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Glyphosate-Resistant Italian Ryegrass (*Lolium perenne*) Populations also Exhibit Resistance to Glufosinate

Wilson V. Avila-Garcia and Carol Mallory-Smith*

Resistance to glufosinate has been confirmed in glyphosate-resistant Italian ryegrass populations collected in hazelnut orchards in Oregon. Dose–response, ammonia accumulation, and enzyme activity studies were conducted to test the sensitivity of three glyphosate-resistant and three susceptible Italian ryegrass populations to glufosinate. The glufosinate rates required to reduce the growth by 50% (GR_{50}) were 0.15, 0.18, and 0.21 for the control populations C1, C2, and C3, respectively, whereas for the resistant populations OR1, OR2, and OR3, the GR_{50} values were 0.49, 0.42, and 0.40 kg ai ha⁻¹, respectively, exhibiting an average resistance index of 2.4. The same trend was observed in ammonia accumulation studies between 48 and 96 h after glufosinate treatment where the susceptible populations accumulated on average two times more ammonia than the resistant populations. The glufosinate concentration required to reduce the glutamine synthetase enzyme activity by 50% (I_{50}) was not different for the resistant and susceptible populations. The I_{50} s ranged from 3.1 to 3.6 μ M for the resistant populations and from 3.7 to 4.3 μ M for the susceptible populations; therefore, an insensitive target site is not responsible for the glufosinate resistance.

Nomenclature: Glufosinate; glyphosate; Italian ryegrass, *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot LOLMU; hazelnut, *Corylus avellana* L.

Key words: Glutamine synthetase, ammonia accumulation, herbicide resistance.

Glufosinate ammonium is a nonselective broad-spectrum herbicide that is used POST in orchards, vineyards, and glufosinate-resistant (Liberty-Link®) crops such as canola (*Brassica napus* L.), corn (*Zea mays* L.), and soybean (*Glycine max* L. Merr.) (Culpepper et al. 2000; Jones et al. 2001). Glufosinate is a potent inhibitor of the enzyme glutamine synthetase (GS), which plays a major role in the pathway that assimilates inorganic nitrogen into organic compounds and ammonia assimilation derived from nitrate reduction and photorespiration (Ray 1989). Inhibition of GS activity leads to a rapid accumulation of high levels of ammonia due to a lack of nitrogen metabolism, as well as depletion of the amino acid glutamine. As a consequence, excess ammonia in the plant causes reduction in photosynthetic activity, disruption of chloroplastic structure, stroma vesiculation, and glyoxylate accumulation, causing inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase and carbon fixation (Devine et al. 1993; Manderscheid 1993; Tachibana et al. 1986). Ammonia accumulation in plants treated with glufosinate has been used widely as a biochemical marker of GS inhibition (Pornprom et al. 2003; Sankula et al. 1998; Tsai et al. 2006;).

Although glufosinate is a nonselective herbicide, there are reports that describe different patterns of sensitivity to glufosinate in weed species (Everman et al. 2009a, 2009b; Skora-Neto et al. 2000). Differential responses in sensitivity to glufosinate have been attributed to three main mechanisms: altered uptake, reduced translocation, and metabolism (Pline et al. 1999; Skora-Neto et al. 2000; Steckel et al. 1997). Recently field and greenhouse dose–response experiments confirmed a 3.4-fold difference between susceptible and resistant biotypes of goosegrass [*Eleusine indica* (L.) Gaertn.] biotype from Malaysia (Jalaludin et al. 2010; Seng et al. 2010).

Glyphosate resistance has been identified in over 21 weed species (Heap 2011), and the most frequently observed mechanism has been limited translocation. Limited translocation has

been identified in horseweed [*Conyza canadensis* (L.) Cronq.] (Koger and Reddy 2005), hairy fleabane [*Conyza bonariensis* (L.) Cronq.] (Dinelli et al. 2008), rigid ryegrass (*Lolium rigidum* Gaudin.) (Wakelin et al. 2004; Wakelin and Preston 2006), and Italian ryegrass (Perez et al. 2004; Perez-Jones et al. 2007).

Italian ryegrass is a widely used forage grass in temperate regions of the world and also is a competitive weed in orchards and crops in the United States (Hoskins et al. 2005; Tucker et al. 2006). The control of Italian ryegrass in orchards is frequently based on the intensive use of glyphosate. As a consequence of the intensive use of glyphosate, seven Italian ryegrass populations have been confirmed to be glyphosate resistant in Oregon. The populations were under glyphosate selection for at least 10 yr with two to three glyphosate applications per year. The glyphosate resistance indices (RI) in these populations ranged from 2.8 to 6.8 (Perez-Jones et al. 2005, 2007).

In 2009, three of the glyphosate-resistant Italian ryegrass populations collected from hazelnut orchards in Oregon were screened using commercial rates of clethodim, glufosinate, imazamox, paraquat, pinoxaden, quizalofop, and pyroxulam. All the herbicides, except glufosinate, controlled the glyphosate-resistant populations. There was no record of the use of glufosinate in the orchards where the populations were collected. Therefore, dose–response, ammonia accumulation and enzyme activity studies were conducted to confirm whether these populations also had evolved resistance to glufosinate.

Material and Methods

Plant Material. Three Italian ryegrass glyphosate-resistant populations (OR1, OR2, and OR3) were collected from hazelnut orchards in Oregon. Glyphosate resistance in the OR1 population is due to reduced glyphosate translocation (Perez-Jones et al. 2005). The mechanism of glyphosate resistance in OR2 and OR3 is not an altered target site because no mutations in the 5-enolpyruvylshikimate-3-phosphate synthase gene have been identified. Three known glyphosate- and glufosinate-susceptible Italian ryegrass populations (C1, C2, and C3) were

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included as controls. The control populations C1 and C2 were from the Willamette Valley in Oregon, whereas C3 was a standard Italian ryegrass-susceptible population provided by an industry partner.

Greenhouse Dose–Response Bioassay. Seeds were germinated in petri dishes containing moistened blotter paper. After 3 d, when the seedling coleoptiles reached on average 1.5 cm, seedlings were transplanted to 267-ml plastic pots containing commercial potting mix.¹ Plants were grown under 25/20 C day/night temperature and natural sunlight in the summer of 2010. At the two- to three-leaf stage, the plants were sprayed with glufosinate² at 0.0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 kg ai ha⁻¹ using an overhead, compressed-air sprayer and an 8003 flat-fan spray nozzle calibrated to deliver 187 L ha⁻¹ at 40 psi. The field rate recommended to control Italian ryegrass is between 0.4 and 0.5 kg ai ha⁻¹. Shoot biomass was harvested 15 d after treatment, dried at 60 C for 72 h, and weighed. Six plants were used per each of the three replications (18 plants total) per herbicide concentration. Resistance index ratios were estimated on the basis of the 50% growth reduction (GR₅₀) values from the susceptible and resistant populations.

After harvesting, the plants were kept in the greenhouse under the same conditions as previously described. Fifteen days after harvesting a visual evaluation of plant regrowth was conducted to estimate the percentage of survivorship per rate and per population. The results are the average percentage of survivorship from two replications.

Ammonia Accumulation. Seeds from resistant and susceptible populations were germinated and seedlings were transplanted and grown in the greenhouse as described previously. At the two- to three-leaf stage, the plants were sprayed with glufosinate at 0.4 kg ai ha⁻¹. Treated and nontreated plants from all populations were assayed for ammonia concentration at 24, 48, 72, and 96 h after treatment (HAT). The experiment was conducted combining the methods proposed by D'Halluin et al. (1992) and Weatherburn (1967). Leaves (250 mg) were chopped, ground in liquid nitrogen, and homogenized in 1 ml of deionized water containing 50 mg of polyvinylpyrrolidone. The samples were centrifuged at 16,100 × *g* rpm for 7 min. An aliquot of 300 μl of the supernatant was added to 700 μl of deionized water and 100 μl of the diluted extract were reacted with 1.5 ml of phenol nitroprusside solution,³ and after mixing, 1.5 ml of an alkaline hydrochlorite solution (2.5 g of sodium hydroxide, 1.6 ml of sodium hypochlorite 5% available chlorine, and 500 ml of distilled water) were added. The samples were incubated at 37 C for 20 min and the optical density was measured spectrophotometrically⁴ at

625 nm. Ammonia accumulation in μg g⁻¹ of fresh weight was determined on the basis of a standard calibration curve. The standard curve was constructed using ammonium chloride with concentrations ranging from 0.004 to 4.0 mg. Four to five plants were used at each evaluation time with two replications per time. The experiment was conducted twice.

Enzyme Activity. The enzyme activity of the total GS enzyme was measured by quantifying the L-glutamine synthesized from ammonia and L-glutamate formed following the protocol proposed by Manderscheid (1993). Studies of enzyme activity were performed with the three resistant populations and two control populations (C1 and C3). Seeds were germinated and seedlings were transplanted and grown in the greenhouse as described previously. At the three- to four-leaf stage, the plants were assayed for GS activity. Leaves (300 mg) were chopped, ground in liquid nitrogen, and homogenized in 1.2 ml of an extraction medium (50 mM Tris[hydroxymethyl]aminomethane, 10 mM of 2-mercaptoethanol, and 10 mM Mg₂Cl) at 4 C and pH 7–8. Polyvinylpyrrolidone (60 mg) was added to the extraction medium to remove phenolic impurities and to improve the GS enzyme stability. The homogenate was centrifuged at 16,100 × *g* for 15 min in a centrifuge precooled at 4 C. Supernatant (0.2 ml) was added to 0.8 ml of medium containing 50 mM Tris(hydroxymethyl)aminomethane buffer (pH 7–8), 50 mM MgSO₄, 20 mM NH₂OH, 3.3 mM L-cysteine, 6 mM adenosine triphosphate, and glufosinate at concentrations ranging from 0.02 to 400 μM. An aliquot of 150 μl of 500 mM of Na-glutamate was added to the medium solution to start the reaction, followed by an incubation of the samples for 40 min at 37 C. The reaction was stopped by the addition of 0.35 ml of a ferric chloride reagent (0.37 M FeCl₃ 6H₂O, 0.67 M HCl, and 0.20 M trichloroacetic acid). Samples were centrifuged at 1,500 × *g* for 10 min and 200 μl of the supernatant were taken to measure absorbance at 540 nm. Absorbance levels were transformed to units of GS activity per gram of fresh weight using a standard curve from known concentrations of L-glutamic acid-γ-monohydroxamate. The results are presented as percentage of the control. Three to five plants were used per each of the four replications per herbicide concentration.

Statistical Analysis. The experiments were conducted twice and arranged in a completely randomized design with either three or four replications. Levene's ANOVA tests for homogeneity of variances were performed in all the experiments.

Two-way ANOVA analysis was performed for ammonia accumulation data and the differences among the populations

Table 1. Parameters estimated from the nonlinear regression analysis of glufosinate dose–response experiments on the basis of aboveground dry weight (percentage of untreated control) of Italian ryegrass populations. Values represent pooled data from two experiments.

Population	Parameters				GR ₅₀ ^a	RI ^b
	<i>b</i> (±SE)	<i>c</i> (±SE)	<i>d</i> (±SE)			
C1	2.97 (±0.59)	10.48 (±2.10)	100.23 (±4.21)		0.15 (±0.01)	–
C2	2.61 (±0.43)	7.60 (±2.16)	100.02 (±4.21)		0.18 (±0.01)	–
C3	1.73 (±0.27)	5.68 (±2.65)	99.89 (±4.21)		0.21 (±0.02)	–
OR1	3.38 (±0.70)	12.28 (±2.63)	105.55 (±2.93)		0.49 (±0.08)	2.7
OR2	2.54 (±0.50)	16.31 (±2.71)	100.81 (±3.54)		0.42 (±0.09)	2.3
OR3	3.35 (±0.94)	11.64 (±2.51)	94.80 (±3.61)		0.40 (±0.09)	2.2

^a Abbreviations: GR₅₀, rate of glufosinate required to reduce plant growth by 50%; RI, resistance index on the basis of the ratio between the average of the GR₅₀ values from the control populations and the GR₅₀ value of each resistant population.

Table 2. Percentage of survivors^a from dose–response experiments 15 d after harvesting of Italian ryegrass populations. Values represent pooled data from two experiments.

Population	Glufosinate rate (kg ai ha ⁻¹)			
	0.5	1.0	2.0	4.0
C1	66%	0%	0%	0%
C2	73%	0%	0%	0%
C3	44%	5%	0%	0%
OR1	100%	100%	28%	12%
OR2	100%	100%	28%	5%
OR3	100%	83%	11%	0%

^a On the basis of the average of 36 plants per rate.

and across time were analyzed using the LSD test at $P = 0.05$ when indicated by ANOVA.

Dose–response curves to estimate the glufosinate GR₅₀ rate were obtained using nonlinear regression on the basis of the equation described by Streibig et al. (1993):

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

where Y represents shoot dry weight at herbicide rate x and e corresponds to the GR₅₀ value. The upper limit is d , the lower limit is c , and b represents the slope of the line at the GR₅₀. Data were analyzed using the R software package⁵ (Knezevic et al. 2007).

The concentration of glufosinate required to inhibit 50% of the GS activity (I_{50}) was calculated using the linear regression model:

$$Y = a + bX \quad [2]$$

where Y correspond to the GS enzyme activity (% of control), a is the intercept, b is the slope, and X is the concentration of glufosinate.

Results and Discussion

Dose–Response Bioassay. There were no differences on the basis of Levene’s ANOVA test for homogeneity of variances between the replications in all experiments; therefore, data were pooled across studies. The GR₅₀ rates of glufosinate ranged from 0.15 to 0.49 kg ai ha⁻¹. The GR₅₀ values for OR1, OR2, and OR3 populations were 0.49, 0.42, and 0.40 kg ai ha⁻¹, respectively, whereas the GR₅₀ values for the control populations C1, C2, and C3 were 0.15, 0.18, and 0.21 kg ai ha⁻¹, respectively (Table 1). RI on the basis of the average of the three control populations were 2.7, 2.3, and 2.2 for OR1, OR2, and OR3, respectively. Although the GR₅₀ values represent the response of the populations, there were recorded on average 23 and 6% resistant individuals that regrew and survived at rates of 2.0 and 4.0 kg ai ha⁻¹,

respectively, in the group of the resistant populations, whereas no survivors were observed in the three control populations even at 1.0 kg ai ha⁻¹ (Table 2). Italian ryegrass is an obligate outcrossing species so the observed survivors at high rates of glufosinate in the resistant populations indicate that the populations are still segregating for resistant and susceptible individuals. Therefore, the GR₅₀ values obtained for the resistant populations could be underestimating the real level of resistance. In the case of the glufosinate-resistant goosegrass biotype reported from Malaysia, the level of resistance was between two- and eightfold (Jalaludin et al. 2010; Seng et al. 2010).

Ammonia Accumulation. Ammonia accumulation is the biochemical indicator of the GS inhibition caused by glufosinate toxicity (Pornprom et al. 2000; Tsai et al. 2006). ANOVA indicated differences for accumulation of ammonia among populations over time and across time. The untreated populations (0 HAT) showed an ammonia concentration that ranged from 11 to 16 µg of ammonia per gram of fresh weight. At 24 HAT, all the populations had increased levels of ammonia; however, the control populations began to accumulate more ammonia than the OR1, OR2, and OR3 populations (Table 3 and Figure 1), and continued this trend at 48, 72, and 96 HAT. Comparing the average ammonia accumulation between susceptible and resistant populations, the susceptible populations accumulated 1.6, 1.9, and 2.6 times more ammonia than the resistant populations at 48, 72, and 96 HAT, respectively, at the rate of 0.4 kg ai ha⁻¹ of glufosinate. It also was observed that the resistant populations reached the maximum peak of ammonia accumulation at 48 HAT and then the ammonia concentration decreased at 72 and 96 HAT. In contrast to the pattern observed in the resistant populations, the three control populations were still accumulating ammonia until 96 HAT. The results of ammonia accumulation were strongly correlated with the results obtained in the dose–response experiments, confirming that ammonia accumulation is a valid indicator for glufosinate resistance. The greatest ammonia accumulation recorded in the control populations was similar in magnitude to the results obtained in ammonium accumulation studies reported in other weed species (Petersen and Hurle 2000; Sellers et al. 2004; Tachibana et al. 1986).

Enzyme Activity. GS enzyme activity was inhibited in all the populations and the inhibition rates were positively correlated with increasing concentrations of glufosinate (Figure 2). The I_{50} values for the resistant and susceptible populations were similar, ranging from 3.7 to 4.3 µM for C3 and C1, and from 3.1 to 3.6 µM for the resistant populations. The similar

Table 3. Ammonia accumulation expressed in µg g⁻¹ of fresh weight in leaves of Italian ryegrass populations treated with glufosinate (0.4 kg ai ha⁻¹). Values represent pooled data from two experiments. Numbers in parentheses are the standard errors of the mean of eight samples.

Population	Time after treatment (h)				
	0 (±SE)	24 (±SE)	48 (±SE)	72 (±SE)	96 (±SE)
C1	13.4 (±0.9)a ^a	224.7 (±21.1)a	304.4 (±18.2)a	332.2 (±16.2)a	427.9 (±12.7)a
C2	12.4 (±0.9)ab	251.3 (±6.1)b	336.5 (±8.1)b	341.6 (±10.0)b	385.8 (±25.8)b
C3	16.7 (±2.7)c	243.8 (±6.9)b	320.2 (±5.4)c	353.1 (±9.1)b	384.5 (±18.2)c
OR1	11.0 (±0.8)b	175.5 (±10.0)ac	196.0 (±5.6)d	173.6 (±12.6)c	148.4 (±7.5)d
OR2	14.7 (±4.0)ad	133.1 (±6.8)d	163.6 (±4.6)e	139.3 (±4.0)c	138.1 (±5.4)e
OR3	15.9 (±5.9)cd	166.3 (±6.0)c	231.3 (±14.4)f	211.7 (±10.2)d	149.4 (±19.6)d

^a Columns followed by the same letter are not significantly different, according to LSD ($\alpha < 0.05$).

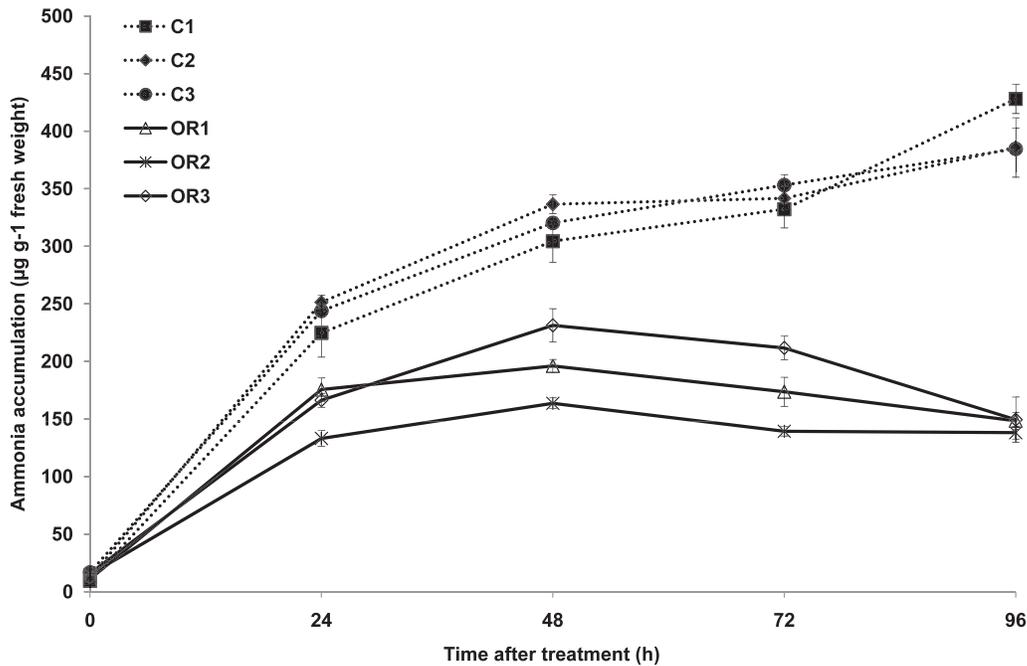


Figure 1. Ammonia accumulation in leaves of Italian ryegrass populations treated with glufosinate ($0.4 \text{ kg ai ha}^{-1}$). C1, C2, and C3 are susceptible populations, and OR1, OR2, and OR3 correspond to the resistant populations. Values represent pooled data from two experiments. Error bars represent the standard errors of the mean from eight samples.

sensitivity of the GS enzyme between the resistant and the susceptible populations suggests that the glufosinate resistance is not conferred by an insensitive target site. Similar levels of enzyme sensitivity to glufosinate were reported in soybean cells by Pornprom et al. (2009).

We hypothesize that reduced herbicide translocation is responsible for resistance to both glyphosate and glufosinate in these populations. Although the sites of action of these two herbicides are different, this does not preclude the possibility that one mechanism could affect the translocation of both herbicides. Our hypothesis is supported by the fact that there

was little or no use of glufosinate in the orchards where the resistant populations were collected, that the resistant populations were not resistant to herbicides with other sites of action, and that there was no difference in GS sensitivity between the resistant and susceptible populations.

Determining if reduced herbicide translocation is the cause of resistance to glufosinate is a key step to understanding the biochemical and physiological basis involved in the evolution of resistance to these two herbicides. In the context of weed management, glufosinate and glyphosate are two of the most important nonselective herbicides used in vineyards and

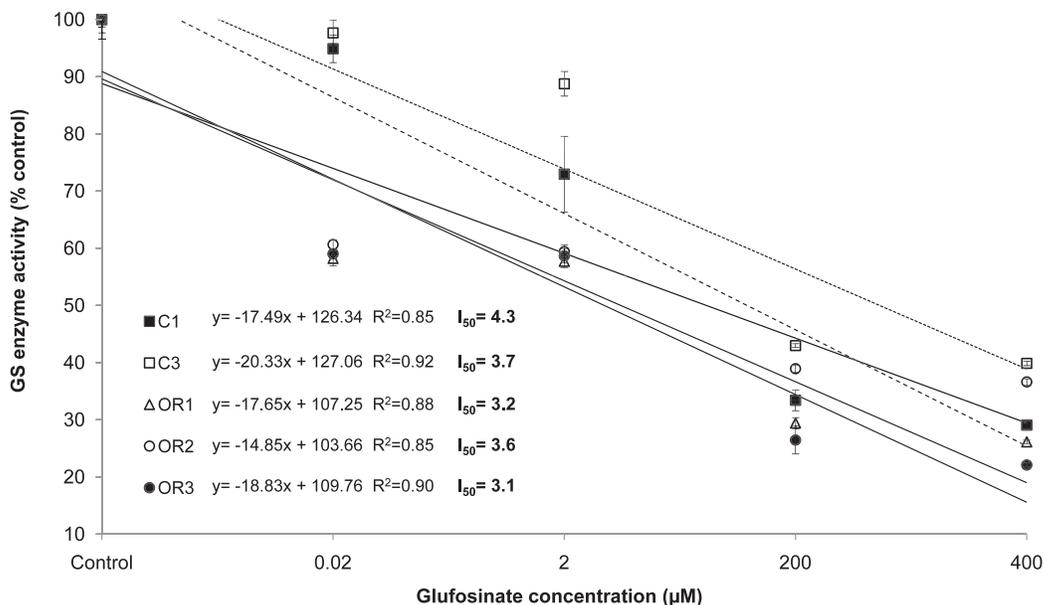


Figure 2. Effect of glufosinate concentration on the glutamine synthetase (GS) enzyme activity extracted from leaves of Italian ryegrass populations. C1 and C3 are susceptible populations, and OR1, OR2, and OR3 correspond to the resistant populations. Values represent pooled data from two experiments. Error bars represent the standard errors of the mean from eight samples.

orchards in the United States. Obviously, the evolution of resistance to these two herbicides reduces the chemical options for weed control in these systems. A more alarming weed management issue is the implication for the evolution of weeds with resistance to both herbicides in the systems where both glyphosate- and glufosinate-resistant crops are grown.

There are no reports of cross-resistance to glufosinate in glyphosate-resistant weeds where resistance is due to reduced herbicide translocation. If in the future more cases of cross-resistance to these two herbicides are identified, new weed management strategies will be required including herbicides with alternative sites of action or nonchemical methods (or both). The use of additional herbicides in these cropping systems will increase the cost and complexity of weed control and decrease the current benefit of these herbicide-resistant crops.

Sources of Materials

¹ Sunshine Mix 1 Potting Mix, Sun Gro Horticulture, Inc., 110th Ave. NE, Suite 490, Bellevue, WA 98004.

² Rely[®] 200, 182 g ai kg⁻¹, Bayer CropScience, 2 T. W. Alexander Dr., Research Triangle Park, NC 27709.

³ Phenol nitroprusside solution, Sigma-Aldrich[®]. 3050 Spruce St., St. Louis, MO 63103.

⁴ VERSAmix[™] tunable absorbance microplate reader, Molecular Devices Corporation, 1311 Orleans Dr., Sunnyvale, CA 94089.

⁵ R statistical software, R development core team, <http://www.r-project.org/>.

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