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# Confirmation and Control of Annual Bluegrass (*Poa annua*) with Resistance to Prodiamine and Glyphosate

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Poor annual bluegrass control was reported in golf course roughs following treatment with prodiamine (1120 g ai $ha^{-1}$) and glyphosate (840 g ae $ha^{-1}$) during hybrid bermudagrass dormancy. Research was conducted to determine if this annual bluegrass phenotype was resistant to both prodiamine and glyphosate and to determine the efficacy of herbicide mixtures for controlling this phenotype in the field. In PRE or POST dose-response experiments, 9 to 31 times more prodiamine or glyphosate was needed to control (or reduce dry biomass of) this resistant phenotype by 50% compared to an herbicide-susceptible phenotype. Moreover, glyphosate-susceptible plants accumulated 50% more shikimic acid (898 mg $kg^{-1}$) 6 d after treatment than those resistant to glyphosate (394 mg $kg^{-1}$). October (fall) applications of herbicide mixtures containing trifloxysulfuron, simazine, *S*-metolachlor, or mesotrione controlled this resistant annual bluegrass phenotype 84 to 98% in April (spring), with no differences detected among treatments. Our findings document the second instance of annual bluegrass evolving multiple resistance in a managed turfgrass system. However, several herbicide mixtures can be used to effectively manage this resistant phenotype.
**Nomenclature:** glyphosate; mesotrione; prodiamine; simazine; *S*-metolachlor; trifloxysulfuron; annual bluegrass, *Poa annua* L.; hybrid bermudagrass, *Cynodon dactylon* × *Cynodon transvaalensis* Burtt-Davy.
**Key words:** Turf, turfgrass, golf course, mitotic-inhibiting herbicide, EPSPS.

Control limitado de *Poa annua* fue reportado en "roughs" de campos de golf después de tratamientos con prodiamine (1120 g ai $ha^{-1}$) y glyphosate (840 g ae $ha^{-1}$) durante el período de dormancia del césped bermuda híbrido. Se realizó una investigación para determinar si este fenotipo de *P. annua* era resistente a prodiamine y glyphosate y para determinar la eficacia de mezclas de herbicidas para controlar este fenotipo en el campo. En experimentos de respuesta a dosis con herbicidas PRE o POST, se necesitó de 9 a 31 veces más prodiamine o glyphosate para controlar (o reducir la biomasa seca) de este fenotipo resistente en 50% en comparación a un fenotipo susceptible a estos herbicidas. Además, plantas susceptibles a glyphosate acumularon 50% más ácido shikimic (898 mg $kg^{-1}$) 6 d después del tratamiento que plantas resistentes a glyphosate (394 mg $kg^{-1}$). Aplicaciones en Octubre (otoño) de mezclas de herbicidas que contenían trifloxysulfuron, simazine, *S*-metolachlor, o mesotrione controlaron este fenotipo resistente de *P. annua* 84 a 98% en Abril (primavera), sin detectarse diferencias entre estos tratamientos. Nuestros resultados documentan la segunda instancia de *P. annua* que evoluciona resistencia múltiple en un sistema manejado de céspedes. Sin embargo, varias mezclas de herbicidas pueden ser usadas para manejar efectivamente este fenotipo resistente.

Annual bluegrass is a common weed of warm-season turfgrass during winter dormancy. Turfgrass managers rely on PRE or POST herbicide applications to selectively control annual bluegrass in warm-season turfgrass given that plants can produce as many as 185,000 viable seeds in the top 2.5 cm of soil, which can germinate at day and night soil temperatures ranging from 19 and 10 C to 39 and 29 C (McElroy et al. 2004; Watschke et al. 1979). High fecundity and adaptability to different growing conditions, along with robust genetic diversity (Mao and Huff 2012), likely couples with herbicide selection pressure to create herbicide-resistant annual bluegrass populations. To date, herbicide resistance has been documented in annual bluegrass more than it has in any other turfgrass weed (Heap 2016).

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Annual bluegrass populations evolving resistance to herbicidal inhibitors of mitosis (e.g., prodiamine), photosystem II (e.g., simazine), acetolactate synthase (e.g., trifloxysulfuron), or enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors (e.g., glyphosate) have all been documented in managed turfgrass systems (Binkholder et al. 2011; Isgrigg et al. 2002; Kelly et al. 1999; McElroy et al. 2013).

Multiple resistance results when weeds have more than one resistance mechanism that confers resistance to multiple herbicides within the same or different modes of action (Vencill et al. 2012). To date, there has only been a single reported case of annual bluegrass evolving multiple resistance in managed turfgrass: a phenotype resistant to simazine and trifloxysulfuron (Brosnan et al. 2015). Genetic sequencing of these annual bluegrass plants revealed a target site mutation (Ser-264-Gly) on the D1 protein that is known to confer resistance to photosystem II–inhibiting herbicides, as well as a new mutation of acetolactate synthase (Ala-205-Phe) that conferred broad spectrum resistance to imidazolinone, sulfonylurea, triazolopyrimidines, sulfonylamino-carbonyl-triazolinones, and pyrimidinyl (thio) benzoate herbicides (Brosnan et al. 2016).

Recent research has found mixing herbicides with different modes of action to be more effective in delaying herbicide resistance than rotating between herbicides with different modes of action over time. However, herbicide mixture components must have efficacy against the target weed individually in order to decrease survival probabilities of the plants and reduce the frequency of resistance alleles in the overall weed population (Evans et al. 2016).

In the spring of 2012, poor annual bluegrass control (<50%) was reported in golf course roughs in Alcoa, Tennessee (35.75°N, 83.88°W) following treatment with a tank mixture of prodiamine (1,120 g $ha^{-1}$) and glyphosate (840 g ae $ha^{-1}$) during hybrid bermudagrass dormancy. This application had been made at this location for over ten consecutive years without rotation (JD Murr, personal communication). We hypothesized that annual bluegrass at this location had evolved resistance to both prodiamine and glyphosate. Therefore, our objectives were 1) to determine if this annual bluegrass phenotype was resistant to both prodiamine and glyphosate, and 2) to determine the efficacy of herbicide mixtures for controlling this phenotype in the field.

## Materials and Methods

**Plant Collection.** One hundred annual bluegrass plants were mechanically harvested from golf course roughs (Alcoa, TN; 35.75°N, 83.88°W) using a Hound Dog weeder (Weed Hound, The Ames Companies Inc., Camp Hill, PA) on March 18, 2014, and transplanted in a glasshouse in Knoxville, Tennessee (35.56°N, 83.56°W). Considering that annual bluegrass is a self-pollinated species (Ellis 1973), harvested plants were given a unique identifier and clonally propagated into a minimum of four single-tiller samples per harvested plant. Tillers were established in 164-$cm^3$ cone-tainers (SC10 Super Cell Cone-tainer, Steuwe & Sons, Tangent, OR) filled with peat moss growing medium (Growing Mix #2, Conrad Fafard, Inc., Agawam, MA). The unique identifier allowed for screening experiments to be conducted using tillers directly associated with a specific plant harvested from the field site. This process produced 890 individual tillers from 100 whole plants. After transplanting, all plant material remained in a glasshouse with day and night temperatures averaging 29 and 19 C, respectively. Plants received irrigation three times a day from an overhead misting system in order to maintain adequate moisture and prevent drought stress. Plants were maintained at an approximate height of 4 cm by cutting with scissors weekly. Nutrients were supplied at a rate of 24 kg N $ha^{-1}$ every 14 d using a complete fertilizer (20-20-20 Soluble Fertilizer with Minor Elements, Southern Agricultural Insecticides Inc., Hendersonville, NC).

**Screening for Resistance Within the Sampled Population.** Screening experiments were conducted in the aforementioned glasshouse in 2014 to determine the percentage of annual bluegrass plants in the sampled population that were resistant to prodiamine, glyphosate, or both.

*Glyphosate Resistance.* On April 29, 2014, clonal tillers of all 100 plants harvested from the field site were treated with glyphosate at 840 g $ha^{-1}$ using a single flat-fan nozzle (8004EVS TeeJet®, Spraying Systems Co., Wheaton, IL) in an enclosed spray chamber (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN) calibrated to deliver 215 L $ha^{-1}$. Plants were at least a single tiller in size when herbicide was applied, and the plants were maintained under the previously described glasshouse



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conditions. Annual bluegrass response to glyphosate was visually assessed on a 0% (no injury) to 100% (complete necrosis) scale relative to a non-treated check 3 wk after treatment. Plants injured ≤30% were placed in a glyphosate-resistant group, while those injured ≥70% were classified as susceptible.

*Prodiamine Resistance.* Tillers of all 100 plants harvested from the field site were screened for prodiamine resistance using hydroponic methods of Brosnan et al. (2014) in the previously described glasshouse. Tillers were transplanted into polyethylene containers (Roughneck®, Rubbermaid Commercial Products LLC, Winchester, VA) filled with 10 L of full-strength Hoagland solution (Hoagland and Arnon 1950) aerated with a blower (VB-007S, Sweetwater, Ft. Collins, CO) and air stones (HAGEN Elite 1" Cube Air Stone, Rolf C. Hagen Corp., Mansfield, MA). Ten holes (0.4 cm diameter) were drilled into the lid of each container at a 5.3 cm spacing. One hundred individual annual bluegrass tillers were transplanted into these holes on April 29, 2014. Root length of all tillers was trimmed to 5 cm before transplanting, and all 100 tillers were placed into containers with roots submerged into the nutrient solution containing 0.04 mM prodiamine. In a previous study, a prodiamine concentration of 0.04 mM was required to reduce the rooting of annual bluegrass from this field location by 50%, compared to a concentration of $2.8 \times 10^{-6}$ mM for a known susceptible phenotype of annual bluegrass (Brosnan et al. 2014). Therefore, all 100 plants in this experiment were exposed to 0.04 mM prodiamine in hydroponic culture to identify prodiamine-resistant and -susceptible individuals within the population sampled from the field site. At 10 days after treatment (DAT), all tillers were harvested and root length was measured. Root growth beyond the 5 cm benchmark was used to classify plants as likely resistant to prodiamine, while root length of 5 cm or less resulted in plants being classified as likely susceptible to prodiamine.

**Confirming Resistance in Progeny.** Research was conducted in the aforementioned glasshouse in 2015 to confirm resistance to prodiamine and glyphosate in the progeny of plants that survived treatment with both of these herbicides during the screening experiments. In all confirmation experiments, irrigation was supplied via an overhead misting system three times a day. Once non-treated plants reached 2.5 cm in height, nutrients were supplied at a rate of 24 kg N $ha^{-1}$ every 14 d using a complete fertilizer. Maximum and minimum air temperature conditions in the glasshouse during confirmation experiments averaged 30 and 20 C, respectively. Chlorantraniliprole (5-bromo-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-2-(3-chloropyridin-2-yl)pyrazole-3-carboxamide; Acelepryn® Turf Insecticide, Syngenta Professional Products, Greensboro, NC) was applied at 0.23 kg $ha^{-1}$ on an as-needed basis to control insect pests. Annual bluegrass control in all experiments was visually assessed on a 0% (no control) to 100% (complete necrosis) scale relative to a non-treated check 35 DAT in prodiamine experiments and 21 DAT in experiments evaluating responses to glyphosate. Once all visual control data were collected, aboveground biomass was measured by harvesting all tissue above the soil line. Plant shoots were then placed in a drying oven at 35 C for 5 d and then weighed. Data are expressed as a percentage of the non-treated control.

*Prodiamine Resistance.* Seeds from plants exhibiting resistance to prodiamine in previously described screening experiments were collected and stored at –20 C during the spring of 2014. After a 4-mo vernalization period, greenhouse pots containing 1,049 $cm^3$ of Sequatchie silt loam soil (fine-loamy, siliceous, semiactive, thermic Humic Hapludult) were surface seeded with prodiamine-resistant germplasm on February 5, 2015. Companion pots were surface seeded with prodiamine-susceptible germplasm (Penn State University, University Park, PA) on the same date. Immediately after seeding, pots were treated with prodiamine at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g $ha^{-1}$ using a previously described spray chamber. Treatments were arranged in a completely randomized design with four replications, and were repeated in time. Applications for the second experiment made on March 13, 2015.

*Glyphosate Resistance.* Seeds from plants exhibiting resistance to glyphosate in screening experiments were collected and stored as previously described for prodiamine. Cone-tainers filled with peat moss growing medium were seeded with glyphosate-resistant germplasm on October 30, 2014. An annual bluegrass phenotype known to be susceptible



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to glyphosate (Penn State University) was seeded in a similar manner on the same date. Cone-tainers were incubated under previously described conditions until plants developed a minimum of three tillers. Resistant and susceptible plants were treated with glyphosate at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g ha$^{-1}$ on February 19, 2015 using the previously described spray chamber calibrated to deliver 215 L ha$^{-1}$. Treatments were arranged in a completely randomized design with four replications, and were repeated in time. Applications for the second experiment made on March 13, 2015.

A separate experiment was conducted to quantify shikimic acid accumulation after exposing resistant and susceptible tillers to glyphosate, similar to that performed by Mueller et al. (2003). Plants were treated with glyphosate at 420 g ha$^{-1}$ using the previously described spray chamber. Annual bluegrass aboveground tissue was harvested using scissors from each cone-tainer at 0, 1, 2, 3, and 6 DAT. Treatments were arranged in a completely randomized design with twenty-sprayed replications of each phenotype on each harvest date. In order to provide sufficient biomass for shikimic acid analysis, these twenty replicates were combined to produce four composite replicates of each phenotype on each harvest date. Aboveground plant tissue harvested 0, 1, 2, 3, and 6 DAT was stored in a freezer (–20 C) within an hour of harvest and remained there until shikimic acid was extracted and analyzed. All samples were extracted and analyzed within 34 d of harvest. Shikimic acid accumulation studies were initiated on February 10, 2015 and repeated on March 3, 2015. Maximum and minimum air temperature conditions in the glasshouse during the time the data were collected averaged 30 and 19 C, respectively.

Annual bluegrass tissue was ground with a mortar and pestle in liquid nitrogen and then weighed in centrifuge tubes. Five milliliters of 1M HCl was added per gram of tissue. The tubes were placed on a reciprocating shaker (Fisher Scientific, Hampton, NH) at 80 rpm for 16 h. HCl extractions were filtered through a 0.45-mm syringe filter into a 4-mL vial for liquid chromatography analysis. All samples were analyzed 1 d after extraction using a liquid chromatograph (Mueller et al. 2011) equipped with an ultraviolet detector (215 nm wavelength). Shikimic acid accumulation in plant tissue (mg shikimic acid per kg annual bluegrass fresh weight) was quantified and plotted over harvest intervals (DAT), with error bars representing standard error of the mean for each phenotype at each harvest interval.

**Statistical Analysis of Confirmation Experiments.** For all confirmation experiments, analysis of variance was performed using SAS® (SAS Institute, Cary, NC) to determine if data from both experimental runs could be combined. No significant interactions with experimental run were detected, allowing data to be combined and subjected to non-linear regression analysis in Prism (Prism 6.0 for Mac OS X, GraphPad Software, La Jolla, CA). Responses for resistant and susceptible annual bluegrass in all experiments were compared using a global sums-of-squares $F$-test at $\alpha = 0.05$.

Annual bluegrass control data were analyzed using the following non-linear regression equation:

$$\text{Control} = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((Rate_{50} - X) \times K)}) \quad [1]$$

In this equation, $Rate_{50}$ represents the herbicide rate ($X$) at which 50% annual bluegrass control was reached. Bottom and Top represent asymptotes that were constrained to 0% and 100%, respectively, as annual bluegrass control could only range from 0% to 100%. $K$ represents the slope of the best-fit line to model the response of resistant and susceptible phenotypes to increasing rates of herbicide.

All dry biomass data were analyzed using the following exponential decay non-linear regression equation:

$$\text{Dry biomass}\,(\%\ \text{non}-\text{treated}) = (\text{Y0} - \text{Plateau}) \times \exp(-\text{K} \times \text{X}) + \text{Plateau} \quad [2]$$

In this equation, Y0 and Plateau were constrained to 100 and 0, respectively, as dry biomass only ranged from 0% to 100% of the non-treated check regardless of herbicide rate. $K$ represented the slope of the best-fit line to model the response of each phenotype to increasing rates of herbicide ($X$). The herbicide rate required to reduce dry biomass to 50% of the non-treated ($GR_{50}$) was calculated in Prism using the formula $GR_{50} = \ln(2)/K$.

Shikimic acid accumulation data were analyzed using the following one-phase exponential association non-linear regression equation:

$$\text{Shikimic acid}\,(\text{mg kg}^{-1}) = \text{Y0} + [(\text{Plateau} - \text{Y0}) \times (1 - \exp[-\text{KX}])] \quad [3]$$



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The parameters of Equation 3 were similar to those of Equation 2, except that Y0 and Plateau were not constrained, and $X$ represented d after glyphosate treatment at 420 g ha$^{-1}$. This approach allowed the maximum shikimic acid concentration accumulated in each phenotype to be calculated (i.e., Plateau). All data and regression analysis files are available at https://dx.doi.org/10.6084/m9.figshare.2066319.v5.

**Efficacy of Herbicide Mixtures.** Field research was conducted in golf course roughs located in Alcoa, TN (35.75°N, 83.88°W) with the objective of evaluating several herbicide mixtures for controlling an annual bluegrass phenotype with putative resistance to mitotic-inhibiting herbicides (e.g., prodiamine) and glyphosate. Soil at this location was a Dewey silty clay loam (fine, kaolinitic, thermic typic Paleudult), while the predominant turf species was hybrid bermudagrass mowed once weekly at 4.5 cm. The site received no supplemental irrigation, and no supplemental nutrition was applied during the experiment.

In order to effectively manage resistance, herbicide mixtures must contain active ingredients that can control the target weed when applied individually (Evans et al. 2016). Given that resistance to mitotic-inhibiting herbicides and glyphosate were both suspected, herbicide mixture treatments in the current study were designed using alternative modes of action. All mixture treatments evaluated in the current study contained herbicides with labeling for annual bluegrass control when applied individually. A complete list of herbicide mixture treatments and application rates is presented in Table 1. All treatments were mixed in water and applied using a $CO_2$-pressurized boom sprayer equipped with 8002 flat-fan spray nozzles (Teejet®, Wheaton, IL) positioned to create a 1.2-m spray swath. In 2014, treatments were applied on October 20 to 1.5 by 2.4 m plots at a carrier volume of 281 L ha$^{-1}$. In 2015, treatments were applied on October 21 to 1.5 by 2.1 m plots at a carrier volume of 374 L ha$^{-1}$. Each year, annual bluegrass had emerged in the plots on the date of herbicide treatment, but the plants had not begun to tiller. In March and April of 2014 and 2015, annual bluegrass control was visually assessed on a 0% (no control) to 100% (complete necrosis) scale relative to a non-treated check. Assessment dates corresponded to 21 and 25 wk after treatment each year.

Experimental design in both years of the study was a randomized complete block with three replications. All annual bluegrass control data were subjected to statistical analysis using R software (version 3.2.3). Expected means squares of McIntosh (1983) determined that data could be combined over years prior to being subjected to analysis of variance using the 'ExpDes' package in R. Means were separated using Fisher's least significant difference test at $\alpha = 0.05$ when appropriate. All data and analysis files are available at https://dx.doi.org/10.6084/m9.figshare.2066319.v5.

## Results and Discussion

**Screening for Resistance Within the Sampled Population.** Eighty-one of the 100 annual bluegrass plants harvested from the field site screened positive for glyphosate and prodiamine resistance, whereas only a single plant was susceptible to both herbicides. A total of 96 of the 100 annual bluegrass plants sampled screened positive for glyphosate resistance, 15 of which were prodiamine susceptible. Conversely, 84 of the 100 plants sampled were classified as likely prodiamine resistant, only three of which were deemed glyphosate susceptible. These

Table 1. Herbicide mixture treatments evaluated for control of annual bluegrass with suspected multiple resistance to glyphosate and prodiamine in hybrid bermudagrass golf course roughs in Alcoa, TN (35.75°N, 83.88°W) during 2014 and 2015.

| Herbicide mixture | Trade names[a] | Application rate g ha$^{-1}$ |
|---|---|---|
| Trifloxysulfuron + *S*-metolachor | Monument® 75WG + Pennant Magnum® | 28 + 5550 |
| Trifloxysulfuron + mesotrione | Monument® 75WG + Tenacity® | 28 + 280 |
| Trifloxysulfuron + simazine | Monument® 75WG + Princep® | 28 + 1120 |
| Trifloxysulfuron + simazine + *S*-metolachlor | Monument® 75WG + Princep® + Pennant® Magnum | 28 + 1120 + 5550 |
| Trifloxysulfuron + simazine + mesotrione | Monument® 75WG + Princep® + Pennant® Magnum | 28 + 1120 + 280 |

[a] All herbicides were manufactured by Syngenta Professional Products (Greensboro, NC) and applied at maximum label rates for annual bluegrass control in managed turf.



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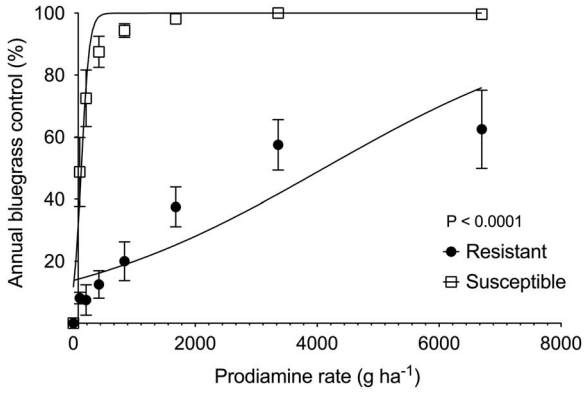


Figure 1. Control of prodiamine-resistant and -susceptible annual bluegrass 35 days after treatment with prodiamine at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g ha$^{-1}$ during the spring of 2015 in a glasshouse (Knoxville, TN; 35.56°N, 83.56°W). Error bars represent standard error of the mean.

responses suggest that the majority of the sampled annual bluegrass population was likely resistant to both prodiamine and glyphosate; however, this response needed to be confirmed from seed in progeny to ensure that these traits were heritable (Burgos et al. 2013).

## Confirming Resistance in Progeny

*Prodiamine Resistance.* Control of resistant and susceptible annual bluegrass phenotypes varied in response to increasing rates of prodiamine (Figure 1, Table 2). Non-linear regression curves fit control data collected on the resistant and susceptible phenotypes, and were significantly different from each other ($P < 0.0001$). Rates of prodiamine required to control the resistant and susceptible phenotypes 50% ($GR_{50}$ values) were 4,117 and 132 g ha$^{-1}$, respectively. Dry biomass data were similar to control assessments in that non-linear regression curves modeling reductions in biomass for the resistant and susceptible phenotypes were significantly different from one another ($P < 0.0001$) (Figure 2, Table 2). Prodiamine rates needed to reduce dry biomass of the resistant and susceptible phenotypes by 50% were 1,006 and 45 g ha$^{-1}$, respectively, equating to a resistance factor of 22 based on dry biomass reductions. These results are similar to research conducted by Cutulle et al. (2009), who identified a phenotype of annual bluegrass 26 times less sensitive to prodiamine than a known susceptible phenotype.

*Glyphosate Resistance.* Significant differences in annual bluegrass control were observed 21 d after treating progeny of resistant and susceptible phenotypes with increasing rates of glyphosate (Figure 3, Table 2). Non-linear regression curves fit control data collected on the resistant and susceptible phenotypes and were significantly different from one another ($P < 0.0001$). Glyphosate rates that resulted in 50% control of the resistant and

Table 2. Regression parameters for control and biomass data, presented in figures, representing annual bluegrass response to increasing rates of prodiamine and glyphosate in glasshouse experiments (Knoxville, TN; 35.56°N, 83.56°W) in 2015.

| Figure[a] | Response | Biotype | $Rate_{50}$ or $GR_{50}$[b] | $Rate_{50}$ or $GR_{50}$ 95% confidence interval | Slope |
|---|---|---|---|---|---|
| | | | g ha$^{-1}$ | | |
| 1 | Prodiamine - Control | Resistant | 4117 | 3,216 to 5,018 | 0.0002 |
| | | Susceptible | 132 | 108 to 155 | 0.0067 |
| 2 | Prodiamine - Dry Biomass | Resistant | 1006 | 600 to 3131 | 0.0007 |
| | | Susceptible | 45 | 41 to 49 | 0.0154 |
| 3 | Glyphosate - Control | Resistant | 3908 | 3,388 to 4,427 | 0.0005 |
| | | Susceptible | 393 | 326 to 460 | 0.0034 |
| 4 | Glyphosate - Dry Biomass | Resistant | 2799 | 2015 to 4,581 | 0.0036 |
| | | Susceptible | 330 | 268 to 431 | 0.0030 |

[a] Control data in Figures 1 and 3 modeled using the equation Control (%) = Bottom + (Top − Bottom)/$(1 + 10^{((Rate_{50}-X) \times K)})$, where $Rate_{50}$ represents the herbicide rate ($X$) at which 50% annual bluegrass control was reached. Bottom and Top represent asymptotes that are constrained to 0 and 100, and $K$ represents the slope of the best-fit line to model the response of resistant and susceptible biotypes to increasing rates of herbicide. Dry biomass data in Figures 2 and 4 modeled using the equation dry biomass (% non-treated) = (Y0 − Plateau) × exp (− $KX$) + Plateau, where Y0 and Plateau are constrained to 100 and 0, $K$ represents the slope of the best-fit line to model the response of each biotype to increasing rates of herbicide ($X$). The herbicide rate required to reduce dry biomass to 50% of the non-treated ($GR_{50}$) was calculated using the formula: $GR_{50} = \ln(2)/K$.

[b] Prodiamine rate (g ha$^{-1}$) required to control annual bluegrass by 50% ($Rate_{50}$) or reduce dry biomass to 50% of the non-treated ($GR_{50}$).



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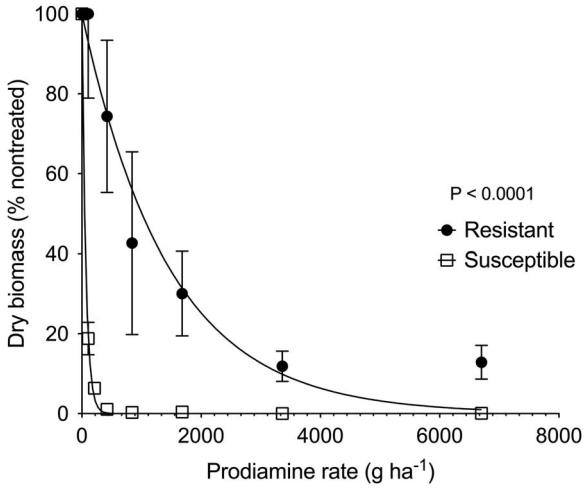


Figure 2. Dry biomass reduction of prodiamine-resistant and -susceptible annual bluegrass 35 days after treatment with prodiamine at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g ha$^{-1}$ during the spring of 2015 in a glasshouse (Knoxville, TN; 35.56°N, 83.56°W). Error bars represent standard error of the mean.

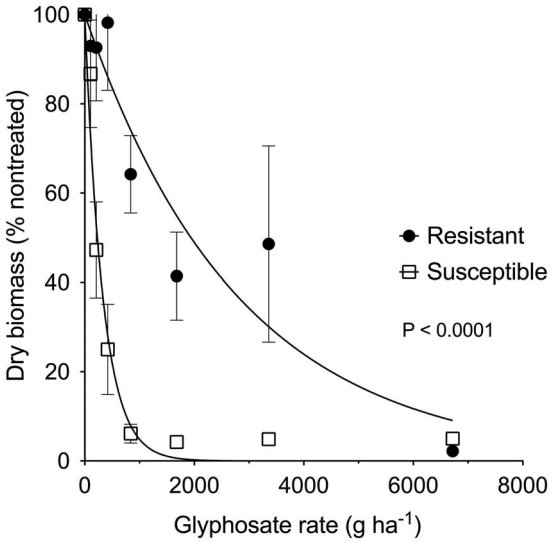


Figure 4. Dry biomass reduction of glyphosate-resistant and -susceptible annual bluegrass 35 days after treatment with glyphosate at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g ha$^{-1}$ during the spring of 2015 in a glasshouse (Knoxville, TN; 35.56°N, 83.56°W). Error bars represent standard error of the mean.

susceptible phenotypes were 3,908 and 393 g ha$^{-1}$, respectively. Dry biomass data supported assessments of annual bluegrass control, as approximately 8.5 times more glyphosate (1,940 g ha$^{-1}$) was required to reduce dry biomass of the resistant phenotype by 50% compared to a known glyphosate-susceptible phenotype (229 g ha$^{-1}$; Figure 4, Table 2). This response is within the range of reduced glyphosate sensitivity (4 to 12 times lower sensitivity) reported by other researchers studying glyphosate-resistant annual bluegrass (Binkholder et al. 2011; Brosnan et al. 2012; Cross et al. 2015).

Shikimic acid accumulation studies also suggest glyphosate resistance in this annual bluegrass phenotype. Glyphosate-susceptible plants accumulated 50% more shikimic acid 6 DAT than those resistant

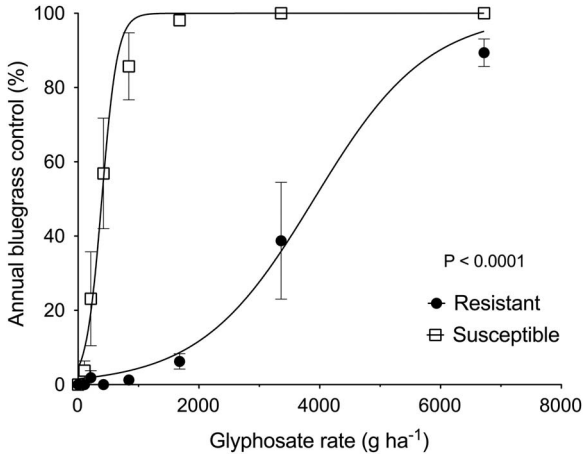


Figure 3. Control of glyphosate-resistant and -susceptible annual bluegrass 35 days after treatment with glyphosate at 0; 105; 210; 420; 840; 1,680; 3,360; and 6,720 g ha$^{-1}$ during the spring of 2015 in a glasshouse (Knoxville, TN; 35.56°N, 83.56°W). Error bars represent standard error of the mean.

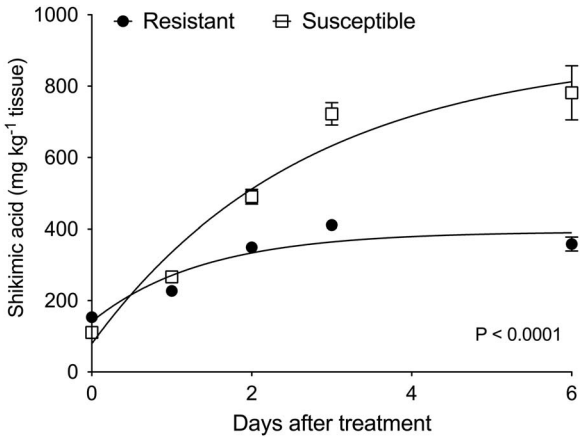


Figure 5. Shikimic acid concentrations (mg kg$^{-1}$) in glyphosate-resistant and -susceptible annual bluegrass following treatment with glyphosate at 420 g ha$^{-1}$. Error bars represent standard error of the mean for each biotype at each timing.



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Table 3. Regression parameters modeling shikimic acid concentrations (mg $kg^{-1}$) in glyphosate-resistant and -susceptible annual bluegrass following treatment with glyphosate at 420 g $ha^{-1}$ in glasshouse experiments (Knoxville, TN; 35.56°N, 83.56°W) in 2015.

| | Model parameters[a] | | | | |
|---|---|---|---|---|---|
| Biotype | Plateau | Plateau 95% confidence interval | Y0 | Y0 95% confidence interval | Slope |
| | mg $kg^{-1}$ | | | | |
| Resistant | 4,117 | 3,216 to 5,018 | 141 | 105 to 177 | 0.72 |
| Susceptible | 132 | 108 to 155 | 79 | −2 to 161 | 0.38 |

[a] Responses modeled using a one-phase exponential association non-linear regression equation, shikimic acid = Y0 + (Plateau − Y0)(1 − exp(− KX)), where Y0 and Plateau were the minimum and maximum shikimic acid accumulation, *K* represented the slope of the best-fit line, and X represented days after treatment with glyphosate at 420 g $ha^{-1}$.

to glyphosate (Figure 5, Table 3). Glyphosate-resistant annual bluegrass accumulated a maximum of 394 mg $kg^{-1}$ shikimic acid 6 DAT, while 898 mg $kg^{-1}$ accumulated in annual bluegrass with susceptibility to glyphosate. Similarly, Brosnan et al. (2012) and Cross et al. (2015) reported shikimate concentrations to be 50% lower in a glyphosate-resistant annual bluegrass compared to susceptible plants at 3 and 6 DAT.

**Efficacy of Herbicide Mixtures.** All POST-applied herbicide mixtures evaluated in the current study effectively controlled annual bluegrass with resistance to prodiamine and glyphosate (Table 4). October applications of herbicide mixtures containing trifloxysulfuron, simazine, *S*-metolachlor, or mesotrione controlled this resistant annual bluegrass biotype 86% to 100% in March and 84% to 98% in April, with no significant differences detected among treatments (Table 4).

**Implications for Turf Managers.** Our findings documented the second instance of annual bluegrass evolving multiple resistance in a managed turfgrass system, and the first case of annual bluegrass with resistance to both PRE and POST herbicides. However, we demonstrated that POST-applied herbicide mixtures can effectively control this annual bluegrass biotype with resistance to prodiamine and glyphosate. However, herbicides included in these mixtures were all active on annual bluegrass and applied at maximum label rate. Our results present a baseline outlining the relative percentage of annual bluegrass plants in the sampled population that were resistant to both prodiamine and glyphosate, either herbicide individually, or neither herbicide. Future research should explore if use of herbicide mixtures evaluated in the current study change the percentage of resistant plants over time.

Our research is limited in that we did not fully determine the mechanisms conferring resistance in

Table 4. Control of annual bluegrass with multiple resistance to glyphosate and prodiamine with herbicide mixtures applied to hybrid bermudagrass golf course roughs in Alcoa, TN (35.75°N, 83.88°W) following applications made in October 2014 and 2015. Means represent combined responses of four replications over two years.

| | | Annual bluegrass control[a] | |
|---|---|---|---|
| Herbicide mixture[b] | Rate | March | April |
| | g $ha^{-1}$ | % | |
| Trifloxysulfuron + *S*-metolachor | 28 + 5550 | 95 | 93 |
| Trifloxysulfuron + mesotrione | 28 + 280 | 86 | 84 |
| Trifloxysulfuron + simazine | 28 + 1120 | 97 | 94 |
| Trifloxysulfuron + simazine + *S*-metolachlor | 28 + 1120 + 5550 | 96 | 97 |
| Trifloxysulfuron + simazine + mesotrione | 28 + 1120 +280 | 100 | 98 |
| $LSD_{0.05}$ | | NS | NS |

[a] Annual bluegrass control assessed on a 0% (no control) to 100% (complete necrosis) scale relative to a non-treated check plot. Assessments made in March and April represent 21 and 25 weeks after treatment, respectively.
[b] Herbicide mixtures were applied on October 20, 2014 and October 21, 2015, and included non-ionic surfactant (Activator-90, Loveland Products, Greeley, CO) at 0.25% v/v.



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this annual bluegrass population. Genetic sequencing work did identify a Pro-106-Ala substitution on the EPSPS gene within this population, a target site alteration that has been shown to confer glyphosate resistance in annual bluegrass (Cross et al. 2015). Complete sequences for EPSPS in the annual bluegrass biotype studied herein are available in GenBank (KU382756 – KU382773).

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