Target-site Mutation and Fitness Cost of Acetolactate Synthase Inhibitor-resistant Annual Bluegrass

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Abstract. Annual bluegrass (Poa annua L.) is an annual weed that is particularly troublesome in managed turfgrass. It has been controlled conventionally with herbicides, including acetolactate synthase (ALS) inhibitors. However, resistance to ALS inhibitors has been documented throughout the southeastern United States since 2012. A rate-response trial was conducted to confirm and determine the resistance level of suspected resistant P. annua biotypes from Mississippi (Reunion), followed by DNA sequencing to determine whether the mechanism of resistance is a target-site mutation. In addition, a fitness assay was conducted together with a susceptible biotype to determine whether resistance to ALS inhibitors is associated with decreased fitness. Reunion was at least 45 times more resistant to foramsulfuron than the standard susceptible biotype based on I50 estimates [I50 is the rate of herbicide giving a 50% response (50% visual necrosis), requiring a predicted 331 g a.i./ha foramsulfuron for 50% control]. DNA sequencing results identified a Trp574-Leu mutation in the ALS gene of the Reunion biotype, which has been shown by other studies to confer resistance to ALS inhibitors. Measurement of fitness parameters among the Reunion and susceptible biotypes demonstrated reduced seed yield, tillering, and flowering time in the resistant Reunion biotype, suggesting that ALS inhibitor resistance is possibly correlated to decreased fitness in P. annua. Alternative methods to control P. annua need to be considered as a result of the evolution of herbicide-resistant biotypes. An integrated management strategy to control P. annua weeds will help prevent further evolution of resistance. Because this study evaluated only the target-site mechanism of resistance, it is also necessary to determine whether the resistant biotype has reduced uptake, translocation, or enhanced metabolism as additional mechanisms of resistance. Consequently, a fitness study encompassing a more comprehensive list of plant parameters will provide conclusions of the fitness costs associated with ALS inhibitor resistance in P. annua. Chemical names: Foramsulfuron [1-(4,6-dimethoxypyrimidin-2-yl)-3-(dimethylcarbamoyl)-5-formamidophenylsulfonyl] urea.

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In the United States, P. annua accounts for more than half of reported herbicide resistance in managed turfgrass system since the 1980s, including populations resistant to photosystem II inhibitors, microtubule inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase inhibitors, and, most recently, ALS inhibitors (Heap, 2012). In 2011, the first P. annua biotype not controlled by ALS inhibitors was reported in Alabama. McElroy et al. (2013) reported that this biotype had an amino acid substitution of Trp574 to Leu in the ALS gene. This specific mutation has been correlated with the most resistant biotypes of P. annua (Cross et al., 2015). Plant fitness can be defined as the capability of a phenotype to produce offspring successfully relative to another phenotype. The fitness cost associated with herbicide resistance is the reduction of plant fitness in a stress-free environment as a result of negative pleotropic effects of herbicide-resistant alleles (Powles and Yu, 2010). Parameters used most often to assess plant fitness associated with herbicide-resistant phenotypes are seed germination, longevity, dormancy, growth rate (plant height), competitive ability, biomass, and seed yield (Holt and Thill, 1994). ALS inhibitor-resistant Lactuca serriola plants containing the Pro197His allele decreased in frequency by as much as 86% over a 3-year period, primarily because of reduced shoot biomass compared with susceptible plants (Alcocer-Ruthling et al., 1992). Similarly, Amaranthus powellii, with the Trp574Leu allele, showed significantly smaller and distorted leaves compared with susceptible plants (Tardif et al., 2010). Roots and shoots of susceptible plants were more developed and had 34% greater biomass than resistant plants (Tardif et al., 2006). These studies indicate the adverse pleotropic effects of resistance-associated mutations on the growth and development of plants, thus reducing overall fitness.

Because certain resistant biotypes are found to have a fitness cost associated with them, the objectives of this study were 1) to confirm and determine the resistance level of the Mississippi biotype, Reunion, compared with a susceptible P. annua biotype; 2) to determine whether resistance is the result of
to a target-site mutation in the ALS gene; and
3) to determine whether resistance to
ALS inhibitors in the resistant Reunion biotype is associated with any fitness penalty.

**Materials and Methods**

**Plant material.** Research was conducted using the resistant *P. annua* biotype Reunion collected from the Reunion Golf and Country Club, Madison, MS. A susceptible biotype collected in Starkville, MS, was used as a standard comparison. Seeds of each biotype were sown in 15-cm-diameter polypropylene containers with a commercial potting mix (Sunshine Mix #1; Sun-Gro Horticulture, Bellevue, WA), followed by incubation in a growth chamber with day and night temperatures of 24 and 19 °C, respectively. Light intensity was 21 μmol·m–2·s–1 with a 16-h photoperiod. Plants matured for ≈12 weeks until they were screened for resistance using 29 and 58 g foramsulfuron/ha (Revolver Herbicide; Bayer Environmental Science, Research Triangle Park, NC), equivalent to one and two times the rates, respectively. Eight potted replicates of each biotype were randomly assigned a 29 or 58 g a.i./ha foramsulfuron herbicide treatment. Plants were allowed to flower and produce seed in preparation for the greenhouse rate–response trial. The seeds were collected, dried, and stored at 4 °C for future testing.

**Rate–response trial.** Two experiments were conducted during 2015. Seeds collected from a previous screening experiment were lightly scarified and planted in 10-cm-diameter plastic pots containing a Marietta silt loam (fine-loamy, siliceous, active; Fluvaquentic Eutrudepts) with pH 6.2 (1:1 soil/H2O) amended with 10% sand and 10% silt loam (fine-loamy, siliceous, active; Flandraivept) in a water carrier volume of 280 L·ha–1. Plants were allowed to dry for 30 min before being returned to the greenhouse. Irrigation was withheld for 24 h. Pots were rerandomized twice weekly to account for variations in greenhouse microclimate.

Percent visual necrosis (PVN) was recorded 4 weeks after treatment (WAT). PVN ratings were based on a 0 to 100% scale, with 0% corresponding to no visible necrosis and 100% corresponding to complete plant necrosis. Data were analyzed by rating date using a log-logistic regression technique described by Seeffeldt et al. (1995), where the 0-kg·ha–1 herbicide rate was assigned artificially at the 0.0001-kg·ha–1 rate. The analysis was conducted with Prism® (GraphPad Software, La Jolla, CA) using the model:

\[ y = C + \frac{D - C}{1 + \left(\frac{X}{I_{50}}\right)^b} \]

where \( y \) is the response (PVN) at herbicide dose \( X \), \( D \) is the upper limit for \( y \), \( C \) is the lower limit for \( y \), \( b \) is the slope of the line at \( I_{50} \), and \( I_{50} \) is the rate of herbicide giving a 50% response (50% visual necrosis). Best-fit parameters were determined by allowing the four parameters (\( D, C, b, \) and \( I_{50} \)) to differ for each biotype. The slope and \( I_{50} \) best-fit parameter estimates were compared between cultivars using an F test analysis of variance with a significance level of 0.05 (Seeffeldt et al., 1995). The tolerance level of biotypes was compared using \( I_{50} \) values (Streibig, 1988).

**Target-site gene sequencing.** Leaf tissue from five plants of each of the Reunion and susceptible biotypes was harvested at the two- to three-tiller stage, frozen in liquid nitrogen, and stored at –80 °C until DNA extraction was performed. Tissue was ground to a fine powder using a mortar and pestle containing liquid nitrogen, and DNA isolation was conducted using a modified hexadeyltrimethylammonium bromide protocol (Dole and Doyle, 1990). The DNA pellet was dissolved in TE buffer (Tris-ethylenediaminetetraacetic acid buffer), and a NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used for DNA quantification before polymerase chain reaction (PCR) amplification of the potential ALS mutation site. PCR was performed using a MyCycler thermal cycler (Bio-Rad, Richmond, CA) in a 25 μL-volume containing LongAmp Taq 2× Master Mix (New England Biolabs Inc., Beverly, MA) (125 U/μL Taq DNA polymerase, 0.3 mM dNTPs), 0.1 μM forward primer (5'-TGG GCG GCT CAG TAT TAC AC-3'), 0.1 μM reverse primer (5'-ATA AGC ACA TGC TCC TG-3') (Yu et al., 2008), 150 ng DNA, and nuclease-free water. The thermocycler protocol started with denaturation at 94 °C for 4 min, followed by 37 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The final extension step continued for 5 min at 72 °C. After amplification, gel electrophoresis was used to confirm success of the PCR amplification and to verify the size of the product. The correct DNA band was then excised from the agarose gel and purified using Wizard SV Gel and the PCR Clean Up Kit (Promega, Madison, WI), and was sequenced (Eurofins MWG Operon, Huntsville, AL).

**Investigation of plant fitness costs associated with ALS gene mutations.** Five seeds each of the resistant biotype (Reunion) and susceptible biotype were placed on a 9 × 9-cm2 petri dish lined with filter paper, moistened with 5 mL distilled water, and placed in a growth chamber with a 16-h photoperiod, 21 μmol·m–2·s–1 light intensity, and day/night temperatures of 24 and 20 °C, respectively. After 1 week of incubation, germinated seedlings from each biotype were transplanted to five 10-cm-diameter pots filled with Sunshine Mix #1 potting soil (Sun Gro Horticulture Inc.), and Miracle-Gro® was supplied weekly. Shoot length and number of tillers were recorded every other day from 7 d after transplanting (DAT) until 25 DAT. Days to flowering were measured. Seed heads were harvested at maturity, and seeds were weighed to determine seed yield. The experiment was conducted in a completely randomized block design with two runs. Statistical analyses were performed using PROC GLM of SAS 9.4 (SAS Institute, Cary, NC) and means were separated by Fisher’s protected least significant difference test at the 0.05 P level.

**Results**

**Greenhouse rate–response trial.** The rate–response trials confirmed foramsulfuron resistance in the Reunion bio Data were similar across experimental timings; therefore, results were pooled for subsequent analysis. A significant biotype-by-herbicide interaction was observed. Resistance parameter estimates were compared between cultivars using an F test analysis of variance with a significance level of 0.05 (Seeffeldt et al., 1995). The tolerance level of biotypes was compared using \( I_{50} \) values (Streibig, 1988).

**Table 1.** Foramsulfuron rates applied to *Poa annua* during rate–response screenings.

| Rate (g a.i./ha) | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 |
|-----------------|------|-----|---|---|---|----|-----|----|
| Times rate | g a.i./ha | 7 | 15 | 29 | 58 | 116 | 232 | 464 | 928 |

A one-time rate was based on label recommendations for a *P. annua* control in field conditions.

**Table 2.** Best-fit regression parameters ±95% confidence intervals of *Poa annua* biotypes describing percent visual necrosis response to foramsulfuron.

| Biotype | \( I_{50} \) | Lower | Upper | Resistant factor | \( R^2 \) |
|--------|--------|------|------|-----------------|-------|
| Susceptible | 7.2 | 5.9 | 8.8 | 0.726 | 0.726 |
| Reunion | 335.1 | 159.1 | 705.9 | 46.0 | 0.815 |

\( I_{50} \) = the rate of herbicide giving a 50% response (50% visual necrosis).
rate interaction \((P < 0.001)\) was detected, indicating biotypes responded differently to foramsulfuron rates. Both biotypes showed increased injury with increasing herbicide rate. The greatest PVN was observed 4 WAT, and no recovery occurred before 4 WAT. However, no rate of foramsulfuron completely controlled all experimental units of the Reunion biotype (Table 2). \(I_{50}\) estimates from PVN (Fig. 1) indicate the Reunion biotype to be at least 45 times more resistant to foramsulfuron than the standard susceptible biotype, requiring a predicted 331 g foramsulfuron/ha for 50% control. The labeled rate of foramsulfuron is 29 g a.i./ha. The susceptible biotype required a predicted 7.2 g foramsulfuron/ha to achieve 50% control.

**Target-site gene sequencing.** The region around amino acid position 574 on the ALS gene was amplified to determine whether there was target-site mutation. The nucleotide sequence for the wild-type susceptible biotype was TGG, which encodes tryptophan (Fig. 2). The Reunion biotype, however, contained the sequence TTG, a missense mutation, requiring a predicted 331 g foramsulfuron/ha for 50% control. The labeled rate of foramsulfuron is 29 g a.i./ha. The susceptible biotype required a predicted 7.2 g foramsulfuron/ha to achieve 50% control.

Herbicide resistance is a global problem and annual bluegrass is reported to have the greatest resistance issues of weeds in turfgrass systems. The Reunion biotype collected from the Reunion Golf and Country Club in Madison, MS, in 2014 was suspected of being resistant to ALS herbicides, and rate–response trails confirmed this hypothesis. Target-site gene sequencing revealed a TGG-to-TTG mutation in Reunion, and this specific mutation has been identified as a mutation conferring resistance to ALS inhibitors not only in *P. annua*, but in other species as well (McElroy et al., 2013; Tranel and Wright, 2002). Patzoldt et al. (2001) observed the Trp 574-to-Leu mutation in the ALS enzyme gene of *Amaranthus powellii*, roots and shoots of susceptible biotypes were four times thicker than the resistant biotype (Tardif et al., 2006). This difference in height and thickness between resistant and susceptible plants is attributed to the slower and reduced differentiation of primary and secondary tissues in resistant plants. In our study, susceptible plants produced five tillers whereas Reunion produced two to three tillers at 23 DAT. Herbicide resistance has an effect on tiller formation in certain weed species. Dinitroaniline and ACCase inhibitor-resistant *Setaria viridis* plants showed a greater number of tillers than susceptible plants (Darmency et al., 1994, 1995). In *Amaranthus powellii*, roots and shoots of susceptible biotypes were four times thicker than the resistant biotype (Tardif et al., 2006). This difference in height and thickness between resistant and susceptible plants is attributed to the slower and reduced differentiation of primary and secondary tissues in resistant plants. In our study, susceptible plants produced five tillers whereas Reunion produced two to three tillers at 23 DAT. Herbicide resistance has an effect on tiller formation in certain weed species. Dinitroaniline and ACCase inhibitor-resistant *Setaria viridis* plants showed a greater number of tillers than susceptible plants (Darmency et al., 2009) studied nontarget-site mechanisms in annual ryegrass (*Lolium rigidum*) that conferred resistance to glyphosate-, ACCase-, and ALS-inhibiting herbicides. They discovered that a reduced translocation of glyphosate led to glyphosate resistance. Gardin et al. (2015) identified five P450 cytochromes that could possibly confer resistance to ALS inhibitors in black-grass (*Alopecurus myosuroides*), an annual weed. However, target-site mutations are the most common cause of ALS inhibitor herbicide resistance, and, according to Tranel and Wright (2002), five amino acid mutations could confer resistance to ALS-inhibiting herbicides in numerous species. Point mutations at Ala122, Pro197, Ala205, Trp574, and Ser653 are found in weeds of many resistant biotypes (Corbett, 2004; Saari et al., 1994).

These mutations associated with resistance generally have minor effects on growth and development of resistant plants (Holt and Thill, 1994; Thompson et al., 1994). In our study, there was no difference in plant height between the resistant Reunion and the susceptible biotypes at 23 DAT. This is in contrast to studies that generally relate resistance alleles to adverse effects on hypocotyl length and plant height (Timpte et al., 1994, 1995). In *Amaranthus powellii*, roots and shoots of susceptible biotypes were four times thicker than the resistant biotype (Tardif et al., 2006). This difference in height and thickness between resistant and susceptible plants is attributed to the slower and reduced differentiation of primary and secondary tissues in resistant plants. In our study, susceptible plants produced five tillers whereas Reunion produced two to three tillers at 23 DAT. Herbicide resistance has an effect on tiller formation in certain weed species. Dinitroaniline and ACCase inhibitor-resistant *Setaria viridis* plants showed a greater number of tillers than susceptible plants (Darmency et al., 2011). However, a competitive study between susceptible and resistant *Brachypodium hybridum* to photosystem II inhibitors.

![Fig. 1. Herbicide rate resulting in 50% visual injury of Poa annua 4 weeks after treatment (WAT). Based on 95% confidence intervals predicted by log-logistic regression.](image1)

![Fig. 2. Chromatogram of DNA sequencing results from each of the Poa annua biotypes tested. The nucleotides coding the position of interest (position 574) on the acetylactate synthase enzyme are boxed in the figure. The respective sequences and accompanying amino acids are enlarged at the top of the figure.](image2)
plants have greater chances of getting
Late flowering has a disadvantage because
DAT) and produced a lower yield (47.5
fecting seed yield negatively.
will ultimately lead to the production of
species might have no fitness cost associated
with herbicide resistance, as often hypothesized
and reported (Frenkel et al., 2017; Panozzo
et al., 2017; Yannicci, et al., 2016). Some
species might have no fitness cost associated
with herbicide resistance; other species have
decreased competitiveness compared with their
susceptible biotypes (Frenkel et al., 2017). All
fitness parameters of the resistant
biotype—seed yield, number of tillers, plant
height, and flowering time—were inferior
compared with the susceptible biotype in this
study. Future research into P. annua control
should include determination of a nonherbi-
cidal means of control, because this weed
develops resistance rapidly to multiple her-
bicide modes of action compared with other
species. Although the Reunion biotype was
shown to have a target-site mutation, addi-
tional testing is required to determine whether
additional target-site mutations or nontarget-
site mechanisms of resistance occurs.
Further research on the mechanism of
herbicide resistance in resistant bio-
types could include determining uptake,
translocation, and metabolism of herbicide
with 14C-labeled experiments using a liquid
scintillation counter. A whole-plant assay to
assess the fitness penalty in ALS inhibitor-
resistant plants using parameters such as seed
yield, photosynthetic efficiency, and root
characterization will provide a comprehen-
spicious picture of the reduced efficiency in the
resistant P. annua biotype.

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