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Characterization of Multiple Herbicide-Resistant Italian Ryegrass (*Lolium perenne* ssp. *multiflorum*) Populations from Winter Wheat Fields in Oregon

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Many Italian ryegrass populations in Oregon are resistant to more than one herbicide; therefore, the resistance patterns of these populations must be determined to identify alternative herbicides for management. Two suspected resistant Italian ryegrass populations (R2 and R4) survived flufenacet plus metribuzin applications under typical winter wheat production conditions. Populations R2 and R4 were resistant to clethodim, pinoxaden, quizalofop, mesosulfuron-methyl, flufenacet, but not to acetochlor, dimethenamid-p, metolachlor, pyroxasulfone, imazapyr, sulfometuron, or glyphosate. R4 was resistant to diuron, but R2 was not. The estimated flufenacet doses required for 50% growth reduction (GR_{50}) were 438 g ai ha^{-1} (R2) and 308 g ai ha^{-1} (R4). Both populations were controlled by pyroxasulfone at rates greater than 15 g ai ha^{-1}. An Asp-2078-Gly substitution in the ACCase gene was found in both populations, while an Ile-2041-Asn was found only in the R4 population. A Ser-264-Gly substitution in *psb*A gene was found in the R4 population. These mutations previously have been reported to provide resistance to ACCase and photosynthetic inhibitors, respectively. No resistance mutations were identified in the acetolactate synthase (ALS) gene of either population. The addition of the P450 inhibitor, chlorpyrifos, increased the injury resulting from mesosulfuron-methyl on both resistant populations providing indirect evidence that the ALS resistance may be metabolic. Multiple herbicide-resistant Italian ryegrass populations were identified in this study with both target site and nontarget site based mechanisms likely involved. However, several herbicides were identified including pyroxasulfone, a herbicide in the same group as flufenacet, which could be used to control these two populations.

Nomenclature: Acetochlor; clethodim; dimethenamid-p; flufenacet; glyphosate; imazapyr; mesosulfuron; metribuzin; metolachlor; pinoxaden; pyroxasulfone; quizalofop; sulfometuron; winter wheat, *Triticum aestivum* L.; Italian ryegrass, *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot.

Key words: Annual ryegrass, flufenacet resistance, pyroxasulfone.

Italian ryegrass is an annual grass native to temperate Europe and is commonly found throughout the United States (Whitson 2006). Because Italian ryegrass has a similar growth habit to winter cereal crops, it is an extremely competitive weed in these crops and has become a significant management problem in winter wheat production in the Pacific Northwest and in the Southeastern United States. Winter wheat grain yield is negatively related to Italian ryegrass density, with up to an 80% reduction reported (Appleby et al. 1976; Appleby and Brewster 1992). The yield loss caused by Italian ryegrass is due to competition for soil nutrients during winter wheat tillering and interference during harvest (Justice et al. 1994; Liebl and Worsham 1987).

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In Oregon, Italian ryegrass management in winter wheat fields relies heavily on the use of PRE and early POST herbicide applications. Flufenacet in mixture with metribuzin is commonly used for Italian ryegrass control in winter wheat (Grey and Bridges 2003; Hulting et al. 2012; Koepke-Hill et al. 2011). Recently, winter wheat growers in Oregon reported reduced control of Italian ryegrass with flufenacet plus metribuzin. These reports raised concern that the populations may have evolved resistance to flufenacet.

Flufenacet inhibits very-long-chain fatty acid biosynthesis (Group 15) (Soltania et al. 2005). Flufenacet is applied PRE- or early POST, either to the soil surface or incorporated, to control annual grasses and broadleaf weeds in corn, soybeans, and winter wheat (Anonymous 2014a; EPA 2014).

Italian ryegrass populations resistant to flufenacet plus metribuzin have been reported previously in other Pacific Northwest states (Rauch et al. 2010). According to a survey conducted in 2006 and

2007, 12% of 75 Italian ryegrass populations from northern Idaho and eastern Washington exhibited varying levels of resistance to flufenacet plus metribuzin (Rauch et al. 2010).

Though herbicides Groups 1, 2, 7, 9, and 15 are commonly used to control Italian ryegrass in various crops and cropping systems in Oregon (Peachy et al. 2015), widespread multiple herbicide-resistant populations increase the difficulty of herbicide rotation (Avila-Garcia and Mallory-Smith 2011; Liu et al. 2014; Rauch et al. 2010). Thus, the herbicide resistance patterns of the resistant populations must be examined before alternative herbicides can be selected.

Pyroxasulfone is a recently introduced herbicide in Group 15 (Anonymous 2014b; Tanetani et al. 2009). It is registered for use in wheat, fallow, corn, and soybean, and has potential uses in other crops (Anonymous 2014b; Sikkema et al. 2008). In Oregon, pyroxasulfone was identified as a potential alternative to flufenacet for control of Italian ryegrass populations in winter wheat production (Hulting et al. 2012). Similar to flufenacet, pyroxasulfone provides PRE and early POST control of many grass and broadleaf weeds (Anonymous 2011; King and Garcia 2008; Knezevic et al. 2009). Pyroxasulfone is regarded as an important alternative herbicide in wheat production because it provides highly selective grass weed control in wheat (Hulting et al. 2012; Tanetani et al. 2013; Walsh et al. 2011).

Though the resistance mechanism to Group 15 herbicides is not well understood, *Lolium* is one of the three genera that have evolved Group 15 resistance (Busi 2014). An experimentally evolved pyroxasulfone-resistant rigid ryegrass (*Lolium rigidum* Gaudin) population was selected via recurrent selection with pyroxasulfone (Busi et al. 2012), and was resistant to other herbicides to which the population had not been exposed (Busi and Powles 2013). Given the potential of pyroxasulfone to replace flufenacet in winter wheat production, the response of the PNW suspected flufenacet-resistant populations to pyroxasulfone must be determined.

The cytochrome P450 enzyme superfamily is responsible for the phase I metabolism of numerous herbicides representing several classes of organic compounds (Siminszky 2006). P450 is believed to be involved in the metabolism of many herbicides, including ALS inhibitors. Organophosphate insecticides such as chlorpyrifos also inhibit P450. When combined with ALS inhibitors, organophosphate insecticides may increase the sensitivity of resistant populations to ALS herbicides (Yu et al. 2009; Yu and Powles 2014). The addition of an organophosphate insecticide to ALS inhibitors can be used to provide indirect evidence that the resistance is based on metabolism if a target-site mutation is not present in a population.

The objectives of this study were to determine if the two Italian ryegrass populations were resistant to flufenacet, to characterize the herbicide resistance patterns of the populations, to identify potential resistance mechanisms, and to identify possible alternative herbicides to manage these resistant populations.

Materials and Methods

Plant Material. Seeds of Italian ryegrass were randomly collected from multiple plants and sites within two fields with populations suspected of being resistant to flufenacet. Seed from population (R2) was collected in 2010 from Linn County, OR, in a field that had a winter wheatgrass grown for seed rotation. Seed from population (R4) was collected in 2011 from a winter wheat field in Washington County, OR, approximately 64 km from the R2 population, in a field that had a winter wheat-red clover (*Trifolium pratense* L.)-field pea (*Pisum sativum* L.) rotation. The crop rotation history at each location varied, but was representative of the crops grown in typical seed production cropping systems in western Oregon. Both locations had a history of extensive use of herbicide Groups 1, 2, 3, 5, 7, 9, and 15 to manage Italian ryegrass in both cereal and broadleaf crops. A known herbicide susceptible Italian ryegrass population (S) was used as the control population.

General Greenhouse Methods. Herbicides were applied at a volume of 187 L ha^{-1} at 276 kPa with an experimental spray chamber. Adjuvants were used as individual herbicide labels required. Annual ryegrass plants were grown under ambient light conditions supplemented by lights to achieve 14-h sunlight greater than 25 mW cm^{-2} per day. Day/night temperatures were maintained at 25/20 C. A preliminary study was conducted to compare the effect of soil type on flufenacet and pyroxasulfone activity on Italian ryegrass control. There was no difference in control whether potting mix (Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004) or a silt loam soil was used (data not shown). Therefore, the commercial potting mix was used in all studies.

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Table 1. Injury ratings for three Italian ryegrass populations and multiple herbicides.[a]

Herbicide	Group	Timing	Rate	Populations[b,c]		
				S	R2	R4
			g ai ha^{-1}			
clethodim	1	POST	136	9.1a	5.5b	5.0b
			68	6.7a	3.8b	3.3b
pinoxaden	1	POST	60	8.8a	3.8b	4.1b
			30	4.6a	2.6b	2.5b
quizalofop	1	POST	184	9.6a	4.5b	3.5b
			92	6.6a	4.2ab	2.5b
imazapyr	2	POST	840[c]	9.1a	10.0a	10.0a
			420[c]	9.3a	9.3a	10.0a
mesosulfuron-methyl	2	POST	15	9.3a	4.1b	4.5b
			7.5	7.0a	4.3b	4.6b
sulfometuron	2	POST	83	10.0a	10.0a	9.5a
			42	9.5a	9.0a	9.0a
diuron	7	PRE	2,000	10.0a	9.3a	4.7b
			1,000	8.2a	7.3a	2.7b
glyphosate	9	POST	1,000[d]	9.6a	8.8a	8.6a
			500[d]	6.7a	8.3b	7.3ab
acetochlor	15	PRE	2,240	9.8a	10.0a	10.0a
			1,120	9.0a	8.1b	9.1ab
dimethenamid-p	15	PRE	1,100	9.8a	10.0a	10.0a
			550	9.0a	8.0a	8.3a
flufenacet	15	PRE	380	9.0a	4.0b	3.4b
			190	8.0a	2.4b	2.9b
s-metolachlor	15	PRE	2,139	10.0a	10.0a	10.0a
			1,069	10.0a	9.3a	9.6a
pyroxasulfone	15	PRE	120	10.0a	10.0a	10.0a
			60	10.0a	10.0a	10.0a

[a] Plant injury value 0 = no injury, 10 = no emergence (PRE) or dead (POST).
[b] Data for R2 and R4 are the average of 36 plants for POST herbicides and 150 plants for PRE herbicides; S data are the average of 72 plants for POST herbicides and 300 plants for PRE herbicides.
[c] Means within the same row followed by the same letter are not significantly different, according to Fisher's Protected LSD at P = 0.05.
[d] g ae ha^{-1}.

Herbicide Screening Assays. *PRE Herbicide Screening.* Twenty-five seeds of R2 or R4 plus 25 seeds of S (50 seeds total) were planted in a 53 by 28 by 5 cm tray filled with commercial potting mix. A completely randomized design with three replications was used and the study was repeated. Six PRE herbicides, acetochlor (Surpass, 768 g L^{-1} acetochlor EC, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268), dimethenamid-p (Outlook, 720 g L^{-1} dimethenamid-p EC, BASF, Crop Science Division, 26 Davis Drive, Research Triangle Park, NC 27709), flufenacet (Define 60% flufenacet by weight DF, Bayer Crop Science, 2T.W. Alexander Drive, Research Triangle Park, NC 27709), metolachlor (Dual II Magnum, 916 g L^{-1} s-metolachlor EC, Syngenta Crop Protection, Inc., 410 Swing Road, Greensboro, NC 27409), pyroxasulfone (Experimental code: KIH-485, 85% pyroxasulfone by weight WP, Kumiai Chemical Industry Co., Ltd., 4-26, Ikenohata 1-Chome, Taitoh-ku, Tokyo 110-8782, Japan), and diuron (Karmex, 80% by weight diuron DF, DuPont, 1007 Market Street, Wilmington, DE 19898) were applied at one-half and at the recommended label rate (Table 1). These rates were chosen based on the results of previous studies (data not shown) because under greenhouse conditions the recommended rate was sufficient to determine whether a population was resistant.

POST Herbicide Screening. Seed from the R2, R4, and S populations were placed in petri dishes containing moistened blotter paper. When the radicles reached approximately 1.5 cm, seedlings (one per plot) were transplanted into a 267-ml plastic pot containing commercial potting mix. Six plants from R2 and R4 and 12 plants of the S populations were treated per herbicide per replication. The experimental design was a completely randomized design with three replications and the study was repeated. Seven POST herbicides, clethodim (SelectMax, 12.6% by weight clethodim EC, Valent USA Corp., P.O.

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Box 8025, Walnut Creek, CA 94596-8025), pinoxaden (Axial XL, 50 g L^{-1} pinoxaden, Syngenta Crop Protection, Inc.), quizalofop (Assure II, 10.3% by weight quizalofop EC, DuPont), imazapyr (Arsenal, 53% by weight imazapyr, BASF, Crop Science Division), mesosulfuron-methyl (Osprey, 4.5% by weight mesosulfuron-methyl WDG, Bayer Crop Science), sulfometuron (OUST, 75% by weight sulfometuron, DuPont), and glyphosate (Roundup Power MAX, 540 g L^{-1} glyphosate EC, Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167) were applied at one half and at the recommended label rate to plants at the two- to three-leaf stage (Table 1).

Herbicide efficacy was estimated visually at 21 d (PRE-) or 14 d (POST-) after herbicide applications. Plant injury was rated on a scale from 0 (no injury) to 10 (death). For PRE studies, the emergence rate of the untreated control for each population was rated as 0, and no emerged seedlings was rated as 10. Fisher's LSD was calculated with a linear model using R software (R statistical software, R development team, http://www.r-project.org/).

Dose–Response Study. Twenty-five seeds of R2 or R4 plus 25 seeds S populations were planted and treatments applied as in the PRE herbicide screening as described previously. Flufenacet was applied at 0, 95, 190, 380, 760, 1,520, and 2,280 g ai ha^{-1}, and pyroxasulfone was applied at 0, 7.5, 15, 30, 60, 120, and 240 g ai ha^{-1}. The experimental design of this study was a completely randomized block with four replications; the study was repeated. The percent emergence relative to the control group was determined at 21 d after herbicide application. Because some seedlings did not grow after emergence, only those greater than 1.5 cm in height were counted. Following the collection of the emergence data, the counted seedlings were cut at the soil surface and dried at 60 C for 72 h. The percent dry weight of the treated plants relative to the control group was calculated. Levene's ANOVA using R software was used to compare differences between treatments. Dry biomass data were fit to a four-parameter logistic dose–response curves (Ritz and Streibig 2005). The four-parameter logistic function formula used was:

$$f[\mathrm{x},(\mathrm{b},\mathrm{c},\mathrm{d},\mathrm{e})] = \mathrm{c} + [(d-c)/1 + \exp\{b[\log(x) - \log(e)]\}],$$

The parameter e is the dose producing a response 50% growth reduction (GR_{50}) between the upper limit, d, and lower limit, c. The parameter b is the relative slope around e. The resistance indices (RI) were calculated as the ratio of GR_{50} of the resistance population to the susceptible population. This model fitting was performed with DRC package (DRC R 2015) in R.

Gene Sequencing. Seedlings from the R2 and R4 populations that survived applications of clethodim, pinoxaden, quizalofop, mesosulfuron-methyl, or diuron in the resistance screening studies were selected for gene sequencing. Fresh plant tissue was harvested from four R2 and R4 plants in each treatment group, and from two untreated plants in the S population. DNA was sequenced from each individual plant. Total DNA was extracted immediately from harvested fresh tissue using a DNeasy Plant Mini kit (Qiagen, Germantown, MD, USA) following the mini protocol. Polymerase chain reactions (PCRs) were performed to amplify the target sites of ALS, ACCase, and *psb*A gene, respectively. The reaction mixture contained 1.15 μL DNA solution, 0.2 μM dntp, 0.5 μM forward primer, 0.5 μM reverse primer, 1 μL 10 × PCR buffer, 0.06 unit *Tag* DNA polymerase, and 6.74 μL water. The primers used in each reaction were dependent on the regions that may potentially contain the resistance mutations (Table 2). The PCR thermal cycler was 37 cycles each consisting of 30 s at 95 C, 45 s at T_m-5 C, and 2 min at 72 C, followed by a final step of 10 min at 72 C. The PCR products were amplified with TOPO TA Cloning Kit for Sequencing following the manufacturer's instructions (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA, USA). Ten clones from each plant were purified using a QIAquick PCR Purification Kit following the vacuum protocol. The final PCR products were sent to the Center for Genome Research and Biocomputing, Oregon State University (Corvallis, OR) for sequencing. Each clone was sequenced in both directions. Sequencing results were edited with Finch TV and aligned using CLC Sequence Viewer version 6.1 (CLC Bio, Waltham, MA, USA).

Herbicide/Insecticide Interaction Assays. Seed from populations R2, R4, and S were planted and treated using the same methods as described previously for the POST bioassay. The treatments were applied to plants at the two- to three-leaf stage. Treatments included the control, 15 g ai ha^{-1} mesosulfuron-methyl, 2.5 g ai ha^{-1} chlorpyrifos (Lorsban Advanced, 40.2% chlorpyrifos, Dow AgroSciences

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Table 2. Primers used for sequencing genes from R2 or R4.

Primer[a]	Gene	Sequence
CP1-F:	ACCase[b]	5′-CAAACTCTGGTGCTCGGATTGGCA-3′
CP1-R:		5′-GAACATAGCTGAGCCACCTCAATATATT-3′
CP4-F:	ACCase[b]	5′-CAGCCTGATTCCCATGAGCGGTC-3′
CP4-R:		5′-CCATGCATTCTTGGAGTTCCTCTGA-3′
psbA-F:	*psbA*[c]	5′-GGATGGTTTGGTGTTTTG-3′
psbA-R:		5′-TAGAGGGAAGTTGTGAGC-3′
ALS122-F:	ALS[d]	5′-GGGCGCCGACATCCTCGTCG-3′
ALS205-R:		5′-CCACCGCCAACATAIAGAAT-3′
ALS376-F:	ALS[d]	5′-ATTCTCTATGTTGGCGGTGG-3′
ALS377-R:		5′-CTTTTCTGCTGCTCCAACTC-3′
ALS547-F:	ALS[d]	5′-TGGGCGGCTCAGTATTACAC-3′
ALS-654-R:		5′-ATAGGCAGCACATGCTCCTG-3′

[a] Abbreviations: F, forward; R, reverse.
[b] Primers from Martins et al. 2014.
[c] Primers from Perez-Jones et al. 2009.
[d] Primers from Yu et al. 2008.

LLC), or 15 g ai ha^{-1} mesosulfuron-methyl plus 2.5 g ai ha^{-1} chlorpyrifos. Chemicals were applied at an application volume of 187 L ha^{-1} at 276 kPa using an experimental spray chamber. The adjuvant (R11, 90% ai NIS surfactant, Wilbur-Ellis, 30665 SW Highway 34, Albany, OR 97321) was used at a concentration of 0.5% v/v.

The percent dry weight of the treated plants relative to the untreated control group was calculated at 28 d after treatment (DAT) following the method described in the Dose–Response Study. The differences between treatments were analyzed by Levene's ANOVA using R software.

Results and Discussion

Herbicide Screening Assays. The R2 and R4 populations had similar, although not identical resistance patterns (Table 1). The two Italian ryegrass populations evaluated in this study were resistant to flufenacet but not to other tested herbicides with the same mode of action. Both populations were resistant to the tested Group 1 herbicides and mesosulfuron-methyl, but not to the other Group 2 herbicides, imazapyr and sulfometuron. R4 was resistant to diuron, but R2 was not. Neither population was resistant to glyphosate.

Dose–Response Study. There was no evidence ($P < 0.05$) of differences between blocks and experiments, according to Levene's ANOVA test for homogeneity of variances. Therefore, data were pooled over the four replications and the two experiments.

R2 and R4 also had similar levels of flufenacet resistance in the dose–response study. Emergence rates were negatively correlated with increasing rates of flufenacet (Figure 1). The GR_{50} values for R2, R4, and S were 438, 308, and 52 g ai ha^{-1}, respectively (Figure 2). The RI values for the R2 and R4 populations were 8.4 and 5.9, respectively, compared to the S population.

For the pyroxasulfone treatments, the S, R2, and R4 populations were controlled with no more than 10 seedlings emerging from the 50 seeds planted at the lowest application rate (7.5 g ai ha^{-1}), which is 0.0625 × of the recommended use rate in winter wheat. There were no survivors in the three populations at higher rates. The lack of survivors prevented the determination of a GR_{50} or any other statistical analysis. These results indicate that these populations of Italian ryegrass were very sensitive to low rates of pyroxasulfone.

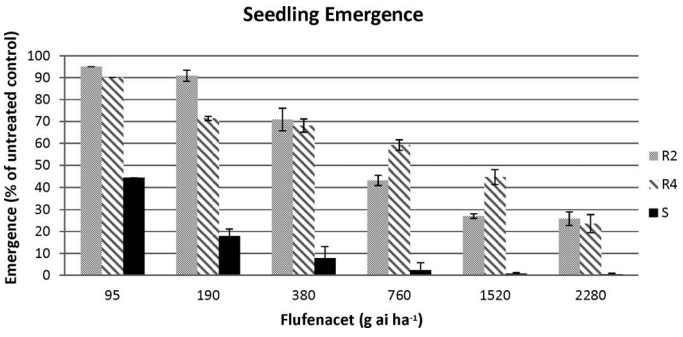

Figure 1. Seedling emergence of three Italian ryegrass biotypes treated with flufenacet. Data for R2 and R4 were pooled over eight replications (200 plants), and data for S were pooled over 16 replications (400 plants) of the same dose. The error bars represent the standard errors in each of the data sets.

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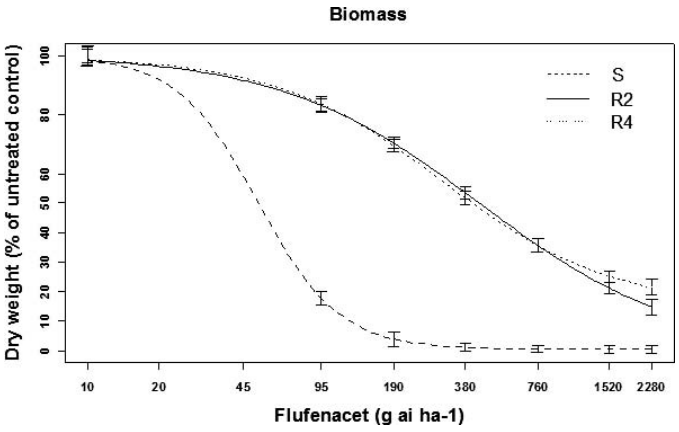

Figure 2. Aboveground biomass dry weight as % of untreated control of three biotypes treated. Data for R2 and R4 were pooled over 200 plants, and data for S were pooled over 400 plants for each dose. Each point is the mean ± SE. The lines are log-logistic curves: R2: $Y = -0.64 + 101.06/(1 + e^{[1.04 \times \log(x) + 0.37]})$; R4: $Y = 14.85 + 84.94/(1 + e^{[1.24 \times \log(x) + 0.63]})$; S: $Y = 0.49 + 99.51/(1 + e^{[2.55 \times \log(x) + 3.28]})$.

Gene Sequencing. Ten clones of each plant were sequenced. Two different ACCase sequences, R4-1 (3, 4, 4, and 5 of 10 clones) and R4-2 (7, 6, 2, and 3 of 10 clones) were present in each of the four R4 plants, indicating that there at least two alleles of ACCase in this population. A single nucleotide change of GAT to GGT was found in the ACCase gene in the R2 population and R4-1 allele of R4 populations (Table 3). This change leads to an Asp-2078-Gly substitution known to confer different levels of ACCase resistance in *Lolium* spp. (Yu et al. 2007). The R4-1 allele contained an ATT to AAT mutation that results in an Ile-2041-Asn substitution. This substitution has been reported previously to confer resistance to aryloxyphenoxypropanoates herbicides of Group 1 in rigid ryegrass (Délye et al. 2003). N2014 was present in all the four plants with R4-1 allele, so it could be a mutation not previously reported. A Ser-264-Gly substitution was found in the *psb*A gene in the R4 population (Table 4). This substitution is caused by a single AGT to GGT mutation in *psb*A gene. The Ser-264-Gly substitution has been widely reported in numerous species to contribute resistance to triazines, triazinones (Group 5), or ureas (Group 7) (Beckie and Tardif 2012; Chandi et al. 2011). Although not tested, the R4 population may be resistant to other herbicides in the triazine, triazinone, or urea families (Siminszky 2006). No other known substitutions were found in Val 219, Phe 255, Leu 275, and Phe 211 of *psb*A gene.

Table 3. Comparison of ACCase gene sequencing results from R2, R4, and S populations.

Amino acid	G2040	I2041	L2042	~[a]	I2077	D2078	S2079
Sequence	GGA	ATT	CTG	~	ATT	GAT	AGC
S	—[b]	ATT	—	~	—	GAT	—
R4-1	—	A**A**T[c]	—	~	—	GAT	—
R4-2	—	ATT	—	~	—	G**G**T	—
R2	—	ATT	—	~	—	G**G**T	—

[a] No substitution was found between L2042 and I2077 in the ACCase gene.
[b] There was no difference of protein coding nucleotides between the two populations.
[c] Boldface indicates the nucleotide change.

Table 4. Comparison of PSII gene sequencing results from R4 and S populations.

Amino acid	Q261	Y262	A263	S264	F265	N266	N267
Sequence	CAA	TAT	GCT	AGT	TTC	AAC	AAC
S	—[a]	—	—	AGT	—	—	—
R4	—	—	—	**G**GT[b]	—	—	—

[a] There was no difference of protein coding nucleotides between the two populations.
[b] Boldface indicates the nucleotide change.

The sequencing of the ALS gene focused on the positions with previously reported substitutions, and surrounding positions. No substitutions were found in positions Ala 122, Pro 197, Ala 205, Asp 376, Arg 377, Trp 574, Ser 653, and Gly 654 of the ALS gene in the R2, R4, or S populations. These results provide evidence that the ALS resistance may be nontarget-site based.

Herbicide/Insecticide Interaction Assays. There was no difference between experiments according to Levene's ANOVA test for homogeneity of variances ($P < 0.05$). Therefore, data were combined by treatment and population (Table 5). All seedlings from the S population were controlled at 28

Table 5. Dry weight as % of control of three populations treated with mesosulfuron and chlorpyrifos.[a]

		Populations[b]		
Chemicals	Rate	S	R2	R4
	g ai ha^{-1}			
chlorpyrifos	15	96.96 a	99.33 a	89.73 a
mesosulfuron	2.5	6.80 b	88.89 b	62.97 b
Mesosulfuron + chlorpyrifos	15 + 2.5	5.75 b	54.01 c	34.17 c

[a] Means within the same column followed by the same letter are not significantly different, according to Fisher's Protected LSD at $P = 0.05$.
[b] Data for R2 and R4 are the average of 48 plants; S data are the average of 96 plants.

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DAT when treated with mesosulfuronmethyl or mesosulfuron-methyl plus chlorpyrifos. More than 80% of seedlings from the R2 or R4 populations survived the mesosulfuron and mesosulfuron plus chlorpyrifos treatments at 28 DAT. However, aboveground biomass of R2 and R4 plants treated with mesosulfuron plus chlorpyrifos was reduced compared to the mesosulfuron only treatment. Only minor injury was caused by the chlorpyrifos application to any of the Italian ryegrass populations. In this study, chlorpyrifos reduced the level of mesosulfuron-methyl resistance in R2 and R4 populations. These results indicated that metabolism likely contributes to mesosulfuron-methyl resistance of these populations.

In summary, the R2 and R4 Italian ryegrass populations were resistant to more than one herbicide group. Even though they were resistant to flufenacet, there was no cross-resistance to other Group 15 herbicides tested. The results of this study reveal the complexity of predicting cross-resistance. Pyroxasulfone is an alternative for Italian ryegrass control in winter wheat production. However, pyroxasulfone resistant rigid ryegrass evolved with repeated applications of pyroxasulfone at initially low doses on a multiple herbicide resistant populations (Busi et al. 2012). In addition, it should not be assumed that cross-resistance between these herbicides will never occur. The lack of cross-resistance indicates that either the site of action is not the same for these herbicides or that the resistance is nontarget-site based, such as enhanced metabolism. Further studies are needed to elucidate the mechanism of resistance and the specific target sites of these herbicides.

Literature Cited

Anonymous (2011) Global technical bulletin: pyroxasulfone. Tokyo, Japan: Kumiai Chemical Industry Co., Ltd

Anonymous (2014a) Define DF™ herbicide supplemental label. Research Triangle Park, NC: Bayer Crop Science. http://www.cdms.net/LDat/ld4EU019.pdf. Accessed November 10, 2014

Anonymous (2014b) Zidua Herbicide Supplemental Label. Research Triangle Park, NC: BASF Corporation. http://www.agproducts.basf.us/products/label-and-msds/zidua-herbicide-label.pdf. Accessed November 1, 2014

Appleby AP, Brewster BD (1992) Seeding arrangement on winter wheat (*Triticum aestivum*) grain yield and interaction with Italian ryegrass (*Lolium multiflorum*). Weed Technol 6:820–823

Appleby AP, Olson PD, Colbert DR (1976) Winter wheat yield reduction from interference by Italian ryegrass. Agron J 68:463–466

Avila-Garcia WV, Mallory-Smith C (2011) Glyphosate-resistant Italian ryegrass (*Lolium perenne*) populations also exhibit resistance to glufosinate. Weed Sci 59:305–309

Beckie HJ, Tardif FJ (2012) Herbicide cross-resistance in weeds. Crop Prot 35:15–28

Busi R (2014) Resistance to herbicides inhibiting the biosynthesis of very-long-chain fatty acids. Pest Manag Sci 70:1378–1384

Busi R, Gaines TA, Walsh MJ, Powles SB (2012) Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: field selection at high doses versus recurrent selection at low doses. Weed Res 52:489–499

Busi R, Powles SB (2013) Cross-resistance to prosulfocarb and triallate in pyroxasulfone-resistant *Lolium rigidum*. Pest Manag Sci 69:1379–1384

Chandi A, York AC, Jordan DL, Beam JB (2011) Resistance to acetolactate synthase and acetyl Co-A carboxylase inhibitors in North Carolina Italian ryegrass (*Lolium perenne*). Weed Technol 25:659–666

Délye C, Zhang XQ, Chalopin C, Michel S, Powles SB (2003) An isoleucine residue within the carboxyl-transferase domain of multidomain acetyl-coenzyme A carboxylase is a major determinant of sensitivity to aryloxyphenoxypropionate but not to cyclohexanedione inhibitors. Plant Physiol 132:1716–1723

DRC package for R, version 2.5-12. https://cran.r-project.org/web/packages/drc/drc.pdf. Accessed June 25, 2015

[EPA] Environmental Protection Agency (2014) Pest fact sheet: flufenacet. http://www.epa.gov/opp00001/chem_search/reg_actions/registration/fs_PC-121903_01-Apr-98.pdf. Accessed November 27, 2014

Grey TL, Bridges DC (2003) Alternatives to diclofop for the control of Italian ryegrass (*Lolium multiflorum*) in winter wheat (*Triticum aestivum*). Weed Technol 17:219–223.

Hulting AG, Dauer JT, Hinds-Cook B, Curtis D, Koepke-Hill RM, Mallory-Smith C (2012) Management of Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) in western Oregon with preemergence applications of pyroxasulfone in winter wheat. Weed Technol 26:230–235

Justice GG, Peeper TF, Solie JB, Epplin FM (1994) Net returns from Italian ryegrass (*Lolium multiflorum*) control in winter wheat (*Triticum aestivum*). Weed Technol 8:317–323

King SR, Garcia JO (2008) Annual broadleaf control with KIH-485 in glyphosate-resistant furrow-irrigated corn. Weed Technol 22:420–424

Knezevic SZ, Datta A, Scott J, Porpiglia PJ (2009) Dose–response curves of KIH-485 for preemergence weed control in corn. Weed Technol 23:34–39

Koepke-Hill RM, Armel GR, Bradley KW, Bailey WA, Wilson HP, Hines TE (2011) Evaluation of flufenacet plus metribuzin mixtures for control of Italian ryegrass in winter wheat. Weed Technol 25:563–567

Liebl R, Worsham AD (1987) Interference of Italian ryegrass (*Lolium multiflorum*) in wheat (*Triticum aestivum*). Weed Sci 35:819–823

Liu M, Hulting AG, Mallory-Smith CA (2014) Characterization of multiple-herbicide-resistant Italian ryegrass (*Lolium perenne* spp. *multiflorum*). Pest Manag Sci 70:1145–1150

Martins BAB, Sánchez-Olguín E, Perez-Jones A, Hulting AG, Mallory-Smith C (2014) Alleles contributing to ACCase-resistance in an Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) population from Oregon. Weed Sci 62:468–473

Peachey E, Ball D, Hulting A, Miller T, Morishita D, Hutchinson P (2015) Pacific Northwest Weed Management Handbook. http://pnwhandbooks.org/weed/agrichemicals/flufenacet. Accessed March 1, 2015

Perez-Jones A, Intanon S, Mallory-Smith C (2009) Molecular analysis of hexazinone-resistant shepherd's-purse (*Capsella*

https://doi.org/10.1614/WS-D-15-00147.1 Published online by Cambridge University Press

bursa-pastoris) reveals a novel *psb*A mutation. Weed Sci 57:574–578
Rauch TA, Thill DC, Gersdorf SA, Price WJ (2010) Widespread occurrence of herbicide-resistant Italian ryegrass (*Lolium multiflorum*) in Northern Idaho and Eastern Washington. Weed Technol 24:281–288
Ritz C, Streibig JC (2005) Bioassay analysis using R. J Stat Software 12:1–22
Sikkema PH, Robison DE, Nurse RE, Soltani N (2008) Preemergence herbicides for potential use in pinto and small red Mexican bean (*Phaseolus vulgaris*) production. Crop Prot 27:124–129
Siminszky B (2006) Plant cytochrome P450-mediated herbicide metabolism. Phytochem Rev 5:445–458
Soltania N, Deenb B, Bowleyb S, Sikkema PH (2005) Effects of pre-emergence applications of flufenacet plus metribuzin on weeds and soybean (*Glycine max*). Crop Prot 24:507–511
Tanetani Y, Ikeda M, Kaku K, Shimizu T, Matsumoto H (2013) Role of metabolism in the selectivity of a herbicide, pyroxasulfone, between wheat and rigid ryegrass seedlings. J Pestic Sci 38:152–156
Tanetani Y, Kaku K, Kawai K, Fujioka T, Shimizu T (2009) Action mechanism of a novel herbicide, pyroxasulfone. Pestic Biochem Physiol 95:47–55
Walsh MJ, Fowler TM, Crowe B, Ambe T, Powles SB (2011) The potential for pyroxasulfone to selectively control resistant and susceptible rigid ryegrass (*Lolium rigidum*) biotypes in Australian grain crop production systems. Weed Technol 25:30–37
Whitson TD, ed. (2006) Weeds of the West. 9th edn. Jackson WO: Grand Teton Lithography. Pp 460–461
Yu Q, Abdallah I, Han H, Owen M, Powles S (2009) Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. Planta 4:713–723
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 145:547–558
Yu Q, Han H, Powles SB (2008) Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. Pest Manag Sci 64:1229–1236
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

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