *Lolium* spp. to increasing quizalofop doses was adequately fitted by the log–logistic equation without any data transformation, with most standard errors of parameters being one order of magnitude lower than the parameters values. Plant survival and fresh weight gave similar results; therefore, only plant survival data are reported. The lack-of-fit *F* test on plant survival indicated that it was not possible to simplify the regressions to a model with a common lower asymptote and slope for all populations. The same results were obtained when grouping susceptible and resistant populations, so a single-curve approach was used.

Overall, data were consistent with the results previously obtained from the preliminary screening. According to the RIs (Table 3), populations 376 and 380 were considered susceptible because the RI based on the LD_{50} was between 1 and 1.5, whereas LD_{80} was between 1.9 and 2.5 and less than 20% of the plants survived the recommended field dose. However, a few plants (16.7 ± 5.56% and 11.1 ± 5.56% for populations 376 and 380, respectively; data not shown) survived the dose 4× (300 g ai ha^{-1}), indicating that the selection process was ongoing in these populations. Populations 375, 377, 378, and 388 were highly resistant to quizalofop, whereas population 379 showed a lower level of resistance. For population 375, it was not possible to calculate either the LD_{50} or the LD_{80} values because of the high plant survival rate (> 50%) at the highest dose sprayed (2,400 g ai ha^{-1}); therefore, the LD values are given as > 2,400 g ai ha^{-1} (Table 3). No relation was detected between the slope and the resistance status.

Target-Site ACCase Point Mutations. Allele variations, compared with susceptible checks sequences, were observed in populations 375, 377, 379, and 388. In particular, three amino acid substitutions were identified by sequencing genomic DNA: Ile-1781–Leu (ATA to TTA) in populations 379 and 388, Trp-2027–Cys (TGG to TGC or TGT) in populations 377 and 388, and Ile-2041–Asn (ATT to AAT) in populations 375, 379, and 388. No amino acid substitutions were found in populations 376, 378, and 380.

All survivors analyzed (10 of 10) of population 375 and 4 of 10 survivors of population 379 carried the amino acid substitution Ile-2041–Asn at a heterozygous status. Scarabel et al. (2011) showed that the Ile-2041–Asn mutation confers high resistance levels to most FOP herbicides, no-to-moderate levels of resistance to the DEN pinox-

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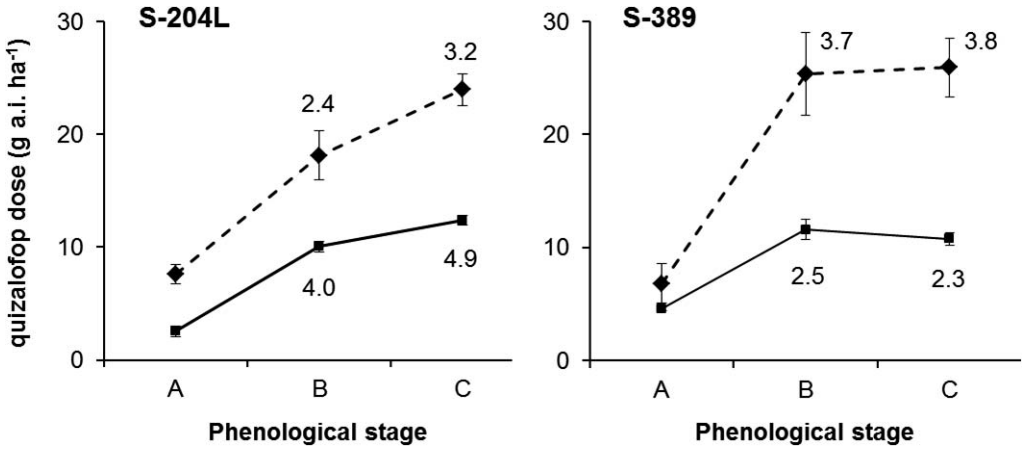

Figure 1. Lethal dose, the herbicide dose that reduce 50% of plant survival (LD_{50} [■] and LD_{90} [◆]) of *Lolium* spp. susceptible checks (S-204L and S-389) treated with quizalofop ethyl ester at different phenological development stages (A, three to four leaves, BBCH scale 13 to 14; B, tillering, BBCH scale 23 to 26; C, regrowth, BBCH scale 25 to 27). Vertical bars represent standard errors, values refer to tolerance indices calculated using the LD_{50} or LD_{90} of the correct phenological development stage (A).

aden, and low-to-moderate levels of resistance to the cyclohexanediones (DIMs) clethodim and sethoxydim (Liu et al. 2007; Martins et al. 2014).

The Trp-2027–Cys was previously reported to confer resistance only to FOP herbicides (Yu et al. 2007). In our results, population 377 carried this substitution in all plants analyzed, both at heterozygous (8 of 10 plants) and homozygous status (2 of 10 plants). This reflects the high resistance status of this population to the FOP herbicide quizalofop but would not explain the loss of susceptibility to the DEN pinoxaden. In fact, this mutation has never been reported alone in *Lolium* spp. resistant to pinoxaden, but it was demonstrated that the Trp-2027–Cys allelic variation can confer resistance to pinoxaden in blackgrass (*Alopecurus myosuroides* Huds) (Petit et al. 2009).

The Ile-1781–Leu mutation was associated with a cross-resistance to all chemical families of ACCase inhibitors (White et al. 2005). In population 379, 1 of 10 plants analyzed carried this mutation, explaining the slight loss of susceptibility (i.e., very few survivors) to all ACCase chemical families tested.

Population 388 showed the highest variability with three different substitutions detected and all in heterozygous status: 1,781 (5 of 10 plants), 2,027 (4 of 10 plants), and 2,041 (1 of 10 plants). This population also showed high resistance to quizalofop as well as several plants surviving pinoxaden and cycloxydim even at the higher dose tested (135 and 480 g ai ha^{-1}, respectively) (Table 2).

Five survivors of population 379 did not display any allelic variation in comparison to the susceptible members. Population 379, together with population 378, in which no mutations were detected, were the more likely candidates to have a nontarget-site resistance mechanism.

Effect of Growth Stage on Quizalofop Efficacy. Plant survival and fresh weight gave similar results in this study, therefore only data on plant survival are presented and discussed. The susceptible rigid ryegrass S-204L, treated at stage A responded differently in the dose–response tests held in the greenhouse at different growth stages and the outdoor experiment. The LD_{50} was 2.5 and 9.6 g ha^{-1} in the indoor and outdoor experiments, respectively. This difference is likely caused by the diverse climatic conditions in the two environments, the herbicides usually being more active in a greenhouse.

As expected, in general, the older the plants, the less susceptible they were to the herbicide (Figure 1). In fact, large differences in quizalofop efficacy were recorded between phenological stages A and B for both populations, whereas the differences in efficacy between plants treated at stages B or C were smaller for population S-204L and negligible for population S-389 (Figure 1). The TI based on the LD_{90} showed that to control the regrowth, it was necessary to use three to four times the herbicide dose suitable for younger plants (Figure 1). A few studies (Christoffoleti et al. 2005), albeit indirectly, have previously stressed the effect of weed age on herbicide efficacy. Kudsk (2014) also recently highlighted that herbicide rate and plant age were important, but often underappreciated, factors.

The results indicate that the more advanced the phenological stage of application, the more difficult

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it was to control susceptible biotypes of Italian ryegrass with quizalofop. In field conditions, it is unlikely that weeds are found at a uniform phenological stage. This variability leads to a difference in the amount of active ingredient that reaches the target (per unit weight or leaf area). Unfortunately, in field conditions, the importance of the phenological stage of weeds is frequently overlooked, which often results in sublethal application rates. This may accelerate the selection of nontarget-site (polygenic) herbicide-resistant mechanisms, such as enzyme overexpression and detoxification, which might initially be hidden by the presence of target-site mutations that usually induce highly resistant biotypes. Several studies have demonstrated that recurrent selection with reduced rates of the P450-metabolisable ACCase herbicide diclofop-methyl results in rapid herbicide resistance evolution in rigid ryegrass (Busi et al. 2012; Manalil et al. 2011; Neve and Powles 2005a,b). If a herbicide is used at a sublethal dose, the more tolerant individuals will survive and possibly reproduce. In this way, resistance alleles will become progressively enriched in successive generations, resulting in a polygenic-based shift toward more resistant individuals. Evidence of sublethal drug dose selection for polygenic resistance is also known for bacteria (Olofsson and Cars 2007), fungi (Shaw 2006), and insects (Roush and McKenzie 1987). In rigid ryegrass, this type of selection was also described using glyphosate (Busi and Powles 2009). It can, therefore, be inferred that, despite the LD_{90} always being below the recommended field rate for quizalofop (75 g ha^{-1}; Figure 1), repeated treatments in fields heavily infested with the regrowth of Italian ryegrass (stage C and beyond) have progressively selected quizalofop-resistant populations. This was confirmed by empirical observations of the alfalfa fields in which the number of quizalofop-treatment survivors progressively increased over the years.

Field Experiments. Bartlett's and Levene's tests revealed that the data were not normally distributed. Despite various types of data transformation, it was not possible to normalize the data; therefore, ANOVA could not be used and only standard errors (which do not require normally distributed data to be applied) are reported on the graphs. The data collected in the 2 yr were very similar and were, therefore, averaged.

Quizalofop efficacy was always very low. Only propyzamide gave satisfactory control of quizalofop-resistant *Lolium*, if the treatment was applied early in the season (i.e., late winter). The same herbicide did not provide a good control when applied in May because it is known to be poorly active on adult plants (Clay et al. 2006) and the environmental conditions in late spring are less favorable for exerting its herbicidal activity (Figure 2).

Weed phenological stage/age influenced the efficacy of all herbicides applied. The VEB recorded in June showed no differences between the plots of the untreated control and quizalofop or imazamox applied in February (i.e., VEB = 100%) because ryegrass regrew from survivors after the forage cut (Figure 2B). A slight decrease in the number of ears was observed in imazamox plots (–23%) sprayed in February (Figure 2A). Fewer ears were counted in all plots treated in May (Figure 2A). The efficacy of the treatments on the regrowth (i.e., May treatment) generally increased compared with the untreated control but remained unsatisfactory.

This study documents that even cropping systems, such as alfalfa, which are considered at low-risk of resistance evolution, if "stressed" by an intensive production system (i.e., dual seed–forage production and intense herbicide use) can be affected by herbicide resistance, particularly when no rotation of SoA is adopted in the overall cropping system. In Europe, no herbicide-resistant weeds have so far been reported in alfalfa crops for forage production only (about 1.5 M ha). It is also clear that herbicide resistance risk increases when dense infestations of species highly prone to evolve herbicide-resistant populations, such as *Lolium* spp., are not properly managed.

Despite alfalfa–wheat rotation being proposed in the literature for managing herbicide-resistant *Lolium* spp. (Doole and Pannell, 2008), the results from this study highlight associated issues. This is likely due to the dual forage and seed alfalfa production system studied. Alfalfa–wheat rotation can be adopted, if herbicides other than FOP herbicides are used in wheat and propyzamide is used in alfalfa. Other rotations between alfalfa and winter crops, such as barley (*Hordeum vulgare* L.) or some vegetables are precluded because only ACCase inhibitors are allowed to control *Lolium* spp. in these crops.

Herbicide resistance management is important to prevent weed seed production (Lutman et al. 2013) and to keep the infestation at a manageable level. Depletion of the soil weed seed bank is an excellent strategy to combat weeds, especially herbicide-resistant ones (Goggin et al. 2012). Different chemistries (i.e., ALS- and PSII-inhibiting herbi-

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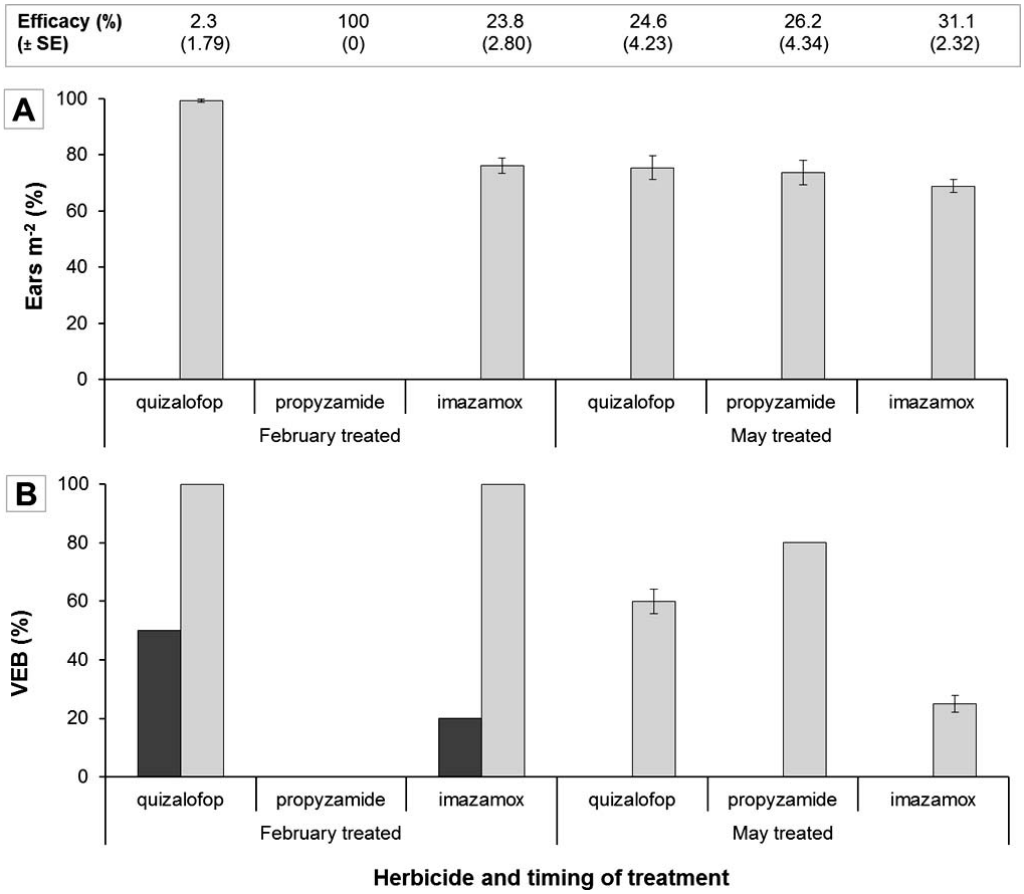

Figure 2. Herbicide efficacy of the field treatments calculated using Abbott's formula, number of ears (A) and visual estimation of biomass (VEB) (B) of Italian ryegrass. All data are expressed as percentages of the untreated control. Treatments were applied in February and in May, 10 d after the first cut. Number of ears was recorded only in June for both treatments (applied in February and May), whereas VEB was recorded in April (dark bars) and June (light bars) for treatments applied in February but only in June (light bars) for treatments applied in May. Vertical bars represent standard errors calculated on the mean value of two experiments.

cides are still effective), as well as nonchemical agronomic practices, should be used to increase diversity in the system. *Lolium* spp. can be managed effectively by avoiding continuous direct seeding or reduced tillage. Potential *Lolium* infestation can also be reduced through stale seedbed preparation when alfalfa is rotated with cereals, and the latter are sown late in the season. Curative chemical measures for FOP-resistant *Lolium* spp. in alfalfa include a timely and careful use of propyzamide or imazamox when alfalfa is not actively growing.

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