Identification of two *Eleusine indica* (goosegrass) biotypes of cool-season turfgrass resistant to dithiopyr

Matthew T Elmore,a,* Katherine H Diehl,a Rong Di,a Jinyi Chen,b Eric L Patterson,b James T Brosnan,c Robert N Trigiano,d Daniel P Tuck,a Sarah L Boggessd and Steven McDonald e

Abstract

Background: Turfgrass managers reported poor *Eleusine indica* control following applications of the mitosis-inhibiting herbicide dithiopyr in cool-season turfgrass. Field, glasshouse, and laboratory experiments were conducted to understand the response of these biotypes to dithiopyr and prodiamine.

Results: In field experiments at two locations with putative dithiopyr-resistant *E. indica*, preemergence applications of dithiopyr provided no *E. indica* control. Single applications of the protoporphyrinogen oxidase (PPO)-inhibitor, oxadiazon, provided > 85% control at these locations. When subjected to agar-based bioassays, root growth of putative resistant biotypes planted with 0.01 mmol L$^{-1}$ dithiopyr was slightly reduced (< 25%) whereas roots were completely inhibited in the susceptible biotype. Glasshouse whole plant rate-response experiments found that the cytochrome P450 inhibitor, piperonyl butoxide (PBO), did not increase the sensitivity of these putative resistant biotypes to dithiopyr. Sequencing of $\alpha$-tubulin 1 (*TUA1*) revealed a Leu-136-Phe substitution in both dithiopyr-resistant populations.

Conclusion: *Eleusine indica* biotypes with resistance to dithiopyr are present in cool-season turfgrass systems in the United States. Resistance is possibly related to a single nucleotide polymorphism (SNP) of an $\alpha$-tubulin gene. If turfgrass managers suspect resistance to dithiopyr, oxadiazon can still be an effective alternative for preemergence control.

© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Keywords: resistance; goosegrass; *Eleusine indica*; microtubule; turfgrass; oxadiazon; dithiopyr; *TUA1*

1 INTRODUCTION

*Eleusine indica* L. (commonly referred to as goosegrass) is C$_4$ grass that behaves as a summer annual in temperate climates. It is problematic in turfgrass systems, especially high traffic areas with limited competition from turfgrass.1,2 Turfgrass managers often rely on preemergence applications of mitosis-inhibiting dinitroaniline herbicides pendimethalin or prodiamine, the mitosis-inhibiting pyridine herbicide dithiopyr, and the protoporphyrinogen oxidase (PPO)-inhibitor oxadiazon to control *E. indica*, crabgrass (*Digitaria* spp.), and other problematic summer annual weeds.3–6 Among the aforementioned preemergence herbicides, oxadiazon is widely considered the most effective against *E. indica* and its use is prevalent in warm-season (C$_4$ photosynthetic pathway) turfgrass of the southern United States where resistance to dinitroaniline herbicides is common.2–4,7,8 In cool-season (C$_3$ photosynthetic pathway) turfgrass of the northern United States where annual crabgrasses (*Digitaria* spp.) were historically the most problematic summer annual grassy weed, dithiopyr and prodiamine use is prevalent as they are more effective against crabgrass than oxadiazon.9–11 Dithiopyr can provide early postemergence crabgrass control, allowing the end-user to delay application until shortly after weed emergence.12

*Eleusine indica* with resistance to dinitroaniline herbicides was first reported by Mudge et al.7 in cotton (*Gossypium hirsutum* L.) after repeated trifuralin applications in South Carolina, USA. Further investigation revealed biotypes with high and intermediate

---

* Correspondence to: M Elmore, Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ 80901, USA. E-mail: matthew.elmore@rutgers.edu

a Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

b Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA
c Plant Sciences Department, The University of Tennessee, Knoxville, TN, USA
d Department of Entomology and Plant Pathology, The University of Tennessee, Knoxville, TN, USA
e Turfgrass Disease Solutions, LLC, Spring City, PA, USA

© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
levels of resistance prevalent in several counties of South Carolina where trifuralin use was common. Dinitroaniline-resistant biotypes have also been reported in warm-season turfgrass of the south-eastern United States. In *E. indica*, the Thr-239-Ile substitution in the α-tubulin gene 1 (TUA1) confers strong resistance to dinitroaniline herbicides, and the Met-268-Thr substitution confers intermediate resistance. Another dinitroaniline-resistant *E. indica* biotype with resistance to dithiopyr was reported in a warm-season turfgrass system in Georgia, USA and is the only reported case of *E. indica* resistance to dithiopyr. Although the severity and mechanism of resistance were not reported, the population was subjected to at least seven consecutive years of dinitroaniline herbicide use. Interestingly, *E. indica* resistance to mitosis-inhibiting herbicides has not yet been reported in cool-season turfgrass.

Generally, herbicide resistance mechanisms involve target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms. Both dithiopyr and dinitroanilines cause dividing cells to not form spindle microtubules and therefore arrest mitosis during prometaphase. While the target site of dinitroanilone herbicides is clearly members of the tubulin protein family, dithiopyr has been reported not to bind tubulin proteins, but instead showed affinity for an unidentified 68-kDa microtubule-associated protein (MAP). No TSR for dithiopyr has been revealed. For NTSR, enhanced herbicide metabolism catalyzed by cytochrome P450 (CYP450) enzymes is a common mechanism of herbicide resistance. A CYP450 inhibitor piperonyl butoxide (PBO) was found to enhance the efficacy of a dithiopyr-related pyridine herbicide, thiazopyr, in other plants. Additionally, in animals, CYP450 metabolism of dithiopyr was found in rat liver.

Poor *E. indica* control following dithiopyr applications at golf courses in East Brunswick and Manalapan, NJ, USA prompted this investigation. Course managers reported applying dithiopyr at 430 to 560 g ha⁻¹ in spring for several consecutive years. Since *E. indica* resistance to dithiopyr or dinitroanilone herbicides has not been reported in cool-season turfgrass, the objectives of this research were to (i) determine the sensitivity of these *E. indica* biotypes to dithiopyr and other herbicides through field experiments and controlled environment assays and (ii) to begin elucidating the mechanism of resistance.

## 2 MATERIAL AND METHODS

### 2.1 Field experiments

Field experiments were conducted in 2017 and 2018 to determine the efficacy of dithiopyr, prodiamine, and oxadiazon for preemergence *E. indica* control. In 2017, an experiment was conducted on a putative-resistant biotype at Tamarack Golf Course in East Brunswick, NJ, USA (biotype TR; 40° 25'35.7"N 74° 27'08.7"W) on a Manahawkin muck (sandy or sandy-skeletal, siliceous, dysic, mesic Terric Haplosaprist). In 2018, three identical experiments were initiated, two on putative resistant sites and another on a site with susceptible *E. indica*. Sites with putative-resistant biotypes were Pine Brook Golf Course in Manalapan, NJ, USA (biotype PB; 40° 19'51.2"N 74° 18'36.1"W) on a Holmedel sandy loam (fine-loamy, mixed, active, mesic Aquic Hapludults) and Walnut Lane Golf Course in Philadelphia, PA, USA (biotype WL; 40° 01'51.2"N 75° 12'11.8"W) on an urban land Chester-complex soil (fine-loamy, mixed, semiactive, mesic Typic Hapludults). The susceptible biotype was located at Rutgers Hort Farm No. 2 (Hort Farm) in North Brunswick, NJ, USA (biotype HF-S; 40° 28'07.0"N 74° 25'24.3"W) on a sandy loam soil of unknown origin.

The Tamarack and Pine Brook sites were similar in that both had severe *E. indica* infestations (~50 plants m⁻²) the previous summer despite the course manager applying dithiopyr preemergence for several years prior. The Walnut Lane site had a moderate *E. indica* infestation (~10 plants m⁻²) the previous summer. The history of preemergence herbicide use at Walnut Lane was not well recorded but is thought to include several years of dinitroaniline herbicide applications. The Hort Farm site did not have a history of preemergence herbicide use.

The Tamarack and Pine Brook sites were *Lolium perenne* L. (perennial ryegrass) maintained at 1.3 cm height of cut, irrigated to prevent wilt and treated with fungicides to prevent fungal disease. The Walnut Lane site was a mixed stand of cool season turfgrass (*L. perenne*, *Poa pratensis* L. and *P. annua* L.) maintained at a 4.0 cm mowing height and irrigated to prevent wilt. The Hort Farm site was *L. perenne* mowed at 1.5 cm and irrigated to optimize *E. indica* survival. Quinclorac (0.56 kg ha⁻¹; Drive® XLR8 0.75 L, BASF Corp., Research Triangle Park, NC, USA) was applied on May 16 and July 12, 2018 at the Hort Farm site to control *Digitaria* spp. without affecting *E. indica*. In 2017 at the Tamarack site, treatments consisted of single or sequential applications of dithiopyr (0.56 kg ha⁻¹; Dimension® 2EW, Dow AgroSciences LLC, Indianapolis, IN, USA), prodiamine (1.12 kg ha⁻¹; Barricade® 65WG, Syngenta Crop Protection LLC, Greensboro, NC, USA), pendimethalin (3.36 kg ha⁻¹; Pendulum® AquaCap™ 3.8MEC, BASF Corp.), and oxadiazon (4.5 kg ha⁻¹; Ronstar® 2G, Bayer Environmental Science, Cary, NC, USA). Sequential application programs applied half the total herbicide rate on May 1 and June 28, 2017, while the full application rate was applied on May 1 for single application programs. These eight treatments were applied to 0.9 by 3.3 m plots arranged in a randomized complete block design with four replications. A non-treated check plot was included in each block for comparison. At Pine Brook, Walnut Lane, and Hort Farm in 2018 treatments included dithiopyr, prodiamine and oxadiazon, but the rates were slightly different than those applied in 2017. Treatments included single oxadiazon application programs (2.24, 2.80, and 3.36 kg ha⁻¹), sequential oxadiazon programs (2.24, 2.80, and 3.36 kg ha⁻¹ followed by 2.24 kg ha⁻¹), single and sequential dithiopyr programs (0.56 kg ha⁻¹ and 0.28 followed by 0.56 kg ha⁻¹, respectively), and single and sequential prodiamine programs (0.73 kg ha⁻¹ and 0.36 followed by 0.36 kg ha⁻¹, respectively). Oxadiazon was applied as 0.67G fertilizer (0–0–50, Harrell’s LLC, Lakeland, FL, USA) formulation and prodiamine and dithiopyr formulations were the same used in 2017. These ten treatments were applied to 0.9 by 2.1 m plots at Pine Brook and Hort Farm and 0.8 by 1.5 m plots at Walnut Lane. The experimental design at all locations was a randomized complete block design with four replications. A non-treated check plot was included in each block for comparison. Initial and sequential applications were made on May 2 and June 19, respectively, at the Hort Farm location, on May 1 and June 20 at the Pine Brook location and on April 9 and May 14 at the Walnut Lane location.

In all experiments, sprayable treatments were applied with water carrier at 420 L ha⁻¹ using a hand-held carbon dioxide (CO₂)-pressurized sprayer equipped with flat-fan nozzles typical of small-plot research. Oxadiazon treatments were granular and applied by hand using a shaker jar. All treatments were integrated into the soil with 0.5 cm of irrigation within 6 h of application. *Elymus indica* control was evaluated visually on a 0 (no control) to
Eleusine indica resistance to dithiopyr

100 (complete control) percent scale relative to non-treated control plots. *E. indica* cover was estimated visually on a 0 (no cover) to 100 (complete cover) percent scale in non-treated check plots to indicate the severity of the *E. indica* infestation. *Eusine indica* cover in the non-treated check plots was estimated to be 50%, 75%, 85%, and 40% at the Tamaraack, Hort Farm, Pine Brook, and Walnut Lane locations, respectively, when herbicide efficacy was evaluated.

The 2017 experiment data were analyzed as a single-factor randomized complete block design. The 2018 field experiments were analyzed as a complete factorial with herbicide (oxadiazon at 2.24, 2.80, and 3.36 kg ha\(^{-1}\), dithiopyr, and prodiame) and number of applications (single or sequential) as main effects. Non-treated check data were removed from the analysis of variance (ANOVA). Model assumptions were tested through residual analysis (Shapiro–Wilk statistic) in SAS (Statistical Analysis Software, Inc., Cary, NC, USA). Data from Pine Brook were subjected to an arcsine square root transformation to improve distribution of transformed values are presented for clarity. Analysis of variance was performed using the mixed-model procedure\(^{29}\) in SAS and Fisher’s Protected least significant difference (LSD) was used to compare means. Treatment effects were fixed while block effects considered random.\(^{30}\) Data from each location were analyzed separately. A contrast was conducted for the 2018 experiments to determine if *E. indica* control provided by a single application of oxadiazon (aggregated across three rates) was greater than two sequential applications (aggregated across three rate programs).

### 2.2 Seed collection

Seeds of *E. indica* surviving dithiopyr treatment in May 2016 were collected in December 2016 from the Tamaraack location (biotype TR) before initiating previously described field studies. *Eusine indica* plants surviving dithiopyr treatment in the 2018 field experiments were harvested from the Walnut Lane (biotype WL) and Pine Brook (biotype PB) locations and cultured in the glasshouse for seed. Susceptible plants were collected from the Hort Farm location (biotype HF-S) in September 2018 and cultured for seed as well. *Eusine indica* seed from field accessions were dried in a forced-air oven at 35 °C and then stored in coin envelopes at 2 °C.

### 2.3 Laboratory assays to confirm resistance

A Murashige and Skoog (MS)\(^{31}\) medium was used in a bioassay to determine the response of each biotype to dithiopyr and prodiame. Cutulle *et al.*\(^{32}\) found that this bioassay was preferred to other methods to detect resistance to mitosis-inhibiting herbicides. Herbicide (prodiame or dithiopyr) was mixed with the media (10 g L\(^{-1}\) agarose and 2.15 g L\(^{-1}\) MS powder with vitamins (PhytoTech Labs, Lenexa, KS, USA)) at 0, 0.01, 0.05, 0.1, 1.0, and 10.0 mmol L\(^{-1}\) concentrations. Seeds of all four biotypes (HF-S, WL, TR, and PB) were surface sterilized by agitation for 20 min at 200 rpm in a solution of 10% (v/v) sodium hypochlorite (NaOCl) and 0.1% polysorbate 20 (Tween\(^{*}\) 20, Sigma Aldrich, St Louis, MO, USA) surfactant, followed by three ethanol washes, and several rinses with sterile deionized water. Ten surface-sterilized seeds were placed on the media within each 10-cm square polystyrene Petri plate. The top 2 cm of media was removed from each plate to allow shoots to grow without contacting the media. Plates were placed at 45° angles to encourage gravitropic root growth. Each concentration was replicated four times. After 21 days, root length was measured using the WinRhizo Arabidopsis System (Regent Instruments Inc., Quebec City, Canada) to determine total root length of each individual plant. On the day of root measurement, non-treated control plants were at the 2- to 3-leaf stage of growth. *Eusine indica* plants were carefully removed from the media and placed into 10 cm by 15 cm trays where roots were separated in a thin film of distilled water. Images of each tray were acquired using a gray scan at 400 dpi. The image area containing only roots was identified. The selected areas were analyzed using Regent’s easy method with the default WinRHIZO settings, to find the total root length for each plant.

Root length data were expressed as a percentage of non-treated check within each main effect and subjected to analysis in SAS. Model assumptions were tested as described earlier. The main factors of *E. indica* biotype and herbicide concentration were fixed effects and individual plants were considered subsamples in a completely randomized factorial design. Least squares non-linear regression analysis was used to calculate the herbicide concentration resulting in 50% growth inhibition (GR\(_{50}\)) values for each population as main effect interactions were significant (\(\sigma = 0.05\)). Root length data were regressed over herbicide rate using a logistic ‘inhibitor versus response’ model in Prism (Prism 9.0, GraphPad Software, San Diego, CA, USA).

### 2.4 Rate response with piperonyl butoxide

To determine whether increased CYP450 enzyme activity contributed to dithiopyr resistance, a glasshouse pot experiment was conducted with the resistant PB and TR biotypes. Treatments were dithiopyr at 0, 10, 50, 100, 500, and 1000 g active ingredient (ai) ha\(^{-1}\) alone and in combination with 1.12 kg a.i. ha\(^{-1}\) of the P450-inhibitor PBO. For comparison to resistant biotypes, the susceptible HF-S biotype was used in the first experimental run, but HF-S data could not be analyzed due to poor germination in all pots including the non-treated controls. This prompted a switch to the WL biotype (determined susceptible in the MS-media bioassay experiment) in the second experimental run. Pots were arranged in a randomized complete block design with four replicates at each rate. The experiment was repeated in time. Experiments were conducted at the Rutgers University New Jersey Agricultural Experiment Station Research Glasshouse in New Brunswick, NJ, USA. The photoperiod was 16 h with supplemental lighting via 400-W high pressure sodium light bulbs when PAR fell below 400 \(\mu\)E m\(^{-2}\) s\(^{-1}\). Air temperatures averaged 21 and 25 °C in the first and second runs, respectively. Daily high and low temperatures averaged 21 and 29 °C, respectively in the first run and 16 and 28 °C in the second run.

Pots (12 cm by 12 cm, filled with silica sand and sphagnum peat moss (4:1, v/v)) were irrigated until saturated, seeded with 25 *E. indica* seeds, and covered with 2 mm of sand/peat mix and lightly irrigated. Seeded pots were treated within 24 h of seeding. Dithiopyr and PBO were applied sequentially with 420 L ha\(^{-1}\) of water carrier through a single flat-fan nozzle (8002 EVS; Spraying Systems Co., Roswell, GA, USA) in a spray chamber (Generation III Research Track Sprayer, DeVries Manufacturing, Hollandale, MN, USA). Treatments were incorporated into the soil with approximately 10 mm of overhead irrigation. Pots were then irrigated lightly to maintain soil moisture but minimize herbicide dissipation. The number of *E. indica* plants in each pot was counted 7 days after seeding emergence at 25 and 18 days after treatment in runs 1 and 2, respectively. After counting, aboveground biomass was harvested from each pot and dried in a laboratory oven at 65 °C for 5 days and weighed. Plant count and biomass

Pest Manag Sci 2022; 78: 499–505 © 2021 The Authors.

wileyonlinelibrary.com/journal/ps

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
Table 1. Eleusine indica control on August 17, 2018 following preemergence herbicide applications in 2018

| Herbicide | Rate (kg ha⁻¹) | Application | Hort farm Percent | Walnut lane Percent |
|-----------|----------------|-------------|-------------------|---------------------|
| Dithiopyr | 0.56           | Single      | 18                | 3                   |
|           | 0.28 f.b. 0.56 | Sequential  | 65                | 83                  |
| Prodiamine| 0.73           | Single      | 64                | 0                   |
|           | 0.36 f.b. 0.36 | Sequential  | 53                | 19                  |
| Oxadiazon | 2.24           | Single      | 76                | 71                  |
|           | 2.24 f.b. 2.24 | Sequential  | 86                | 91                  |
|           | 2.80           | Single      | 86                | 91                  |
|           | 2.80 f.b. 2.24 | Sequential  | 86                | 91                  |
|           | 3.36           | Single      | 86                | 75                  |
|           | 3.36 f.b. 2.24 | Sequential  | 85                | 95                  |
|           |                | LSD₀.₀⁵      | 20                | 24                  |

P-Value: 0.002 < 0.001
Contrast: Oxadiazon single versus sequential

Field experiments were conducted at Walnut Lane Golf Course (Walnut Lane) in Philadelphia, PA, USA on a suspected microtubule-inhibitor resistant biotype and at Rutgers Hort Farm No. 2 (Hort Farm) in North Brunswick, NJ, USA on a known susceptible biotype.

Control was evaluated on a 0 (no control) to 100 (complete control) percent scale relative to the non-treated control.

Treatments for the single application program were applied on May 2 and April 9, 2018 at the Hort Farm and Walnut Lane locations, respectively. The sequential application programs consisted of two applications on May 2 and June 19, 2018 at the Hort Farm location and on April 9 and May 14, 2018 at the Walnut Lane location.

Abbreviation: f.b., followed by.

Note: *, **, significant when α ≤ 0.05, 0.01, respectively.

2.5 Tubulin sequencing and molecular analysis

Seeds of susceptible (S) and herbicide resistant (PB and TR biotypes) E. indica were planted as described in section 2.4. The main factors of E. indica biotype, herbicide rate, and CYP450 inhibitor were considered fixed effects while block and experimental run were considered random. Least squares non-linear regression analysis was used to calculate GR₅₀ values for each population where main effect interactions were significant (α = 0.05). Biomass data were regressed over herbicide rate using logistic regression in Prism. To determine if GR₅₀ values differed due to biotype or CYP450 inhibitor treatment, a lack of fit F test was conducted in Prism. A different curve was calculated for each biotype and CYP450 inhibitor treatment when the F test indicated it was appropriate.

3 RESULTS AND DISCUSSION

3.1 Field experiments

In 2017 at the Tamarack location, single and sequential dithiopyr applications provided 0% E. indica control compared to > 85% control with oxadiazon (data not presented). A single application of prodiamine provided 46% control, whereas sequential applications provided 23% control. Pendimethalin provided < 20% control regardless of application regimen.

In 2018, sequential applications of dithiopyr provided 65 and 83% E. indica control at the Hort Farm and Walnut Lane locations, respectively (Table 1). The moderate dithiopyr efficacy we observed has been reported in other research and is attributed to a severe E. indica infestation at the test site where turfgrass density was intentionally poor. Oxadiazon provided greater efficacy than dithiopyr, which aligns with findings of Johnson. Comparatively at the Pine Brook location, single and sequential applications of dithiopyr and prodiamine provided 0% E. indica control on August 17, 2018 following preemergence herbicide applications in 2018.

DNA polymerase chain reaction (PCR) was conducted with DreamTaq DNA polymerase (Thermo Fisher Scientific) to amplify the internal 591 bp cDNA fragment encompassing nucleotides #235 to #825 (amino acid residues #79 to #275) with forward primer 5’ AGGA CTGGCACCTACCAGCCAG 3’ and reverse primer 5’ CACTGGCGC GTAGGATGAAAG 3’. The PCR was conducted in a 50 μL volume, consisting of 2 μL of cDNA product from the RT reaction, 0.8 μL of each primer, 4 μL of 2.5 mmol L⁻¹ dNTP mix, 0.3 μL DreamTaq (Thermo Fisher Scientific) DNA polymerase, 5 μL of 10X buffer and water. The PCR was run in the Veriti thermocycler (Applied Biosystems) with the following program: The PCR fragments were cloned into pGEMT-easy and sequenced with T7 promoter primer (Quintara BioScience, Cambridge, MA, USA). Two to four clones were sequenced from each plant.

3 RESULTS AND DISCUSSION

3.1 Field experiments

In 2017 at the Tamarack location, single and sequential dithiopyr applications provided 0% E. indica control compared to > 85% control with oxadiazon (data not presented). A single application of prodiamine provided 46% control, whereas sequential applications provided 23% control. Pendimethalin provided < 20% control regardless of application regimen.

In 2018, sequential applications of dithiopyr provided 65 and 83% E. indica control at the Hort Farm and Walnut Lane locations, respectively (Table 1). The moderate dithiopyr efficacy we observed has been reported in other research and is attributed to a severe E. indica infestation at the test site where turfgrass density was intentionally poor. Oxadiazon provided greater efficacy than dithiopyr, which aligns with findings of Johnson. Comparatively at the Pine Brook location, single and sequential applications of dithiopyr and prodiamine provided 0% E. indica control on August 17, 2018 following preemergence herbicide applications in 2018.

DNA polymerase chain reaction (PCR) was conducted with DreamTaq DNA polymerase (Thermo Fisher Scientific) to amplify the internal 591 bp cDNA fragment encompassing nucleotides #235 to #825 (amino acid residues #79 to #275) with forward primer 5’ AGGA CTGGCACCTACCAGCCAG 3’ and reverse primer 5’ CACTGGCGC GTAGGATGAAAG 3’. The PCR was conducted in a 50 μL volume, consisting of 2 μL of cDNA product from the RT reaction, 0.8 μL of each primer, 4 μL of 2.5 mmol L⁻¹ dNTP mix, 0.3 μL DreamTaq (Thermo Fisher Scientific) DNA polymerase, 5 μL of 10X buffer and water. The PCR was run in the Veriti thermocycler (Applied Biosystems) with the following program: The PCR fragments were cloned into pGEMT-easy and sequenced with T7 promoter primer (Quintara BioScience, Cambridge, MA, USA). Two to four clones were sequenced from each plant.

3 RESULTS AND DISCUSSION

3.1 Field experiments

In 2017 at the Tamarack location, single and sequential dithiopyr applications provided 0% E. indica control compared to > 85% control with oxadiazon (data not presented). A single application of prodiamine provided 46% control, whereas sequential applications provided 23% control. Pendimethalin provided < 20% control regardless of application regimen.

In 2018, sequential applications of dithiopyr provided 65 and 83% E. indica control at the Hort Farm and Walnut Lane locations, respectively (Table 1). The moderate dithiopyr efficacy we observed has been reported in other research and is attributed to a severe E. indica infestation at the test site where turfgrass density was intentionally poor. Oxadiazon provided greater efficacy than dithiopyr, which aligns with findings of Johnson. Comparatively at the Pine Brook location, single and sequential applications of dithiopyr and prodiamine provided 0% E. indica control on August 17, 2018 following preemergence herbicide applications in 2018.
Table 2. *Eleusine indica* control on August 17, 2018 following pre-emergence herbicide applications in 2018 at Pine Brook Golf Course (Pine Brook) in Manalapan, NJ, USA where a putative microtubule-inhibitor resistant biotype was present

| Herbicide    | Rate (kg ha⁻¹) | Pine Brook |
|--------------|----------------|------------|
| Dithiopyr    | —              | 0 b b      |
| Prodiamine   | —              | 0 b        |
| Oxadiazon    | 2.24           | 94 a       |
| Oxadiazon    | 2.80           | 93 a       |
| Oxadiazon    | 3.36           | 95 a       |
| P-Value      | < 0.001        |            |
| Application  | Single         | 54 b       |
| regimen      | Sequential     | 58 a       |
| P-Value      | < 0.001        |            |
| Contrast     | Oxadiazon single versus sequential | *** |

Means are presented across main effects of herbicide (oxadiazon at 2.24, 2.80, and 3.36 kg ha⁻¹, dithiopyr, and prodiamine) and number of applications (single or sequential). Single oxadiazon application programs consisted of one application at 2.24, 2.80, and 3.36 kg ha⁻¹. Sequential oxadiazon programs consisted of one application at 2.24, 2.80, and 3.36 kg ha⁻¹ followed by an application at 2.24 kg ha⁻¹. Single dithiopyr and prodiamine programs consisted of one application at 0.56 and 0.73 kg ha⁻¹, respectively. The sequential dithiopyr program consisted of an application at 0.28 kg ha⁻¹ followed by 0.56 kg ha⁻¹. The sequential prodiamine programs consisted of two applications at 0.36 kg ha⁻¹. Initial applications were made on May 1 and sequential applications on June 20, 2018.

Control was evaluated on a 0 (no control) to 100% (complete control) scale relative to the non-treated control. Within main effects of herbicide and application regimen, means followed by the same letter do not differ according to Fisher’s Protected LSD (α = 0.05).

Note: ***, significant when α ≤ 0.001, respectively.

control whereas oxadiazon controlled *E. indica* > 85% (Table 2). Results from 2 years of field experiments suggest that *E. indica* at our Tamarack and Pine Brook locations warranting more investigation.

Oxadiazon generally provided similar *E. indica* control regardless of application rate in all experiments. Generally, these results agree with previous cool-season turfgrass research reporting good to excellent *E. indica* control from single applications of oxadiazon at rates as low as 2.2 kg ha⁻¹. Single degree of freedom contrasts highlighted that sequential oxadiazon applications provided more *E. indica* control than single applications. Although McCullough et al. detected no benefit to sequential applications at higher oxadiazon rates (4.5 kg ha⁻¹) than those used in this experiment, the work of Dernoeden et al. found sequential applications provided better control than single applications at 1.8 kg ha⁻¹ where *E. indica* infestations were severe due to poor turfgrass cover.

### 3.2 Laboratory assay to confirm resistance

Prodiamine treatments resulted in no measurable root growth in any of the four biotypes tested at all concentrations (data not presented). Non-treated mean root lengths for the HF-S, WL, TR, and PB biotypes were 2.3 cm (N = 37), 7.6 cm (N = 37), 2.9 cm (N = 23), and 4.9 cm (N = 19), respectively. The lowest dithiopyr concentration (0.01 mmol L⁻¹) resulted in no measurable root growth for the WL and HF-S biotypes (Fig. 1). In previous research 0.01 mmol L⁻¹ dithiopyr completely inhibited susceptible *P. annua* root growth. At 0.01 mmol L⁻¹ dithiopyr, TR and PB root length was > 75% of the non-treated. Dithiopyr GR₅₀ values were calculated to be 0.014 and 0.015 mmol L⁻¹ for TR and PB biotypes, respectively. These results align with observations from our field experiments where dithiopyr provided no control of TR and PB biotypes. It is not possible to determine the magnitude of...
resistance in this experiment given that the lowest dithiopyr concentration completely inhibited root growth of susceptible biotypes (WL and HF-S). However, comparing our results to experiments conducted with putative resistant E. indica biotypes of warm-season turfgrass suggests the magnitude of resistance may be similar. For example, in a whole plant hydroponics assay, exposure to 0.001 mmol L\(^{-1}\) prodiamine had minimal effect on root growth (94% of non-treated) and concentrations of 0.01 mmol L\(^{-1}\) were needed to reduce root growth to < 10% of the non-treated.\(^{14}\) In another whole plant hydroponics assay, Murphy and Smith\(^{16}\) observed pendimethalin and dithiopyr concentrations as high as 1.0 μmol L\(^{-1}\) did not cause root growth of susceptible biotypes. CYP450 belongs to a large superfamily composed of about 50 sub-families with hundreds of members (e.g. 244 in Arabidopsis and 356 in rice).\(^{33}\) One CYP450 inhibitor can only inhibit certain members within the family. Therefore, it is still possible that some CYP450 enzymes are involved in dithiopyr resistance.

### 3.4 Tubulin sequencing and molecular analysis

Sequencing results of the cDNA clones from five S plants, four resistant PB plants and three resistant TR plants did not show the Thr-239-Ile or Met-268-Thr mutations known to confer resistance to dinitroaniline herbicides.\(^{19}\) However, a CTT to TTT mutation resulting in the amino acid substitution of Leu by Phe at residue 136 was identified in all clones of the resistant plants (Fig. 3), while all susceptible plants sequenced were 136-Leu.

Thus far, the Leu-136-Phe α-tubulin mutation has been reported to confer dinitroaniline resistance in weed species like Setaria viridis (green foxtail) and Alopecurus aequalis (shortawn foxtail).\(^{34,35}\) In protozoa, Tetrahymena thermophila and Toxoplasma gondii strains with 136-Phe tubulin mutation exhibited resistance to oryzalin.\(^{36,37}\) However, there is no report on tubulin mutations conferring resistance to dithiopyr, and it has previously been shown that dithiopyr probably does not bind tubulin directly.\(^{22}\) We did not detect resistance to prodiamine in these dithiopyr-resistant biotypes even though prodiamine provided no control in field experiments. It is likely that prodiamine concentrations used in the MS media bioassay (≥ 10 μmol L\(^{-1}\)) were too high to detect a resistant phenotype. We expect this concentration would detect resistance of Thr-239-Ile mutants based on previous research.\(^{14,32}\) However, research with green foxtail Leu-136-Phe mutants used a much lower 0.2 μmol L\(^{-1}\) trifluralin to discriminate among resistant and susceptible phenotypes. It is possible that the Leu-136-Phe confers a lower level of resistance dinitroanilines than dithiopyr.

There are three possible explanations for our results: (i) Even if dithiopyr does not bind directly to α-tubulin, it selects for this Leu-136-Phe mutation at rates commonly used in the field by practitioners. Research with protozoa found purified mutant Leu-136-Phe tubulin heterodimers free of high-molecular-weight MAPs have greater affinity for each other, even in the absence of microtubule-inhibiting herbicides.\(^{33}\) This greater affinity may overcome the effects of dithiopyr. (ii) It is possible that repeated dinitroaniline from before dithiopyr was commercialized selected for this Leu-136-Phe mutation. (iii) Given that the dithiopyr site of action is unknown, there is an unexplained connection between dinitroaniline resistance and dithiopyr resistance as it relates to Leu-136-Phe mutants. Further research on the association between the 136 α-tubulin mutation and dithiopyr resistance is warranted.

### 4 CONCLUSION

_Eleusine indica_ populations in our study exhibited resistance to the microtubule-inhibiting herbicide dithiopyr. Resistance was observed in rate-response experiments and these populations were not controlled by registered use rates of dithiopyr and prodiamine in field studies. The PPO-inhibitor oxadiazon effectively controlled these dithiopyr-resistant populations in field experiments. In the MS media assays these resistant populations were less sensitive to dithiopyr. Rate-response assays in glasshouse experiments found that the CYP450 inhibitor PBO did not reduce dithiopyr resistance. The α-tubulin gene cloning and sequencing found a Leu-136-Phe mutation in two dithiopyr-resistant biotypes. This Leu-136-Phe mutation may be responsible for the dithiopyr-resistant phenotype observed. Future research should explore the prevalence of this mutation in populations where...
poor *E. indica* control follows dithiopyr applications and better characterize the sensitivity of Leu-136-Phe mutants to other pre-emergence herbicides.

**ACKNOWLEDGEMENTS**

This research was supported by funding from the Rutgers Center for Turfgrass Science. The authors are grateful to Andrew Petryna, Liam Ryan and Alex Coward for assistance with the research.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**REFERENCES**

1. Arrieta C, Busey P and Daroub SH, Goosegrass and bermudagrass competition under compaction. *Agron J* 101:11–16 (2009).
2. McElroy JS, Head WB, Wehtje GR and Spak D, Identification of goosegrass (*Eleusine indica*) biotypes resistant to pre-emergence-applied oxadiazon. *Weed Technol* 31:675–681 (2017).
3. Dernoeden PH, Watschke TL and Mathias JK, Goosegrass (*Eleusine indica*) control in turf in the transition zone. *Weed Sci* 32:4–7 (1984).
4. Johnson BJ, Reduced herbicide rates for large crabgrass (*Digitaria sanguinalis*) and goosegrass (*Eleusine indica*) control in bermudagrass (*Cynodon dactylon*). *Weed Sci* 45:283–287 (1997).
5. Johnson BJ, Herbicide programs for large crabgrass and goosegrass control in Kentucky bluegrass turf. *HortScience* 29:876–879 (1994).
6. McCullough PE, Yu J and de Barreda DG, Efficacy of pre-emergence herbicides for controlling a dinitroaniline-resistant goosegrass (*Eleusine indica*) in Georgia. *Weed Technol* 27:639–644 (2013).
7. Mudge LC, Gossett BJ and Murphy TR, Resistance of goosegrass (*Eleusine indica*) to diquat and 2,4-D. *Weed Sci* 32:591–594 (1984).
8. Reicher ZJ, Throsell CS and Lefton JL, Annual grass control in cool season turf with sequential applications of unlike pre-emergence herbicides. *Weed Technol* 5:387–391 (1991).
9. Bhownik PC and Bingham SW, Pre-emergence activity of dinitroaniline herbicides used for weed control in cool-season turfgrasses. *Weed Technol* 4:387–393 (1990).
10. Gannon TW, Jeffries MD, Brosnan JT, Breeden GK, Tucker KA and Henry GM, Pre-emergence herbicide efficacy for crabgrass (*Digitaria ssp.*) control in common bermudagrass managed under different mowing heights. *HortScience* 50:546–550 (2015).
11. Proctor CA, Sousek MD, Patton AJ, Weisenberger DV and Reicher ZJ, Combining pre-emergence herbicides in tank mixes or as sequential applications provides season-long crabgrass control in the upper Midwest. *HortScience* 47:1159–1162 (2012).
12. Enache AJ, Ilinicki RD and BAS, 514 and dithiopyr for weed control in cool-season turfgrasses. *Weed Technol* 5:616–621 (1991).
13. Vaughn K, Vaughan MA and Gossett BJ, A biotype of goosegrass (*Eleusine indica*) with an intermediate level of diuron resistance. *Weed Technol* 4:157–162 (1990).
14. Breeden SM, Brosnan JT, Breeden GK, Vargas JJ, Eichberger G, Tresch S et al., Controlling dinitroaniline-resistant goosegrass (*Eleusine indica*) in turfgrass. *Weed Technol* 31:883–889 (2017).
15. Brosnan JT, Elmore MT and Bagavathiannan MV, Herbicide-resistant weed in turfgrass: current status and emerging threats. *Weed Technol* 34:424–430 (2020).
16. Murphy TR and Smith AE, Detection and control of dinitroaniline-resistant *Eleusine indica* (L.) Gaertn. in *Cynodon dactylon*. *Int Turfgrass Soc Res J* 8:1051–1057 (1997).
17. Anthony RG, Waldin TR, Ray JA, Bright SW and Hussey PJ, Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin. *Nature* 393:260–263 (1998).
18. Yamamoto E, Zeng L and Vance Baird W, α-Tubulin missense mutations correlate with antimicrotubule drug resistance in *Eleusine indica*. *Plant Cell* 10:297–308 (1998).
19. Armbruster BL, Molin WT and Bugg MW, Effects of the herbicide dithiopyr on cell division in wheat root tips. *Pestic Biochem Physiol* 39:110–120 (1991).
20. Vaughn KC, Cytological studies of dinitroaniline-resistant *Eleusine*. *Pestic Biochem Physiol* 26:66–74 (1986).
21. Vaughn KC and Lehnen LP, Mitotic disruptor herbicides. *Weed Sci* 39:450–457 (1991).
22. Molin WT, Lee TC and Bugg MW, Purification of a protein which binds MON 7200. *Weed Sci Soc Am Abstr* 28:97 (1988).
23. Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* 61:317–347 (2010).
24. Yu Q and Powles S, Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol* 166:1106–1118 (2014).
25. Rao SR, Feng PCC and Schafer DE, Enhancement of thiazopyr bioefficacy by inhibitors of monoxygenases. *Pestic Sci* 45:209–213 (1995).
26. Feng PCC, Inhibition of plant oxidative deactivation: a mechanism to enhance efficacy and manage resistance to thiazopyr herbicide, in *Regulation of Enzymatic Detoxifying Xenobiotics in Plants*, ed. by Hatzios KK. Kluwer Academic Publishers Group, Amsterdam, pp. 51–66 (1997).
27. Feng PCC and Solsten RT, In vitro transformation of dithiopyr by rat liver enzymes: conversion of methylothienoesters to acids by oxyge- nases. *Xenobiotica* 21:1265–1271 (1991).
28. Zawierucha JE and Penner D, The relationship of goosegrass (*Eleusine indica*) stage of growth to quinclorac tolerance. *Weed Technol* 15:216–219 (2001).
29. Saxton AM, Danda A. SAS: design and analysis macro collection version 2.11. University of Tennessee, Knoxville, (2018).
30. McIntosh MS, Analysis of combined experiments. *Agron J* 75:153–155 (1983).
31. Murashige T and Skoog F, A revised medium for rapid growth and bio- assays with tobacco tissue cultures. *Physiol Plant* 15:473–497 (1962).
32. Cuttulue MA, McElroy JS, Millwood RW, Sorohan JC and Stewart CN, Selection of bioassay method influences detection of annual blue- grass resistance to mitotic-inhibiting herbicides. *Crop Sci* 49:1088–1095 (2009).
33. Nelson DR, Schuler MA, Paquette SM, Werck-Reichhart D and Bak S, Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. *Plant Physiol* 135:756–772 (2004).
34. Dely C, Menchari Y, Michel S and Darmency H, Molecular bases for sensitivity to tubulin-binding herbicides in green foxtail. *Plant Physiol* 136:3920–3932 (2004).
35. Hashim S, Jan A, Sunohara Y, Hachinohe M, Ohdan H and Matsumoto H, Mutation of alpha-tubulin genes in trifluralin-resistant water foxtail (*Alopecurus aequalis*). *Pest Manag Sci* 68:422–429 (2012).
36. Morrissette NS, Mitra A, Sept D and Sibley LD, Dinitroanilines bind α-tubulin to disrupt microtubules. *Mol Biol Cell* 15:1960–1968 (2004).
37. Lyons-Abbott S, Sackett DL, Wloga D, Gaertig J, Morgan RE, Werbovetz KA et al., α-tubulin mutations after oryzalin affinity and microtubule assembly properties to confer dinitroaniline resistance. *Eukaryot Cell* 9:1825–1834 (2010).