Research Article

Received: 29 October 2019 Revised: 2 January 2020 Accepted article published: 11 January 2020 Published online in Wiley Online Library: 5 February 2020

(wileyonlinelibrary.com) DOI 10.1002/ps.5740

Annual bluegrass (*Poa annua*) resistance to indaziflam applied early-postemergence

James T Brosnan,[a*] José J Vargas,[a] Bruce Spesard,[b] Derek Netzband,[c] John M Zobel,[d] Jinyi Chen[e] and Eric L Patterson[e]

Abstract

BACKGROUND: Indaziflam is an alkylazine herbicide used to control annual bluegrass (*Poa annua* L.). Several locations in the southeastern USA reported poor annual bluegrass control following treatment with indaziflam during autumn 2015. A series of controlled environment experiments were conducted to confirm putative resistance to indaziflam in annual bluegrass collected from these field locations.

RESULTS: Indaziflam (25 g ha^{-1}) effectively controlled all putative-resistant annual bluegrass collections when applied pre-emergence (PRE), but was ineffective when applied early-postemergence (< 2.5 cm plant height; BBCH scale = 1; EPOST). In agarose-based plate assays, EPOST I_{50} values for putative-resistant collections ranged from 2424 to 4305 pM compared with 633 pM for the herbicide-susceptible control; therefore, resistance indexes (R/S) ranged from 3.8 to 6.8. Resistant collections were not controlled by foramsulfuron, flumioxazin, glyphosate, glufosinate, metribuzin, pronamide, or simazine applied EPOST. Indaziflam content in herbicide-susceptible annual bluegrass was greater than a resistant collection from 0 to 10 days after treatment (DAT). Susceptibility was not restored when resistant collections were treated with indaziflam plus 1-aminobenzotriazole (10 mg L^{-1}), tebuconazole (1510 g ha^{-1}), or malathion (400 g ha^{-1}).

CONCLUSIONS: This is a first report of resistance to indaziflam in any plant. Additionally, we confirm that these annual bluegrass collections are resistant to several other herbicidal modes-of-action. It is unclear if this multi-herbicide resistance is due to a single resistance gene, multiple resistance genes, non-target site mechanisms, or a combination thereof. Additional research to better understand resistance mechanisms in these annual bluegrass collections is warranted.

Keywords: annual bluegrass; cellulose biosynthesis; indaziflam; non-target site resistance (NTSR); resistance; turfgrass

1 INTRODUCTION

Indaziflam is an alkylazine herbicide used for preemergence (PRE) control of annual grass and broadleaf weeds.[1] In managed turfgrass systems (i.e. golf courses, lawns, etc.) indaziflam has been shown to effectively control annual weeds such as crabgrass[2] (*Digitaria* spp.), goosegrass[3] (*Eleusine indica* L. Gaertn.), and annual bluegrass[4] (*Poa annua* L.) when applied PRE at rates of 30 to 70 g ha^{-1}. Additionally, efficacy for controlling juvenile annual bluegrass plants with early-postemergence (EPOST) applications of indaziflam has been reported.[4,5] Utility of indaziflam for weed management extends beyond managed turfgrass. For example, in rangeland systems, Sebastian *et al.*[6] reported that indaziflam was an effective option for controlling invasive winter annual grasses such as downy brome (*Bromus tectorum* L.), feral rye (*Secale cereale* L.), and Japanese brome (*Bromus japonicus* Thunb), allowing for increases in biomass and species richness 2 years after treatment. Other studies have reported similar findings with indaziflam in rangeland systems.[7–10]

Although cellulose biosynthesis inhibition is not a new mode of action, the mechanisms by which indaziflam controls susceptible weeds differ from other cellulose biosynthesis-inhibiting herbicides (CBI) such as isoxaben or dichlobenil. Brabham *et al.*[11] demonstrated that root tissue of indaziflam-treated annual bluegrass and *Arabidopsis thaliana* exhibited radial swelling and abnormal lignification consistent with CBI treatment. However, the researchers confirmed that isoxaben-resistant *Arabidopsis thaliana* mutants were not cross-resistant to indaziflam; they suggested that indaziflam may have a different molecular target than isoxaben which ultimately causes differential effects on the velocity of cellulose synthase proteins clearing the plasma membrane in treated plants.

Annual bluegrass populations have evolved resistance to nine different sites of action, third most of all weed species.[12] Indaziflam is an effective option for controlling many herbicide-resistant

* Correspondence to: JT Brosnan, Department of Plant Sciences, University of Tennessee, 2505 EJ Chapman Drive, Knoxville, TN 37996, USA. E-mail: jbrosnan@utk.edu

a Department of Plant Sciences, University of Tennessee, Knoxville, TN, USA

b Bayer Crop Science Division, Cary, NC, USA

c Bayer Crop Science Division, Chesterfield, MO, USA

d Department of Forest Resources, University of Minnesota, St. Paul, MN, USA

e Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI, USA

annual bluegrass populations. For example, PRE applications of indaziflam at 52.5 to 70 g ha^{-1} controlled dinitroaniline-resistant annual bluegrass 88–97% rated 180 days after treatment (DAT).[13] Similar responses have been reported following PRE applications of indaziflam to control annual bluegrass with resistance to acetolactate synthase (ALS) and photosystem II (PSII) inhibiting herbicides.[14,15]

It is important to note that most cases of herbicide resistance in annual bluegrass populations of turfgrass have been target-site based. To date, there is only one published report of annual bluegrass surviving PSII-inhibiting herbicide treatment via non-target site resistance mechanisms.[16] The researchers in this report evaluated several populations of annual bluegrass with resistance to PSII-inhibiting herbicides and identified a population from Oregon (DR3) that survived treatment with diuron, atrazine, and amicarbazone similar to those with a Ser264→Gly substitution on the D1 protein targeted by PSII-inhibiting herbicides. Further experiments elucidated that this biotype from Oregon exhibited reduced atrazine absorption and translocation compared to those with target site mutations, along with greater metabolism.

Several locations in the southeastern USA reported poor annual bluegrass control following treatment with indaziflam at 32 to 50 g ha^{-1} during the autumn of 2015, as well as treatment with foramsulfuron at 29 g ha^{-1} during spring 2016. A series of controlled environment experiments were conducted from 2016 to 2019 to confirm putative resistance to indaziflam in annual bluegrass collected from these field locations.

2 MATERIALS AND METHODS

2.1 Plant collection and seed increase

Annual bluegrass collections from five locations in the southeastern USA were sent to the University of Tennessee Weed Diagnostics Center (WDC; Knoxville, TN, USA) for analysis from 28 March to 15 April 2016. Two collections (Site 2 and Site 11) were obtained from golf course fairways after surviving treatment with indaziflam (Specticle FLO; Bayer Environmental Science, Cary, NC, USA) in autumn 2015 as well as foramsulfuron (Revolver; Bayer Environmental Science) during spring 2016. The Site 2 collection had infested a hybrid bermudagrass (*Cynodon dactylon (L.) Pers. x Cynodon transvaalensis Burtt Davy*, cv. 'Tifway') golf course fairway in Kingwood, TX. The Site 11 collection was obtained from a zoysiagrass (*Zoysia japonica* Steud, cv. 'Meyer') golf course fairway in Memphis, TN. Two collections were obtained from golf course roughs (Site 3A) and fairways (Site 3B) established with Tifway hybrid bermudagrass in Cumming, GA. These collections survived treatment with indaziflam in autumn 2015 and had putative resistance to PSII-inhibiting herbicides. The final collection, Site 5, was obtained from a residential lawn in Wake Forest, NC that received no herbicide applications from 2005 to 2017. This was included as a negative control.

Whole plants from each location were harvested using a golf course cup cutter (108 mm diameter). Upon receipt at the WDC, plants were labeled with a unique identifier, washed free of soil, and transplanted into greenhouse pots filled with a peat-moss-based growing medium (PRO MIX BX General Purpose; Premier Tech Horticulture, Quakertown, PA, USA). After transplanting, annual bluegrass was cultured in a glasshouse at the University of Tennessee to generate seed for experimentation. Culture included supplying irrigation via an overhead misting system as well as applying nutrients with a complete fertilizer (20 N–20 P_2O_5-20 K_2O; Peter's 20–20–20. JR PETERS, Inc., Allentown, PA, USA) at 49 kg N ha^{-1} every 14 days. Insecticide (a commercial mixture of bifenthrin, zeta-cypermethrin, imidacloprid, and beta cyfluthrin) was applied at label rate on an as-needed basis to manage insect pests while plants were being propagated for seed collection. Scissors were used to remove panicles from each plant once spikelets exhibited a straw color. Harvested panicles were dried in a forced-air oven at 35 °C and screened using a 0.76 mm sieve (WS Tyler Company, Mentor, OH, USA). Seeds passing through the sieve were collected in a high-density polyethylene bottle (Nalgene; Thermo-Fisher Scientific, St. Louis, MO, USA) and stored at 0 °C.

2.2 Responses to indaziflam applied PRE and EPOST

Separate growth chamber experiments were conducted to evaluate annual bluegrass response to indaziflam applied PRE as well as EPOST (BBCH scale = 1). Annual bluegrass collections in these experiments included those submitted to the WDC (Site 2, Site 3A, Site 3B, Site 5, and Site 11) as well as a herbicide-susceptible control (S1) native to University Park, PA. This herbicide-susceptible control had no history of exposure to indaziflam or ALS-inhibiting herbicides used for annual bluegrass control.

Annual bluegrass was established in greenhouse trays containing 98 cells (26.2 cm^3 in volume) filled with Sequatchie silt loam soil (fine-loamy, siliceous, semiactive, thermic humic Hapludult; pH 6.0, organic matter 2.1%) local to Knoxville, TN. Trays were surface-seeded by uniformly distributing 4 g of germplasm across the surface, ensuring that each cell contained a minimum of one seed. Collections were established in separate trays effectively creating 98 experimental units per collection. Trays were placed inside a walk-in growth chamber set to a constant temperature of 16 °C, configured to provide a 16 : 8 h light/dark photoperiod at a photosynthetic photon flux density of 512 µmol m^{-2} s^{-1}. Sub-irrigation was used to provide sufficient moisture for annual bluegrass seed germination and emergence.

Indaziflam (Specticle FLO; Bayer Environmental Science) was applied PRE on 5 August 2016 at 25 g ha^{-1} inside an enclosed spray chamber (Generation III Research Sprayer; DeVries Manufacturing, Hollandale, MN, USA) calibrated to deliver 215 L ha^{-1} via a single flat-fan nozzle (8004EVS. TeeJet; Spraying Systems Co., Wheaton, IL, USA). An overhead misting system was used to activate the herbicide during the initial 12 h after treatment. Each treated tray was paired with an accompanying non-treated tray for comparison. Percent survival data were collected 56 DAT by tabulating the number of cells within each treated tray that contained emerged annual bluegrass plants.

Indaziflam (25 g ha^{-1}) was applied EPOST when cells contained emerged annual bluegrass with at least one true leaf and no tillers; overall plant height at application measured < 2.5 cm (BBCH scale = 1). Cells devoid of annual bluegrass that conformed to these parameters were marked and not included in assessments of percent survival. The first collection to meet the criteria for EPOST application was treated on 19 August 2016 with the final collection treated on 7 September 2016. Percent survival was assessed on 6 October 2016 after herbicide application by tabulating the number of cells within each treated tray that contained living annual bluegrass plants.

Fisher's exact test ($\alpha = 0.05$) was conducted using the 'fisher.test' function in R (v. 3.5.1)[17] to determine if the relationships in survival following indaziflam applied PRE and EPOST were consistent across collections in this experiment.

2.3 Dose–response experiments

Dose–response experiments were conducted at Michigan State University (East Lansing, MI, USA) during 2019 to better understand the response of these putative-resistant annual bluegrass collections (Site 3A, Site 3B, Site 11) and the herbicide-susceptible population S1, to increasing doses of indaziflam. Seeds from each collection were imbibed on moistened filter papers at 22 °C. Differences in time to germination among collections were normalized by pre-germinating seeds from Site 3B, Site 11, Site 3A, and S1 for 8, 6, 6, and 5 days, respectively. Seedlings were transplanted onto the surface of 1.5% agar (Acumedia 7558A, Neogen® culture media; Neogen Food Safety, Lansing, MI, USA) mixed with different concentrations of technical grade indaziflam (99.6% from Bayer Environmental Science). The S1 annual bluegrass collection was treated with indaziflam at 0, 100, 200, 400, 800, and 1600 pM, whereas all three putative-resistant collections were treated with indaziflam at 0, 400, 800, 1600, 3200, and 6400 pM. Agar-filled plates were sealed with Parafilm®, and vertically placed in a growth chamber with a 12 : 12 h light/dark photoperiod and photon flux density of 600 μmol m^{-2} s^{-1}. Each agar plate contained ten seedlings of each collection and there were three plates per each indaziflam concentration.

Root length (the distance from the apical meristem to the bottom of the longest root) was measured 7 DAT with a ruler. The length of each treated seedling was divided by the average length of corresponding plants grown on agar containing 0 pM indaziflam. The indaziflam rate causing 50% inhibition (I_{50}) of plants root growth was calculated using SigmaPlot® (version 13.0, 2014; Systat Software, Inc., San Jose, CA USA) software with non-linear regression analysis. The data were fit to a three-parameter logistic model:

$$y = \frac{D}{1 + (x/x0)^b}$$

where D was the upper limit representing plant root length at low herbicide rates close to non-treated controls, x_0 is the I_{50} and b is the slope around x_0. The resistance index was calculated by dividing the I_{50} of the three resistant collections by the I_{50} of the susceptible (S1) collection. Significant differences in the estimated I_{50} values between collections were determined via t-test using Prism (version 5.0; GraphPad Software, Inc., San Diego, CA, USA).

2.4 Responses to other modes of action

Separate growth chamber experiments were conducted to evaluate the efficacy of several modes of action for PRE and EPOST control of annual bluegrass with resistance to indaziflam. Experimental conditions were identical to those described previously. Resistant (Site 3A, Site 3B, and Site 11) and herbicide-susceptible (S1) collections of annual bluegrass were established in 98-cell trays filled with Sequatchie silt loam and placed inside a walk-in growth chamber set to 16 °C and a 16 : 8 h light/dark photoperiod. Trays were surface seeded on 7 September 2017. Collections were established in separate trays effectively creating 98 experimental units per collection.

PRE experiments were initiated 24 h after seeding by applying the following herbicide treatments: prodiamine (Barricade; Syngenta Professional Products, Greensboro, NC, USA) at 1680 g ha^{-1}; oxadiazon (Ronstar FLO, Bayer Environmental Sciences) at 3360 g ha^{-1}; pronamide (Kerb SC; Dow AgroSciences, Indianapolis, IN, USA) at 1150 g ha^{-1}; *S*-metolachlor (Pennant Magnum; Syngenta Professional Products) at 2800 g ha^{-1}; and indaziflam at 50 g ha^{-1}. A non-treated tray of each collection was established for comparison. These treatments were selected to explore plant response to an array of mode-of-action groups labeled for PRE control of annual bluegrass in turf; rates selected represented label maximums for a single application.

EPOST experiments evaluated response of the same resistant and susceptible collections to the following herbicides: simazine (Princep; Syngenta Professional Products) at 1120 g ha^{-1}; pronamide (Kerb SC; Dow AgroSciences) at 1150 g ha^{-1}; glyphosate (Roundup Pro; Bayer Environmental Science) at 1120 g ha^{-1}; glufosinate (Finale; BASF Corp., Research Triangle Park, NC, USA) at 1680 g ha^{-1}; metribuzin (Sencor; Bayer Environmental Science) at 557 g ha^{-1}; foramsulfuron (Revolver; Bayer Environmental Science) at 29 g ha^{-1}; flumioxazin (SureGuard; NuFarm Americas, Alsip, IL, USA) at 430 g ha^{-1}; and indaziflam at 50 g ha^{-1}. Metribuzin, flumioxazin, and simazine were mixed with non-ionic surfactant (Activator-90; Loveland Products, Greeley, CO, USA) at 0.25%. Non-treated control trays of each collection were established within the chamber for comparison. These treatments were selected to explore plant response to an array of mode-of-action groups labeled for EPOST control of annual bluegrass in turf; rates selected represented label maximums for a single application. EPOST experiments were initiated when plants in each cell measured < 2.5 cm in height and contained at least one true leaf (BBCH scale = 1). Cells devoid of annual bluegrass that conformed to these parameters were marked and not included in assessments of percent survival after herbicide application. The first collection to meet benchmark requirements for EPOST application was treated on 16 October 2017 with the final collection treated on 19 October 2017.

In both PRE and EPOST experiments, herbicide was applied in a previously described spray chamber using a single flat fan nozzle. Percent survival data were collected 42 DAT in the PRE experiment by tabulating the number of cells within each treated tray that contained emerged annual bluegrass plants. The same assessment was made in the EPOST experiment 31 DAT. Fisher's exact test ($\alpha = 0.05$) was performed using the 'fisher.test' function in R to compare effects of PRE herbicides tested on each collection.

2.5 Exploring non-target site resistance

2.5.1 Responses to indaziflam applied with inhibitors of cytochrome P450

Chemicals that synergize with herbicides and target cytochrome P450 monooxygenase(s) have been used to elucidate metabolism-based herbicide resistance in weeds.[18] To that end, a growth chamber experiment was conducted evaluating the efficacy of indaziflam applied EPOST with several synergists known to target cytochrome P450 monooxygenases. Experimental conditions were the same as those previously described with resistant annual bluegrass (Site 3A, Site 3B, and Site 11) established in 98-cell trays filled with Sequatchie silt loam. A herbicide-susceptible collection (S1) was established similarly for comparison. Collections were established in separate trays effectively creating 98 experimental units per collection.

Trays were treated with herbicides and synergists at an EPOST timing when cells contained annual bluegrass measuring < 2.5 cm in height that had at least one true leaf (BBCH scale = 1). Treatments included the following: indaziflam at 25 g ha^{-1}; indaziflam (25 g ha^{-1}) plus 1-aminobenzotriazole (ABT; 10 mg L^{-1}); indaziflam (25 g ha^{-1}) plus tebuconazole (1510 g ha^{-1}); and

Table 1. Mass spectrometry parameters for detecting indaziflam and 1-fluoroethyl triazinediamine (FDAT; the primary metabolite of indaziflam) content in tissues of herbicide-susceptible and -resistant annual bluegrass (*Poa annua* L.)

Analyte	Molecular ion (amu)	Base peak (amu)	Scan time (s)	Collision energy (keV)	Retention time (min)
Indaziflam	302.21	138.09	0.20	29	4.4
Indaziflam-$^{15}N_4$	306.34	142.06	0.20	29	4.4
1-Fluoroethyl triazinediamine	158.16	138.16	0.20	16	2.4
1-Fluoroethyl triazinediamine-$^{15}N_5$-$^{13}C_2$	165.09	145.16	0.20	16	2.4

Isotropic standards were used to detecting indaziflam (indaziflam-$^{15}N_4$) and FDAT (1-fluoroethyl triazinediamine-$^{15}N_5$-$^{13}C_2$) in each sample.

indaziflam (25 g ha^{-1}) plus malathion (400 g ha^{-1}). A non-treated control tray of each collection was also established in the chamber for comparison. All herbicides and synergists were applied as foliar sprays using a previously described spray chamber except ABT; a stock solution of ABT was prepared at 262 mg L^{-1} with 1 mL of this solution delivered to the soil surface of each cell using a pipette. Herbicide was applied immediately following synergist application with percent survival data collected 15 DAT. Fisher's exact test ($\alpha = 0.05$) was performed using the 'fisher.test' function in R to compare effects of applied treatments (i.e., indaziflam and indaziflam + synergists) on each collection.

2.5.2 *Metabolism of indaziflam in annual bluegrass*

A growth chamber experiment was conducted to quantify indaziflam metabolism in a herbicide-susceptible (S1) and -resistant collection (Site 3B) of annual bluegrass. Collections were seeded in 98-cell trays filled with Sequatchie silt loam soil on 25 July 2018 and placed inside a walk-in growth chamber configured to maintain a constant temperature of 16 °C and a 16 : 8 h light/dark photoperiod. Trays were sub-irrigated to facilitate seed germination and emergence. Indaziflam (25 g ha^{-1}) was applied EPOST on 13 August 2018 using the previously described spray chamber. Within each tray, cells devoid of annual bluegrass with the morphological parameters for EPOST treatment (i.e., < 2.5 cm height, minimum of one true leaf, no tillers; BBCH scale = 1) were marked and excluded from analysis. Aboveground biomass (i.e., leaf + stem tissue) of each collection was harvested with sterilized scissors at the soil line 0, 0.5, 1, 2, 5, and 10 days after herbicide treatment. After harvesting, biomass was flash-frozen with liquid N and stored at −80 °C for analysis.

Indaziflam and 1-fluoroethyl triazinediamine (FDAT, primary metabolite of indaziflam) content in harvested tissues were quantified using high-performance liquid chromatography (HPLC)-electrospray ionization/tandem mass spectrometry (LC–MS/MS). Harvested annual bluegrass tissue from each time point was blended in a mixture of acetonitrile and water (4 : 1 ratio) for 3 min. This mixture was then filtered into a 100 mL mixing cylinder. This process was repeated and filtered material was placed into the same 100 mL mixing cylinder where combined filtrates were amended with a mixture of isotropic standards of indaziflam-$^{15}N_4$ and 1-fluoroethyl-triazinediamine-$^{15}N_5$-$^{13}C_2$ (Bayer CropScience, St. Louis, MO, USA). A 1.25 mL aliquot of this combined sample was passed through an octadecyl solid-phase extraction cartridge (C_{18} SPE, 50 mg, Varian Bond Elute; Agilent Technology. Santa Clara, CA, USA). Eluate from the cartridge was evaporated with sample residue reconstituted in a solution of methanol (0.100 mL) and 10 mM ammonium bicarbonate (0.900 mL), mixed well, and transferred into a HPLC vial for analysis via LC–MS/MS. The samples were analyzed for indaziflam and FDAT by LC–MS/MS with quantification based on a comparison of peak areas with those of known standards.

The LC–MS/MS method was developed on a ThermoFinnigan Surveyor mass spectrometer with a Thermo LC auto-sampler (Thermo-Fisher Scientific, Waltham, MA, USA); the instrument was equipped with a 5 µm particle size HPLC column that measured 50 × 2.0 mm (Phenomenex, Torrance, CA, USA) and used in a column oven set at 40 °C. A 20 µL injection loop was used which was overfilled. A gradient program was used, at injection, with the mobile phase consisting of 100% methanol/10 mM ammonium bicarbonate (2 : 98 v/v) switching to 100% methanol after 5.5 min at a flow rate of 250 µL min^{-1}.

MS scanning was conducted in single reaction monitoring (positive ion) mode at a capillary temperature of 380 °C and spray voltage of 4200 V. Nitrogen was used as the sheath (80 psi),

Table 2. Percent survival of herbicide-susceptible and putative-resistant annual bluegrass (*Poa annua* L.) following treatment with indaziflam (25 g ha^{-1}) at preemergence (PRE) and early-postemergence (EPOST) timings in growth chamber experiments

Collection[a,b]	Survival (%) Indaziflam (25 g ha^{-1}) PRE	EPOST
Site 2	4	0
Site 3A	1	70
Site 3B	0	78
Site 5	0	0
Site 11	0	19
S1	0	0

Percent survival for each collection was calculated after treating 98 individual plants with indaziflam and tabulating those containing living annual bluegrass 56 days after PRE treatment and 28 days after EPOST treatment.

[a] Collections were submitted to the University of Tennessee Weed Diagnostics Center (WDC) after surviving treatment with indaziflam in autumn 2015 as well as foramsulfuron during spring 2016. One collection (Site 5) was submitted to the WDC after being collected from an area with no herbicide use history. S1 is a herbicide-susceptible collection native to University Park, PA with no history of exposure to indaziflam.

[b] Fisher's exact test determined that the relationships in percent survival for the PRE and EPOST timings were not consistent across collections ($P < 0.0001$).

Figure 1. Symptoms of early-postemergence (EPOST) indaziflam treatment on annual bluegrass (*Poa annua*) with resistance to indaziflam. Note that the bud leaf appears unaffected by the herbicide, whereas the secondary and tertiary leaves show symptoms of herbicide treatment.

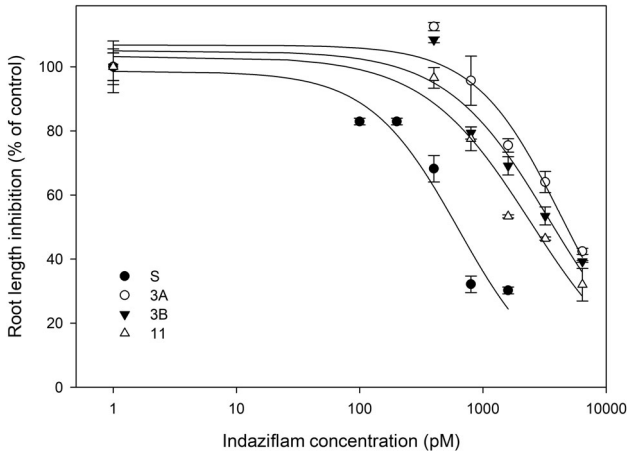

Figure 2. Dose–response curves of herbicide-susceptible (S1: ●) and three putative-resistant annual bluegrass (*Poa annua* L.) collections (Site 3A: ○; Site 3B: ▼; Site 11: Δ) 7 days after indaziflam treatment in agar culture. Each data point represents the mean ± standard error of three replicates in a single dose–response experiment.

auxiliary (8 mL min^{-1}), and sweep gas (5 mL min^{-1}). Collision gas pressure was 1.7 mTorr. HPLC effluent entered the mass spectrometer interface from 1 to 6.25 min. MS parameters for detecting indaziflam and FDAT are presented in Table 1.

Indaziflam content (ng g^{-1} fresh weight) in annual bluegrass tissues of a herbicide-susceptible (S1) and -resistant collection (Site 3B) from 0 to 10 DAT were fit to a lognormal Gaussian model in Prism (version 8.2. GraphPad Prism, La Jolla, CA, USA). The primary metabolite of indaziflam, FDAT, was quantified over the same period using identical methodology and fit to a quadratic model. Responses of S1 and Site 3B annual bluegrass were compared using a global sums-of-squares *F*-test at $\alpha = 0.05$. Overall recovery of indaziflam and FDAT in sampled tissues ranged from 92% to 95%.

3 RESULTS

3.1 Responses to indaziflam applied PRE and EPOST

Survival following PRE and EPOST treatment with indaziflam (25 g ha^{-1}) in growth chamber experiments varied among collections ($P < 0.0001$). For all collections, survival following PRE application of indaziflam at 25 g ha^{-1} in this research was low (0 to 4%; Table 2). This response aligns with previous reports (and current labeling) of indaziflam for PRE control of annual bluegrass in warm-season turfgrass.[4,19] EPOST applications yielded different responses from the collections; three collections (Site 3A, Site 3B, Site 11) survived indaziflam treatment 19–78% compared to 0% for the herbicide-susceptible control (Table 2). Annual bluegrass surviving EPOST treatment with indaziflam had similar symptomology regardless of collection; plants were able to produce new, actively growing leaf tissue without injury symptoms, whereas secondary and tertiary leaf tissues were chlorotic (Fig. 1).

3.2 Dose–response experiments

Significant differences were detected when comparing response of annual bluegrass collections to increasing doses of indaziflam in agar, EPOST (Table 3 and Fig. 2). The concentration of indaziflam to yield a 50% reduction in root growth (I_{50}) for all putative-resistant collections (i.e., Site 3A, Site 3B, and Site 11) was significantly different from the herbicide-susceptible (S1) control (Table 3 and Fig. 2). I_{50} values for these putative-resistant collections ranged from 2424 to 4305 pM compared with 633 pM for the herbicide-susceptible control. Resistance indexes for the Site 3A, Site 3B, and Site 11 collections were 6.8, 5.4, and 3.8, respectively (Table 3).

Table 3. Parameter estimates from non-linear logistic regression analysis of four annual bluegrass *(Poa annua* L.*)* collections response to increasing doses of indaziflam applied early-postemergence

Collection[a]	*D*[b]	*b*[b]	I_{50}[b]	r^2	*P*[c]	Resistance index[d]
S1	98.6 (8)	1.2 (0.3)	633 (155)	0.95	—	—
Site 3A	106.8 (7.5)	1.2 (0.4)	4305 (937)	0.93	0.018	6.8
Site 3B	105 (9)	1 (0.3)	3420 (970)	0.92	0.047	5.4
Site 11	103 (7)	1 (0.2)	2424 (565)	0.96	0.038	3.8

Standard error values are listed in parentheses.
[a] Collections were submitted to the University of Tennessee Weed Diagnostics Center (WDC) after surviving treatment with indaziflam in autumn 2015 as well as foramsulfuron during spring 2016. S1 is a herbicide-susceptible collection native to University Park, PA with no history of exposure to indaziflam.
[b] *D* is the upper limit representing plant root length at low herbicide concentrations close to non-treated controls, I_{50} is the concentration of indaziflam to cause a 50% reduction in root growth, and *b* is the slope around I_{50}.
[c] Significance of a *t*-test comparison of I_{50} values between the S and each putative-resistant collection.
[d] Resistance index was calculated as the ratio of R to S I_{50} values.

Table 4. Percent survival of herbicide-susceptible and -resistant annual bluegrass (*Poa annua* L.) following treatment with preemergence (PRE) herbicides in growth chamber experiments

	Survival (%)					
Collection[a]	Indaziflam (50 g ha^{-1})	Oxadiazon (3360 g ha^{-1})	Prodiamine (1680 g ha^{-1})	Pronamide (1150 g ha^{-1})	S-metolachlor (2800 g ha^{-1})	Fisher's exact test[b]
Site 3A	7	1	95	0	0	—[c]
Site 3B	1	0	53	3	0	—[c]
Site 11	1	1	80	2	0	—[c]
S1	0	0	0	0	0	NS

Percent survival for each collection was calculated after treating 98 individual plants with herbicide and tabulating those containing living annual bluegrass 42 days after treatment.
[a] Collections were submitted to the University of Tennessee Weed Diagnostics Center (WDC) after surviving treatment with indaziflam in autumn 2015 as well as foramsulfuron during spring 2016. S1 is a herbicide-susceptible collection native to University Park, PA.
[b] Fisher's exact test conducted using 'fisher.test' function in R to compare effects of applied herbicides tested on each collection.
[c] Significant at $P \leq 0.0001$. NS, not significant at $\alpha = 0.05$.

3.3 Responses to other modes of action

Survival of annual bluegrass collections with indaziflam resistance (i.e., Site 3A, Site 3B, Site 11) varied in response to PRE herbicides applied in this experiment (Table 4). Although prodiamine PRE effectively controlled herbicide-susceptible annual bluegrass (0% survival), the herbicide was ineffective (53–95% survival) at controlling annual bluegrass from Site 3A, Site 3B, and Site 11. Oxadiazon, pronamide, *S*-metolachlor, and indaziflam applied PRE resulted in ≤ 7% survival of these indaziflam-resistant collections 42 DAT, similar to a herbicide-susceptible control (0% survival; Table 4).

In EPOST experiments, efficacy of other modes of action for controlling indaziflam-resistant annual bluegrass was poor (Table 5). For example, 99% of Site 3A and Site 3B annual bluegrass survived EPOST treatment with indaziflam at 50 g ha^{-1}; these collections exhibited 58–100% survival following treatment with foramsulfuron, glyphosate, metribuzin, pronamide, or simazine as well. Flumioxazin and glufosinate were marginally more effective; they reduced percent survival to between 17% and 69%. Similar trends were apparent in percent survival data collected on the Site 11 collection; however, this collection was more affected by indaziflam, flumioxazin, and metribuzin than the Site 3A and Site 3B collections which, in the case of indaziflam, aligns with conclusions of our dose–response experiments. All herbicides controlled the herbicide-susceptible collection ≥ 97% (0–3% survival) when applied EPOST.

3.4 Exploring non-target site resistance

3.4.1 Responses to indaziflam applied with inhibitors of cytochrome P450s

Few statistically significant differences in survival were detected among indaziflam and indaziflam + synergist treatments applied to Site 3A, Site 3B, and Site 11 annual bluegrass collections (Table 6). Survival following those applications ranged from 72 to 100% with no statistically significant differences detected among treatments applied to each collection. Interestingly, a statistically significant difference in survival was detected among treatments applied to herbicide-susceptible (S1) annual bluegrass; treatment with indaziflam + malathion resulted in 18% survival compared with 0% survival for indaziflam alone, indaziflam + ABT, and indaziflam + tebuconazole.

3.4.2 Metabolism of indaziflam after EPOST application

Lognormal Gaussian models effectively fit indaziflam content data collected from herbicide-susceptible (S1) and -resistant (Site 3B) annual bluegrass in our metabolism experiment; fit of

Table 5. Percent survival of herbicide-susceptible and -resistant annual bluegrass (*Poa annua* L.) following treatment with early-postemergence (EPOST) herbicides in growth chamber experiments

	Survival (%)								
Collection[a]	Indaziflam (50 g ha^{-1})	Flumioxazin (430 g ha^{-1})	Foramsulfuron (29 g ha^{-1})	Glyphosate (1120 g ha^{-1})	Glufosinate (1680 g ha^{-1})	Metribuzin (557 g ha^{-1})	Pronamide (1150 g ha^{-1})	Simazine (1120 g ha^{-1})	Fisher's exact test[b]
Site 3A	99	69	70	68	17	70	100	99	—[c]
Site 3B	99	26	98	58	24	70	91	98	—[c]
Site 11	34	6	99	99	21	14	90	97	—[c]
S1	1	0	0	0	0	0	3	2	NS

Percent survival for each collection was calculated after treating 98 individual plants with herbicide and tabulating those containing living annual bluegrass 31 days after treatment. EPOST applications were made to plants measuring < 2.5 cm in height with at least one true leaf.
[a] Collections were submitted to the University of Tennessee Weed Diagnostics Center (WDC) after surviving treatment with indaziflam in autumn 2015 as well as foramsulfuron during spring 2016. S1 is a herbicide-susceptible collection native to University Park, PA.
[b] Fisher's exact test conducted using 'fisher.test' function in R to compare effects of applied herbicides tested on each collection.
[c] Significant at $P \leq 0.0001$. NS, not significant at $\alpha = 0.05$.

Table 6. Percent survival of herbicide-susceptible and -resistant annual bluegrass (*Poa annua* L.) following treatment with early-postemergence (EPOST) herbicides and synergists in growth chamber experiments

	Survival (%)				
Collection[a]	Indaziflam	Indaziflam + ABT[b]	Indaziflam + tebuconazole	Indaziflam + malathion	Fisher's exact test[c]
Site 3A	100	98	100	99	NS
Site 3B	78	82	84	72	NS
Site 11	99	100	99	100	NS
S1	0	0	0	18	—[d]

Percent survival for each collection was calculated after treating 98 individual plants with herbicide and tabulating those containing living annual bluegrass 15 days after treatment. EPOST applications were made to plants measuring < 2.5 cm in height with at least one true leaf.
[a] Collections were submitted to the University of Tennessee Weed Diagnostics Center (WDC) after surviving treatment with indaziflam in autumn 2015 as well as foramsulfuron during spring 2016. S1 is a herbicide-susceptible collection native to University Park, PA.
[b] 1-Aminobenzotriazole (ABT) was applied to the soil surface of each cell using a pipette. A stock solution of ABT was prepared at 262 mg L^{-1} with 1 mL of this solution delivered to the soil surface prior to indaziflam treatment. Tebuconazole (1510 g ha^{-1}) and malathion (400 g ha^{-1}) were applied to trays as foliar sprays immediately preceding indaziflam treatment.
[c] Fisher's exact test conducted using 'fisher.test' function in R to compare effects of applied herbicides tested on each collection.
[d] Significant at $P \leq 0.0001$. NS, not significant at $\alpha = 0.05$.

Table 7. Regression models used to fit indaziflam and 1-fluoroethyl triazinediamine (primary metabolite of indaziflam) content in foliar tissue of a resistant (Site 3B) and herbicide-susceptible (S1) collection of annual bluegrass (*Poa annua* L.) from 0 to 10 days after treatment (DAT) with indaziflam at 25 g ha^{-1}

Collection	Indaziflam (ng g^{-1})	*P*-value	1-Fluoroethyl triazinediamine (ng g^{-1})	*P*-value
S1	$y = (105\ 751/DAT)*e^{-0.5*(\ln(DAT/4263)/\ln(17.42))^2)}$	0.001	$y = -1.76 + 3.08*DAT + 0.25*DAT^2$	<0.0001
Site 3B	$y = (3935/DAT)*e^{-0.5*(\ln(DAT/12.47)/\ln(4.38))^2)}$		$y = -0.50 + 0.65*DAT + 0.06*DAT^2$	

For both indaziflam and 1-fluoroethyl triazinediamine, models for S1 and Site 3B collections were compared using a global sums-of-squares *F*-test at $\alpha = 0.05$.

these models on S1 (R^2 = 0.93) and Site 3B annual bluegrass (R^2 = 0.98) was excellent (Fig. 3 and Table 7). A global sums-of-squares *F*-test determined that indaziflam content significantly varied between the S1 and Site 3B collections from 0 to 10 DAT (Fig. 3 and Table 7). Models were used to determine that both peak and mean indaziflam content in S1 annual bluegrass tissues were greater than those measured in tissues of Site 3B annual bluegrass from 0.5 to 10 DAT (Fig. 3).

Quadratic models effectively fit FDAT (the primary metabolite of indaziflam) content data well (Fig. 3; Table 7). Fit of these models on S1 ($R^2 = 0.99$) and Site 3B annual bluegrass ($R^2 = 0.98$) was excellent and a global sums-of-squares *F*-test determined that FDAT content significantly varied between S1 and Site 3B annual bluegrass over the time course of the experiment. By 10 DAT, FDAT content was greater in susceptible annual bluegrass (54 ng g^{-1}) than the collection with resistance (11.8 ng g^{-1}; Fig. 3).

4 DISCUSSION

Growth chamber experiments were conducted to better understand poor annual bluegrass control with indaziflam reported in the field during autumn 2015. Two application timings, PRE and EPOST, were studied to align with current indaziflam labeling for annual bluegrass control in turfgrass.[19] Data from our initial experiment highlighted that 25 g ha^{-1} indaziflam was efficacious when applied PRE, but ineffective on the Site 3A, Site 3B, and Site 11 collections when applied EPOST. An increase in indaziflam rate to 50 g ha^{-1} yielded similar results when applied EPOST, particularly for Site 3A and Site 3B. Dose–response data illustrated that resistance indexes for the Site 3A, Site 3B, and Site 11 annual bluegrass were 6.8, 5.4, and 3.8, respectively.

Target site resistance was not investigated in these collections because the site of action of indaziflam is currently unknown. We did observe extensive multiple resistance in annual bluegrass collections that survived treatment with indaziflam EPOST. These resistant collections were not effectively controlled by foramsulfuron, flumioxazin, glyphosate, glufosinate, metribuzin, pronamide, or simazine. It is not clear if survival of the Site 3A, Site 3B, and Site 11 collections in our experiment is the result of a single resistance trait, multiple resistance traits, non-target site mechanisms, or combinations thereof. However, multiple resistance to varying modes of action via non-target site mechanisms has been reported in *Amaranthus* and *Lolium* species.[20,21] Moreover, differential susceptibility of resistant weeds to PRE *versus* POST herbicide applications (similar to what was observed in our experiments with these annual bluegrass collections) has been reported in Palmer amaranth (*Amaranthus palmeri* S. Watson) in agronomic crops.[22]

We hypothesize that survival of the resistant annual bluegrass collections in these experiments may be the result of a non-target site mechanism considering the extent of multiple resistance observed and that both indaziflam and FDAT (the primary metabolite of indaziflam) content in tissues of a resistant collection were less than that of herbicide-susceptible annual bluegrass. Given that several inhibitors of cytochrome P450 monooxygenase were not able to restore susceptibility, changes in absorption of indaziflam may be culpable for causing resistance. For example, our dose–response work in agar suggests that less indaziflam enters

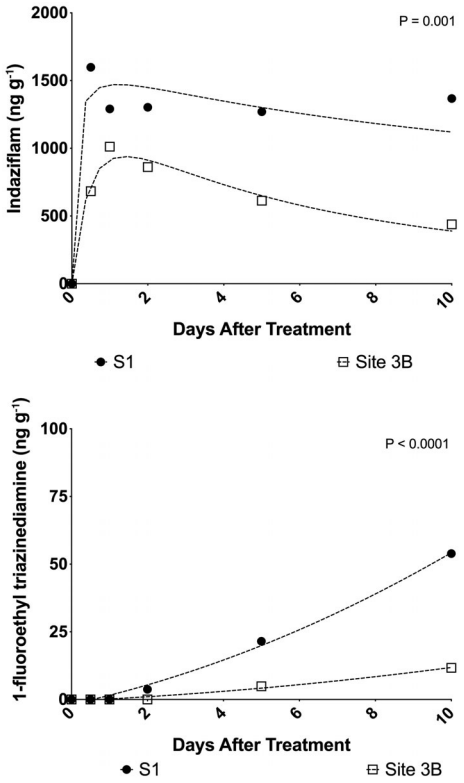

Figure 3. Indaziflam and 1-fluoroethyl triazinediamine (FDAT, primary metabolite of indaziflam) content in foliar tissue of a resistant (Site 3B) and herbicide-susceptible (S1) collection of annual bluegrass (*Poa annua* L.) following early-postemergence treatment with indaziflam at 25 g ha^{-1}. Indaziflam data were fit to a lognormal Gaussian model, whereas FDAT data were fit to a quadratic model. Model parameters are presented in Table 7. Global sums of squares *F*-tests at $\alpha = 0.05$ were used to compare response of S1 and Site 3B annual bluegrass from 0 to 10 days after treatment. Values are presented as ng g^{-1} fresh weight.

the root of resistant annual bluegrass plants (i.e., Site 3A, Site 3B. Site 11). However, it should also be noted that failure of various cytochrome P450 monooxygenase inhibitors to restore susceptibility could mean that other metabolic enzymes may be active in these indaziflam-resistant annual bluegrass plants. The cytochrome p450 inhibitors used in our experiments may not have sufficiently inhibited target enzymes at the doses applied or they may not target the enzymes involved in metabolizing indaziflam. Additional research to better understand the mechanisms of resistance in these annual bluegrass collections is warranted. A combination of radiolabeled (^{14}C) herbicide and transcriptomics experiments could provide a more detailed understanding of absorption, translocation, and/or metabolite identification in indaziflam-resistant plants.

5 CONCLUSIONS

This is a first report of annual bluegrass resistance to indaziflam applied early-postemergence. Evolution of annual bluegrass with resistance to indaziflam is concerning given that the herbicide is an important tool for turfgrass managers controlling annual bluegrass with resistance to mitotic inhibitor, PSII-, and ALS-inhibiting herbicides.[13,15] The scope of multiple resistance documented herein is perhaps more concerning given that annual bluegrass surviving EPOST treatment with indaziflam was also able to survive EPOST treatment with six other modes of action. Additional research to better understand mechanisms of resistance in these annual bluegrass collections is well warranted and could lead to the development of a diagnostic test for resistance in annual bluegrass similar to that developed for blackgrass (*Alopecurus myosuroides*) in cereals.[23]

ACKNOWLEDGEMENTS

The authors would like to acknowledge Doug Karcher for assistance in non-linear regression analysis of metabolism data. Several individuals also assisted in maintaining plant material used in these experiments: Greg Breeden, Roberto Viggiani, Robert Trigiano, Sarah Boggess, Dallas Taylor, and Mitchell Riffey. The authors would like to thank Qin Yu for reviewing this manuscript prior to submission. Lastly, the authors would like to thank Bayer Environmental Science as well as the University of Tennessee Office of AgResearch for their financial support of this research. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the University of Tennessee.

REFERENCES

1 U.S. Environmental Protection Agency, *Pesticide Fact Sheet—Indaziflam*. U.S. Environmental Protection Agency, Washington, DC, p. 108 (2010).
2 Brosnan JT, McCullough PE and Breeden GK, Smooth crabgrass control with indaziflam at various spring timings. *Weed Technol* **25**:363–366 (2011).
3 McCullough P, Yu J and De Barreda D, Efficacy of preemergence herbicides for controlling a dinitroaniline-resistant goosegrass (*Eleusine indica*) in Georgia. *Weed Technol* **27**:639–644 (2013).
4 Brosnan JT, Breeden GK, McCullough PE and Henry GM, PRE and POST control of annual bluegrass (*Poa annua*) with indaziflam. *Weed Technol* **26**:48–53 (2012).
5 Brosnan JT and Breeden GK, Annual bluegrass (*Poa annua*) control with indaziflam at various stages of growth. *Int Turf Soc Res J* **12**: 671–674 (2013).
6 Sebastian DJ, Flemming MB, Patterson EL, Sebastian JR and Nissen SJ, Indaziflam: a new cellulose biosynthesis inhibiting herbicide provides long-term control of invasive winter annual grasses. *Pest Manag Sci* **73**:2149–2162 (2017).
7 Sebastian DJ, Nissen SJ, Sebastian JR, Meiman PJ and Beck KG, Pre-emergence control of nine invasive weeds with aminocyclopyrachlor, aminopyralid, and indaziflam. *Invasive Plant Sci. Manage* **10**: 99–109 (2017).
8 Sebastian DJ, Nissen SJ and De Souza Rodrigues J, Pre-emergence control of six invasive winter annual grasses with imazapic and indaziflam. *Invasive Plant Sci. Manage* **9**:308–316 (2016).
9 Sebastian DJ, Sebastian JR, Nissen SJ and Beck KG, A potential new herbicide for invasive annual grass control on rangeland. *Rangeland Ecol Manage* **69**:195–198 (2016).
10 Clark SL, Sebastian DJ, Nissen SJ and Sebastian JR, Effect of indaziflam on native species in natural areas and rangeland. *Invasive Plant Sci. Manage* **12**:60–67 (2019).
11 Brabham C, Lei L, Gu Y, Stork J, Barrett M and DeBolt S, Indaziflam herbicidal action: a potent cellulose biosynthesis inhibitor. *Plant Physiol* **166**:1177–1185 (2014).
12 Heap I, *International Survey of Herbicide Resistant Weeds*. [Online]. Available: http://www.weedscience.org [24 September 2019].
13 Brosnan JT, Reasor EH, Vargas JJ, Breeden GK, Kopsell DA, Cutulle MA *et al.*, A putative prodiamine-resistant annual bluegrass population is controlled by indaziflam. *Weed Sci* **62**:138–144 (2014).
14 Brosnan JT, Breeden GK, Vargas JJ and Grier L, A biotype of annual bluegrass (*Poa annua* L.) in Tennessee is resistant to inhibitors of ALS and photosystem II. *Weed Sci.* **63**:321–328 (2015).
15 Brosnan JT, Breeden GK, Vargas JJ, Trigiano RN, Boggess SL and Staton ME, Confirmation and control of annual bluegrass resistant

to photosystem II inhibiting herbicides. *Int Turfgrass Soc Res J* **13**: 675–680 (2017).
16 Syvantek AW, Aldahir P, Chen S, Flessner ML, McCullough PE, Sidhu SS *et al*., Target and non-target resistance mechanisms induce annual bluegrass (*Poa annua*) resistance to atrazine, amicarbazone, and diuron. *Weed Technol* **30**:773–782 (2016).
17 R Core Team (2018). *R: A language and environment for statistical computing. R Foundation for Statistical Computing*, Vienna, Austria.
18 Busi R, Gaines TA and Powles SB, Phorate can reverse P450 metabolism-based herbicide resistance in *Lolium rigidum*. *Pest Manag Sci* **73**:410–417 (2017).
19 Anonymous. *Specticle FLO Herbicide Label*. [Online]. Available: http://www.cdms.net/ldat/ldANH006.pdf. [24 September 2019].
20 Varanasi V, Brabham C, Korres N and Norsworthy J, Non-target site resistance in Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] confers cross-resistance to protoporphyrinogen oxidase-inhibiting herbicides. *Weed Technol* **33**:349–354 (2019).
21 Yu Q, Abdallah I, Han H, Owen M and Powles S, Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. *Planta* **230**:713–723 (2009).
22 Houston M, Norsworthy J, Barber T and Brabham C, Field evaluation of preemergence and postemergence herbicides for control of protoporphyrinogen oxidase-resistant Palmer amaranth (*Amaranthus palmeri S. Watson*). *Weed Technol* **33**:610–615 (2019).
23 Stafford RS. Glutathione transferase (AmGSTF1) as a biomarker of multiple herbicide resistance in *Alopecurus myosuroides* (black-grass). PhD thesis, Newcastle University (2017). Available: http://theses.ncl.ac.uk/jspui/handle/10443/3767.

