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α-Tubulin mutation Thr-239-Ile in annual bluegrass (*Poa annua*) induces variable responses to prodiamine and dithiopyr

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Research Article

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Abstract

Mitotic-inhibiting herbicides, like prodiamine and dithiopyr, are used to control annual bluegrass (*Poa annua* L.) preemergence in managed turfgrass; however, resistance to mitotic-inhibiting herbicides has evolved due to repeated applications of herbicide from a single mechanism of action. Three suspected resistant populations (R1, R2, and R3) were collected in Alabama and Florida and screened for resistance to prodiamine. Part of the *α-tubulin* gene was sequenced for known target-site mutations. Target-site mutations were reported in all three R populations, with each containing an amino acid substitution at position 239 from threonine to isoleucine (Thr-239-Ile). Previous research has indicated that the Thr-239-Ile mutation confers resistance to dinitroaniline herbicides in other species. Dose–response screens using prodiamine and dithiopyr were conducted and I_{50} values were calculated for R1, R2, and R3 using regression models based on seedling emergence. For prodiamine, I_{50} values for R1, R2, and R3 were 35.3, 502.7, and 91.5 g ai ha^{-1}, respectively, resulting in 2.9-, 41.9-, and 7.6-fold resistance, respectively, when compared with a susceptible (S) population. For dithiopyr, I_{50} values for R1, R2, and R3 were 154.0, 114.2, and 190.1 g ai ha^{-1}, respectively, resulting in 3.6-, 2.7-, and 4.5-fold resistance, respectively, when compared with an S population. When comparing I_{90} values with the highest labeled use rates, R2 had a 2.9-fold level of resistance to prodiamine, and R1, R2, and R3 had a 2.4-, 2.0-, and 3.2-fold levels of resistance to dithiopyr, respectively. This is the first report of a variable response in *P. annua* to prodiamine despite each R population possessing the same mutation.

Introduction

Annual bluegrass (*Poa annua* L.) is a cool-season grass that can be considered either a weed or a beneficial turfgrass (Wu and Harding 1992). According to a 2020 survey conducted by the Weed Science Society of America (WSSA), *P. annua* is considered the most troublesome weed in turfgrass in North America (Van Wychen 2020). It also has highly variable morphological and biological characteristics due to various ecological pressures and turfgrass management regimes (McElroy et al. 2002). *Poa annua* is an allotetraploid whose genome formed as the result of a cross between weak bluegrass (*Poa infirma* Kunth) and supine bluegrass (*Poa supina* Schrad.) followed by a genome duplication event (Mao and Huff 2012). Although *P. annua* is native to Europe, it has naturalized on every continent (Chwedorzewska 2008).

Mitotic-inhibiting herbicides (WSSA/HRAC Group 3) are commonly used as preemergence herbicides to control annual grasses and small-seeded broadleaves (McElroy and Martins 2013). These herbicides result in inhibition of shoot and root development by preventing the polymerization of microtubules, protein dimers composed of α- and β-tubulin, which separate the chromosomes during mitosis (Nogales et al. 1998; Shaner 2014). Mitotic-inhibiting herbicides ultimately arrest cell division in prometaphase; however, the mechanism varies by herbicide family. Dinitroaniline herbicides like prodiamine prevent microtubule polymerization by binding directly to the α-tubulin protein, while dithiopyr, a pyridine herbicide, binds to microtubule-associated proteins that help stabilize the microtubules (Shaner 2014; Vaughn and Lehnen 1991). However, research on dithiopyr's mode of action has not been thoroughly vetted.

Prodiamine and dithiopyr are often used as preemergence controls for *P. annua* and have been shown to reduce swards of *P. annua* when applied correctly (Cutulle et al. 2009; Reicher et al. 2017). Repeated use has resulted in resistance evolving to these herbicides. *Poa annua*

resistance to dinitroaniline herbicides was first observed in 2002 in a North Carolina population exhibiting a 6-fold level of resistance to prodiamine (Isgrigg et al. 2002). In 2009 and 2017, two populations of *P. annua* with 26- and 22-fold resistance to prodiamine, respectively, were also reported (Breeden et al. 2017; Cutulle et al. 2009). *Poa annua* has also been reported with a 1.5-fold resistance to dithiopyr when compared with a susceptible population; however, the resistance level was marginal and was not studied further (Cutulle et al. 2009). In an additional case, *P. annua* was evaluated for resistance to pronamide when the suspected resistant population in question was not controlled by a field rate of dithiopyr; however, the population was not further evaluated for potential resistance to dithiopyr (McCullough et al. 2017).

Even though resistance to prodiamine has been reported in *P. annua*, the mechanism of resistance is not often reported. However, there are mutations on the *α-tubulin* gene that have been reported to confer resistance to dinitroaniline herbicides in other species. Mutations have been reported at positions Leu-125, Leu-136, Val-202, Thr-239, Arg-243, and Met-268 on the *α-tubulin* gene (Chu et al. 2018; Délye et al. 2004; Hashim et al. 2012; Yamamoto et al. 1998). A recent study revealed that out of 82 *P. annua* populations that were resistant to mitotic-inhibiting herbicides, 75 populations possessed the Thr-239-Ile mutation (Rutland et al. 2022). Currently there are no reports of target-site mutations that result in resistance to dithiopyr. But there have been two reported cases in goosegrass [*Eleusine indica* (L.) Gaertn.] that indicate that *α-tubulin* mutations could result in dithiopyr resistance even though α-tubulin is not the proposed target site. Recently, three *E. indica* populations were discovered to be resistant to dithiopyr. Each of these populations possessed a mutation at the Leu-136 position on the *α-tubulin* gene (Elmore et al. 2022; Russell et al. 2022). However, there is not enough research to confirm that mutations on the *α-tubulin* gene result in dithiopyr resistance.

Target-site mutations exist in resistant populations of *P. annua*, but they are hard to document. This is because sequencing *α-tubulin* for target-site mutations using standard sequencing methods, like capillary sequencing, is challenging (Rutland et al. 2022). Amplicon sequencing offers a way to overcome the nucleotide conflictions that pose a challenge for capillary sequencing (Rutland et al. 2022). Therefore, the objective of this research was to sequence part of the *α-tubulin* gene and determine whether the mutations discovered confer varying levels of resistance to prodiamine and confer cross-resistance to dithiopyr.

Materials and Methods

Poa annua populations with suspected resistance to dinitroaniline herbicides were collected across the state of Alabama and the Florida Panhandle. Roughly 5 to 10 whole plants were collected from areas that were treated with a dinitroaniline herbicide. Once collected, these populations were transplanted into flats filled with potting medium (Scotts Miracle-Gro Products, Marysville, OH) and were fertilized (28-6-16 Miracle-Gro Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products) twice a month until plants were healthy and established. Ten plants from each population were used to screen for prodiamine resistance using a hydroponic screen similar to the one reported in Cutulle et al. (2009). Treated plants that exhibited root growth similar to nontreated plants were labeled as resistant and sequenced for known target-site mutations on the *α-tubulin* gene (Figure 1).

Table 1. Primer sequences used for amplification and sequencing of *α-tubulin* gene in *Poa annua*.

Primer	Sequence 5′ to 3′	Length	Target sites captured
Tua_ampseq_1F Tua_ampseq_1R	GRCACCARTCSACRAACTGGA GTABGGSACMAGRTTGGTCTG	474 bp	Leu-125, Leu-136, Val-202, Thr-239, Arg-243
Tua_ampseq_2F Tua_ampseq_2R	CCWACCTACACCAACCTSAAC GRCACCARTCSACRAACTGGA	379 bp	Thr-239, Arg-243, Met-268

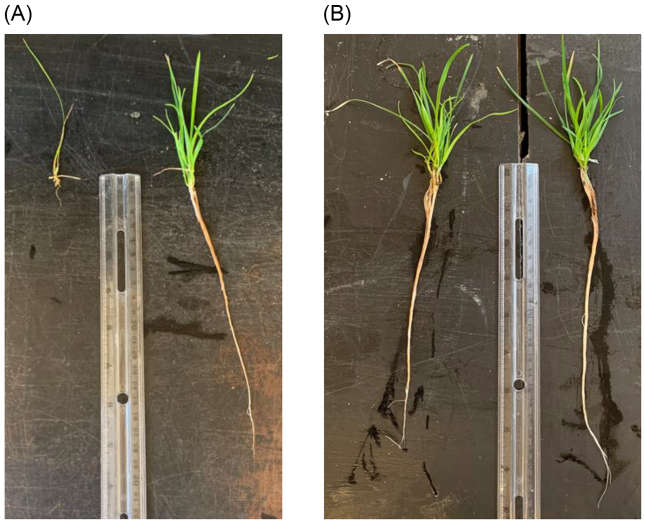

Figure 1. Example of susceptible (A) and resistant (B) populations after the hydroponic screen. In each image, the plant on the left was treated with prodiamine and the plant on the right was nontreated.

Resistant populations were propagated for seed. Seeds were collected from these plants and combined, then dried for 48 h and stored at 4 C for future use.

α-Tubulin Sequencing

Amplicon sequencing was used to determine whether the populations identified as resistant to prodiamine in the initial hydroponic screen had any known target-site mutations. RNA was extracted from 100 to 150 mg of leaf tissue collected from the newest fully developed leaves of a single suspected resistant plant (Direct-zol RNA Kits, Zymo Research, Irvine, CA). RNA was then converted into complementary DNA (cDNA) (qScript cDNA SuperMix, Quantabio, Beverly, MA). A Thermo Scientific Invitrogen Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to check quality and quantity of the cDNA. Two sets of degenerate primers were designed to capture all the reported regions that contain potential target-site mutations (Table 1). Primer 1 covered a 474-bp region on *α-tubulin* including the target sites Leu-125, Leu-136, Val-202, Thr-239, and Arg-243. Primer 2 covered a 379-bp region on *α-tubulin* including the target sites Thr-239, Arg-243, and Met-268. For PCR amplification, roughly 150 ng of cDNA was added to a standard 25 µl PCR rear reaction mix containing 10X standard Taq reaction buffer (New England BioLabs, Ipswich, MA), dNTPs (Promega Corporation, Madison, WI), forward and

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reverse primers, and Taq DNA polymerase (New England BioLabs, Ipswich, MA). Amplification was carried out using a Biometra TOne thermal cycler (Analytik Jena, Jena, Germany) with the following conditions: 30-s denaturing at 95 C; 35 cycles of 30-s denaturation at 95 C, 30-s annealing at 58 C, and 60-s elongation at 68 C, and a final extension step for 10 min at 68 C. The remaining product was then cleaned up for sequencing using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek, Norcross, GA). The DNA was sent for sequencing at GeneWiz using Amplicon-EZ (GeneWiz, South Plainfield, NJ). Sequencing data were analyzed using Snakemake-pipeline (Hall 2020) and CLC Genomics Workbench 20 (Qiagen, Germantown, MD). Putative sequences were read-mapped to the *P. annua* transcriptome (Chen et al. 2016). Sequencing reads for the resistant populations were submitted to NCBI under BioProject number PRJNA847601.

Dose–Response Screen

Three populations of *P. annua* were selected for dose–response screening after sequencing, as they possessed known target-site mutations on the *α-tubulin* gene. Resistant populations were collected from a golf course putting green at the Fort Walton Beach Golf Course in Fort Walton Beach, FL (R1), from the Robert Trent Jones Golf Course in Opelika, AL (R2), and from a golf course fairway at the General Golf Course in Rogersville, AL (R3). A susceptible (S) population was collected from a field next to Crestline Elementary School in Mountain Brook, AL, and screened to confirm that it was susceptible to dithiopyr and prodiamine.

Dose–response screens were conducted in a glasshouse environment from September 2020 to November 2020. No supplemental light was provided, and the greenhouse conditions were 22 ± 2 C throughout the experiment. The trials were conducted at the same time but were separated by space. Dose–response screens were conducted to evaluate prodiamine (Barricade® 4FL, Syngenta Crop Protection, Greensboro, NC) and dithiopyr (Dimension® 2EW, Dow AgroSciences, Indianapolis, IN). Both herbicides had seven rates and a nontreated control for comparison. The rates were the same for each herbicide: 0.01, 0.1, 1.0, 10.0, 100.0, 1,000.0, and 10,000.0 g ai ha^{-1}. These herbicide rates were chosen with an ascending logarithmic scale. Field use rates for prodiamine and dithiopyr are 1681.5 g ai ha^{-1} and 560.5 g ai ha^{-1}, respectively. The experiment was arranged as a completely randomized block design with three replicates. The experiment was repeated in time. The pots were filled with 230 cm^3 of the surface horizon Marvyn loamy sand (fine-loamy, kaolinitic, thermic Typic Kanhapludults) with pH 6.4 and 0.9% organic matter collected from the top 15 cm in an area with no previous presence of *P. annua*. Each population was planted in a separate pot, with 20 seeds in each pot. Soil was added (~2-mm depth) to lightly cover seeds after planting. Pots were sprayed the following day using a CO_2-pressurized backpack sprayer that was equipped with TeeJet® TP 8002 flat-fan nozzles (TeeJet Technologies, Glendale Heights, IL). The sprayer was calibrated to apply 280 L ha^{-1} at 206 kPa. Pots were fertilized (28-6-16 Miracle-Gro Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products) every 2 wk for the duration of the experiment. Pots were irrigated three times daily by an elevated misting system. At 6 wk after treatment, the treated pots were compared with the nontreated control. The number of emerged seedlings was recorded for each pot.

Data Analysis

Dose–response data were subjected to ANOVA at a significance level of $P < 0.05$ using the PROC GLM procedure of SAS v. 9.4 (SAS Institute, Cary, NC). Interactions and main effect of populations, herbicide, herbicide rate, and experimental runs were analyzed. Seedling emergence data for dithiopyr and prodiamine were converted to percent relative to the nontreated. Means and standard errors were generated using the LSMEANS procedure in SAS. Means and standard errors were modeled, and I_{50} values were generated using Prism v. 9.0.0 (GraphPad Software, San Diego, CA). Before modeling, the eight rates for prodiamine and dithiopyr (including the nontreated) were log transformed to log rates with the nontreated set to −3 to maintain equal spacing between treatments. The log-transformed rates were −3, −2, −1, 0, 1, 2, 3, 4, corresponding to 0, 0.01, 0.1, 1.0, 10.0, 100.0, 1,000.0, 10,000.0 g ai ha^{-1} for each herbicide. Seedling emergence control ratings for prodiamine and dithiopyr were modeled using a log(dose) versus response curve equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{[(\log I_{50} - X) * Hillslope]}} \quad [1]$$

where Y is the seedling emergence (%), X is the log rate of the herbicide, Top and Bottom are plateaus, $\log I_{50}$ is the log rate of the herbicide that is needed to reduce the seedling emergence by 50%, and HillSlope is the steepness of the curve. Concentrations to inhibit 50% and 90% of seedling emergence (I_{50} and I_{90}), R^2, and Top and Bottom values were calculated for all populations and herbicides based on regression models (Table 2). I_{90} values were calculated separately for each population as it was not inherent to the model.

Results and Discussion

α-Tubulin Sequencing

Sequencing data revealed that each of the three suspected resistant populations contained a single-nucleotide polymorphism that resulted in an amino acid substitution from threonine to isoleucine at the known target-site of position 239 (Thr-239-Ile) on *α-tubulin* (Figure 2). Although this mutation has yet to be reported to confer resistance to dinitroaniline herbicide in *P. annua*, it has been previously reported in other grass species. Mutations at Thr-239-Ile have been reported to confer resistance to dinitroaniline herbicides in *E. indica*, green foxtail [*Setaria viridis* (L.) P. Beauv.], and rigid ryegrass (*Lolium rigidum* Gaudin) (Anthony et al. 1998; Délye et al. 2004; Fleet et al. 2018). Anthony et al. (1998) reported an *E. indica* population with a Thr-239-Ile mutation that was 60 and 42 times more resistant to oryzalin and trifluralin, respectively, when compared with a sensitive population. Délye et al. (2004) reported a Thr-239-Ile mutation in *S. viridis* that had increased survival rates compared with a susceptible population when treated with trifluralin. Fleet et al. (2018) reported a population of *L. rigidum* with a Thr-239-Ile mutation that was 17 times more resistant to trifluralin than a susceptible population.

Mutations on the *α-tubulin* gene (Leu-136-Phe) have been reported in *E. indica* resistant to dithiopyr (Elmore et al. 2022; Russell et al. 2022). However, there is not enough research on the interaction between dithiopyr's target protein and the α-tubulin protein to confirm whether mutations on *α-tubulin* confer resistance to dithiopyr. Therefore, we are unable to confirm

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Table 2. I_{50} values and I_{90} values, or the concentration that inhibits 50% and 90%, respectively, of seedling emergence, R^2, Top, Bottom, and HillSlope values (see Eq. 1) for resistant (R) and susceptible (S) populations from dose–response screens for both prodiamine and dithiopyr.

Population	I_{50}	I_{90}	R^2	Top	Bottom	HillSlope	R/S ratio[a]
	g ai ha^{-1}			Prodiamine			
R1	35.3	268	0.9686	96.4	4.77	−1.10	2.9
R2	502.7	4,909	0.8832	108.8	4.79	−0.75	41.9
R3	91.5	479	0.9380	114.7	23.45	−5.73	7.6
S	12.0	37	0.9345	134.4	1.23	−5.30	NA
				Dithiopyr			
R1	154.0	1,358	0.9913	98.5	−1.17	−1.98	3.6
R2	114.2	1,130	0.9883	108.5	0.80	−5.94	2.7
R3	190.1	1,799	0.9438	104.7	−0.35	−3.01	4.5
S	42.6	321	0.9333	85.6	−2.51	−0.89	NA

[a]R/S ratio individually compares the I50 values of each R population with the S population for each herbicide.

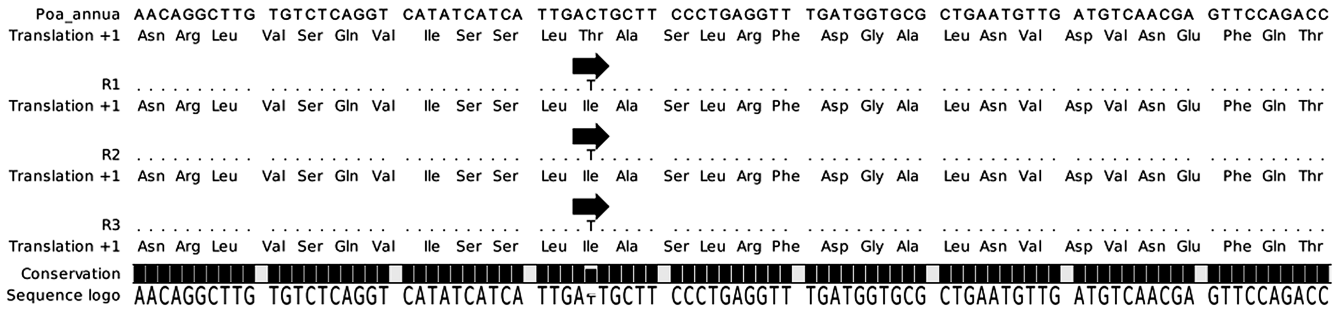

Figure 2. Suspected resistant populations R1, R2, and R3 *α-tubulin* contigs aligned with *Poa annua*. The R populations possess the amino acid substitution Thr-239-Ile.

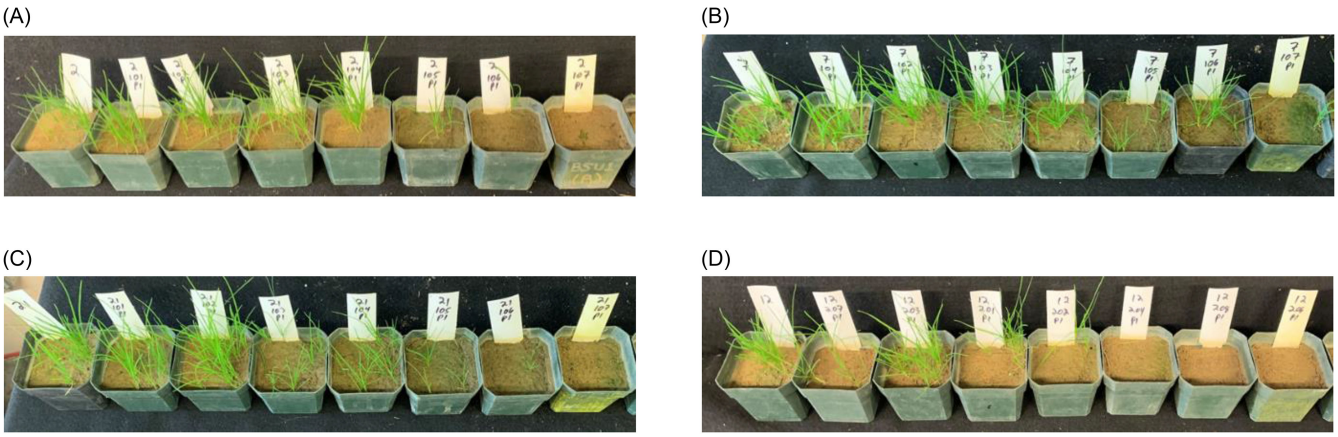

Figure 3. Seedling emergence response for suspected resistant populations R1 (A), R2 (B), R3 (C), and susceptible (S) population (D) to increasing rates of prodiamine.

whether the Thr-239-Ile mutation observed in the three R populations is the causal mechanism of dithiopyr resistance.

Dose–Response Screen

R and S populations responded differently to both herbicides in the dose–response screens (Figures 3 and 4). More seedlings of R populations emerged at higher prodiamine concentrations compared with S (Figure 5). Based on the I_{50} values, the level of resistance varied for the different R populations. I_{50} values for seedling emergence response to prodiamine were 35.3, 502.7, and 91.5 g ai ha^{-1} for R1, R2, and R3, respectively, resulting in 2.9-, 41.9-, and 7.6-fold greater resistance, respectively, than S (I_{50} 12.0 g ai ha^{-1}), based on seedling emergence response. R populations did not vary as greatly in response to dithiopyr (Figure 5). I_{50} values for seedling emergence in response to dithiopyr were 154.0, 114.2, and 190.1 g ai ha^{-1} for R1, R2, and R3, respectively, resulting in 3.6-, 2.7-, and 4.5-fold greater resistance to dithiopyr, respectively, than S (I_{50} 42.6 g ai ha^{-1}). Although variation was observed between R populations for response to both herbicides, the differences were more pronounced with respect to prodiamine response.

Comparisons between I_{90} values and recommended use rates for prodiamine and dithiopyr were made for the R populations. Recommended use rates for prodiamine and dithiopyr can vary widely based on target weeds and desired turfgrass species. For simplicity, the highest application rate for each herbicide was selected for the comparison. These application rates were 1,681.5 g ai ha^{-1} for prodiamine and 560.5 g ai ha^{-1} for dithiopyr. I_{90} values were calculated based on regression curves and were compared with prodiamine and

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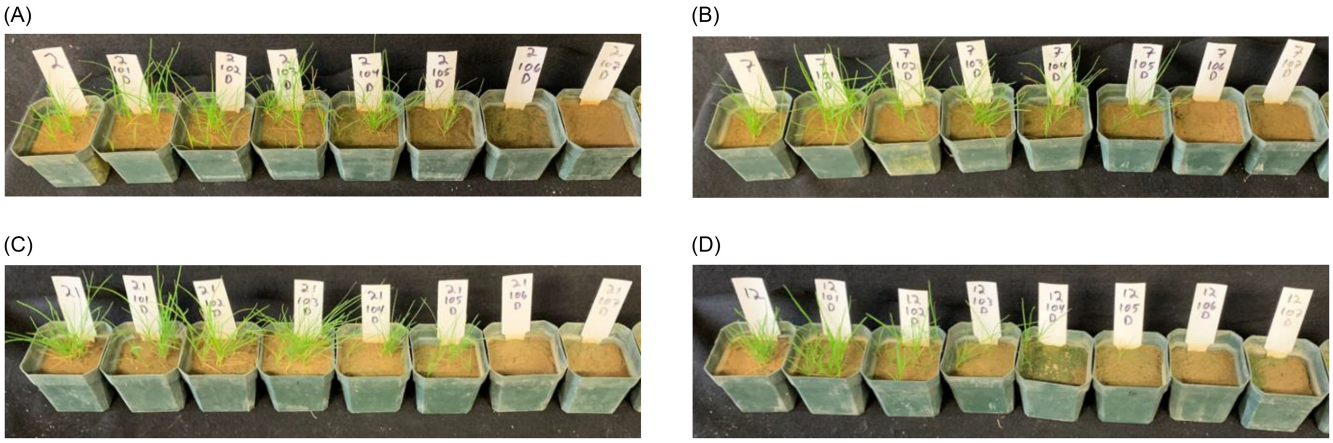

Figure 4. Seedling emergence response for suspected resistant populations R1 (A), R2 (B), R3 (C), and susceptible (S) population (D) to increasing rates of dithiopyr.

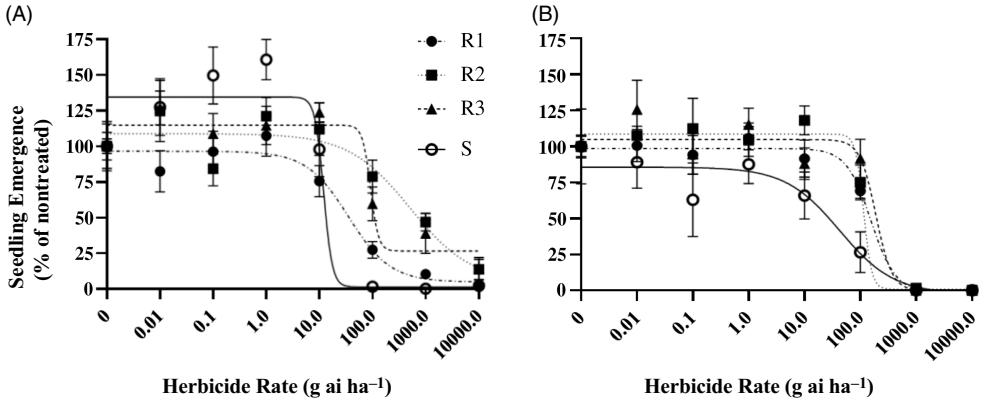

Figure 5. Seedling emergence response of suspected resistant (R1, R2, and R3) and susceptible (S) populations to increasing rates of prodiamine (A) and dithiopyr (B). Seedling emergence is relative to the nontreated. Field use rates for prodiamine and dithiopyr are 1,681.5 g ai ha^{-1} and 560.5 g ai ha^{-1}, respectively. Vertical bars are standard errors of individual means.

dithiopyr application rates to determine whether the population could still be controlled by a high field application rate. For prodiamine, the I_{90} values were 268, 4,909, and 479 g ai ha^{-1} for R1, R2, and R3, respectively. This resulted in R2 having a 2.9-fold level of resistance to the highest labeled rate of prodiamine. While R1 and R3 had I_{90} values less than the highest labeled rate, the labeled rate in some turf species for prodiamine can be as low as 420 g ai ha^{-1}. So, this rate would potentially still control most of R1, but R3 would have less than ideal control at that lower rate. For dithiopyr, I_{90} values were 1,358, 1,130, and 1,799 g ai ha^{-1} for R1, R2, and R3, respectively. This resulted in 2.4-, 2.0-, and 3.2-fold levels of resistance to dithiopyr for R1, R2, and R3, respectively. These data show that even at the highest application rate, R2 would not be adequately controlled with prodiamine, and R1, R2, and R3 would not be adequately controlled with dithiopyr, indicating that there is potential cross-resistance. This reveals that these herbicides are no longer useful when it comes to controlling these populations and that other herbicide modes of actions are needed.

Variation in resistance level to different herbicides in the same family or across different species is common. As seen in previous research, the level of resistance to dinitroaniline herbicides confered by the Thr-239-Ile mutation varies among species and different dinitroaniline herbicides. However, it is interesting that there is variation in the level of resistance to a single herbicide present between three R populations of *P. annua*, even though they all possess the same target-site mutation (Thr-239-Ile). This variation in resistance could be due to *α-tubulin* expression, *β-tubulin* mutation, non–target site resistance mechanisms, or a combination of these factors (Schibler and Huang 1991). An *α-tubulin* study in corn revealed that gene expression occurred at different locations, with *tua*1 being more expressed in pollen and the root apex, while *tua*3 was expressed in the immature embryo and the vascular cylinder of the root (Uribe et al. 1998). The differences in where certain *α-tubulin* genes are expressed could explain why R1 possessed a known mutation but was more susceptible to prodiamine, especially if the mutated gene is not expressed in the roots. Also, increased metabolism of the herbicide or reduced absorption could affect the resistance level of these populations. The goal of this research was focused on finding known target-site mutations, but future research needs to be focused on understand *α-tubulin* copy number and expression throughout the plant and how non–target site mechanisms could affect resistance level.

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research was conducted without any commercial or financial interactions that could be interpreted as likely conflicts of interest.

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