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Target-site and non–target site mechanisms of pronamide resistance in annual bluegrass (*Poa annua*) populations from Mississippi golf courses

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Research Article

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Abstract

The mitotic-inhibiting herbicide pronamide controls susceptible annual bluegrass (*Poa annua* L.) pre- and postemergence, but in some resistant populations, postemergence activity is compromised, hypothetically due to a target-site mutation, lack of root uptake, or an unknown resistance mechanism. Three suspected pronamide-resistant (LH-R, SC-R, and SL-R) and two pronamide-susceptible (BS-S and HH-S) populations were collected from Mississippi golf courses. Dose–response experiments were conducted to confirm and quantify pronamide resistance, as well as resistance to flazasulfuron and simazine. Target sites known to confer resistance to mitotic-inhibiting herbicides were sequenced, as were target sites for herbicides inhibiting acetolactate synthase (ALS) and photosystem II (PSII). Pronamide absorption and translocation were investigated following foliar and soil applications. Dose–response experiments confirmed pronamide resistance of LH-R, SC-R, and SL-R populations, as well as instances of multiple resistance to ALS- and PSII-inhibiting herbicides. Sequencing of the α-tubulin gene confirmed the presence of a mutation that substituted isoleucine for threonine at position 239 (Thr-239-Ile) in LH-R, SC-R, SL-R, and BS-S populations. Foliar application experiments failed to identify differences in pronamide absorption and translocation between the five populations, regardless of harvest time. All populations had limited basipetal translocation—only 3% to 13% of the absorbed pronamide—across harvest times. Soil application experiments revealed that pronamide translocation was similar between SC-R, SL-R, and both susceptible populations across harvest times. The LH-R population translocated less soil-applied pronamide than susceptible populations at 24, 72, and 168 h after treatment, suggesting that reduced acropetal translocation may contribute to pronamide resistance. This study reports three new pronamide-resistant populations, two of which are resistant to two modes of action (MOAs), and one of which is resistant to three MOAs. Results suggest that both target site– and translocation-based mechanisms may be associated with pronamide resistance. Further research is needed to confirm the link between pronamide resistance and the Thr-239-Ile mutation of the α-tubulin gene.

Introduction

Annual bluegrass (*Poa annua* L.) is one of the most problematic weeds in turfgrass systems (Van Wychen 2020). It decreases the quality and playability of golf courses because of its light-green color, abundant seedhead production, and rapid summer decline that leaves aesthetically unpleasing brown patches in turfgrass (Beard 1969; Christians 1996; McCarty and Miller 2002; Yelverton 2015). *Poa annua* is genetically diverse (Christians 1996; Lush 1989) and may vary between annual and perennial growth cycles (Carroll et al. 2021; Huff 2003). It is adapted to many habitats (Heide 2001; Vargas and Turgeon 2003), and although it is considered a winter weed, it may germinate under a wide variety of conditions (Christians 1996).

Poa annua is commonly controlled with preemergence and postemergence herbicides along with commonly employed nonchemical control strategies. Effective preemergence herbicides

include bensulide, benefin, cumyluron, DCPA, diphenamid, dithiopyr, ethofumesate, fenarimol, indaziflam, oxadiazon, methiozolin, pendimethalin, prodiamine, pronamide, and simazine (Askew and McNulty 2014; Bingham et al. 1969; Callahan and McDonald 1992; Dernoeden 1998; Dickens 1979; McCarty and Miller 2002; Stier et al. 2013; Yelverton 2015). Sulfonylurea herbicides applied preemergence provide limited residual control of *P. annua* (McElroy et al. 2011) and are typically applied postemergence (Stier et al. 2013; Toler et al. 2007). *Poa annua* may be selectively controlled with a wide variety of postemergence herbicides in dormant non-overseeded bermudagrass [*Cynodon dactylon* (L.) Pers.] (Toler et al. 2007). However, postemergence herbicides for the selective control of *P. annua* in cool-season turfgrass are limited (Coats and Krans 1986).

Pronamide, alternatively referred to as propyzamide (Group 3 WSSA/HRAC), is a mitotic-inhibiting herbicide that provides effective pre- and postemergence control of susceptible *P. annua* populations in warm-season turfgrasses (Burt and Gerhold 1970; Johnson 1975; Shaner 2014; Toler et al. 2007). Pronamide shortens microtubules—polymers formed of α- and β-tubulin (Nogales et al. 1998)—in the kinetochore region during mitosis, subsequently disrupting cell division in susceptible species (Akashi et al. 1988). Bartels and Hilton (1973) reported that pronamide causes the loss of spindle and cortical microtubules of root cells in wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.), presumably due to the inhibition of microtubular protein synthesis or interference with the microtubule assembly mechanism. Pronamide accumulates primarily in meristematic tissue (Smith et al. 1971). Smith et al. (1971) reported that young leaves of pronamide-treated quackgrass [*Elymus repens* (L.) Gould] plants died 2 wk after application, while older leaves died after 8 wk.

The evolution of herbicide-resistant weeds is a worldwide concern. *Poa annua* is first in a list of resistant weeds ranked by number of sites of action (Heap 2023). It has developed resistance to 12 different herbicide sites of action globally. In the last 5 yr, 18 new cases of *P. annua* herbicide resistance have been reported worldwide. Pronamide-resistant *P. annua* was first reported on a golf course in Georgia (McCullough et al. 2017). Recently, there have been reports of multiple-resistant *P. annua*: two populations collected from golf courses in Texas exhibited multiple resistance to photosystem II (PSII) inhibitors, acetolactate synthase (ALS) inhibitors, and pronamide (Singh et al. 2021). Brosnan et al. (2020) reported multiple resistance to glyphosate (5-enolpyruvylshikimate-3-phosphate synthase [EPSPS] inhibitor), foramsulfuron, and simazine in *P. annua* collections from Tennessee. Three populations from golf courses in Australia exhibited multiple resistance to acetyl-CoA carboxylase (ACCase) inhibitors, ALS inhibitors, microtubule inhibitors, serine-threonine protein phosphatase inhibitors (endothall), and PSII inhibitors (Barua et al. 2020).

Herbicide resistance can be conferred by two general mechanisms: target-site resistance (TSR) and non–target site resistance (NTSR) (Petit et al. 2010; Yuan et al. 2007). TSR is due to a deletion of an amino acid or substitutions of different amino acids in the herbicide target protein, which may prevent the occurrence of herbicide interactions (Dayan et al. 2018; Kukorelli et al. 2013; Petit et al. 2010). Target-site mutations contribute to *P. annua* resistance to ACCase, ALS, microtubule-assembly, PSII, and EPSPS inhibitors (Barua et al. 2020; Cross et al. 2015; Délye and Michel 2005; McElroy et al. 2013; Svyantek et al. 2016; Tseng et al. 2019). Target-site mutations reported for mitotic-inhibiting herbicides confer resistance to the dinitroaniline herbicides. Target-site mutations for pronamide resistance have not been reported. Mutations on the α-tubulin gene conferring dinitroaniline herbicide resistance are reported at position 125 from leucine to methionine (Hashim et al. 2012), at position 136 from phenylalanine to leucine (Délye et al. 2004), at position 202 from valine to phenylalanine (Fleet et al. 2018; Hashim et al. 2012), at position 239 from threonine to isoleucine (Anthony et al. 1998; Breeden et al. 2017a; Délye et al. 2004; Fleet et al. 2018; Russell 2021; Yamamoto et al. 1998), at position 243 from arginine to methionine and arginine to lysine (Chu et al. 2018), and at position 268 from methionine to threonine (Yamamoto et al. 1998),

NTSR involves a change in a plant's physiological response to herbicides and can occur due to decreased uptake or translocation, sequestration, or metabolic detoxification of the herbicide in the plant (Délye 2013; Van Eerd et al. 2003; Yuan et al. 2007). According to some, herbicide detoxification may be the most threatening NTSR mechanism, because it can bestow multiple-herbicide resistance to numerous herbicide modes of action (Ma et al. 2013; Preston 2004; Preston et al. 1996). It is characterized by elevated enzymatic response by enzymes such as cytochrome P450 monooxygenases (P450s) and glutathione-*S*-transferases (GSTs) (Brazier et al. 2002; Breaux 1987; Breaux et al. 1987; Farago et al. 1993; Kaundun 2014; Yuan et al. 2007). Several studies have reported similarities in pronamide metabolism between resistant and susceptible species (McCullough et al. 2017; Mersie 1995; Yih et al. 1970).

Although pronamide has both pre- and postemergence activity on susceptible *P. annua* populations, postemergence activity in some resistant populations is hypothetically compromised due to target-site mutations, the lack of root uptake and translocation, or an unknown resistance mechanism. Little is known regarding uptake and translocation of pronamide within susceptible or resistant *P. annua* populations. Only one pronamide-resistant population has been characterized in the literature (McCullough et al. 2017); a population from a golf course in Georgia was controlled when pronamide was applied preemergence but exhibited >10-fold resistance to pronamide compared with the susceptible population when it was applied postemergence. Reduced absorption and translocation were reported to be the NTSR mechanisms associated with resistance. The resistant population absorbed 32% less radioactivity from ^{14}C-labeled pronamide and translocated 10% less radioactivity to the shoots relative to the susceptible population after 72 h in hydroponic culture.

Whole-plant dose–response experiments were conducted on three suspected pronamide-resistant *P. annua* populations to confirm and quantify the level of resistance to pronamide, as well as resistance to flazasulfuron and simazine. Target sites known to confer resistance to mitotic-inhibiting herbicides were sequenced, as were target sites for herbicides inhibiting ALS and PSII. The dynamics of pronamide at four different harvest times were investigated after foliar-only and soil-only applications.

Materials and Methods

Dose–Response Experiment

Poa annua populations were screened for herbicide resistance at the Mississippi State University R.R. Foil Plant Science Research Center near Starkville, MS (33.47°N, 88.78°W) to determine resistance to the mitotic-inhibiting herbicide pronamide (Kerb® 3.3SC, Corteva Agriscience, Indianapolis, IN), the ALS-inhibiting herbicide flazasulfuron (Katana® 0.25WG, PBI Gordon

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Table 1. Herbicides and application rates for whole-plant dose–response experiments.[a]

Active ingredient	Trade name	Manufacturer	Application rate[b]
			—kg ai ha^{-1}—
Pronamide	Kerb®	Corteva Agriscience, Indianapolis, IN	0, 0.28, 0.56, **1.12**, 3.36, 6.7, 20.2
Flazasulfuron	Katana®	PBI Gordon Corporation, Shawnee, KS	0.01, 0.02, **0.04**, 0.13, 0.26, 0.79
Simazine	Princep®	Syngenta Professional Products, Greensboro, NC	0, 0.28, 0.56, **1.12**, 3.36, 6.7, 20.2

[a]Treatments were applied in an enclosed spray chamber to *Poa annua* plants at the 2- to 3-leaf stage. Plants were grown in controlled greenhouse conditions at the Mississippi State University R.R. Foil Plant Science Research Center near Starkville, MS.
[b]1× label rates are indicated in bold.

Corporation, Shawnee, KS), and the PSII-inhibiting herbicide simazine (Princep® 4L, Syngenta Professional Products, Greensboro, NC) using rate–response studies (Seefeldt et al. 1995; Table 1).

Seed for all populations were collected from multiple plants having survived typical field-level herbicide rates and programs throughout the winter of 2018 to 2019. Before rate–response screens, twenty-five 1-tiller plants were first-pass screened in greenhouse conditions at 2× the labeled rates for herbicide resistance to seven different postemergence herbicide treatments. For the research conducted herein, 1× labeled rates for pronamide, flazasulfuron, and simazine are considered 1.12, 1.12, and 0.04 kg ai ha^{-1}, respectively. Screens included known susceptible populations and nontreated controls for reference. Seeds from surviving suspected-resistant populations were bagged, dried (36 C for at least 1 wk), sieved, stored (4 C), and propagated for rate–response assays.

Rate–response assays were conducted in January to April 2020 as follows. Single-tiller plants from two confirmed pronamide-susceptible (S) *P. annua* populations (Battle Sod Farm in Tunica, MS [34.66°N, 90.36°W] and Humphreys High School in Belzoni, MS [33.18°N, 90.48°W]), and three suspected pronamide-resistant (R) *P. annua* populations (Lion Hills Golf Club in Columbus, MS [33.52°N, 88.40°W], Starkville Country Club in Starkville, MS [33.41°N, 88.80°W], and Shell Landing Golf Club in Gautier, MS [30.38°N, 88.67°W]) (Table 2), were transplanted per pot (10-cm diameter) containing native Marietta silt loam soil (fine-loamy, siliceous, active, Fluvaquentic Eutrudepts) with a pH of 6.3. Plants were grown in controlled greenhouse conditions with day/night temperatures of 21/10 C and natural irradiance. Plants were fertilized weekly using a water-soluble complete fertilizer (24-8-16; Miracle-Gro® Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Marysville, OH) at a rate of 24 kg N ha^{-1} and were watered as needed to maintain adequate soil moisture and prevent drought stress.

The experiment was arranged as a completely randomized design with five replications and was repeated twice in time. When plants reached the 2- to 3-tiller stage of growth, treatments were applied using an enclosed spray chamber (Generation III track sprayer, DeVries Manufacturing, Hollandale, MN) equipped with two spray nozzles (AIXR 11003, TeeJet® Spraying Systems, Glendale Heights, IL), 48 cm apart and placed at a height of 50 cm from the plants, delivering 374 L ha^{-1}. Pressure was 241 kPa and speed was 4.4 km h^{-1}. *Poa annua* control was visually evaluated at 4 wk after treatment (WAT) on a scale from 0% to 100% (0 = no control, 100 = complete control) relative to the nontreated control. At 4 WAT, foliage was harvested and oven-dried at 60 C for 1 wk before foliar dry mass was recorded.

Dose response was modeled with a nonlinear sigmoidal variable slope regression model using GraphPad Prism (v. 7.04, GraphPad Software, San Diego, CA). Models were compared using pairwise *F*-tests ($\alpha = 0.05$) and 95% confidence intervals of doses causing 50% injury or growth reduction (GR_{50}). Dose–response models were determined using Equation 1:

$$Y = \text{Bottom} \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{\text{LogEC50}-X} * \text{Hill Slope})} \quad [1]$$

where Y is the response, X is the logarithm of the concentration, Top and Bottom are the plateaus in the same units as Y, logEC_{50} is the log rate of the amount of herbicide needed for 50% growth reduction or 50% visual injury, and Hill Slope is the steepness of the curve.

Evaluation of Prodiamine Resistance

Prodiamine (Barricade®, Syngenta Professional Products, Greensboro, NC) resistance was assessed using a rapid whole-plant assay within a hydroponic system with a 1.0 mM herbicide solution (Brosnan et al. 2014; Cutulle et al. 2009). The experiment was conducted as a randomized complete block design (four replications blocked by hydroponic vessel) and was replicated twice in time. Plants were maintained at 23 C with a photoperiod of 9 h under LED growth lights (Model P2500, Viparspectra, Richmond, CA) providing 250 µmol m^{-2} s^{-1} of illumination. Root growth was assessed at 14 d after initiation.

Preemergence efficacy of prodiamine was assessed using a seedling germination experiment. Seeds (20) from suspected resistant populations and of known susceptible standards were sown in mixed sand/peat (90/10) soil in 10-cm-diameter pots before application of 1.12 kg ha^{-1} using the previously described spray chamber. Herbicide was allowed to dry, and pots were covered with 2 mm of the same soil mixture used for top-dressing the seedbed. Pots were maintained in growth chambers at 18 C with supplemental light (10/4 day/night cycle). The experiment was conducted as a completely randomized design (three replications) and was conducted only once. Surviving plants were counted 28 d after germination to confirm resistance.

Target-Site Gene Sequencing

Common mutations in the target sites of ALS-, PSII-, and mitotic-inhibiting herbicides were sequenced for the R and S populations. Polyploidy of *P. annua* and the presence of multiple α-tubulin gene copies (Chen et al. 2021; Patterson et al. 2019) has previously hindered the description of target site–related mitotic-inhibiting herbicide resistance. The combination of amplicon sequencing (AmpSeq) and degenerate primers—instead of Sanger sequencing with a single primer pair—allowed description of all α-tubulin gene copies (Rutland et al. 2022). Populations resistant to PSII (simazine) and ALS (flazasulfuron) inhibitors were sequenced using capillary sequencing, while α-tubulin binding site disruptors (prodiamine and pronamide) and ALS-resistant populations that

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Table 2. Characterization of herbicide resistance (R) and susceptibility (S) of five Mississippi *Poa annua* populations to flazasulfuron, prodiamine, pronamide, and simazine, as well as relevant target-site mutations and a summary of absorption and translocation for each.

Mississippi location	Latitude, longitude	Designation	Documented herbicide resistance (R) or susceptibility (S)	Dose–response[a] GR_{50} values (in kg ai ha^{-1}) or assay confirmation[b]	Relevant target-site mutations[c]	Pronamide absorption and translocation relative to susceptible populations
Lion Hills Golf Course	33.52°N, 88.40°W	LH-R	Flazasulfuron-S	—[c]	Wild-type	Reduced acropetal translocation relative to the S populations
			Prodiamine-R	Hydroponics assay	α-Tubulin: Thr-239-Ile	
			Pronamide-R	6.62		
			Simazine-R	2.59	*psbA*: not present	
Starkville Country Club	33.41°N, 88.80°W	SC-R	Flazasulfuron-S	—[c]	Wild-type	Foliar absorption, basipetal translocation, and acropetal translocation similar to those in S populations
			Prodiamine-R	Hydroponics assay	α-Tubulin: Thr-239-Ile	
			Pronamide-R	6.82		
			Simazine-S	—	Wild-type	
Shell Landing Golf Club	30.38°N, 88.67°W	SL-R	*Flazasulfuron-R*	>0.79	*ALS*: Trp-574-Leu	Foliar absorption, basipetal translocation, and acropetal translocation similar to those in S populations
			Prodiamine-S	Hydroponics assay	α-Tubulin: Thr-239-Ile	
			Pronamide-R	>20.2		
			Simazine-R	1.39	*psbA*: not present	
Battle Sod Farm	34.66°N, 90.36°W	BS-S	Flazasulfuron-S	0.01	Wild-type	Foliar absorption, basipetal translocation, and acropetal translocation similar to those in the Humphrey's S population
			Prodiamine-S	Hydroponics assay	α-Tubulin: Thr-239-Ile	
			Pronamide-S	0.19		
			Simazine-S	0.56	Wild-type	
Humphreys High School Athletic Field	33.18°N, 90.48°W	HH-S	Flazasulfuron-S	0.01	Wild-type allele for all	—
			Prodiamine-S	Hydroponics assay		
			Pronamide-S	0.32		
			Simazine-S	0.12		

[a]Resistance to flazasulfuron, pronamide, and simazine was validated using replicated dose–response experiments.
[b]Resistance to prodiamine was confirmed with hydroponic and germination assays.
[c]LH and SC populations were only screened at a 2× rate for flazasulfuron resistance and were confirmed susceptible; therefore, they were not rate–response screened.

failed capillary sequencing were sequenced using the AmpSeq methods described by Rutland et al. (2022). All populations were analyzed for target-site mutations using CLC Genomics Workbench v. 21.0 (QIAGEN, Hilden, Germany).

Pronamide Absorption and Translocation

Experiments were conducted to evaluate the absorption and translocation of pronamide in susceptible and resistant *P. annua* populations. The experiments were performed twice between September and December 2021 under controlled conditions with a completely randomized design with five replications. Two experiments—foliar-only and soil-only application of pronamide—were conducted using similar methodology.

Foliar-only Application of Pronamide

Poa annua plants from the same five populations characterized with rate–response screens (LH-R, SC-R, SL-R, BS-S, and HH-S) were transplanted in pots (10-cm diameter), each pot containing a single tiller and 410 cm^3 of a commercial potting mix (Promix® BX general purpose, Premier Tech Horticulture, Quakertown, PA). Plants were fertilized weekly with a water-soluble fertilizer (24-8-16; Miracle-Gro® Water Soluble All Purpose Plant Food, Scotts Miracle-Gro Products) at a rate of 24.4 kg N ha^{-1} and were watered as needed to maintain adequate moisture and prevent drought stress. Seed heads were removed weekly with scissors or by hand. Plants were maintained at 23 C with a photoperiod of 9 h using LED lights (Model P2500, Viparspectra) providing 250 µmol m^{-2} s^{-1} of illumination.

Immediately before treatment, the soil surface of the pots was covered with aluminum foil to prevent herbicide from contacting the soil. Pronamide was applied at 1.16 kg ha^{-1} using an enclosed spray chamber, similar to methods previously described. Plants were treated at the 2- to 3-tiller stage of growth and a height of 6.5 cm when foliar mass was estimated to be >0.1 g pot^{-1}.

After application, plants were watered directly on the soil surface with a disposable plastic syringe to prevent movement of herbicide from foliage to the soil surface. Plants were destructively harvested at 8, 24, 72, and 168 h after treatment (HAT). Foliage was harvested at soil level with scissors, and roots were washed free of soil with tap water and blotted dry with paper towels. Herbicide wash of leaves was performed following the methods of Bradley et al. (2001). Foliage samples were washed twice for 30 s by shaking them in plastic bags containing 10 ml of 10% ethanol to remove the herbicide solution deposited on the foliage but not absorbed. The resulting 20-ml solution was combined in vials (Fisher Scientific, Pittsburgh, PA), resulting in one composite sample for each experimental unit (e.g., each pot). Samples were stored at 3 C until further processing. Foliage and root samples were stored at −80 C until further processing.

Pronamide was extracted using a method similar to that of Zangoueinejad et al. (2020). Foliage and root samples were cut into 5-mm segments with scissors and placed in 2-ml microcentrifuge tubes (Avantor, Radnor, PA) and were weighed to 0.10 g using an analytical scale (Mettler Toledo AE260, Marshall Scientific, Hampton, NH). Three 2.8-mm ceramic beads (Avantor) were added to each microcentrifuge tube for effective tissue disruption.

Root and foliage samples were individually homogenized (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France) for 1 min (two 20-s cycles with a 20-s pause between cycles) at 6,000 rpm. Methanol (900 µl) was added as the extraction

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solution to each microcentrifuge tube. Samples were further homogenized and centrifuged at 13,200 rpm (Eppendorf 5415D Digital Centrifuge, Marshall Scientific) for 1 min at room temperature. Leaf-wash samples were placed in 2-ml microcentrifuge tubes and subjected to centrifugation. Because these samples were not homogenized, beads and methanol were not added to the microcentrifuge tubes. The supernatant of each sample was filtered through a 0.2-µm-pore, 13-mm-diameter syringe filter (Fisher Scientific) and transferred to 2-ml vials (Avantor). Samples were stored at −80 C until mass spectrometric (liquid chromatography–mass spectrometry [LC/MS]) analysis.

Pronamide was quantified using high-performance liquid chromatography (HPLC) (Agilent 6470, Agilent Technologies, Santa Clara, CA) coupled to a mass spectrometer (Agilent 1290) with a reversed-phase column (Agilent Zorbax Eclipse Plus C18, RR HT, 50 mm by 2.1 mm, 1.8-µm particle size) maintained at 45 C with a flow rate of 0.3 ml min^{-1} and an injection volume of 2.00 µl. Mobile phase A consisted of 95% water (Optima™ LC/MS grade, Thermo Fisher Scientific, Fair Lawn, NJ) and 5% acetonitrile (0.1% formic acid + 5 mM ammonium formate; Optima™ LC/MS grade), and mobile phase B consisted of 95% acetonitrile (0.1% formic acid + 5 mM ammonium formate; Optima™ grade, Thermo Fisher Scientific) and 5% water (Optima™ grade, Thermo Fisher Scientific). Mobile phase A decreased from 90% to 10% over 2 min. The mobile phase ratio was held for 1 min before a post-run was used to equilibrate the instrument for the next injection. The HPLC–mass spectrometer was held at a source temperature of 400 C with drying gas (nitrogen) flow and nebulizer pressure at 7 L min^{-1} and 310.3 kPa, respectively, in positive ion electrospray mode (capillary voltage at 3500 V). Sheath gas flow was 11 L min^{-1} held at a temperature of 300 C. Agilent MassHunter software (Agilent Technologies) was used for method development and data acquisition. Pronamide was measured using the precursor ion 256.0 to the product ion 189.9 and confirmed with 256.0 to 172.9 ions. Sample concentrations were estimated with linear regression using quantitative analysis software (Mass Hunter QQQ Analysis, Agilent Technologies). The calibration curve was determined using varying concentrations of 10 pronamide standard solutions that covered the range of herbicide levels found in different plant parts. The calibration curve was represented by linear regression according to Equation 2:

$$y = mx + b \quad [2]$$

where y is the peak area of each herbicide and x is the herbicide concentration. The detection limit was 35 ppb pronamide.

Pronamide absorption, as a percentage of the amount applied, was determined as the total amount of pronamide detected inside the plant (roots + foliage) relative to the total amount of pronamide detected inside and outside (roots + foliage + leaf wash). Therefore, the foliar absorption of pronamide was calculated based on the quantification of the pronamide level inside the plants and in the ethanol used to remove the unabsorbed herbicide deposited on the plants according to Equation 3:

$$\text{Total absorption (\%)} = (\text{roots} + \text{foliage})/(\text{roots} + \text{foliage} + \text{leafwash}) \quad [3]$$

with total absorption being the percentage of herbicide absorbed by the foliage, roots being the herbicide translocated to the roots (root samples), foliage being the herbicide absorbed by the foliage (foliage samples), and leaf wash being the herbicide deposited on the leaf surface (leaf-wash samples).

Translocation of pronamide to roots (basipetal translocation), as a percentage of the amount absorbed, was calculated by dividing the pronamide concentration detected in roots by the total pronamide concentration detected in the whole plant (roots + foliage). Pronamide distribution, as a percentage of the amount applied, was calculated by dividing the pronamide concentration detected in the respective sample (roots, foliage, or leaf wash) by the total pronamide concentration detected in all samples (roots + foliage + leaf wash).

Soil-only Application of Pronamide

Research was conducted to evaluate the fate of pronamide when applied to the *P. annua* root zone by quantifying the total pronamide detected inside the plant (roots + foliage). The conditions of this experiment were similar to those previously described in the foliar-only application, except for the soil type and the application method. Plants of the same populations were transplanted into a native Marietta silt loam soil (fine-loamy, siliceous, active, and Fluvaquentic Eutrudepts) with a pH of 6.8 and an organic matter content of 0.45% (determined by dry-combustion method). Pronamide was directly applied to soil at 1.160 kg ha^{-1} in 20 ml of distilled water with a syringe.

Translocation of pronamide to foliage (acropetal translocation), as a percentage of the amount absorbed, was calculated by dividing the total pronamide concentration detected in foliage by the pronamide concentration detected in the whole plant (roots + foliage). Pronamide distribution, as a percentage of amount absorbed, was calculated by dividing the pronamide concentration detected in the respective sample (roots or foliage) by the total pronamide concentration detected in all samples (roots + foliage).

Statistical Analysis

Absorption and translocation data were subjected to ANOVA ($\alpha = 0.05$), and pairwise means comparison was performed with Fisher's protected LSD test using the PROC GLM procedure of SAS v. 9.4 (SAS Institute, Cary, NC) at $\alpha = 0.05$. Absorption and translocation data were also analyzed with simple linear regression performed in GraphPad Prism (v. 9.0, GraphPad Software). The slopes of absorption and translocation were compared using pairwise *F*-tests ($\alpha = 0.05$) to determine whether harvest time affected herbicide recovery parameters.

Results and Discussion

Evaluation of Pronamide, Simazine, and Flazasulfuron Resistance Levels

Whole-plant dose–response experiments confirmed postemergence pronamide resistance in each of the three suspected R populations, simazine resistance in LH-R and SL-R populations, and flazasulfuron resistance in the SL-R population (Figure 1). The estimated GR_{50} values for visual injury in response to pronamide of LH-R, SC-R, and SL-R populations were 6.62, 6.82, and >20.2 kg, ha^{-1}, respectively, which were 4 to 12 times the maximum single-use rate of 1.12 kg pronamide ha^{-1} on golf course putting greens (Anonymous 2020). By comparison, the estimated GR_{50} values of the BS-S and HH-S populations were 0.19 and 0.32 kg, ha^{-1}, respectively. Based on the R/S GR_{50} ratio, the level of resistance to pronamide of the LH-R, SC-R, and SL-R populations were

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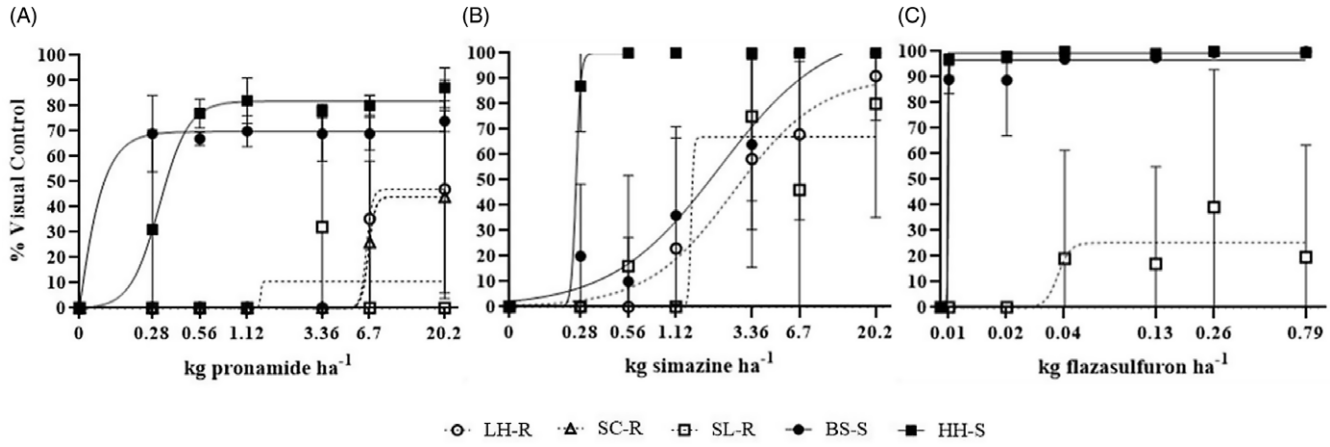

Figure 1. Visual control at 42 d after treatment of *Poa annua* plants from resistant (R) and susceptible (S) populations in response to increasing rates of pronamide, simazine, and flazasulfuron relative to the nontreated control. Dose response was modeled with a nonlinear sigmoidal variable slope model. Models were compared using pairwise *F*-tests ($\alpha = 0.05$) and 95% confidence intervals of doses causing 50% injury or growth reduction (GR_{50}).
Abbreviations: LH-R, Lion Hills Golf Club (pronamide-resistant); SC-R, Starkville Country Club (pronamide-resistant); SL-R, Shell Landing Golf Club (pronamide-resistant); BS-S, Battle Sod Farm (pronamide-susceptible); and HH-S, Humphreys High School (pronamide-susceptible). Error bars indicate the standard error of the mean.

35, 36, and >106 times more than that of the BS-S population and 20, 20, and >63 times more than that of the HH-S population, respectively. Plants from both S populations were not completely controlled with pronamide, presumably due to favorable greenhouse conditions and a final assessment/foliar harvest date of only 4 wk—in research since that time, that date has been prolonged to more than 6 wk for adequate plant death. Under standard field conditions, these populations would likely be completely controlled. Neither population has a history of pronamide application.

The estimated GR_{50} values for visual injury in response to simazine were 2.59 and 1.39 kg, ha^{-1} for LH-R and SL-R populations, respectively, while the estimated GR_{50} values of BS-S and HH-S populations were 0.12 and 0.56 kg, ha^{-1}, respectively. By comparison, the maximum onetime application rate of simazine is 2.24 kg ha^{-1} (Anonymous 2021b), which suggests resistance of the LH-R and SL-R populations. Population SC-R was only screened at a 2× rate for simazine resistance and was confirmed susceptible; therefore, it was not rate–response screened.

The estimated GR_{50} value for visual injury in response to flazasulfuron of SL-R was >0.79 kg, ha^{-1}, while it was 0.01 kg, ha^{-1} for both BS-S and HH-S populations. By comparison, typical onetime application rates to control *P. annua* are between 0.044 and 0.053. kg ha^{-1} (Anonymous 2021a). Populations LH-R and SC-R were confirmed susceptible by 2× rate screens for simazine resistance and were not rate–response screened.

Hydroponic Assays and Preemergence Germination Tests

Both hydroponic assays and seedling germination tests confirmed that the LH-R and SC-R populations were resistant to prodiamine, while SL-R, BS-S, and HH-S were susceptible (Table 2). Roots of all suspected resistant populations were unaffected by prodiamine in hydroponic solution (1.0 mM herbicide solution).

Target-Site Gene Sequencing

Sequencing data revealed that each of the three R populations has an amino acid substitution of isoleucine for threonine at position 239 (Thr-239-Ile) on the α-tubulin gene—a mutation commonly associated with resistance to dinitroaniline herbicides, including prodiamine, but in this case, presumably also pronamide. Results were convoluted by the discovery that the BS-S population contained the same target-site mutation yet was susceptible to postemergence applications of pronamide (Table 2), as well as to preemergence prodiamine in hydroponic assays and germination tests. Thr-239-Ile is associated with prodiamine resistance in the LH-R and SC-R populations and may also be responsible for pronamide resistance in the LH-R, SC-R, and SL-R populations.

Foliar-only Application of Pronamide: Absorption and Translocation

Absorption of foliar-applied pronamide in all five populations was similar at 8, 24, and 168 HAT (26% to 32%, 33% to 44%, and 23% to 31%, respectively). The only exception was that the pronamide-susceptible HH-S population absorbed more pronamide from the foliar application than did the two R populations, LH-R and SL-R (40% vs. 27% and 24%, respectively), at 72 HAT (Table 3; Figure 2). Pronamide foliar absorption did not exceed 44%, regardless of population and harvest time (31% averaged over populations at all harvest times). Over the course of the experiment, the R populations LH-R, SC-R, and SL-R absorbed 30%, 32%, and 28%, respectively, of the applied pronamide, while the S populations BS-S and HH-S absorbed 31% and 34%, respectively. Maximum absorption occurred at 24 HAT in R populations SC-R, SL-R, and S population HH-S, whereas absorption of LH-R and BS-S populations was similar across harvest times.

Most of the foliar-applied pronamide (69% averaged over populations at all harvest times) was recovered from the outside of the plant when washed off, followed by within the foliage (29% averaged over populations at all harvest times), and then the roots (2% averaged over populations at all harvest times) (Table 4). This trend was consistent for all populations at all harvest times, indicating that R and S populations did not differ in the distribution pattern of foliar-applied pronamide. Carlson (1972) evaluated the foliar uptake of [^{14}C]pronamide by *E. repens* plants and reported that almost all the herbicide (99.5%) recovered from the plants at 24 HAT was washed off the leaves and less than 1% came from the roots and foliage (0.1% and 0.4%, respectively); the author concluded that lack of foliar activity was due to poor cuticular penetration. In this study, pronamide foliar absorption

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Table 3. Foliar absorption of pronamide by *Poa annua* populations following foliar application.[a]

	Means comparison between populations[b]					Means comparison within each populations[c]				
	Population[d]					Population[d]				
h after treatment	LH-R	SC-R	SL-R	BS-S	HH-S	LH-R	SC-R	SL-R	BS-S	HH-S
8	28 a	26 a	31 a	32 a	28 a	28 a	26 A	31 ab	32 A	28 ab
24	33 A	44 A	34 A	34 A	40 A	33 a	44 A	34 a	34 A	40 a
72	27 b	33 ab	24 b	34 ab	40 a	27 a	33 AB	24 bc	34 A	40 a
168	31 A	23 A	23 A	25 A	26 A	31 a	23 A	23 c	25 A	26 b

[a]Pronamide was applied at 1.160 kg ha^{-1} using an enclosed spray chamber. Plants were treated at the 2- to 3-tiller stage of growth and a height of 6.5 cm when foliar mass was estimated to be >0.1 g pot^{-1}. Plants were grown in controlled greenhouse conditions at the Mississippi State University R.R. Foil Plant Science Research Center near Starkville, MS. Data were pooled across the two runs of the study. Means were compared using Fisher's protected LSD test at the $\alpha = 0.05$ significance level.
[b]Different letters in the same row indicate significant differences between populations.
[c]Different letters in the same column indicate significant differences between harvest times.
[d]LH-R, Lion Hills Golf Club (pronamide-resistant); SC-R, Starkville Country Club (pronamide-resistant); SL-R, Shell Landing Golf Club (pronamide-resistant); BS-S, Battle Sod Farm (pronamide-susceptible); and HH-S, Humphreys High School (pronamide-susceptible).

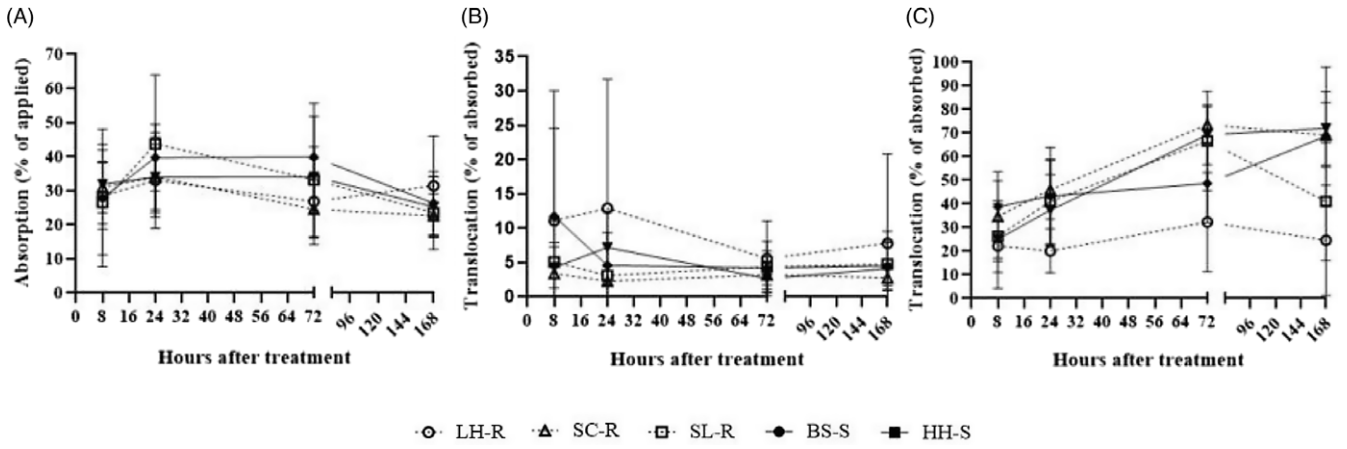

Figure 2. Pronamide absorbed by foliage (A), basipetally translocated (B), and acropetally translocated (C) in *Poa annua* plants from each population at 8, 24, 72, and 168 h after treatment.
Abbreviations: LH-R, Lion Hills Golf Club (pronamide-resistant), SC-R, Starkville Country Club (pronamide-resistant), SL-R, Shell Landing Golf Club (pronamide-resistant), BS-S, Battle Sod Farm (pronamide-susceptible), and HH-S, Humphreys High School (pronamide-susceptible). Error bars indicate the standard error of the mean.

was similar between R and S populations across harvest times, which suggests that pronamide resistance is unlikely to be associated with reduced foliar absorption.

All five populations translocated similar amounts of pronamide from foliage to roots at 8, 72, and 168 HAT. Only the LH-R population translocated more pronamide than SC-R, SL-R, and HH-S populations at 24 HAT (Table 5; Figure 2). The pronamide-susceptible BS-S was the only population that differed in translocation depending on harvest time, having translocated 7% of the absorbed pronamide to roots by 24 HAT, which decreased to an average of 3% by 72 HAT. Across populations, basipetal translocation was 3% to 13% (5.5% averaged over populations at all harvest times). Results suggest that foliar-applied pronamide is retained on the outside of leaves or within the aerial foliage of *P. annua* and does not readily move downward into roots. Basipetal translocation was similar across harvest times in R and S populations and did not appear to be associated with pronamide resistance in the three R populations.

Soil-only Application of Pronamide: Absorption and Translocation

Acropetal translocation of pronamide generally did not differ between the S populations and the pronamide-resistant SC-R and SL-R populations across harvest times (Table 5; Figure 2). This result agrees with the findings of Mersie (1995), who studied pronamide absorption, translocation, and metabolism in seedlings of tolerant witloof chicory (*Cichorium intybus* L.) and sensitive common amaranth (*Amaranthus retroflexus* L.) at 24, 48, and 72 h after root treatment to determine whether any of these processes caused differences in sensitivity between species. The author concluded that these processes are unlikely to be the basis of differential response to pronamide between these two species.

The LH-R population translocated less pronamide from roots to foliage than the S populations at 24, 72, and 168 HAT and was the population with the lowest acropetal translocation (<33%), regardless of harvest time (Table 5; Figure 2). Averaged over the course of the experiment, R populations LH-R, SC-R, and SL-R translocated 25%, 44%, and 56% of the absorbed pronamide from roots to foliage, while S populations BS-S and HH-S translocated 51% and 50%, respectively. Therefore, on average, the S populations translocated to foliage twice as much pronamide as the LH-R population. Similarly, McCullough et al. (2017) attributed differences in pronamide control between resistant and sensitive populations to differences in the absorption and translocation of the herbicide.

Acropetal translocation was similar across harvest times in the LH-R population. Acropetal translocation in SC-R, SL-R, and

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Table 4. Distribution of pronamide in samples (leaf wash, roots, and foliage) from different *Poa annua* populations following foliar and soil applications.[a]

Application method	Population[b]	8 HAT			24 HAT			72 HAT			168 HAT		
		Leaf wash	Roots	Foliage	Leaf wash	Roots	Foliage	Leaf wash	Roots	Foliage	Leaf wash	Roots	Foliage
Foliar	LH	72 a	3 A	25 a	67 A	4 a	29 B	73 A	1 a	26 B	69 a	3 A	28 a
	SC	74 a	1 A	25 a	56 A	1 ab	43 A	67 AB	1 a	32 AB	78 a	1 AB	22 a
	SL	69 a	1 A	30 a	66 A	1 b	33 AB	75 A	1 a	24 B	77 a	0 B	22 a
	BS-S	68 a	1 A	31 a	66 A	3 ab	31 AB	66 AB	1 a	33 AB	75 a	1 AB	24 a
	HH-S	72 a	1 A	27 a	60 A	2 ab	38 AB	60 B	2 a	38 A	74 a	1 AB	25 a
Soil	LH		78 a	22 B		80 A	20 b		68 A	32 c		75 a	24 B
	SC		74 ab	26 AB		59 B	41 a		33 C	67 a		59 a	41 B
	SL		65 ab	35 AB		54 B	46 a		26 C	74 a		31 b	69 A
	BS-S		75 a	25 B		63 B	37 a		31 C	69 a		28 b	72 A
	HH-S		61 b	39 A		57 B	43 a		51 B	49 b		31 b	69 A

[a]Pronamide was applied at 1.160 kg ha^{-1} using an enclosed spray chamber. Plants were treated at the 2- to 3-tiller stage of growth and a height of 6.5 cm when foliar mass was estimated to be >0.1 g pot^{-1}. Plants were grown in controlled greenhouse conditions at the Mississippi State University R.R. Foil Plant Science Research Center near Starkville, MS. Data were pooled across two study runs. Means were compared using Fisher's protected LSD test at the $\alpha = 0.05$ significance level. Different letters in the same column indicate significant differences between populations. HAT, hours after treatment.
[b]LH-R, Lion Hills Golf Club (pronamide-resistant); SC-R, Starkville Country Club (pronamide-resistant); SL-R, Shell Landing Golf Club (pronamide-resistant); BS-S, Battle Sod Farm (pronamide-susceptible); and HH-S, Humphreys High School (pronamide-susceptible).

Table 5. Translocation of pronamide in *Poa annua* populations following foliar (basipetal translocation) and soil (acropetal translocation) applications.[a]

	Basipetal translocation					Acropetal translocation				
	Population[b]									
	Means comparison between populations[c]					Means comparison within each population[c]				
h after treatment	LH-R[c]	SC-R	SL-R	BS-S	HH-S	LH-R	SC-R	SL-R	BS-S	HH-S
8	11 a	5 a	4 a	4 a	12 a	22 B	26 AB	35 AB	25 B	39 A
24	13 A	3 B	3 B	7 AB	5 B	20 b	41 a	46 a	37 a	43 a
72	6 a	5 a	3 a	3 a	4 a	32 C	67 A	74 A	69 A	49 B
168	8 A	5 A	3 A	4 A	5 A	24 b	41 b	69 a	72 a	69 a
	Means comparison between populations[d]					Means comparison witdin each population[d]				
8	11 a	5 A	4 a	4 AB	12 a	22 A	26 b	35 B	25 c	39 B
24	13 a	3 A	3 a	7 A	5 a	20 A	41 b	46 B	37 b	43 B
72	6 a	5 A	3 a	3 B	4 a	32 A	67 a	74 A	69 a	49 B
168	8 a	5 A	3 a	4 AB	5 a	24 A	41 b	69 A	72 a	69 A

[a]Data were pooled across two study runs. Means were compared using the Fisher's protected LSD test at the $\alpha = 0.05$ significance level.
[b]LH-R, Lion Hills Golf Club (pronamide-resistant); SC-R, Starkville Country Club (pronamide-resistant); SL-R, Shell Landing Golf Club (pronamide-resistant); BS-S, Battle Sod Farm (pronamide-susceptible); and HH-S, Humphreys High School (pronamide-susceptible).
[c]Different letters in the same row indicate significant differences between populations.
[d]Different letters in the same column indicate significant differences between harvest times.

BS-S populations was 37% to 46% at 24 HAT and increased to 67% to 74% at 72 HAT (Table 5; Figure 2). The pronamide-susceptible HH-S population translocated similar amounts of pronamide at 8, 24, and 72 HAT (39%, 43%, and 49%, respectively), which increased to 69% at 168 HAT. Carlson (1972) reported that 81% of root-applied pronamide was recovered from the foliage of *E. repens* plants at 24 HAT, and 19% from the roots. Overall, these data indicate that acropetal translocation is not associated with pronamide resistance in the SC-R and SL-R populations but may contribute to pronamide resistance in the LH-R population.

Foliar versus Soil Application of Pronamide

Acropetal translocation exceeded basipetal translocation of pronamide, regardless of population and harvest time (45% vs. 5.5% averaged over all populations at all harvest times; $P < 0.0001$). Pronamide is a systemic herbicide; however, it appears to be translocated mostly via the xylem (Carlson 1972). These results are consistent with those of Carlson (1972), who reported acropetal, but no basipetal, movement of pronamide following foliar penetration. *Elymus repens* plant leaves were treated with [^{14}C] pronamide and divided into basipetal, central, and acropetal sections at 24 HAT. While most of the [^{14}C]pronamide recovered from within the leaves was from the acropetal sections (0.24%), the central sections contained 0.15% and the basipetal sections only 0.01% of the radioactivity. Thus, the author concluded that the small amount of pronamide absorbed by the leaves moved through the xylem.

The Thr-239-Ile mutation of the α-tubulin gene in each of the three Mississippi R populations is the most likely contributor to prodiamine resistance in LH-R and SC-R populations and may be associated with pronamide resistance in all three R populations. This is the first report linking a target-site mutation to pronamide resistance. Previous reports of mutations on the α-tubulin gene were for dinitroaniline herbicide resistance in goosegrass [*Eleusine indica* (L.) Gaertn.] (Anthony et al. 1998; Breeden et al. 2017a; Yamamoto et al. 1998), green foxtail [*Setaria viridis*

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(L.) P. Beauv.] (Délye et al. 2004), and rigid ryegrass (*Lolium rigidum* Gaudin) (Chen et al. 2018; Fleet et al. 2018). Several studies have reported resistance to prodiamine, a dinitroaniline herbicide, in *P. annua* (Breeden et al. 2017b; Brosnan et al. 2014; Cutulle et al. 2009; Isgrigg et al. 2002). The first reported case of a target-site mutation conferring resistance to mitotic-inhibiting herbicides in *P. annua* was in Alabama. Russell (2021) reported that the Thr-239-Ile mutation conferred varying levels of resistance to prodiamine and cross-resistance to dithiopyr in three *P. annua* populations.

The high levels of pronamide resistance in the SL-R population observed in the dose–response experiments and the lack of reduced absorption and translocation of pronamide suggest that the Thr-239-Ile mutation is likely responsible for pronamide resistance in SL-R. It is interesting, however, that hydroponic assays failed to confirm prodiamine resistance in the SL-R population—with root growth stunted similarly as in susceptible populations. Further investigations are needed to elucidate the contribution of the Thr-239-Ile mutation to pronamide resistance in this population.

Alternatively, reduced acropetal pronamide translocation of the LH-R population suggests that NTSR may be contributing to resistance, although the presence of the Thr-239-Ile mutation is strongly suggestive of an accompanying TSR mechanism. Estimation of the relative contribution of reduced translocation to the overall pronamide resistance is difficult, because the reduced acropetal translocation could be masked by the Thr-239-Ile mutation. Importantly, if Thr-239-Ile is responsible for conferring resistance in the LH-R population, then this study is the first to report the presence of both TSR and NTSR to mitotic-inhibiting herbicides in the same *P. annua* population. The occurrence of both TSR and NTSR mechanisms in the same population of weed species is increasing and is usually masked by TSR. The first reported coexistence of TSR and

NTSR to mitotic-inhibiting herbicides in the same population was in Australia (Chen et al. 2020). The authors reported that the α-tubulin mutation Val-202-Phe and enhanced metabolism were responsible for dinitroaniline resistance in an *L. rigidum* population. Other studies have reported herbicide resistance due to both TSR and NTSR mechanisms in corn poppy (*Papaver rhoeas* L.), waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Délye et al. 2011; Guo et al. 2015; Nakka et al. 2017). Surprisingly, the Thr-239-Ile mutation was also discovered in the BS-S population, suggesting that this mutation might be associated with resistance to prodiamine. Yet the BS-S population is not resistant to prodiamine. Sufficient data are lacking about how the Thr-239-Ile mutation may contribute to pronamide resistance.

If the Thr-239-Ile mutation is responsible for pronamide resistance in R populations, determination of the relative contribution of this mechanism to the overall resistance to pronamide in each of the R populations is difficult, because the level of resistance could differ between populations. Uribe et al. (1998) reported that the α-tubulin gene is expressed at different levels and locations within the plant. Russell (2021) reported that three *P. annua* populations were 1.6-, 16.5-, and 4.6-fold resistant to prodiamine relative to the susceptible population and concluded that the variation in resistance levels between populations could be explained by both gene copy variation and intra-plant variation in gene expression. This same rationale could also be extended to the absence of prodiamine resistance in BS-S; however, gene expression was not measured in our study.

The process for determining TSR in *P. annua* with standard methods is challenging and can be convoluted, because *P. annua* is an allotetraploid species. The R populations tested in this study may contain other target-site mutations on the α-tubulin gene or on a different gene that confer resistance to pronamide, which needs to be further investigated. Although these results confirm the presence of reduced acropetal translocation in LH-R and suggest that the Thr-239-Ile mutation might be associated with pronamide resistance in the three R populations, other mechanisms of resistance cannot be ruled out. For instance, Hess and Putnam (1971) reported that resistant lettuce (*Lactuca sativa* L.) metabolized pronamide at a greater rate than susceptible oats (*Avena sativa* L.). Our study did not directly investigate metabolism-based resistance.

To conclude, the results of this study suggest that the same Thr-239-Ile amino acid substitution that leads to dinitroaniline resistance may also contribute to pronamide resistance in the three R populations. Pronamide absorption and translocation are similar in both S populations and two R populations (SC-R and SL-R). Pronamide resistance of the LH-R population may be due to reduced acropetal translocation, but it shares the Thr-239-Ile amino acid substitution with other R populations.

According to Heap (2023), only three pronamide-resistant *P. annua* populations have been reported (Barua et al. 2020; McCullough et al. 2017; Singh et al. 2021). This study reports three new pronamide-resistant populations from Mississippi, some of which are cross-resistant to the mitotic inhibitor prodiamine and/or are resistant to inhibitors of ALS and/or PSII. Results indicate that both target site– and translocation-based mechanisms may be associated with pronamide resistance. Further studies should evaluate whether P450 and GST enzymes are involved in pronamide resistance in the populations tested. Additionally, studies evaluating more pronamide-resistant *P. annua* populations are needed to confirm the association between the Thr-239-Ile mutation and pronamide resistance; likewise, research on the α-tubulin gene expression level and where it is expressed in the plant could confirm that this TSR mechanism is a cause of pronamide resistance.

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