A bioassay to determine *Poa annua* responses to indaziflam

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Herbicide resistance within *Poa annua* is widespread in managed turfgrass systems. In 2020, a *P. annua* collection from a golf course in the southeastern United States was reported to be resistant to indaziflam as well as six other mode-of-action groups. This first report in 2020 suggests that turfgrass managers would benefit from a bioassay to screen other collections with putative indaziflam resistance. A dose-response experiment was conducted with ten concentrations of indaziflam (0, 250, 500, 667, 1000, 1143, 1333, 2000, 4500, and 9000 pM) in Gelrite® culture during 2021 and 2022. An herbicide-susceptible (S1) collection of *P. annua*, a resistant standard (Site 3A), and a collection with putative resistance to indaziflam (Site 18) were included in this experiment. Petri dishes were filled with 80 mL of Gelrite® (3.75 g L^{-1}) containing technical grade ($\geq$ 98%) indaziflam and rifampicin (1000 μg mL^{-1}). Each plate was sealed with parafilm after placing 15 seeds of a single collection on the Gelrite® surface. At 14 days after seeding (DAS), the length of the radicle (mm) protruding from each seed was recorded with digital calipers. Indaziflam concentrations required to reduce root growth by 70% (EC$_{70}$) were calculated via non-linear regression. Statistically significant differences were detected among *P. annua* collections with the EC$_{70}$ values for the herbicide-susceptible collection measuring 708 pM [95% confidence interval (CI) = 656 to 764 pM] compared to 2130 pM (CI = 1770 to 2644 pM) for Site 3A and 4280 pM (CI = 3464 to 5442) for Site 18. Given that resistant collections exhibited longer root length in the absence of herbicide, confocal microscopy analysis was used to explore differences in root cell count among resistant and susceptible *P. annua* collections; however, few differences in cell count were detected. Overall, these findings indicate that a discriminatory dose of 708 pM (95% CI = 656 to 764 pM) can be used to differentiate among susceptible and resistant *P. annua* collections from field sites where poor control is observed following broadcast applications of indaziflam.

KEYWORDS

herbicide, resistance, cellulose biosynthesis, turfgrass, weeds

1 Introduction

Poa annua is an allotetraploid species within the Poaceae family that resulted from a hybridization event between the diploid species *Poa supina* (male parent) and *Poa infirma* (female parent) (Huff et al., 2003; Mao and Huff, 2017). *P. annua* can be found on all seven continents globally (Chwedorzewska, 2008) and is referred to by various common names, including: annual meadowgrass, annual bluegrass, wintergrass, junegrass, suffolkgrass, speargrass, and walkgrass (Gibeault, 1971). Morphological diversity among *P. annua* is wide-ranging and its characterization as an annual species has been questioned, given that it does not exhibit monocarpic senescence (Carroll et al., 2021a; Carroll et al., 2022).

P. annua is the most troublesome weed of turfgrass (Van Wychen, 2020), commonly infesting golf courses, sports fields, and lawns. A survey conducted by the Golf Course Superintendents Association of America reported that *P. annua* was the third most abundant species found on United States golf courses in 2015, behind *Cynodon* spp. and *Poa pratensis* L. (Gelernter et al., 2017). Sole reliance on herbicides to control *P. annua* infestations in turfgrass has resulted in extensive selection for herbicide-resistant biotypes (Brosnan et al., 2020a), including herbicidal inhibitors of acetolactate synthase (ALS; Cross et al., 2013; McElroy et al., 2013; Brosnan et al., 2015; Brosnan et al., 2016; Singh et al., 2021), cellular mitosis (Isgrigg et al., 2002; Cutulle et al., 2009; Brosnan et al., 2014; McCullough et al., 2017; Singh et al., 2021), photosystem II (PSII, Kelly et al., 1999; Mengistu et al., 2000; Hutto et al., 2004; Brosnan et al., 2020b), 5-enolpyruvate-shikimate-3-phosphate-synthase (Binkholder et al., 2011; Brosnan et al., 2012; Cross et al., 2015; Breeden et al., 2017), and protoporphyrinogen oxidase (Yu et al., 2018). Multiple resistance in *P. annua* is an emerging concern to turfgrass managers as these populations become uncontrollable by chemical methods (Brosnan et al., 2020a). Reports of *P. annua* with resistance to ALS and PS-II inhibitors as well as pre- and postemergence (prodiamine and glyphosate, respectively) herbicides have been documented on golf courses in the United States transition zone (Brosnan et al., 2016; Breeden et al., 2017). Multiple resistance to five modes of action was identified in *P. annua* from an Australian golf course (Barua et al., 2020). In Texas, Singh et al. (2021) identified a population of *P. annua* resistant to simazine, amicarbazone, trifloxysulfuron, foramsulfuron, and preemergence applications of pronamide.

The possibility of simultaneous co-evolution of non-target and target-site resistance within the self-pollinating species *P. annua* has been explored (Laforest et al., 2021). In working with a *P. annua* collection that contained target site mutations conferring resistance to both PS-II and ALS-inhibiting herbicides, Laforest et al. (2021) confirmed that treatment with an ALS-inhibiting herbicide (trifloxysulfuron) resulted in differential expression of genes associated with non-target site resistance, particularly those associated with oxidative stress. Interestingly, resistant plants also exhibited constitutive expression of genes regulating transmembrane transport prior to treatment. The researchers suggested that continued use of an ALS-inhibitor after evolution of target site resistance may lead to an accumulation of resistance

mechanisms affecting other modes of action. For example, *P. annua* plants characterized by Laforest et al. (2021) were less affected by applications of flumioxazin, indaziflam, and pronamide than herbicide-susceptible controls.

Cellulose biosynthesis inhibiting herbicides (HRAC Group 29) target the cellulose synthase complex (CSC) where cellulose polymers are synthesized and then released to the cell wall (Mutwil et al., 2008; Bashline et al., 2015). Within the CSC, 18 to 24 catalytic cellulose synthase A (CESA) proteins could be targets of cellulose biosynthesis inhibiting herbicides (Jarvis, 2013; Brabham et al., 2014; Tateno et al., 2016). Inhibiting the function of any of these proteins leads to radial root swelling caused by the loss of anisotropic growth in cells undergoing expansion (Brabham et al., 2014). Indaziflam is an alkylazine inhibitor of cellulose biosynthesis with an unknown site-of-action. Brabham et al. (2014) reported that indaziflam increased CESA particle density in the plasma membrane and inhibited polymerization by reducing CESA particle velocity by 65% (Brabham et al., 2014). Indaziflam is also a unique herbicide as it is efficacious on both monocots and dicots, whereas other Group 29 herbicides (e.g., isoxaben) only exhibit dicot activity (Sabba and Vaughn, 1999). Furthermore, plants with target-site resistance to isoxaben are not cross-resistant to indaziflam, indicating a unique site of action (Brabham et al., 2014). When indaziflam is applied to a susceptible plant, radial root swelling and ectopic lignification will occur leading to chlorosis and eventual mortality (Brabham et al., 2014). In *Arabidopsis*, indaziflam inhibited cellulose production within one hour of treatment in a dose-dependent manner (Brabham et al., 2014).

Indaziflam is a lipophilic herbicide with low water solubility, a dissociation constant (pK_a) of 3.5, an octanol/water partition coefficient of 2.8 at pH 7, and residual activity for controlling annual weeds in soil (EPA, 2010; Sebastian et al., 2017). In managed turfgrass, indaziflam (Specticle® FLO. Bayer Environmental Sciences, St. Louis, MO) is used to control problematic grassy weeds such as *P. annua*, *Eleusine indica*, and *Digitaria* spp., as well as annual sedges and broadleaf weeds. Considering that few Group 29 herbicides are used for weed control in turfgrass, indaziflam offered turfgrass managers a new mode of action for managing *P. annua* populations that have evolved resistance to other modes of action (Brosnan et al., 2014; Brosnan et al., 2015; Brosnan et al., 2017a). For example, a *P. annua* population with target site resistance to PS-II inhibiting herbicides was controlled ≥ 98% with indaziflam (Brosnan et al., 2017a). Similarly, indaziflam effectively controlled *P. annua* with resistance to mitotic and ALS-inhibiting herbicides (Brosnan et al., 2014; Brosnan et al., 2015).

The first case of any plant evolving resistance to indaziflam was identified in *P. annua* collected from a golf course and confirmed via greenhouse and laboratory bioassays (Brosnan et al., 2020c). Plants were identified and collected after escaping preemergence applications of indaziflam (Specticle® FLO. Bayer Environmental Sciences. St. Louis, MO) at maximum label rate (48.7 g ha⁻¹) in the field. *P. annua* surviving indaziflam treatment (applied early-postemergence to non-tillered plants< 2.5 cm height) in greenhouse and laboratory bioassays also exhibited extensive multiple resistance to several other herbicide mode-of-action groups, including #2 (foramsulfuron), #3 (pronamide), #5

(metribuzin and simazine), #9 (glyphosate), #10 (glufosinate), and #14 (flumioxazin). Inhibitors of cytochrome P450 monooxygenase, including 1-aminobenzotriazole (10 mg L^{-1}), tebuconazole (1510 g ha^{-1}), or malathion (400 g ha^{-1}) could not restore indaziflam susceptibility in these *P. annua* collections suggesting that metabolism may not be the sole mechanism of resistance in these plants. Interestingly, Brosnan et al. (2020c) demonstrated that concentrations of indaziflam and 1-fluoroethyl triazinediamine (the primary metabolite of indaziflam) were lower in resistant plants compared to susceptible controls and suggested that altered herbicide absorption may be a mechanism of resistance to be further explored in these collections.

Evolution of indaziflam resistance within *P. annua* is concerning considering that the herbicide helped turfgrass managers control infestations with resistance to other mode of action groups (Brosnan et al., 2014; Brosnan et al., 2015, Brosnan et al., 2017a). There is a need to develop a bioassay to rapidly screen many collections of *P. annua* for resistance to indaziflam. A gel-based, *in vitro*, bioassay could help turfgrass managers understand how plants will respond to indaziflam treatment in as few as 14-days compared to the 42 days required for whole-plant testing in a soil medium (Brosnan et al., 2020c). Similar bioassays have been developed for other herbicidal mode of action groups with an array of plant species, including *P. annua* (Bourgeois et al., 1997; Claude et al., 2004; Cutulle et al., 2009; Kaundun et al., 2011; Kaundun et al., 2014; Brosnan et al., 2017b; Messelhäuser et al., 2021). Moreover, a gel bioassay could identify indaziflam resistance regardless of mechanism (Kaundun, 2021), which is important given that the indaziflam target site is currently unknown. This research aims to develop a bioassay to determine *P. annua* responses to indaziflam that can be used to discern amongst resistant and susceptible accessions.

2 Materials and methods

2.1 Plant culture

Laboratory research was conducted at the University of Tennessee in 2021 and 2022 to develop a bioassay to screen *P. annua* germplasm response to indaziflam. Herbicide susceptible (S1) and one resistant biotype (Site 3A) used in this experiment were the same as those described by Brosnan et al. (2020c). Collections S1 and Site 3A are from University Park, PA and a golf course fairway in Cummings, GA respectively. Additionally, a collection of *P. annua* with putative resistance to indaziflam (Site 18) was also included. This collection was submitted to the University of Tennessee Weed Diagnostics Center after escaping treatment with preemergence applications of indaziflam (Specticle® FLO; Bayer Environmental Sciences, St. Louis, MO) on a golf course fairway in Thomasville, GA. Plants were cultured in a glasshouse to produce seed required for research. Plants were placed in greenhouse pots filled with a peat moss growing media (PRO MIX BX General Purpose; Premier Tech Horticulture, Quakertown, PA, USA) and treated with complete fertilizer (20 N–20 P$_2$O$_5$-20 K$_2$O. Peter's 20–20–20. JR PETERS, Incorporated.

Allentown, PA, USA) at 49.5 kg N ha^{-1} wk^{-1}. Mature seed from each collection were harvested with scissors, dried in a forced-air oven (Laboratory Oven; The Grieve Corporation, Round Lake, IL), and sieved (Standard Test Sieve; Fisher Scientific Company LLC, Hampton, NH) to remove chaff. After processing, seed were stored in glass vials (40 mL Scintillation Vial; Thermo-Fisher Scientific, St. Louis, MO, USA) at −20°C for a minimum of four weeks to break any potential dormancy (Carroll et al., 2021b).

2.2 Method development

A natural gelling agent (Gelrite®; Duchefa Biochemie, Haarlem, The Netherlands) was used to culture plants in this research. This agent was prepared by adding 7.5 g Gelrite® to 8.9 g MS Salts & Vitamins (Murashige & Skoog M519 Basal Medium with Vitamins; Phyto Tech Labs, Lenexa, KS) in 1966 mL of distilled H$_2$O in sterilized Erlenmeyer flasks; MS Salts & Vitamins was added to distilled H$_2$O prior to the Gelrite®. The pH of this solution was measured with a pH conductivity meter (Multimeter Model 250; Denver Instrument, Bohemia, NY) and adjusted to 6.5 by adding either hydrochloric acid (HCl) or sodium hydroxide (NaOH) to lower or raise the pH, respectfully. The resultant solution went through a liquid 20 cycle that raised temperature to 121°C in an autoclave (Amsco Century V120; Steris, Mentor, OH) for 20 minutes. After the autoclave cycle was finished, sealed flasks were placed on a hot plate (FisherBrand Stirring Hot Plate; Thermo Orion Incorporated, Chelmsford, MA) and stirred until cooling to 80°C. Rifampicin (1000 µg mL^{-1}; Rifamipicin Molecular Biology Reagent; Alfa Aesar, Ward Hill, MA) and technical indaziflam (≥ 98%; Sigma-Aldrich, St. Louis, MO) were added at this temperature to create ten indaziflam concentrations for evaluation: 0, 250, 500, 667, 1000, 1143, 1333, 2000, 4500, 9000 picomolar (pM). These concentrations were selected based results of Brosnan et al. (2020c) who reported that early-postemergence exposure to 633 pM indaziflam in agar culture could differentiate between indaziflam resistant and susceptible collections of *P. annua*. Technical indaziflam (150 mg) was diluted in 99.5 mL dimethyl sulfoxide with a concentration of 5000 µM. This stock solution was frozen to keep concentrations uniform across all trials. On the day of trial initiation, this bottle was allowed to thaw to facilitate adding the appropriate amount of indaziflam to each flask for each concentration. Serial dilutions using deionized H$_2$O were used to deliver intended pM concentrations of indaziflam to each autoclaved solution.

After adding rifampicin and indaziflam, 80 mL of the resultant solution was added to square petri dishes (120 x 15 mm) within a sterile fume hood (1300 Series A2 Fume Hood; Thermo-Fisher Scientific, St. Louis, MO, USA) using a dispensette (Dispensette S Bottle Top Dispenser; BrandTech Scientific Incorporated, Essex, CT). Plates were left open in the fume hood for 12 min in order to cool before being covered, sealed with parafilm (2 IN All-Purpose Laboratory Film; Amcor, Menasha, WI), and placed in a growth chamber (G1000-Germinator; Conviron, Winnipeg, Manitoba, Canada) set to constant 16°C temperature and 16 h photoperiod. Plates were unsealed to facilitate adding *P. annua* seed (15 seeds,

one collection per plate) to the Gelrite® surface using sterilized tweezers; seed were pressed into the Gelrite® surface with sterilized spatulas after plating. Each petri dish was then re-sealed with parafilm after seeding and placed in the aforementioned growth chamber at a constant temperature of 16°C.

2.3 Data collection and statistical analysis

Fourteen days after seeding (DAS), responses to indaziflam were evaluated by assessing the presence (or absence) of root or shoot growth (i.e., 1 = yes, 0 = no). Additionally, the length of the radicle (mm) protruding from each seed was measured via digital calipers (CD-6" AX Digimatic Caliper; Mitutoyo Corporation, Kawasaki, Kanagawa, Japan). The experiment was arranged in a completely randomized design with five replications of each indaziflam concentration. Within a given concentration, the 15 seeds per collection were considered sub-samples and used to calculate mean root length values for each *P. annua* collection that were expressed as a percentage of the non-treated. The experiment was repeated as previously described in January 2022 using five replications of each indaziflam concentration and 15 sub-samples. Data from each experimental run were combined following ANOVA in R (v. 3.6.2) with root length values subjected to non-linear regressions in Prism (Version 9.0.0. GraphPad Prism. La Jolla, CA.) using the 'EC Anything' model described below:

$$Y = Bottom + (Top - Bottom)/(1 + (\frac{EC_{70}}{X})^{HillSlope})$$

In this model, Bottom and Top represent asymptotes that were constrained to 0 and 100 percent, respectively. Y represented root growth as a percentage of the non-treated, and HillSlope represented the steepness of the curve. X represented the indaziflam concentration to reduce root length 70% (EC_{70}). Best-fit parameters for modeled responses were compared using a global sums-of-squares F-test at α = 0.05 with 95% confidence intervals used to compared EC_{70} concentrations of indaziflam among resistant and susceptible *P. annua* collections. EC_{70} values were used to discern amongst potential resistant and susceptible accessions to provide a more stringent filter than EC_{50} values commonly used in resistance screening (Burgos et al., 2013).

2.4 Microscopy analysis of *P. annua* root growth

Roots from indaziflam-resistant (Site 3A, Site 18) and -susceptible collections used in Gelrite® assay development were analysed using microscopy to evaluate the cause of apparent increased root length in indaziflam resistant populations. Additional indaziflam-resistant (Site 3B, FL) collections submitted to the University of Tennessee Weed Diagnostics Center were also included, along with an additional indaziflam-susceptible collection

(S5) originating from Wake Forest, NC to better support this observation. All seeds were sterilized using 70% ethanol for 5 min, followed by 40 min exposure to a solution of 10% bleach + 0.01% triton X-100, followed by three rinses with distilled water. Plants were grown in vertical, square (10 cm x 10 cm), petri plates, with 1% agar + ½ strength Murashige & Skoog M519 Basal Medium, similar to Brosnan et al. (2020c). Preliminary germination assays showed variability among resistant and susceptible collections. To that end, plates were grown in the dark for three days before being exposed to light (16/8 day/night cycle) in the growth chamber in order to synchronize root emergence.

Five days after light exposure, confocal root tissue images for each *P. annua* collection were taken using an inverted laser scanning microscope (Nikon-A1RSi. Nikon Instruments. Melville, NY). The microscope was configured using a 20x Plan Apo objective (NA 0.75) and 10X ocular lenses for a total magnification of 200x. A z-series with a step size of 1.9 um was taken for all root images. A 32 µM stock solution of N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide dye (FM4-64. Invitrogen. Waltham, MA) was applied to root tissue for 3 min and then washed (three times) using distilled water to stain cell membranes. Root tissue from each seedling was placed on a glass slide and seperated from shoot tissue with a scalpel. Post-acquisition image analysis was performed using ImageJ (Schneider et al., 2012) equipped with a cell counter plugin. Images were enhanced using enhance contrast function of Fiji (Schindelin et al., 2012) using a saturated pixels value of 0.35% and the equalize histogram function. The depth of root tissue selected for analysis was determined by drawing five segmented lines of 100 pixels (1 micron = 1.6 pixels, 500 pixels = 312.5-micron length) from the quiescent center of the root tip; the number of cells was counted from quiescent layer to the end of line within the outermost anterior and posterior epidermal layers of each root. Cell count data from each *P. annua* collection were subjected to a one-way ANOVA using Minitab software (Minitab Statistical Software, 2010) with mean values for each collection compared using a pairwise T-test at α = 0.05.

3 Results

3.1 Method development

Significant *P. annua* collection-by-indaziflam concentration interactions were detected in root length data from each experimental run; therefore, data from both were combined before being subjected to non-linear regression analysis. A non-linear model fit the combined data set with a global sums-of-squares F-test detecting highly significant (F = 117, P< 0.0001) differences among *P. annua* collections (Figure 1).

While root length reduced for all *P. annua* collections as indaziflam concentration increased, there were significant differences in the concentration of indaziflam required to reduce root length 70% (EC_{70}) among *P. annua* collections in this
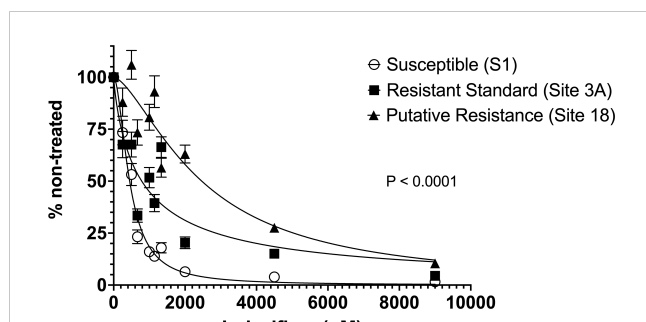
FIGURE 1
Root length of *P. annua* collections subjected to increasing concentrations of indaziflam (0, 250, 500, 667, 1000, 1143, 1333, 2000, 4500, and 9000 pM) in Gelrite® culture for 14 days. Root length (mm) measurements were expressed as a percentage of the non-treated (0 pM) for each collection. Error bars presented illustrate the standard error of the mean for each collection (N = 150).

experiment (Table 1). EC_{70} values for the herbicide-susceptible collection measured 708 pM [95% confidence interval (CI) = 656 to 764 pM] compared to 2130 pM (CI = 1770 to 2644 pM) for Site 3A and 4280 pM (CI = 3464 to 5442) for Site 18 (Table 1). These values can be used to calculate resistance indices (i.e., R/S ratios) of 3 and 6 for the Site 3A and Site 18 P. *annua* collections, respectively. Low-level resistance within the *P. annua* collections evaluated in this study provides further evidence that non-target site mechanisms may be affecting indaziflam efficacy given that non-target site mechanisms are often continuous as opposed to discreet for

target-site mutations (Tranel and Wright, 2002; Yu and Powles, 2014).

3.2 Microscopy analysis of *P. annua* root tissue

Root length values for both indaziflam-resistant collections (Site 3A and Site 18) were greater than the susceptible *P. annua* collection used in this study (Table 2). Microscopy analysis was conducted in order to explore possible differences at the cellular level between the *P. annua* collections. We hypothesized that enhanced root cell division in indaziflam-resistant collections may be causing the constitutive differences between indaziflam-resistant and indaziflam-susceptible collections in control plates (0 pM indaziflam). Cell count data from indaziflam-resistant and susceptible *P. annua* collections are presented in Figure 2 with sample microscopy images used for analysis presented in Figure 3. No significant differences were detected in cell number (in the zone of cell division) among the indaziflam-resistant and -susceptible *P. annua* collections included in this experiment.

4 Discussion

Indaziflam resistance indices in the current study align with previous research using Site 3A and S1 *P. annua* germplasm (Brosnan et al., 2020c); the researchers determined indaziflam

TABLE 1 *P. annua* collections subjected to increasing doses of indaziflam (0, 250, 500, 667, 1000, 1143, 1333, 2000, 4500, and 9000 pM) in Gelrite® culture for 14 days.

Collection[a]	EC_{70} [b]	Resistance Index[c]	95% CI[d]
S1	708	–	656 to 764
Site 3A	2130	3.0	1770 to 2644
Site 18	4280	6.0	3464 to 5442

[a]S1 collection was herbicide-susceptible standard from University Park, PA with no exposure history to indaziflam. Site 3A and Site 18 were collected from hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *Cynodon transvaalensis* Burtt Davy, cv. 'Tifway') on golf courses after surviving field applications and challenges with 48.7 g ha^{-1} in the glasshouse
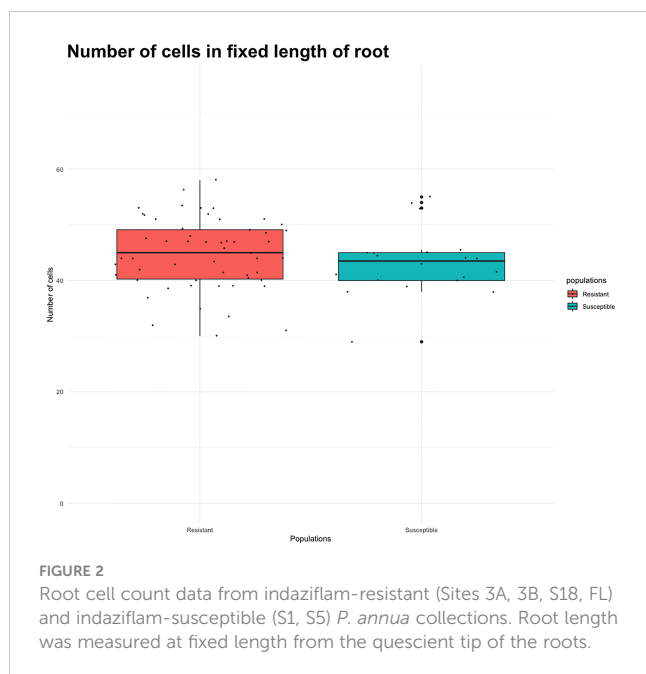[b]EC_{70} = concentration of indaziflam required to reduce root growth from seed by 70% in Gelrite® culture.
[c]Resistance indices calculated by dividing the EC_{70} value for Site 3A and Site 18 by the EC_{70} value for the susceptible control (S1).
[d]95% Confidence Interval (CI) for EC_{70} values determining the concentration of indaziflam required to reduce root growth by 70%. Root length (mm) measurements were expressed as a percentage of the non-treated (0 pM) for each collection. The concentration of indaziflam to reduce root length 70% (EC_{70}) was used to calculate a resistance index.

TABLE 2 Mean root length (mm) data for indaziflam-resistant (Site 3A and Site 18) and -susceptible (S1) collections of *P. annua* in laboratory assays evaluating root growth in in Gelrite® culture for 14 days.

	Indaziflam Concentration (pM)									
Collection[a]	0	250	500	667	1000	1143	1333	2000	4500	9000
	—mm—									
S1	5.97	4.38	3.18	1.39	0.96	0.83	1.07	0.38	0.23	0.10
Site 3A	13.94	9.43	9.42	4.66	7.21	5.50	9.25	2.83	2.11	0.62
Site 18	14.14	12.43	14.97	10.38	11.41	13.16	7.98	8.91	3.90	1.47

[a]S1 collection was herbicide-susceptible standard from University Park, PA with no exposure history to indaziflam. Site 3A and Site 18 were collected from hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *Cynodon transvaalensis* Burtt Davy, cv. 'Tifway') on golf courses after surviving field applications and challenges with 48.7 g ha^{-1} in the glasshouse.

FIGURE 2
Root cell count data from indaziflam-resistant (Sites 3A, 3B, S18, FL) and indaziflam-susceptible (S1, S5) *P. annua* collections. Root length was measured at fixed length from the quescient tip of the roots.

concentrations required to reduce root growth of newly emerged *P. annua* plants 50% (I_{50} values) and calculated a resistance index of 6.8 for Site 3A. The difference in resistance factor for collection Site 3A between experiments can most likely be attributed to differences in plant maturity at the time of indaziflam exposure. Brosnan et al. (2020c) calculated a resistance index following early-postemergence indaziflam exposure to emerged seedlings (a labeled use pattern for indaziflam in turfgrass; Anonymous, 2023), whereas preemergence exposure was evaluated in this research. Additionally, EC_{70} values were used to calculate resistance indices in the current study compared to EC_{50} values commonly used by other researchers (Cutulle et al., 2009; Kaundun et al., 2014; Brosnan et al., 2020c). Given that there is only a single report of indaziflam resistance in published literature to date (Brosnan et al., 2020c), EC_{70} values were

selected to provide a more stringent filter among putative resistant and susceptible *P. annua* collections. A limitation of this research is that only a single susceptible collection was included in the dose-response experiments. Future research to refine this assay using additional susceptible *P. annua* collections is warranted.

A collection of *P. annua* with putative resistance to indaziflam (Site 18) was included in this research after escaping treatment with preemergence applications of indaziflam on a golf course fairway. *P. annua* from Site 18 exhibited nearly 2x the level of indaziflam resistance than Site 3A, the resistant standard used in the experiment (Table 1, Figure 1). This represents the highest level of indaziflam resistance reported in the peer-reviewed scientific literature (to date) and a first report of resistance to indaziflam applied preemergence.

Evolution of indaziflam resistance in *P. annua* is concerning given that many turfgrass managers have effectively used this herbicide to control *P. annua* biotypes that evolved resistance to other modes of action, including those with multiple resistance. After screening 1,500 plants across ten concentrations, 708 pM indaziflam (95% confidence interval of 656 to 764 pM) consistently separated resistant collections from a susceptible control in gel culture. Sebastian et al. (2017) reported that 251 pM indaziflam reduced root growth of feral rye (*Secale cereale* L.) in agar culture by 50%. In the current study, 448 pM of indaziflam was required to yield a similar reduction in root growth of herbicide susceptible *P. annua* in gel culture. The discriminatory concentration identified in this research successfully identified other accessions of *P. annua* with putative resistance to indaziflam that escaped control in the field (Figure S1). This assay could be used to determine if indaziflam can be a component of an effective herbicide mixture for *P. annua* control or potentially be expanded to screen response of *P. annua* accessions to herbicide mixtures. Hulme (2022) explored multiple resistance across an array of weed species and reported that certain mode of action groups cluster together, with a main cluster including ALS and PS-II inhibitors as well as glyphosate. Given widespread reports of resistance to these herbicides in *P. annua*,
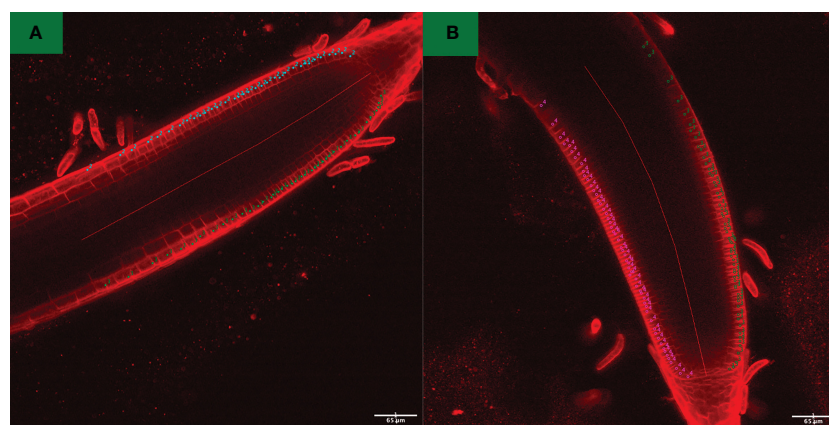
FIGURE 3
Confocal fluorescence images of **(A)** indaziflam-resistant (Site 18) and **(B)** indaziflam-susceptible (S1) *P. annua*, treated with FM4-64 dye and post-acquisition processed using FIJI function enhance contrast, with equalize histogram enabled. Cell counter plugin was utilized for counting the number of cells in the fixed length (500 pixels) from the quescient center of roots. These count data are presented in Figure 2.

challenging resistant plants with indaziflam using this assay would help determine if the herbicide could be a component of an effective mixture for resistance management or falls within the same multiple resistant cluster.

In addition, it is worth noting that root length values for both indaziflam-resistant collections used in this study (Site 3A and Site 18) were significantly greater (>2x) than the susceptible *P. annua* collection under no indaziflam treatment (Table 2). Microscopy analysis did not show an increased number of cells in the zone of division for these resistant plants suggesting that these differences are not the result of increased cell division. It may be that cells in the resistant plants are elongating more and/or faster than susceptible plants, but this was not explored. Future research exploring this and other growth phenotypes associated with mechanisms of indaziflam resistance in *P. annua* is warranted as the resistance mechanism may cause or be linked with traits that may be disadvantageous for resistant plants.

5 Conclusion

A gel bioassay was developed to discern *P. annua* responses to indaziflam in a 14-day period once seed is available. Comparatively, traditional screening via greenhouse pot studies can require months to complete. This novel bioassay allows turfgrass managers to screen *P. annua* plants for indaziflam resistance within the same season of management to determine if indaziflam will be an effective component of their *P. annua* management plans. This bioassay will also aid those challenged with indaziflam resistant *P. annua* in other cropping systems. For example, indaziflam resistance in *P. annua* was recently reported in hazelnut (*Corylus avellana* L.) orchard production (Miranda and Moretti, 2023). One *P. annua* collection screened using this assay (Site 18) exhibited the highest level of indaziflam resistance reported in the peer-reviewed scientific literature (to date) and serves as a first report of resistance to indaziflam applied preemergence. Future work should be conducted to determine the mechanism of resistance in the indaziflam-resistant *P. annua* collections described in this research. Doing so would allow for molecular assays to identify indaziflam resistance to be developed that could return results even more rapidly.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Dose-response experiments were designed by JB, EP, and BP and conducted by BP and JV. Analysis of dose-response data and supplemental information was conducted by JB and BP. Root microscopy experiments were designed by EP, conducted by MM, with data analyzed by MM and EP. JB and EP provided insights on overall research effort. BP drafted the original manuscript, which was reviewed, edited, and revised by JB and EP. All authors agreed to the submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2023.1182528/full#supplementary-material

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