Improved Understanding of Mitotic-Inhibiting Herbicide Resistance in *Poa annua* and *Eleusine indica*

by

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Abstract

Poa annua is a problematic weed that infests golf courses, sports fields, and home lawns. Mitotic-inhibiting herbicides are often used to control *Poa annua* in these affected areas. However, resistance to mitotic-inhibiting herbicides has developed due to unaltered herbicide regimes. Suspected resistant populations were collected from across the state of Alabama and screened for resistance to prodiamine. Populations were then sequenced for known target-site mutations located on the α-tubulin gene. The mutation Thr239-Ile on the α-tubulin gene was discovered in each of the three suspected resistant populations tested. The results from this study indicated that these mutations confer resistance to prodiamine and cross-resistance to dithiopyr. The level of resistance to prodiamine for the R populations were 1.6, 16.5, and 4.6 times more than the susceptible population based on seedling emergence response and 1.8, 59.2, and 1.4 times more than the susceptible population based on biomass reduction response. The level of resistance to dithiopyr for the R populations were 4.6, 5.0, and 6.8 times more than the susceptible population based on seedling emergence response and 3.9, 9.1, and 11.2 times more than the susceptible population based on biomass reduction response.

Goosegrass (*Eleusine indica*) is a summer annual grass that is a problematic weed in turfgrass. Dithiopyr and dinitroanilines are mitotic-inhibiting herbicides that are commonly used as a preemergent application to control goosegrass. A suspected resistant goosegrass population was collected from a golf course putting green and was evaluated for possible resistance to dithiopyr and prodiamine. After rate response

evaluation, the α-tubulin gene was sequenced for known target-site mutations that have been reported to confer resistance to mitotic-inhibiting herbicides. A mutation was discovered that resulted in an amino acid substitution at position 136 from leucine to phenylalanine (Leu136-Phe). Previous research has indicated that Leu136-Phe does confer resistance to dinitroaniline herbicides. The level of resistance indicated by regression models and I50 values indicates that there is a 54.1-, 4.7-, >100-, and >100-fold resistance to dithiopyr, prodiamine, pendimethalin, and oryzalin, respectively when compared to the susceptible population based on seedling emergence response and 88.4-, 7.8-, >100-, and >100-fold resistance to dithiopyr, prodiamine, pendimethalin, and oryzalin, respectively when compared to the susceptible population based on biomass reduction response.

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Table of Contents

Abstract ii

Acknowledgments iv

List of Tables vii

List of Figures viii

List of Abbreviations x

Literature Review 1

Mitotic-Inhibiting Herbicides 1

Resistance Causing Mutations 3

Poa annua 7

Eleusine indica 9

Thesis Objectives 12

Unique α-Tubulin Mutations in *Poa annua* Induce Variable Response to Prodiamine and Dithiopyr 14

Introduction 14

Materials and Methods 16

Results and Discussion 21

Research Implications 23

Identification of Goosegrass (*Eleusine indica*) Resistant to Dithiopyr and Dinitroaniline Herbicides 37

Introduction 37

Materials and Methods.....39
Results and Discussion.....44
Research Implications.....46
Literature Cited.....60

List of Tables

Table 1. Collection Site of Suspected Resistant and Susceptible Populations 26

Table 2. Primer Sequences 26

Table 3. Parameters for Seedling Emergence and Biomass Reduction in Response Prodiamine and Dithiopyr..... 27

Table 4. Mutations and Subgenome Location for the Suspected Resistant Populations..... 27

Table 5. Parameters for Seedling Emergence and Biomass Reduction in Response to Dithiopyr, Prodiamine, Pendimethalin, and Oryzalin..... 50

List of Figures

Figure 1.

A. Symptomology Associated with Dinitroaniline Herbicides 28

B. Comparison in Root Growth of Resistance and Susceptible Populations 29

Figure 2.

A. α-Tubulin Contig Alignment for R1 30

B. α-Tubulin Contig Alignment for R2 31

C. α-Tubulin Contig Alignment for R3 32

Figure 3.

A. Seedling Emergence Response to Prodiamine 33

B. Seedling Emergence Response to Dithiopyr 34

C. Biomass Reduction Response to Prodiamine 35

D. Biomass Reduction Response to Dithiopyr 36

Figure 4.

A. Seedling Emergence Response to Dithiopyr 51

B. Biomass Reduction Response to Dithiopyr 52

C. Seedling Emergence Response to Prodiamine 53

D. Biomass Reduction Response to Prodiamine 54

E. Seedling Emergence Response to Pendimethalin 55

F. Biomass Reduction Response to Pendimethalin 56

G. Seedling Emergence Response to Oryzalin 57

H. Biomass Reduction Response to Oryzalin 58

Figure 5. α-Tubulin Contig Alignment for the R population 59

List of Abbreviations

α	Alpha
β	Beta
Arg	Arginine
bp	Base pair
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
g ai ha^{-1}	Grams of Active Ingredient per Hectare
Ile	Isoleucine
Lys	Lysine
MAP	Microtubule-associated protein
Met	Methionine
Phe	Phenylalanine
POST	Postemergence
PRE	Preemergence
RNA	Ribonucleic acid
Thr	Threonine
TSR	Target-site resistance
Val	Valine

Chapter 1: Literature Review

Mitotic-Inhibiting Herbicides

Mitotic-inhibiting herbicides [Herbicide Resistance Action Committee (HRAC) Classification Code: K1] are preemergence herbicides that are often used to control small-seeded annual weeds (McElroy and Martins 2013). Common mitotic-inhibiting herbicide families are dinitroanilines, pyridines, and benzamides (Shaner 2014). These three herbicide families cause the inhibition of shoot and root development by preventing the polymerization of microtubules which separate the chromosomes during mitosis (Shaner 2014). This results in mitosis of susceptible plants being arrested in prometaphase (Vaughn and Lehnen 1991). While the dinitroanilines, pyridine, and benzamide families result in the same end goal of inhibiting proper cell division, the mechanism varies between the different families.

Dinitroanilines. The dinitroaniline family is the largest family within the mitotic-inhibiting herbicides and as a result has more information about them than the other mitotic-inhibiting herbicide families. (Vaughn and Lehnen 1991). Dinitroanilines are often used as a preemerge control method to control small-seeded grass weeds in both turf and crop systems (McCullough et al. 2013; Vaughn and Lehnen 1991). This family inhibits the polymerization of microtubules by binding directly to the tubulin protein (Vaughn and Lehnen 1991). This results in cells being void of all microtubules (Vaughn and Lehnen 1991). Without microtubules, spindle fibers do not form, and chromosome are unable to move to the poles of the cell during mitosis (Vaughn and

Lehnen 1991). The cells then expand isodiametrically instead of elongating (Vaughn and Lehnen 1991). Ultimately this results in inhibition of root and shoot growth and causes clubbed or swollen root tips (Shaner 2014).

Pyridine. While most active ingredients within the pyridine family are classified as auxin mimics [HRAC Code: O], dithiopyr is one of the active ingredients in the pyridine family that inhibits mitosis (Shaner 2014). Dithiopyr prevents the polymerization of microtubules which leads to the inhibition of root and shoot development. The symptomology of dithiopyr is very similar to dinitroaniline herbicides and it results in the same inhibition of root and shoot growth and swelling of root tips (Shaner 2014). Dithiopyr does share characteristics with mitotic-inhibiting herbicides, however it has a distinctive effect on microtubule organization and stability (Lehnen and Vaughn 1991). Dithiopyr inhibits mitosis by binding to microtubule-associated proteins (MAPs), unlike the dinitroanilines (Shaner 2014). MAPs are proteins that are essential to the stabilization of microtubules (Cutulle et al. 2009). When MAPs are not allowed to stabilize microtubules, the microtubules are unable to properly polymerize (Cutulle et al. 2009). This results in shortened spindle fibers. Spindle fibers help pull the chromosomes to the poles during mitosis, so if the spindle fibers are shortened the cell is unable to divide properly leading to the cell being arrested in prometaphase (Cutulle et al. 2009). If the concentration of dithiopyr is high, complete lack of microtubules is observed like in cells treated with dinitroaniline herbicides (Hoffman and Vaughn 1994). However, at normal rates tufts of microtubules can usually be found surrounding the kinetochores (Hoffman and Vaughn 1994).

Benzamide. Pronamide (propyzamide) is an active ingredient within the benzamide family that inhibits mitosis (McCullough et al. 2017). Pronamide can be

used as either a PRE or a POST control method to control small-seeded weeds in turf (Barua et al. 2020). Like the other mitotic-inhibiting herbicides, pronamide inhibits root and shoot development resulting in swollen root tips (Shaner 2014). Pronamide affects the stability of the microtubules, which inhibits the polymerization of microtubules (Akashi et al. 1988). However, unlike other mitotic-inhibiting herbicides, pronamide is unable to depolymerize the microtubules that were already formed (Akashi et al. 1988). This results in short microtubules located around the kinetochore region within the cell (Vaughn and Lehnen 1991). Even though some cells will become arrested in prometaphase, most cells will also begin to reform after attempting the first division resulting in polymorphic nuclei (Vaughan and Vaughn 1987).

Resistance Causing Mutations

Microtubules are protein dimers that are composed of alpha (α) and beta (β) tubulin (Nogales et al. 1998). Dinitroanilines, pyridine, and benzamide herbicides inhibit plant growth by inhibiting the formation of microtubules (Shaner 2014). These herbicide families result in very similar symptomology which usually are swollen root tips due to cells inability to divide properly (Anthony and Hussey 1999). At a molecular level the three herbicide families have different ways of disrupting mitosis and the α- and β-tubulin protein dimer. Dinitroanilines bind to the tubulin protein thus preventing the polymerization of the tubulin protein dimer (Anthony and Hussey 1999). Dithiopyr (pyridine family) targets MAPs, microtubule-associated proteins, these proteins help stabilize microtubules (Shaner 2014). Pronamide (benzamide family)

affects the stability of the microtubules resulting in shortened tufts around the kinetochores (Vaughan and Vaughn 1987). Mutations on the α-tubulin gene have been reported to confer resistance to mitotic-inhibiting herbicides. While there are reported cases of herbicide resistance to dithiopyr and pronamide, there has not been any target-site mutations that have resulted in resistance to those herbicides. All target-site mutations reported for the three mitotic-inhibiting herbicide families have resulted in resistance to the dinitroaniline family. The first mutations on the α-tubulin gene were reported in *Eleusine indica* (L.) Gaertn. in 1998 (Anthony et al. 1998; Yamamoto et al. 1998). A mutation at position 239 from threonine to isoleucine was confirmed to confer a high level of resistance to dinitroaniline herbicides in *Eleusine indica* (Anthony et al. 1998). Also, in 1998, Yamamoto et al. (1998) discovered two different mutations on the α-tubulin gene that conferred resistance to dinitroaniline herbicides. The first mutation was Thr239-Ile, and it conferred a high level of resistance in goosegrass (Yamamoto et al. 1998). The second mutation was located on the α-tubulin gene at position 268 (Yamamoto et al. 1998). This mutation resulted in an amino acid substitution from methionine to threonine and resulted in an intermediate level of resistance to dinitroaniline herbicides in *Eleusine indica* (Yamamoto et al. 1998). In 1999, a double mutation in *Eleusine indica* containing mutations at positions 239 (threonine to isoleucine) and 268 (methionine and threonine) was used to transform maize calli (Anthony and Hussey 1999). The maize calli was used to confirm that these two mutations were able to confer resistance to dinitroanilines. The results revealed that the maize calli that contained the before mentioned mutations were resistant to dinitroaniline herbicides while the maize calli that did not have the mutations was

unable to grow in the presence of the herbicide (Anthony and Hussey 1999). A few years later a novel mutation was reported in green foxtail (*Setaria viridis* (L.) P. Beauv). A mutation at position 136 on the α-tubulin gene that resulted in an amino acid substitution from leucine to phenylaniline was confirmed to confer resistance to dinitroaniline herbicides (Délye et al. 2004). The mutation from threonine to isoleucine at position 239 on the α-tubulin gene was also discovered in green foxtail (Délye et al. 2004). This research confirmed that the mutations on the α-tubulin gene at positions 136 and 239 confirmed resistance to dinitroaniline herbicides by looking at the survival rates between susceptible plants (did not have mutations) and resistant plants (had a mutation). The susceptible plants had leucine at position 136 and threonine at position 239, while the resistant plants had phenylalanine at position 136 or isoleucine at position 239 (Délye et al. 2004). The first resistant population possessed the Leu136-Phe mutation, and the second resistant population possessed the Thr239-Ile mutation (Délye et al. 2004). The survival rates of the populations that contained either mutation were higher than the susceptible population (Délye et al. 2004). In 2011, three mutations were reported in different populations of water foxtail (*Alopecurus aequalis* Sobol.). These mutations were determined to confer varying levels of resistance to trifluralin, a herbicide in the dinitroaniline family (Hashim et al. 2011). All of the mutations were found on the α-tubulin gene with the first mutation discovered at position 125 that resulted in an amino acid substitution from leucine to methionine, the second mutation was at position 202 and it resulted in an amino acid substitution from valine to phenylalanine, and the third mutation reported was located at position 136 and resulted in an amino acid substitution from a leucine to phenylalanine (Hashim et al

2011). There was a 30.7-fold increase in level of resistance to dinitroaniline herbicides in the population of water foxtail that contained the mutations Leu136-Phe and Val202-Phe (Hashim et al. 2011). A 5.7-fold level of resistance to dinitroaniline herbicides was also discovered in a different population of water foxtail that contained the mutations Leu125-Met and Val202-Phe (Hashim et al. 2011). In 2017, a mutation was reported in goosegrass at position 239 from threonine to isoleucine that conferred resistance to prodiamine (Breeden et al. 2017). In 2018, a novel mutation was discovered in *Lolium rigidum* Gaudin (rigid ryegrass) that was determined to confer resistance to dinitroaniline herbicides (Chu et al. 2018). Two mutations were discovered at the same position on the α-tubulin gene. The mutations were at position 243 and the resulting amino acid changes were from arginine to methionine and arginine to lysine (Chu et al. 2018). While the mutations did confer resistance to trifluralin and other dinitroaniline herbicides, there was a significant fitness cost associated with these mutations (Chu et al. 2018). This is suspected because although position 243 is located at the target-site of dinitroaniline herbicides on the α-tubulin gene, its highly conserved nature indicates that it is also crucial to microtubule function (Chu et al. 2018). The two mutations at position 243 were determined to confer roughly an eight-fold level of resistance in rice calli. However, the mutations were hard to test in the plants due to the poor growth and low germination rate of the plants that possessed the mutations (Chu et al. 2018). Another study in 2018 looked at two mutations found in two resistant population of rigid ryegrass (*Lolium rigidum*) (Fleet et al. 2018). The mutations that they found were on the α-tubulin gene. The first mutation was at position 239 with an amino acid substitution from threonine to isoleucine and the second mutation was at position 202

with an amino acid substitution from valine to phenylaniline (Fleet et al. 2018). The population of rigid ryegrass that contained the mutation Thr239-Ile was tested and determined to have a 17-fold level of resistance to dinitroaniline herbicides (Fleet et al. 2018). The population that contained the mutation Val202-Phe was not tested to determine its level of resistance (Fleet et al. 2018).

Poa annua

Poa annua L.*,* annual bluegrass, is a winter annual grass that can be described as either an undesired weed or a desired turfgrass (Wu and Harding 1992). While annual bluegrass can be considered a turfgrass, it is more often looked at as a weed that infests golf courses, sports fields, and home lawns (Brosnan et al. 2014). Annual bluegrass is a prolific weed and is able to adapt to many environments. With the discovery of natural populations in 2005 in Antarctica, *Poa annua* can now be found on every continent (Chwedorzewska 2008). According to a survey conducted by the Weed Science Society of America (WSSA) in 2017 concluded that in North America annual bluegrass was considered the 4th most troublesome weed in turfgrass (Van Wychen 2017). *Poa annua* is a very adaptive and has morphological characteristics that are highly variable due to the many ecological pressures and management regimes that it faces (McElroy et al. 2002). In turfgrass management systems that have bermudagrass (*Cynodon dactylon*) as their main turfgrass, mitotic-inhibiting herbicides applied in late summer provides a control for annual bluegrass that germinates in the fall (Isgrigg III et al. 2002). However, consistent yearly applications of only mitotic-inhibiting herbicides have led to the herbicide resistance evolving in annual bluegrass. There have been reported cases of

mitotic-inhibiting herbicide resistance in *Poa* annua over the years, however, there have not been any discovered mutations in the resistant populations. Sequencing annual bluegrass using standard sequencing methods such as Sanger Sequencing (Capillary Action Sequencing) is very difficult because annual bluegrass is an allotetraploid. The genome of *Poa annua* is the result of a cross between *Poa infirma* and *Poa supina* (Mao and Huff 2012). After the cross there was a genome doubling event resulting in annual bluegrass becoming a tetraploid (Mao and Huff 2012). In 2012, research using DNA from both *Poa infirma* and *Poa supina* they were able to determine that the maternal genome was *Poa infirma* and the paternal genome was *Poa supina* (Mao and Huff 2012). The first case of resistance to dinitroaniline herbicides was reported in 2002 in North Carolina (Isgrigg III et al. 2002). The study reported a sixfold resistance to prodiamine for root growth inhibition and a 105-fold resistance to prodiamine for shoot growth inhibition when compared to a susceptible population (Isgrigg III et al. 2002). In 2009, a resistant population was determined to be roughly 26 times more resistant to prodiamine than a control population when using a hydroponic screening method (Cutulle et al. 2009). In 2014, another population of annual bluegrass was found that was resistant to prodiamine. The population was sprayed in the field with rates up to 1400 g ai ha^{-1}, which failed to control the resistant population (Brosnan et al. 2014). A population of annual bluegrass was confirmed to be resistant in 2017 to both prodiamine and glyphosate. It was determined that it was 22 times more resistant to prodiamine than the susceptible control population (Breeden et al. 2017). In 2019 in Tennessee, suspected resistant populations were screened for resistance as part of the *Poa annua* SCRI project. One hundred different populations were tested using hydroponics as the screening method. Forty-two populations were ranked as

susceptible, 49 were determined to be segregating for resistance, and 9 populations were ranked as resistant to prodiamine (Brosnan et al. 2020). Resistance to dithiopyr in *Poa annua* has only been reported twice. In 2009, a population on *Poa annua* was marginally resistant to dithiopyr when it was screened using hydroponics. However, the resistance level was so low that it was not studied any further (Cutulle et al. 2009). Dithiopyr also failed to control a pronamide resistant population in 2017 when a standard rate was applied (McCullough et al. 2017). The first reported case of pronamide resistance in *Poa annua* came in 2017. A population from a golf course in Georgia was controlled by a PRE application of pronamide but had a greater than 10-fold resistance to pronamide when it was applied POST (McCullough et al. 2017). This was then supported by a report from Australia that found that resistant populations were controlled by a PRE application, but failed to control it with a POST application. There were four resistant populations and their level of resistance to POST applied pronamide ranged from 2-fold up to 4.1-fold (Barua et al. 2020). There was one population though that did have a 2.7-fold resistance to pronamide applied PRE (Barua et al. 2020). In Texas, two populations of *Poa annua* were not controlled by a POST application of pronamide having a 4.3 and 5.2-fold resistance (Singh et al. 2020). These populations were controlled with PRE application, but they were less sensitive to it than the susceptible population screened with them (Singh et al. 2020).

Eleusine indica

Goosegrass, *Eleusine indica*, is a summer annual grass weed in turfgrass. The unsightly seed heads and the coarse leaf texture can not only severely affect the playing

surface on golf courses but can also reduce the aesthetics and the quality of any turfgrass in which it is present (McCullough et al. 2013). *Eleusine indica* was the first grass to develop resistance to dinitroaniline herbicides. In 1984, a biotype of *Eleusine indica* was discovered in South Carolina that was resistant to seven dinitroaniline herbicides (Mudge et al. 1984). It was discovered in the cotton growing regions of seven counties and is believed to have emerged due to the repeated used of trifluralin in these areas for roughly 10 years (Mudge et al. 1984). The susceptible biotype of goosegrass was able to be controlled at the recommended rate for all seven of the dinitroaniline herbicides (benefin, ethalfluralin, fluchloralin, isopropalin, oryzalin, pendimethalin, and trifluralin) while the resistant biotype was able to withstand two times the recommended rates for all of the dinitroaniline herbicides tested (Mudge et al. 1984). Trifluralin was the exception with the resistant population withstanding rates up to six times the recommended rate (Mudge et al. 1984). In 1990, two different biotypes of *Eleusine indica* from South Carolina were studied because of their resistance to dinitroaniline herbicides. The first biotype (labeled as the "R" biotype) was highly resistant to trifluralin. It ranged from 1,000 to 10,000-fold resistance to trifluralin and was cross-resistant to all other dinitroaniline herbicides. The other biotype (labeled as the "I" biotype) was determined to have an intermediate level of resistance to dinitroaniline herbicides and had a 50-fold resistance to trifluralin. As far as cross resistance goes, the intermediate biotype had high levels of cross resistance to some dinitroaniline herbicides and very low cross resistance to others (Vaughn et al. 1990). In their tests they determined that the I and R biotypes responded similarly to the controls. At higher concentrations the R biotype was still similar to the control while the I biotype was affected by the increased concentration, but it was not affected as much as the

susceptible (Vaughn et al. 1990). In 1998, a mutation on the α-tubulin gene of a biotype of *Eleusine indica* from threonine to isoleucine at position 239 was discovered (Anthony et al. 1998). This mutation was determined to confer resistance to dinitroaniline herbicides. The resistant biotype had a 60-fold level of resistance to oryzalin indicated by dose response studies (Anthony et al. 1998). A 42-fold level of resistance to trifluralin was also reported for the Thr239-Ile mutation in the same resistant population (Anthony et al. 1998). Also, in 1998, a study was published that took an in-depth look at mutations on the α-tubulin gene of two different biotypes of goosegrass (Yamamoto et al. 1998). The first biotype (or the "R" biotype) contained a mutation at position 239 from threonine to isoleucine and the second biotype (or the "I" biotype) contained a mutation at position 268 from methionine to threonine (Yamamoto et al. 1998). Both of these mutations were confirmed to confer resistance to dinitroaniline herbicides in goosegrass. They also resulted in resistance to antimicrotubular drugs in bacteria (Yamamoto et al. 1998). Then, in 1999, mutations in the α-tubulin gene at position 239 and 268 were transformed into maize calli to confirm if these mutations confer resistance to dinitroaniline herbicides (Anthony and Hussey 1999). The single mutations Thr239-Ile and Met268-Thr were both confirmed to be resistant to dinitroaniline herbicides on their own, but when they were combined within the maize calli, the herbicide tolerance was similar to the sum of the tolerance of the individual mutations (Anthony and Hussey 1999). Another goosegrass population that was resistant to mitotic-inhibiting herbicides was discovered in Georgia in 2013. The population had less than 7% control in the field when the recommended rate of prodiamine was applied (McCullough et al. 2013). A report in 2017 involved a

population of goosegrass that was resistant to prodiamine. The resistant population was sequenced and a mutation on the α-tubulin gene at position 239 from threonine to isoleucine was discovered (Breeden et al. 2017). There has only been one reported case of dithiopyr resistance in goosegrass. A recommended rate was applied in the field and it achieved less than 20% control of the population in question (McCullough et al. 2013). Currently there are no reported cases of pronamide resistance in *Eleusine indica*.

Thesis Objectives

The purpose of this thesis study is to better understand resistance to mitotic-inhibiting herbicides in *Poa annua* and *Eleusine indica*. This thesis can be divided into two studies. The first study focused on mitotic-inhibiting herbicide resistance in *Poa annua* while the second study focused on mitotic-inhibiting herbicide resistance in *Eleusine indica*.

Three objectives were defined to study mitotic-inhibiting herbicide resistance in *Poa annua*. The first objective was to use an initial screening method to determine potential resistance to prodiamine in populations of *Poa annua*. The second objective was to develop a method to efficiently sequence the α-tubulin gene of *Poa annua*. The third objective was to use rate response screens to test for differences in target-site mutations discovered in different populations of *Poa annua* in response to prodiamine and dithiopyr.

Two objectives were defined to study mitotic-inhibiting herbicide resistance in *Eleusine indica*. The first objective was to conduct rate response screens with dithiopyr,

prodiamine, pendimethalin, and oryzalin to determine if the suspected resistant population was resistant to dithiopyr and cross-resistant to select dinitroaniline herbicides. The second objective was to sequence the α-tubulin gene of *Eleusine indica* to determine if there were any known target-site mutations on the α-tubulin gene that confer resistance to mitotic-inhibiting herbicides.

Chapter 2: Unique α-Tubulin Mutations in *Poa annua* Induce Variable Response to Prodiamine and Dithiopyr

Introduction

Annual bluegrass (*Poa annua* L.) is a cool-season grass that can be considered a weed or a beneficial turfgrass (Wu and Harding 1992). According to a 2017 survey conducted by the Weed Science Society of America (WSSA), annual bluegrass is considered the fourth most troublesome weed in turfgrass in North America (Van Wychen 2017). It also has highly variable morphological and biological characteristics due to the various ecological pressures and turfgrass management regimes (McElroy et al. 2002). Annual bluegrass is an allotetraploid and the genome is the result of a cross between *Poa infirma* and *Poa supina* followed by a genome doubling event (Mao and Huff 2012). As a species, annual bluegrass could be as young as 10,000 years old, and while native to Europe, it has naturalized on every continent (Chwedorzewska 2008; Mao and Huff 2012).

Mitotic-inhibiting herbicides ($3^{(k_1)}$) are commonly used as preemergence herbicides to control annual grasses and small seeded broadleaves (McElroy and Martins 2013). These herbicides result in inhibition of shoot and root development by preventing the polymerization of microtubules which help separate the chromosomes during mitosis (Shaner 2014). Mitotic-inhibiting herbicides arrest cell division in prometaphase. However, the mechanism varies by herbicide family. Dinitroaniline herbicides prevent microtubule polymerization by binding directly to the tubulin protein

(Vaughn and Lehnen 1991). Pyridine herbicides bind to microtubule-associated proteins (MAPs) which help stabilize the microtubules (Shaner 2014). Prodiamine and dithiopyr are used as preemergence control for annual bluegrass and have been shown to reduce swards of annual bluegrass when applied correctly (Cutulle et al. 2009; Reicher et al. 2017).

Resistance to mitotic-inhibiting herbicides has been reported since 1973 (Heap 2021). Annual bluegrass resistance to dinitroaniline herbicides was first reported in 2002 in North Carolina when a sixfold level of resistance to prodiamine was observed (Isgrigg III et al. 2002). In 2009 and 2017, two populations of *Poa annua* that had a 26-fold and a 22-fold resistance to prodiamine were also reported (Breeden et al. 2017; Cutulle et al. 2009). Annual bluegrass was also reported to be resistant to dithiopyr, however the resistance level was marginal and was not studied further (Cutulle et al. 2009). In an additional case, annual bluegrass was being evaluated for resistance to pronamide when it was noticed that the suspected resistant population in question was unable to be controlled by a field rate of dithiopyr, however the population was not further evaluated for potential resistance to dithiopyr (McCullough et al. 2017).

Even though resistance to prodiamine have been reported in annual bluegrass, the mechanism of resistance has not been reported. Mutations at positions Leu 125, Leu 136, Val 202, Thr 239, Arg 243, and Met 268 on the α-tubulin gene have been reported to confer resistance to dinitroaniline herbicides. In water foxtail (*Alopecurus aequalis* Sobol.), the mutations Lue125-Met and Val202-Phe resulted in a 30.7-fold level of resistance to trifluralin (Hashim et al. 2011). In green foxtail (*Setaria viridis* (L.) P. Beauv.), an increase in survival rates in comparison to a susceptible population was observed in plants that contained the mutation Lue136-Phe (Délye et al. 2004). Two

mutations were confirmed in goosegrass (*Eleusine indica* (L.) Gaertn.) with Thr239-Ile conferring a high level of resistance and Met268-Thr conferring an intermediate level of resistance to dinitroaniline herbicides (Yamamoto et al. 1998). A mutation Met243-Arg/Lys was discovered in rigid ryegrass (*Lolium rigidum* Gaudin) in 2018, and while it did confer resistance to trifluralin, a dinitroaniline herbicide, the fitness costs associated with this mutation made testing the level of resistance difficult (Chu et al. 2018). Target-site resistance mutations exist in the discovered resistant populations of annual bluegrass but sequencing the α-tubulin gene to confirm these mutations using capillary sequencing is challenging. The objective of this research was to determine if the mutations discovered confer varying levels of resistance to prodiamine and confer cross resistance to dithiopyr.

Materials and Methods

Annual bluegrass (*Poa annua*) populations with suspected resistance to dinitroaniline herbicides were collected across the state of Alabama. Once collected, these populations were planted into flats filled with potting medium (Scotts Miracle-Gro Products Inc., Marysville, OH) and were fertilized (28-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food; Scotts Miracle-Gro Products Inc., Marysville, OH) as needed until the plants were healthy and established. These populations were screened for resistance using an initial hydroponic screen (described below) and sequenced for known target-site mutations. Three populations with suspected resistance to prodiamine were then selected for rate-response screens (Table 1).

Initial Hydroponic Screen. A hydroponic assay was used as a first phase testing procedure to determine if a rate response screen was warranted. The system was modeled after the hydroponics assay by Cutulle et al. (2009) that compared different bioassay methods for mitotic-inhibiting herbicides. The containers (Husky 3 Gallon Heavy-Duty Tote, Home Depot, Atlanta, GA) were filled with 11.4 L of water. An aquarium air pump (Tetra Whisper 10, Spectrum Brands Pet, LLC, Blacksburg, VA) was used to aerate the hydroponic solution. Nutrients (1.2 g per container) were added using a premade hydroponic fertilizer (10-5-14, MaxiGro, General Hydroponics, Santa Rosa, CA). Two containers were prepared for each population tested, one container was treated with a 1 μM solution of prodiamine (Barricade 4FL, Syngenta Crop Protection, Inc., Greensboro, NC) and the other was a non-treated control. Ten healthy plants were placed in each container. The plants were allowed to grow for two weeks. After two weeks the plants were rated on a binary scale as either susceptible or resistant based on root growth and morphology in comparison to the non-treated plants (data not shown). Plants displaying symptomology associated with dinitroaniline herbicides, such as swollen root tips and stunted root growth, were ranked as susceptible. Plants displaying no altered growth or morphology compared to non-treated plants were ranked as resistant. After the screening process, plants identified as resistant were planted back into flats and propagated for seed for future use. Seeds were collected from these plants and dried for 48 h and stored at 4 ºC. Plants identified as prodiamine resistant were further researched for α-tubulin mutations and rate response screens using prodiamine and dithiopyr were conducted.

α-Tubulin Sequencing. Amplicon sequencing was conducted to determine if the populations identified as resistant to prodiamine in the initial hydroponic screen

had any known mutations that confer resistance to mitotic-inhibiting herbicides. RNA was extracted from the leaf tissue of a single suspected resistant plant (Direct-zol RNA Kits, Zymo Research, Irvine, CA). The RNA was then converted to cDNA (qScript cDNA SuperMix, Quantabio, Beverly, MA). Two sets of degenerate primers were designed to capture all of the reported regions that contain potential target-site mutations (Table 2). Primer 1 covered a 474 bp region on the α-tubulin gene, and it covered the target-sites at position Leu 125, Leu 136, Val 202, Thr 239, and Arg 243. Primer 2 covered a 379 bp region of the α-tubulin gene and covered the target-site mutations at position Thr 239, Arg 243, and Met 268. For PCR amplification, roughly 150 ng of cDNA was added to a standard 25 µL PCR rear reaction mix containing 10x standard Taq reaction buffer (New England BioLabs Inc., Ipswich, MA), dNTPs (Promega Corporation, Madison, WI), forward and reverse primers, and Taq DNA polymerase (New England BioLabs Inc., Ipswich, MA). Amplification was carried out using a Biometra TOne thermal cycler (Analytik Jena, Jena, Germany) with the following conditions: 30 s denaturing at 95 ºC; 35 cycles of 30 s denaturation at 95 ºC, 30 s annealing at 58 ºC, and 60 s elongation at 68 ºC, and a final extension step for 10 min at 68 ºC. PCR product was prepared with 10x loading dye (Amresco, Solon, OH) and visualized on ethidium bromide-stained 1.5% agarose gels. The samples were electrophoresed in 1x TAE buffer and photographed under UV light. The DNA fragment sizes were estimated by comparing to a 100 bp DNA ladder (New England BioLabs Inc., Ipswich, MA). The rest of the product was then cleaned up for sequencing using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek, Inc., Norcross, GA). The DNA was then sent for sequencing at GeneWiz using Amplicon-EZ (GeneWiz, South Plainfield, NJ). Sequencing data was analyzed using Snakemake-pipeline (Hall 2020). The sequences were visualized using CLC Genomics Workbench

20 (Qiagen, Germantown, MD) and read mapped to Chen et al. (2016) *Poa supina* and *Poa infirma* transcriptomes in order to determine the subgenome location.

Rate Response Screen. Three different populations of annual bluegrass were selected for rate response screens based on unique amino acid substitutions present. Resistant populations were collected from a golf course putting green at the Fort Walton Beach Golf Course in Fort Walton Beach, Florida (R1), from Robert Trent Jones Golf Course in Opelika, Alabama (R2), and from a golf course fairway at The General Golf Course in Rogersville, Alabama (R3). A susceptible population was collected from a field next to Crestline Elementary School in Mountain Brook, Alabama (S1).

Rate response screens were conducted to evaluate the response of the R populations to prodiamine (Barricade 4FL, Syngenta Crop Protection, Inc., Greensboro, NC) and dithiopyr (Dimension 2EW, Dow AgroSciences LLC, Indianapolis, IN). Both herbicides had seven treatments and a nontreated control for comparison. The rates were the same for each herbicide and the rates were 0.01, 0.1, 1.0, 10.0, 100.0, 1000.0, and 10000.0 g ai ha^{-1}. The experiment was arranged as a completely randomized block design with three replicates. The experiment was repeated in time. Twenty seeds were planted in each pot. The pots were filled with 230 cm^3 of the surface horizon Marvyn loamy sand (Fine-loamy, kaolinitic, thermic Typic Kanhapludults) with pH 6.4 and 0.9% organic matter. Soil was added (~2mm depth) to lightly cover seeds after planting. Pots were sprayed the following day using a CO_2 pressurized backpack sprayer that was equipped with TeeJet TP 8002 flat fan nozzles (TeeJet Technologies, Glendale Heights, IL). The sprayer was calibrated to apply 280 L ha^{-1} at 206 kilopascals. Pots were fertilized (28-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food; Scotts Miracle-Gro Products Inc., Marysville, OH) following the manufactures instructions every two

weeks for the duration of the experiment. Pots were irrigated three times daily by an elevated misting system. After six wk the treated pots were compared to the non-treated control. For each pot, the number of germinated seedlings and above ground biomass were recorded six wk after treatment.

Data Analysis. Data were subjected to ANOVA analysis at a significance level of $P<0.05$ using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Interactions and main effect of populations, herbicide, herbicide rate, and runs were analyzed. Seedling emergence and biomass data for dithiopyr and prodiamine were converted to percent relative to the non-treated. Means and standard errors were generated using LSMEANS procedure in SAS. Means and standard errors were modeled, and I50 values were generated using Prism 9.0.0 (GraphPad Software, San Diego, CA). Prior to modeling, the eight rates for prodiamine and dithiopyr (including the non-treated) were log transformed to log rates with the non-treated set to -3 to maintain equal spacing between treatments. The spacing was -3, -2, -1, 0, 1, 2, 3, 4. Seedling emergence control ratings for prodiamine and dithiopyr were modeled using a log(dose) vs response curve equation,

$$Y=Bottom + (Top-Bottom)/(1+10^(X-LogI50) \quad [Eq. 1]$$

where Y is the seedling emergence (%), X is the log rate of the herbicide, Top and Bottom are plateaus, and LogI50 is the log rate of the herbicide that is needed to reduce the seedling emergence by 50%. Biomass reduction control ratings in response to prodiamine and dithiopyr modeled using a log(dose) vs response curve equation,

Y=Bottom + (Top-Bottom)/1+10^(LogI50-X) [Eq. 2]

where Y is the biomass reduction (%), X is the log rate of the herbicide, Top and Bottom are plateaus, and LogI50 is the log rate of the herbicide that is needed to reduce the biomass by 50%. Concentration to induce 50% of seedling emergence or biomass reduction, I50, R squared, and Top and Bottom values were calculated for all populations and herbicides based on regression models (Table 3).

Results and Discussion

The hydroponic screen distinguished potential dinitroaniline resistant populations of annual bluegrass based off morphological response characteristics (Figure 1). Populations that were ranked as susceptible displayed symptoms of clubbed and shortened roots which are often associated with dinitroaniline herbicides and the roots were very different in length and size when compared to the nontreated check. The populations that were ranked as suspected resistant did not display any symptoms associated with dinitroaniline herbicides and the root length and size were visually similar to that of the nontreated check.

Sequencing data revealed that each of the three suspected resistant populations contained mutations at known target-sites on the α-tubulin gene. The mutations were located at position Thr 239 on the α-tubulin gene. The mutations were also located in different subgenomes, either *Poa supina* and/or *Poa infirma* (Table 4). For population R1, a mutation at position 239 from threonine to isoleucine was located on the *supina* subgenome (Figure 2). For population R2, two mutations were found at position 239

from threonine to isoleucine with one located on the *infirma* subgenome and the other located on the *supina* subgenome. For population R3, a mutation was found at position 239 from threonine to isoleucine located on the *supina* subgenome. The target-site mutation that we discovered have been reported in other grass species and have been confirmed to confer herbicide resistance to dinitroanilines. The mutation at Thr 239 was reported to confer a high level of resistance in goosegrass, green foxtail, and rigid ryegrass (Anthony et al. 1998; Délye et al. 2004; Fleet et al. 2018).

R and S populations responded differently in the rate response screens to both herbicides with the R populations emerging more seedlings and producing more above ground biomass at higher herbicide concentrations than the S population (Figure 3). Variation was also observed between R populations for response to both herbicides, however the differences were more pronounced with respect to prodiamine response. Based on the I50 values, the level of resistance to each herbicide varied for the different R populations. I50 values for seedling emergence in response to prodiamine were 36.47, 368.2, and 101.9 g ha^{-1} for the suspected resistant populations R1, R2, and R3, respectively. Suspected resistant populations R1, R2, and R3 were 1.6, 16.5, and 4.6 times more resistant to prodiamine than the susceptible population, respectively, based on seedling emergence response. I50 values for biomass reduction in response to prodiamine were 44.98, 1503, and 30.41 g ha^{-1} for the suspected resistant populations R1, R2, and R3, respectively. Suspected resistant population R1, R2, and R3 were 1.8, 59.2, and 1.4 times more resistant to prodiamine than the susceptible population, respectively, based on biomass reduction response. I50 values for seedling emergence in response to dithiopyr were 194.5, 211.4, and 289.1 g ha^{-1} for the suspected resistant populations R1, R2, and R3, respectively. Suspected resistant populations R1, R2, and

R3 were 4.6, 5.0, and 6.8 times more resistant to dithiopyr than the susceptible population, respectively, based on seedling emergence response. I50 values for biomass reduction in response to dithiopyr were 111.3, 258.4, and 316.9 g ai ha^{-1} for the suspected resistant populations R1, R2, and R3, respectively. Suspected resistant populations R1, R2, and R3 were 3.9, 9.1, and 11.2 times more resistant to dithiopyr than the susceptible population, respectively, based on biomass reduction response.

Research Implications

The rate response screens indicated a consistent level of resistance to dithiopyr among the resistant populations, while the level of resistance to prodiamine was variable. Resistant populations were able to consistently emerge and accumulate biomass at higher levels of dithiopyr when compared to the susceptible population. However, the level of resistance was not consistent for the resistant populations when comparing seedling emergence and biomass reduction in response to prodiamine to the susceptible population.

For dithiopyr the I50 values for seedling emergence and biomass reduction were similar to the R populations. The lowest I50 values for seedling emergence and biomass reduction were 194.5 and 111.3 g ai ha^{-1}, respectively and the highest I50 values were 289.1 and 316.9 g ai ha^{-1}, respectively. Numerical ranked, the level of resistance for the R populations in comparison to the S population were consistent with R1 < R2 < R3 in both the seedling emergence data and biomass reduction data.

For prodiamine, the I50 values for seedling emergence and biomass reduction were variable. The lowest and highest I50 values for seedling emergence were 36.47 and

368.2 g ai ha^{-1}, respectively and biomass reduction were 30.41 and 1503 g ai ha^{-1}, respectively. When numerically ranked, the level of resistance when compared to the S population for seedling emergence was different than biomass reduction. First, the R1 population did not appear to be resistant to prodiamine, and the I50 values for both seedling emergence and biomass reduction were similar to that of the susceptible. In both seedling emergence and above ground biomass accumulation, similar levels of resistance were seen for each resistant population. Second, the R2 population had very different levels of resistance for seedling emergence and biomass reduction. The high I50 value for the biomass reduction data indicates that the R2 population was able to accumulate biomass very well even at high concentrations of prodiamine. Third, the R3 population indicated resistance in the seedling emergence data but the results were not echoed in the biomass reduction data. This could indicate that the R3 population was able to germinate but unable to accumulate biomass properly at higher concentrations of prodiamine. The overall level of resistance for prodiamine was variable across populations. This difference reveals the variability of annual bluegrass.

This variation in resistance to prodiamine could be due to the level of gene expression or where the α-tubulin genes are expressed. A study on α-tubulin genes in corn revealed that different genes were expressed at different levels reporting that the *tua*1 gene was nearly 100 times more abundant than the *tua*2 gene (Uribe et al. 1998). The location within the plant where these genes were expressed was also different. For example, *tua*1 was expressed in pollen and the root apex while *tua*3 was only expressed in the immature embryo and the vascular cylinder of the root (Uribe et al. 1998). This could explain why R1 and R3 do not have a high level of resistance to prodiamine, despite possessing the Thr239-Ile mutation. If the gene copy that had the resistant

mutation was not expressed in the roots, we would not expect a high level of resistance. However, more research testing expression level of the α-tubulin gene containing the mutation and where it is expressed in the plant is needed.

Table 1: The location and site where the suspected resistant and susceptible populations used in the rate response screens were collected from.

Population	Site Name	Location
R1	Fort Walton Beach Golf Course	Fort Walton Beach, Florida
R2	Robert Trent Jones Golf Course	Opelika, Alabama
R3	The General Golf Course	Rogersville, Alabama
S1	Crestline Elementary School	Mountain Brook, Alabama

Table 2: Primer sequences used for amplification and sequencing of α-tubulin gene in annual bluegrass.

Primer	**Sequence 5' to 3'**	**Length**	**Target Sites Captured**
Tua_ampseq_1F	GRCACCARTCSACRAACTGGA	474 bp	Leu 125, Leu 136, Val 202, Thr 239, Arg 243
Tua_ampseq_1R	GTABGGSACMAGRTTGGTCTG		
Tua_ampseq_2F	CCWACCTACACCAACCTSAAC	379 bp	Thr 239, Arg 243, Met 268
Tua_ampseq_2R	GRCACCARTCSACRAACTGGA		

Table 3. I50 values, R squared, Top, and Bottom values for the suspected resistant and susceptible populations for both prodiamine and dithiopyr.

Herbicide	Prodiamine				Dithiopyr			
Biotype	I50	R squared	Top	Bottom	I50	R squared	Top	Bottom
	% Seedling Emergence							
R1	36.47	0.9680	96.76	3.700	194.5	0.9827	100.0	-8.228
R2	368.2	0.8776	107.8	14.08	211.4	0.9718	109.7	-9.166
R3	101.9	0.9281	115.7	19.70	289.1	0.9192	107.3	-11.82
S1	22.26	0.9101	136.0	-6.413	42.25	0.9327	85.01	-1.378
	% Biomass Reduction							
R1	44.98	0.9101	101.4	14.08	111.3	0.9660	104.4	12.39
R2	1503	0.9881	101.9	3.779	258.4	0.9768	109.5	3.637
R3	30.41	0.9402	90.88	11.36	316.9	0.9296	111.0	6.235
S1	25.39	0.9491	100.7	0.5125	28.30	0.8626	101.8	21.51

Table 4. The mutations and the subgenome location for each suspected resistant population of annual bluegrass.

Population	**Mutation**	**Subgenome**
R1	Thr239-Ile	*supina*
R2	Thr239-Ile	*infirma & supina*
R3	Thr239-Ile	*supina*

Figure 1.

A. Clubbed and shortened roots symptomology associated with dinitroaniline herbicides that was seen in the susceptible populations.

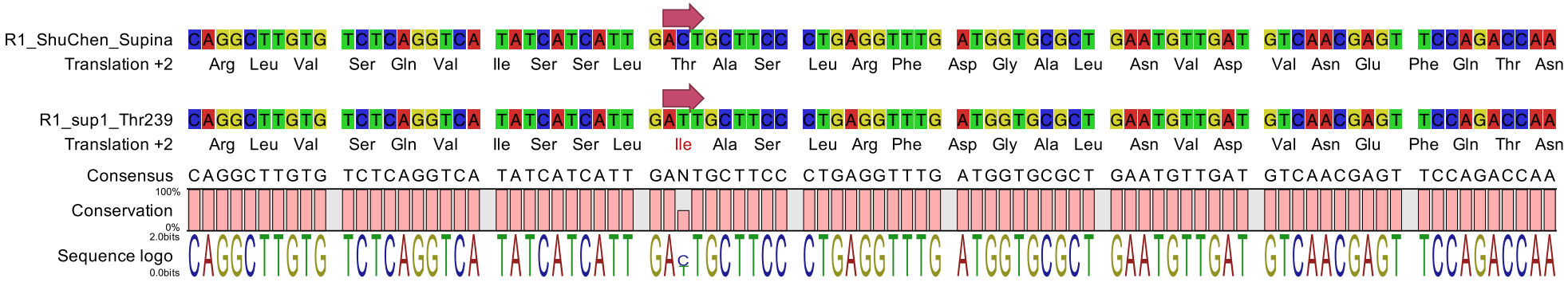
B. The difference in root growth for a suspected resistant population and a susceptible population. Left image, a treated plant (left) in comparison to a nontreated plant (right) from population R1. Right image, a treated plant (left) in comparison to a nontreated plant (right) from population S1.

Treated | Non-Treated

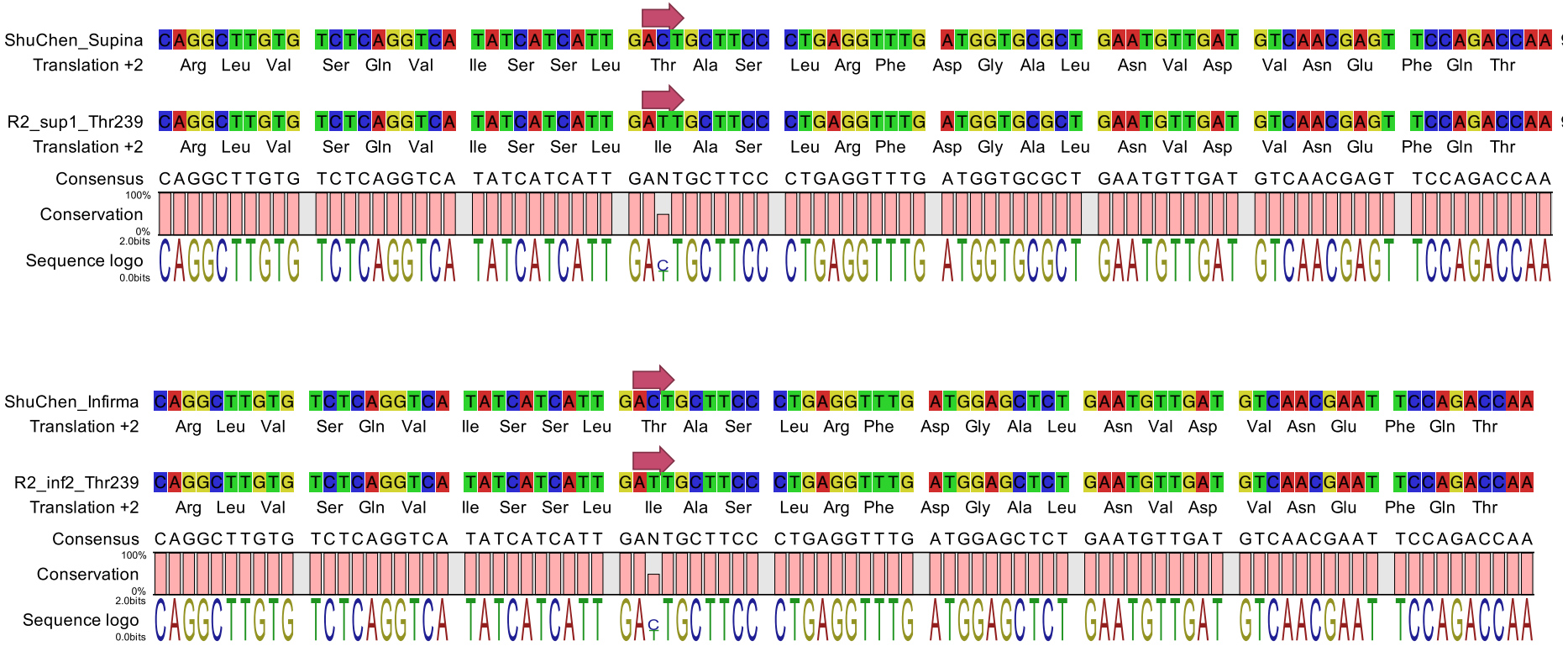
Treated | Non-Treated

Figure 2.

A. R1 sequence read mapped to the *supina* subgenome revealing the mutation Thr239-Ile.



B. Top image: R2 sequence read mapped to the *infirma* subgenome revealing the mutation Thr239-Ile. Bottom image: R2 sequence read mapped to the *supina* subgenome revealing the mutation Thr239-Ile.



C. R3 sequence read mapped to the *supina* subgenome revealing the mutation Thr239-Ile.

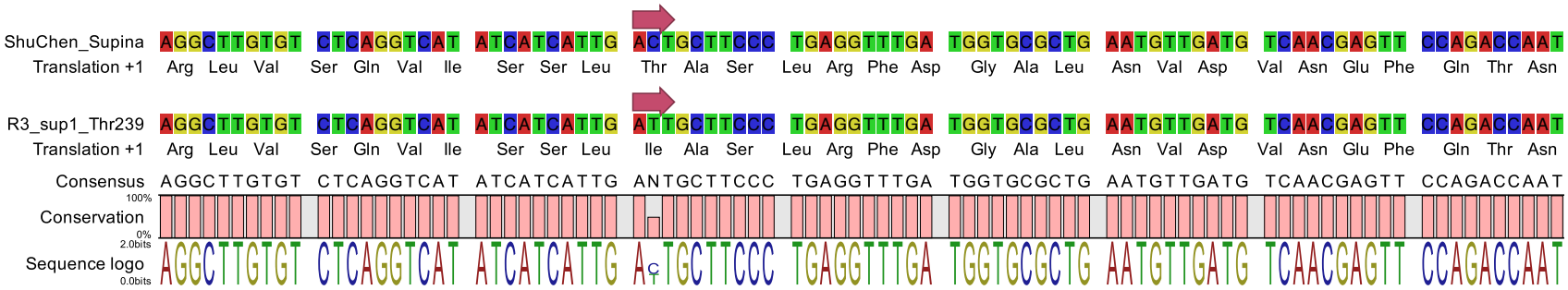
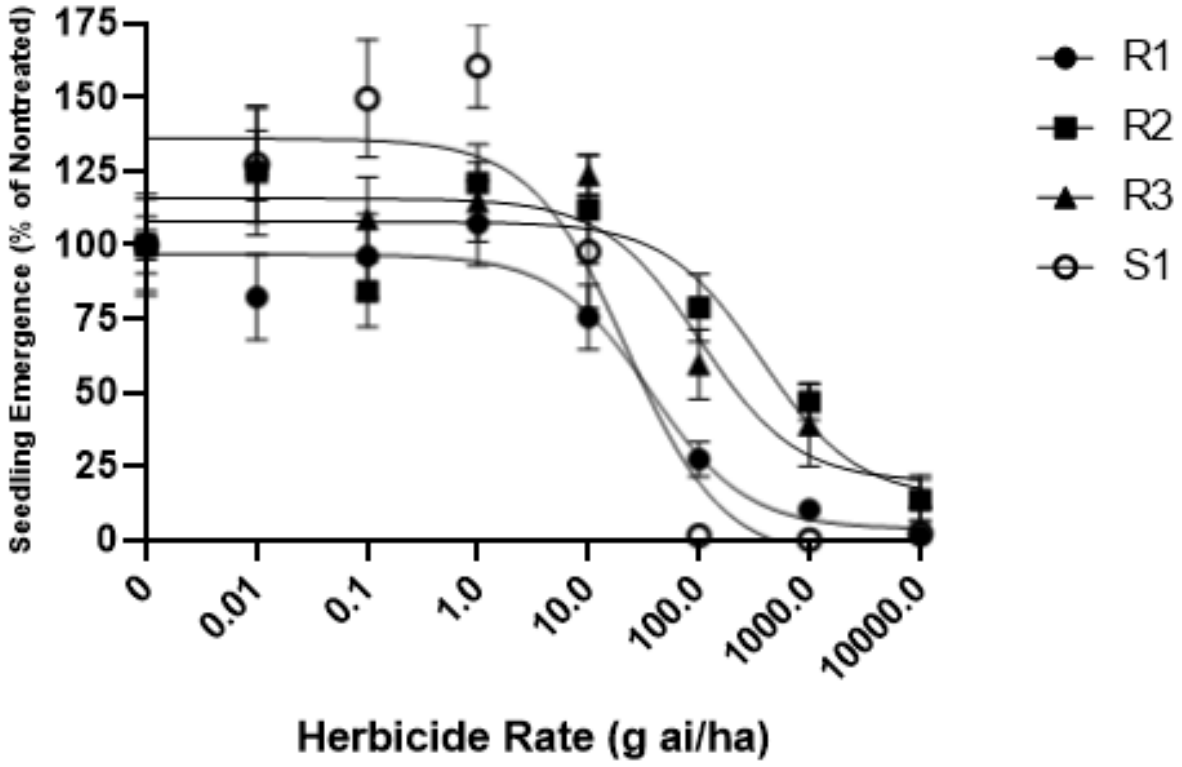
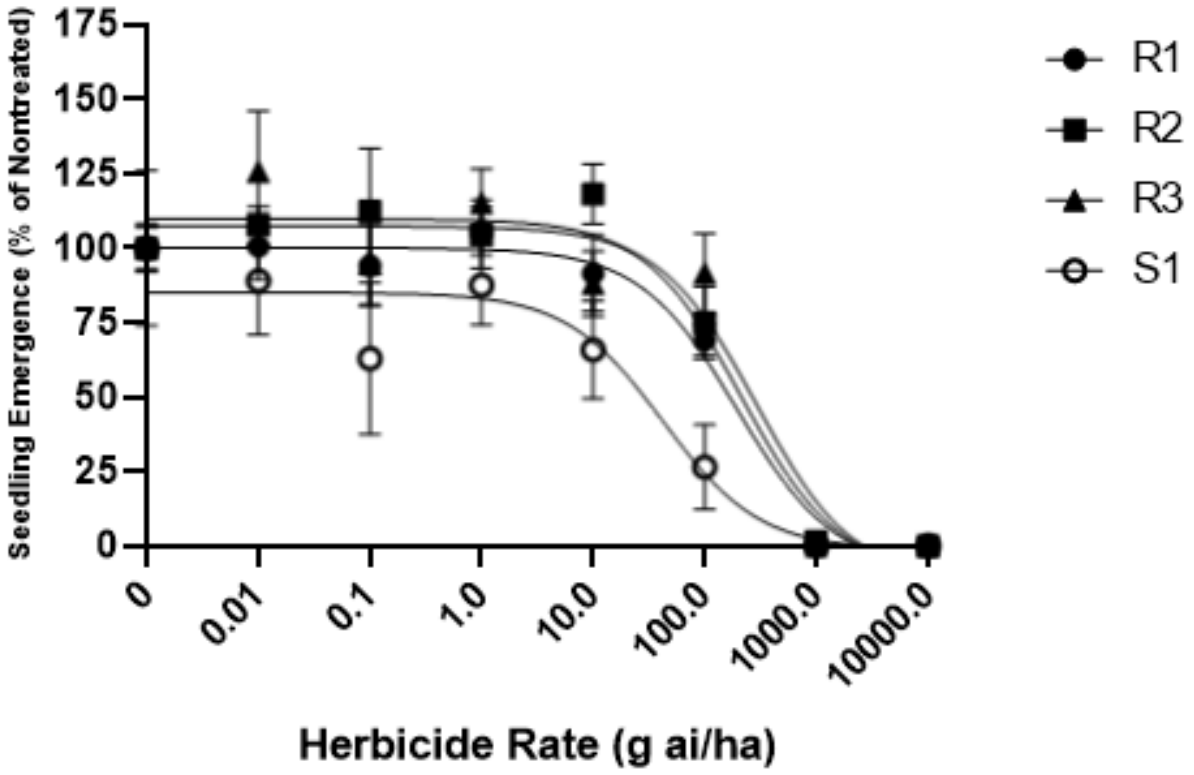


Figure. 3

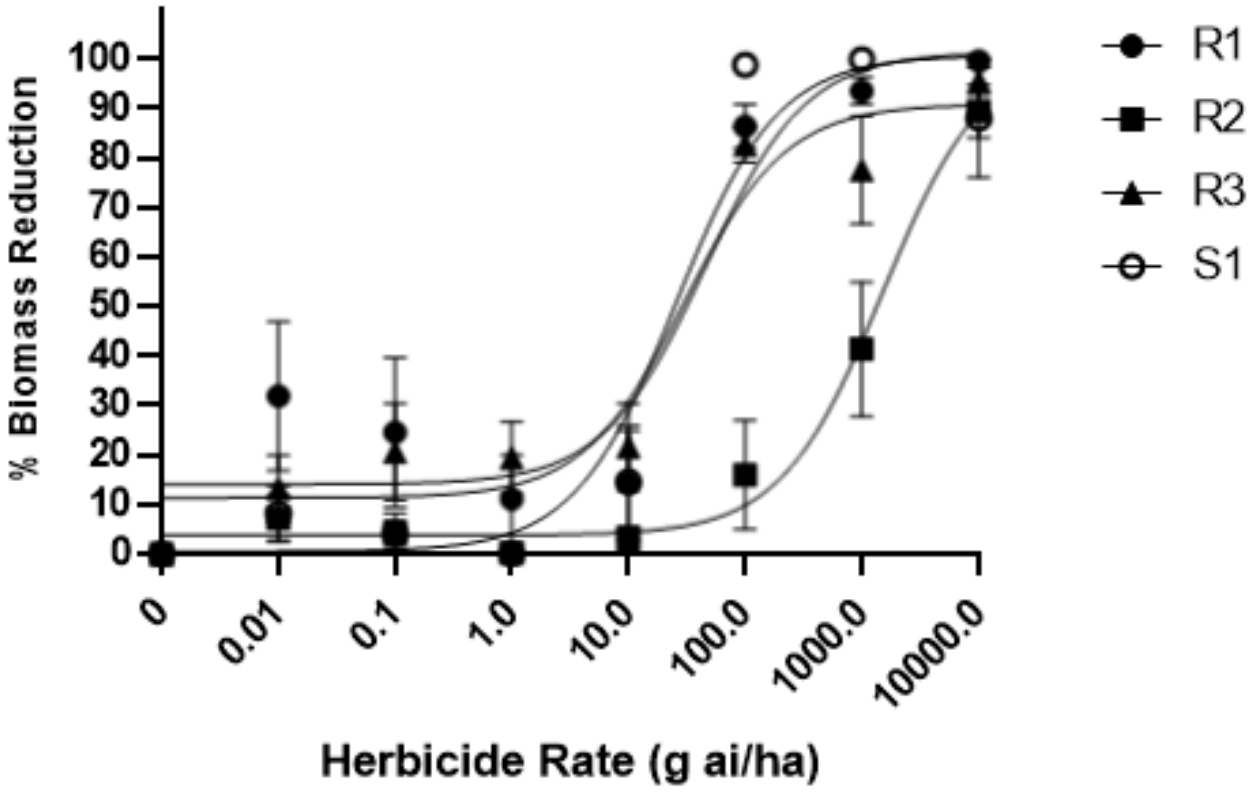
A. Seedling emergence response of R and S populations to increasing rates of prodiamine. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



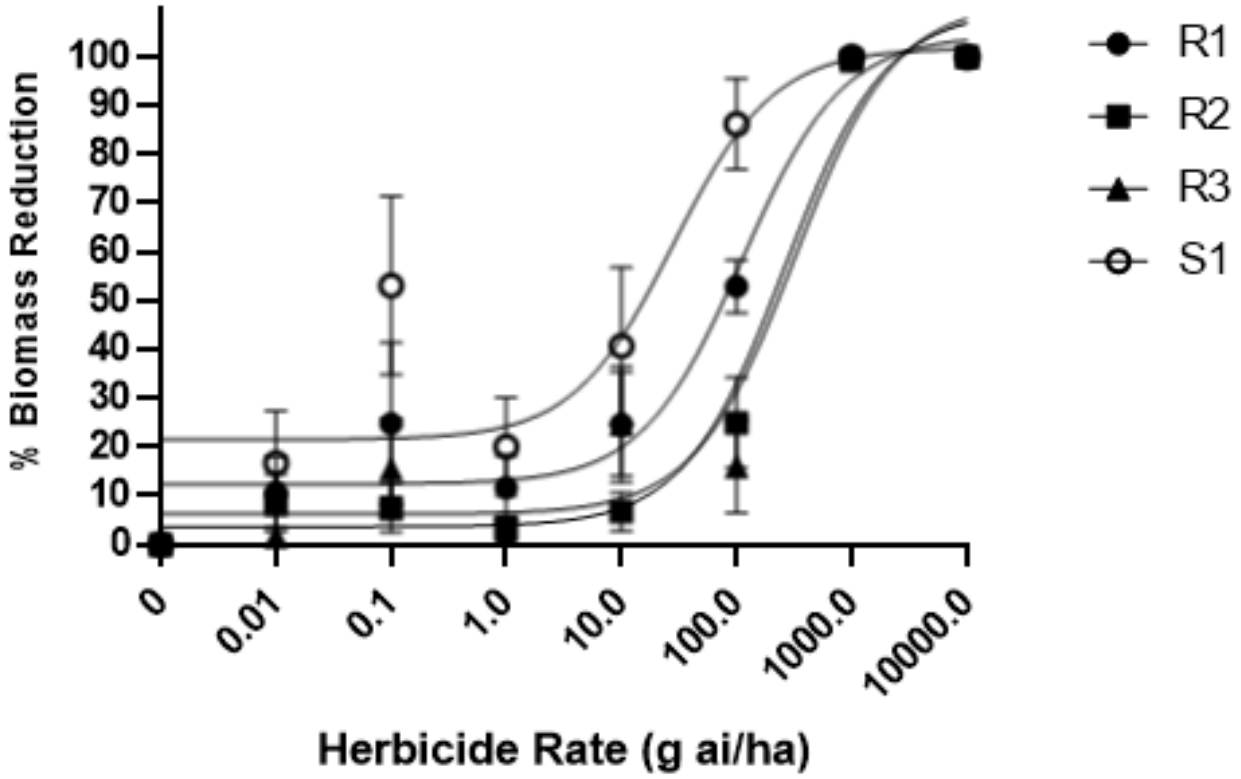
B. Seedling emergence response of R and S populations to increasing rates of dithiopyr. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



C. Biomass reduction response of R and S populations to increasing rates of prodiamine. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.



D. Biomass reduction of R and S populations to increasing rates of dithiopyr. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.



Chapter 3: Identification of Goosegrass (*Eleusine indica*) Resistant to Dithiopyr and Dinitroaniline Herbicides

Introduction

Goosegrass (*Eleusine indica* (L.) Gaertn.) is a problematic summer annual grass weed that is difficult to control in turfgrass. Preemergence herbicides are often preferred for control as postemergence herbicide options are limited (McCullough et al. 2013). Dinitroaniline and pyridine are two families of mitotic-inhibiting herbicide that are commonly used for preemergence control of annual weeds in turfgrass systems (Breeden et al. 2017; McElroy and Martins 2013). The dinitroaniline family consists of active ingredients such as prodiamine, pendimethalin, and oryzalin, while the pyridine family consists of the active ingredient dithiopyr. Mitotic-inhibiting herbicides inhibit the polymerization of microtubules which affects root and shoot development (McElroy and Martins 2013). Dinitroanilines bind directly to the tubulin protein which prevents the polymerization of the tubulin protein dimer (Vaughn and Lehnen 1991). Dithiopyr on the other hand inhibits the polymerization of microtubules by binding to microtubule associated proteins (MAPs) that aid in the stabilization of microtubules (Cutulle et al. 2009). Mitotic-inhibiting herbicides result in swollen root tips as cells at the growing points are unable to divide properly (Vaughn and Lehnen 1991). Unaltered herbicide regimes revolving around the use of dinitroanilines or dithiopyr as the only preemergence method for controlling goosegrass has resulted in the development of mitotic-inhibiting herbicide resistance in goosegrass.

Goosegrass was first reported as having resistance to mitotic-inhibiting herbicides in 1984 (Mudge et al. 1984). This resistant biotype was able to withstand up to 6 times the recommended rate of the dinitroaniline herbicide trifluralin (Mudge et al. 1984). In 1990, two different biotypes of goosegrass in South Carolina were reported as having varying levels of resistance to dinitroaniline herbicides (Vaughn et al. 1990). The first biotype had reported levels of resistance that ranged from 1,000 to 10,000-fold and the second biotype had reported levels of resistance at roughly 50-fold (Vaughn et al. 1990). However, it was not until 1998 that mutations were reported in *Eleusine indica* that revealed target-site resistance to dinitroaniline herbicides. The reported mutations were found at two different locations on the α-tubulin gene that codes for the α-tubulin protein. A high level of resistance was reported for a mutation at the 239 position from threonine to isoleucine (Thr239-Ile) in goosegrass (Anthony et al. 1998). Thr239-Ile mutation has also been reported to cause resistance to dinitroaniline herbicides in *Lolium rigidium* (Fleet et al. 2018). Methionine to threonine at position 268 has also been reported to confer intermediate resistance to dinitroaniline herbicides in goosegrass (Yamamoto et al. 1998). These are currently the only two mutations that have been reported in goosegrass that are known to confer resistance to dinitroaniline herbicides. Resistance to dithiopyr has not been reported in *Eleusine indica*, nor has cross resistance been reported from biotypes that are resistant to dinitroaniline herbicides.

There have been other mutations on the α-tubulin gene that have been confirmed to confer resistance to mitotic-inhibiting herbicides. A mutation at position 136 was discovered in green foxtail from phenylalanine to leucine that conferred resistance to dinitroaniline herbicides (Délye et al. 2004). In 2011, two new mutations were

discovered on the α-tubulin gene of water foxtail, one at position 125 that resulted in an amino acid substitution from leucine to methionine and one at position 202 that resulted in an amino acid substitution from valine to phenylalanine, that conferred resistance to the dinitroaniline herbicide trifluralin (Hashim et al. 2011). In 2018, a novel mutation was discovered in rigid ryegrass, Arg243-Meth/Lys, that conferred resistance to dinitroaniline herbicides (Chu et al. 2018). These target-site mutations have not been reported in goosegrass.

A suspected dithiopyr-resistant goosegrass population was collected from a golf course putting green in 2018. An initial treatment revealed a recommended field rate of dithiopyr and prodiamine failed to control the resistant population. The objective of this research was to determine the resistance level in the suspected resistant population and to determine if target-site mutations exist. We hypothesized that it was resistant to dithiopyr as well as cross resistant to dinitroaniline herbicides.

Materials and Methods

The suspected resistant goosegrass population was collected in 2018 from Limestone Springs Golf Course in Oneonta, Alabama. The population was placed into a flat filled with potting medium (Scotts Miracle-Gro Products Inc., Marysville, OH), fertilized (28-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food; Scotts Miracle-Gro Products Inc., Marysville, OH) as needed, and irrigated three times daily by an elevated misting system. A known susceptible population was also collected from the Alabama Agricultural Experiment Station, Plant Breeding Unit in Tallassee, Alabama.

Both populations were propagated for seed. Seeds were collected from these plants, dried for 48 h, and stored at 4 °C.

Initial Hydroponic Screen. A hydroponic screen was conducted to determine if further evaluation was warranted. Seeds were planted into flats of potting medium (Scotts Miracle-Gro Products Inc., Marysville, OH). After germination, seedlings were fertilized (28-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food; Scotts Miracle-Gro Products Inc., Marysville, OH) as needed. Three plastic bins (Husky 3 Gallon Heavy-Duty Tote, Home Depot, Atlanta, GA) were filled with 11.4 L of water. An aquarium air pump was used to provide air to the water for the plants (Tetra Whisper 10, Spectrum Brands Pet, LLC, Blacksburg, VA). Nutrients (1.2 g per container) were added using a premade hydroponic fertilizer (10-5-14, MaxiGro, General Hydroponics, Santa Rosa, CA). Herbicide treatments were added to the bins next. The first bin was a non-treated control, so it did not receive any herbicide. The second bin was treated with prodiamine (Barricade 4FL, Syngenta Crop Protection, Inc., Greensboro, NC) to create a 1 µM solution. The third bin was treated with dithiopyr (Dimension 2EW, Dow AgroSciences LLC, Indianapolis, IN) to create a 1 µM solution. The herbicide and fertilizer were then evenly mixed until there was no fertilizer residue at the bottom. Next ten seedlings from the known susceptible populations and ten seedlings from the suspected resistant population were placed into each bin. The plants were allowed to grow for two weeks. The root growth of the treated plants was compared to the nontreated plants. Treated plants were also checked for typical symptomology associated with dithiopyr and prodiamine such as swollen root tips.

Rate Response Screen. A rate response screen was conducted on the suspected resistant population and compared to the susceptible population. The rate

response was conducted using dithiopyr (Dimension 2EW, Dow AgroSciences LLC, Indianapolis, IN), prodiamine (Barricade 4FL, Syngenta Crop Protection, Inc., Greensboro, NC), pendimethalin (Pre-M AquaCap, LESCO, Inc, Cleveland, OH), and Oryzalin (Surflan A.S., United Phosphorus, Inc., King of Prussia, PA). There were seven treatments for each herbicide and the treatments were 0, 0.1, 1.0, 10.0, 100.0, 1000.0, 10000.0 g ai ha^{-1}. Pots were filled with 230 cm^3 of Marvyn loamy sand (Fine-loamy, kaolinitic, thermic Typic Kanhapludults). Roughly 20 seeds were planted in each pot and soil was added (~2mm depth) to lightly cover the seeds. The pots were irrigated three times daily by an elevated misting system. The pots were fertilized after planting (28-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food; Scotts Miracle-Gro Products Inc., Marysville, OH) and were fertilized every two weeks after for the remainder of the experiment. The pots were then sprayed the following day. The herbicides were applied using a hand-held CO_2 pressurized sprayer that was equipped with TeeJet TP 8002 flat fan nozzles (TeeJet Technologies, Glendale Heights, IL). The sprayer was calibrated to apply 280 L ha^{-1} at 206 kilopascals. There were three replications per treatment and the experiment was repeated in time. The treatments were compared to the non-treated control six wk after treatment. For each pot, the number of germinated seedlings and the above ground biomass was recorded six weeks after treatment.

α-Tubulin Sequencing. Transcriptome sequencing was conducted to determine if there were any target-site mutations known to confer resistance to mitotic-inhibiting herbicides. Freshly collected leaf tissue of about 100 mg was grounded using bead mill homogenizer (Omni International, Kennesaw, GA). RNeasy Plant Mini kit (Qiagen, Hilden, Germany) was used to extract RNA following manufacturer

instruction. DNA digestion was performed using turbo DNA-free kit (Applied Biosystems, Foster City, CA) to eliminate any genomic DNA content in the samples. RNA concentration and quality were checked on Nano drop 2000 (ThermoFisher Sci., Waltham, MA), Qubit 2.0 Fluorometer (ThermoFisher Sci., Waltham, MA) and RNA integrity was determined using electrophoresis in 2% (w/v) agarose gel. RNA was sequenced via Illumina NovaSeq 6000 instrument by Novogene (Beijing, China) yielding approximately 45 million 150 bp, paired-end reads. Data were assembled using Trinity (https://anaconda.org/bioconda/trinity) and the resulting assembly was annotated using Trinotate (https://anaconda.org/bioconda/trinotate). Putative α-tubulin contigs were extracted based on BLASTp (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn) annotation utilizing some goosegrass α-tubulin contigs (CAA06619). α-tubulin contigs were aligned with closely related grass species (XP_025791387 and XP_004981922), including other goosegrass sequences.

Data analysis. Rate response data were subjected to ANOVA analysis at a significance level of $P<0.05$ using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) to test for significance ($P<0.05$) of biotypes, herbicide rate, and runs with seedling emergence and biomass reduction variables. Means and standard errors were generated using LSMEANS procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Means and standard errors were graphed, and I50 values were generated using Prism 9.0.0 (GraphPad Software, San Diego, CA). Prior to modeling, the eight rates for prodiamine and dithiopyr (including the non-treated) were log transformed to log rates with the non-treated set to -3 to maintain equal spacing between treatments. The log

transformed rates were -3, -2, -1, 0, 1, 2, 3, 4, corresponding to 0, 0.01, 0.1, 1.0, 10.0, 100.0, 1000.0, 10000.0 g ha^{-1} for each herbicide. Four models were used to analyze the data. Seedling emergence control ratings for both populations in response to dithiopyr, prodiamine, and pendimethalin were modeled using a log(dose) vs response curve equation,

Y=Bottom + (Top-Bottom)/(1+10^(X-LogI50) [Eq. 1]

where Y is the seedling emergence (%), X is the log rate of the herbicide, Top and Bottom are plateaus, and LogI50 is the log rate of the herbicide that is needed to reduce the seedling emergence by 50%. Biomass reduction control ratings for both populations in response to dithiopyr and pendimethalin and the R population in response to prodiamine were modeled using a log(dose) vs response curve equation,

Y=Bottom + (Top-Bottom)/1+10^(LogI50-X) [Eq. 2]

where Y is the biomass reduction (%), X is the log rate of the herbicide, Top and Bottom are plateaus, and LogI50 is the log rate of the herbicide that is needed to reduce the biomass by 50%. A line equation was used to model seedling emergence control rating for both populations in response to oryzalin and to model the biomass reduction control rating for the S population in response to oryzalin. The line equation is,

Y=Slope*X + YIntercept [Eq. 3]

where Y is either seedling emergence (%) or biomass reduction (%), Slope is the slope of the line, and YIntercept is where the line intersects the Y axis. The last equation used was an exponential plateau equation that was used to model biomass reduction for the S population in response to both prodiamine and oryzalin. The equation used was,

$$Y=YM-(YM-Y0)*\exp(-k*X) \quad [Eq. 4]$$

where Y is the biomass reduction (%), YM is the maximum, Y0 is the starting point, k is the rate constant, and X is the log rate of the herbicide. Concentration to induce 50% of seedling emergence or biomass reduction, I50, R squared values, and the other parameters for each equation were calculated for all populations and herbicides based on regression models. If not inherent to the model, I50 values were calculated for each equation. Seedling emergence and biomass reduction data for dithiopyr, prodiamine, pendimethalin, and oryzalin were converted to percent relative to the non-treated.

Results and Discussion

Our initial hypothesis was that the suspected resistant population would be resistant to dithiopyr and cross resistant to the tested dinitroaniline herbicides. Rate response screens revealed that the R population responded differently than the S population. The R population germinated more seedlings and produced more above ground biomass than the S population when treated with dithiopyr, prodiamine, pendimethalin, or oryzalin (Figure 4). Based on the I50 values for both seedling emergence and biomass reduction, the R population is highly resistant to dithiopyr,

pendimethalin, and oryzalin (Table 5). However, the resistance level to prodiamine appears to be marginal.

The I50 values indicated that a much higher concentration in g ai ha^{-1} was needed to control the resistant population with dithiopyr, pendimethalin, and oryzalin than for prodiamine. For seedling emergence, I50 values for dithiopyr, pendimethalin, and oryzalin were 919.2, 7640, and $1.32*10^{17}$ g ai ha^{-1}, respectively, while prodiamine I50 value was only 73.60 g ai ha^{-1}. The level of resistance was also much lower for prodiamine than the other herbicides when compared to I50 value of the susceptible population. The level of resistance for prodiamine was 4.7-fold while the level of resistance for dithiopyr, pendimethalin, and oryzalin were 54.1-, >100-, and >100-fold, respectively. This was also observed with biomass reduction with dithiopyr, pendimethalin, and oryzalin having I50 values at 285.9, 8885, and 5907 g ai ha^{-1}, respectively, but the I50 value for prodiamine was low again at 25.25 g ai ha^{-1}. When compared to the I50 of the S population the resistance level of the R population to prodiamine was again lower than that of the other herbicides. The resistance level to prodiamine compared to the S population was 7.8-fold, while the resistance level of the R population to dithiopyr, pendimethalin, and oryzalin were 88.4-, >100-, and >100-fold, respectively.

A mutation at position 136 on the α-tubulin gene that resulted in an amino acid substitution from leucine to phenylalanine was observed for the R population of goosegrass (Figure 5). This mutation at position 136 was first reported in green foxtail (*Setaria viridis* (L.) P. Beauv.) in 2004 and was confirmed to confer resistance to dinitroaniline herbicides (Déyle et al. 2004). It was reported again in 2011 when it was discovered in water foxtail (*Alopecurus aequalis* Sobol.) (Hashim et al. 2011). The

mutation was confirmed to confer resistance to dinitroaniline herbicides. However, this mutation has yet to be reported in goosegrass. While this mutation has been previously reported to confer resistance to dinitroaniline herbicides, this mutation, or any other known mutation on the α-tubulin gene, has not been associated with resistance to dithiopyr.

Research Implications

Goosegrass was the first species confirmed as resistant to mitotic-inhibiting herbicides, with the first case reported in 1973 (Heap, 2021). Mudge et al. (1984), first published on goosegrass resistance reporting that the R population was not controlled by 2 times the recommended rate of pendimethalin and oryzalin and 6 times the recommend rate of trifluralin. Anthony et al. (1998), first identified TSR as the causal mechanism of resistance reporting a 42-fold level of resistance to trifluralin and a 60-fold level of resistance to oryzalin. Subsequent TSR was reported by Yamamoto et al. (1998). Since that time, other target-site mutations reported in different grass species. In water foxtail, different mutations resulted in a 5.7- and 30.7-fold level of resistance to trifluralin (Hashim et al. 2011). In rigid ryegrass, three different target-site mutations resulted in 8-, 4-, and 17-fold level of resistance to trifluralin (Chu et al, 2019; Fleet et al. 2018).

Despite the over forty years of known resistance, our research demonstrates two unknown points. First, TSR mutations in α-tubulin do not confer equal levels of cross resistance to all DNA herbicides. This can be seen with the mutation Thr239-Ile. This mutation has different levels of resistance reported that vary across herbicides and

species with a 42-fold resistance to trifluralin and 60-fold resistance to oryzalin reported in goosegrass and a 17-fold resistance to trifluralin in rigid ryegrass. Each of these resistant populations contain the same mutation, but the level of resistance differs (Anthony et al. 1998; Fleet et al. 2018).

Second, no mutations on the α-tubulin gene have ever been associated with dithiopyr resistance. Dithiopyr is suspected to bind to microtubule-associated proteins (MAPs) instead of the tubulin protein. MAPs aid in microtubule stability. In the presence of dithiopyr, MAPs are unable to function properly resulting in shortened microtubules. However, no functional assay has definitively proven that dithiopyr binds to MAPs. Dithiopyr does bind to a protein that is 65 kDa, but they have not directly identified the protein (Lehnen and Vaughn 1991). Although dithiopyr does share characteristics with mitotic-inhibiting herbicides, it has a distinctive effect on microtubule organization and stability (Lehnen and Vaughn 1991). Without more information of how dithiopyr interacts with the target protein and with the microtubules, it cannot be determined if target-site mutations on the α-tubulin gene result in resistance to dithiopyr. So, while we cannot definitively say if the mutation Leu136-Phe is the causal mechanism of dithiopyr resistance, it seems that the greatest probable causal mechanism is the Leu136-Phe mutation.

The resistant biotype exhibited resistance to dithiopyr, pendimethalin, and oryzalin, but a marginal resistance to prodiamine when compared to the other herbicides tested. When the I50 values are compared with each other, the amount of prodiamine needed to control the R population is less than the other three herbicides tested. When the I50 value for the R population is compared to the I50 value for the S population, the level of resistance to prodiamine is also much lower than the level of

resistance of the other three herbicides tested. This low I50 value for the S population in response to prodiamine could be the result of poor germination. The R and S populations had varying responses to both pendimethalin and oryzalin. This could be due to varying germination rates of the populations.

The target-site mutation on the α-tubulin one gene was identified and determined to be the cause of resistance to the tested herbicides. This mutation had been previously reported to confer resistance to trifluralin, a dinitroaniline herbicide. This resistance seems to be true for the dinitroaniline herbicides pendimethalin and oryzalin, but not so much so for prodiamine. This mutation also seems to confer resistance to dithiopyr, which was not reported in the previous experiments involving the mutation at position 136. While the two previous experiments involving the target-site mutation Leu136-Phe observed resistance to trifluralin, the level of resistance that was reported was much lower than what was observed in the current study. Déyle et al. (2004) did not publish any data indicating how resistant the resistant population was to the dinitroaniline herbicides that it was tested against. However, the population that possessed the Leu136-Phe mutation did have a high survival rate when treated with either pendimethalin or trifluralin. Hashim et al. (2011) reported a 5.7-fold resistance to trifluralin in a resistant population that contained the Leu136-Phe mutation. However, this resistant population also possessed a Val202-Phe mutation. This second mutation could have affected the resistance level to trifluralin.

While variation to mitotic-inhibiting herbicides exists, it is unclear if there are differences in field response to prodiamine. McCullough et al. (2013) reported on variation in shoot reduction of goosegrass resistant to prodiamine. In greenhouse studies shoot reduction ranged from 4 to 63% at 6 weeks after treatment (WAT)

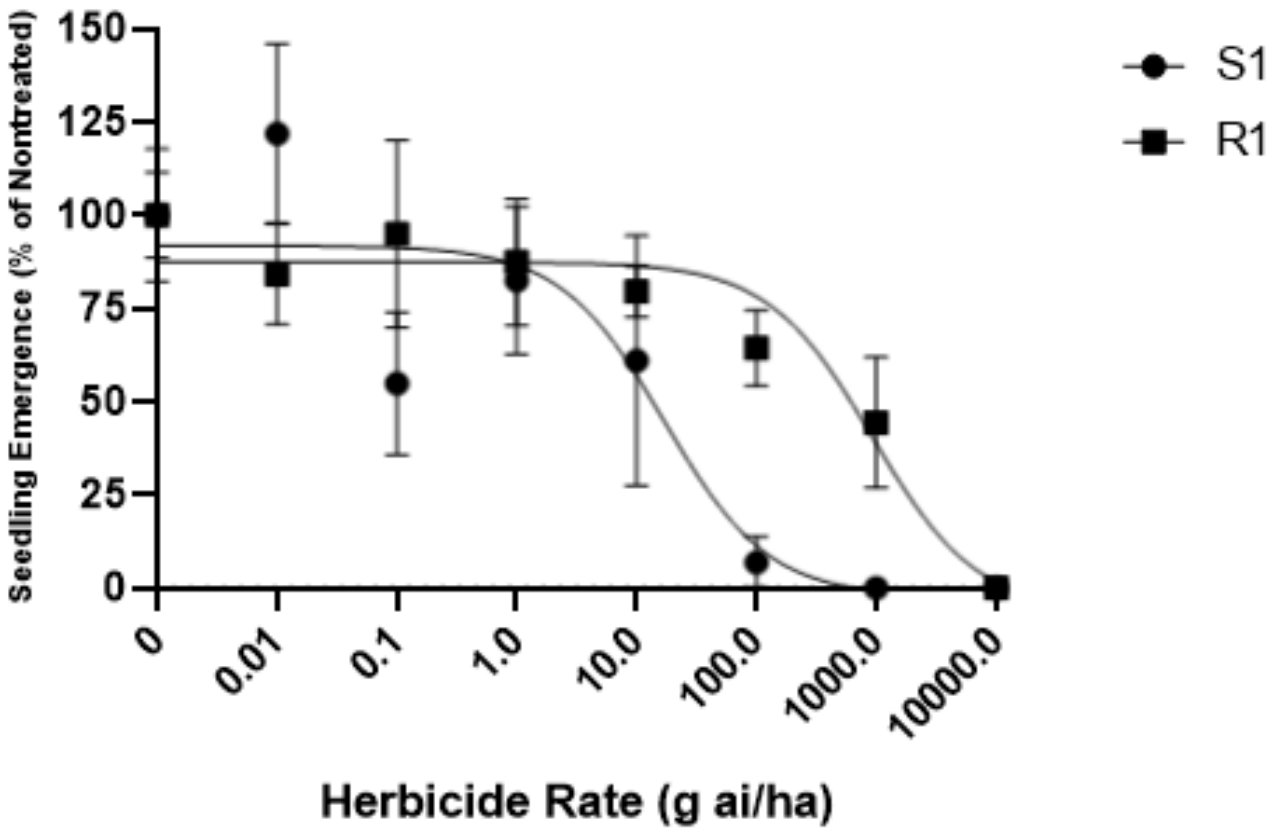
(McCullough et al. 2013). Overall control of the resistant population with prodiamine was similar in both greenhouse and field experiments, but there was still a difference with greenhouse and field experiment control levels being <35 and <7%, respectively (McCullough et al. 2013). Without more research, the poor control seen in our greenhouse rate response screens to prodiamine cannot be translated to control in a field setting.

Table 5. Rate at which 50% of seedling emergence and biomass is reduced for R and S populations for dithiopyr, prodiamine, pendimethalin, and oryzalin. I50 is in g ai ha^{-1}.

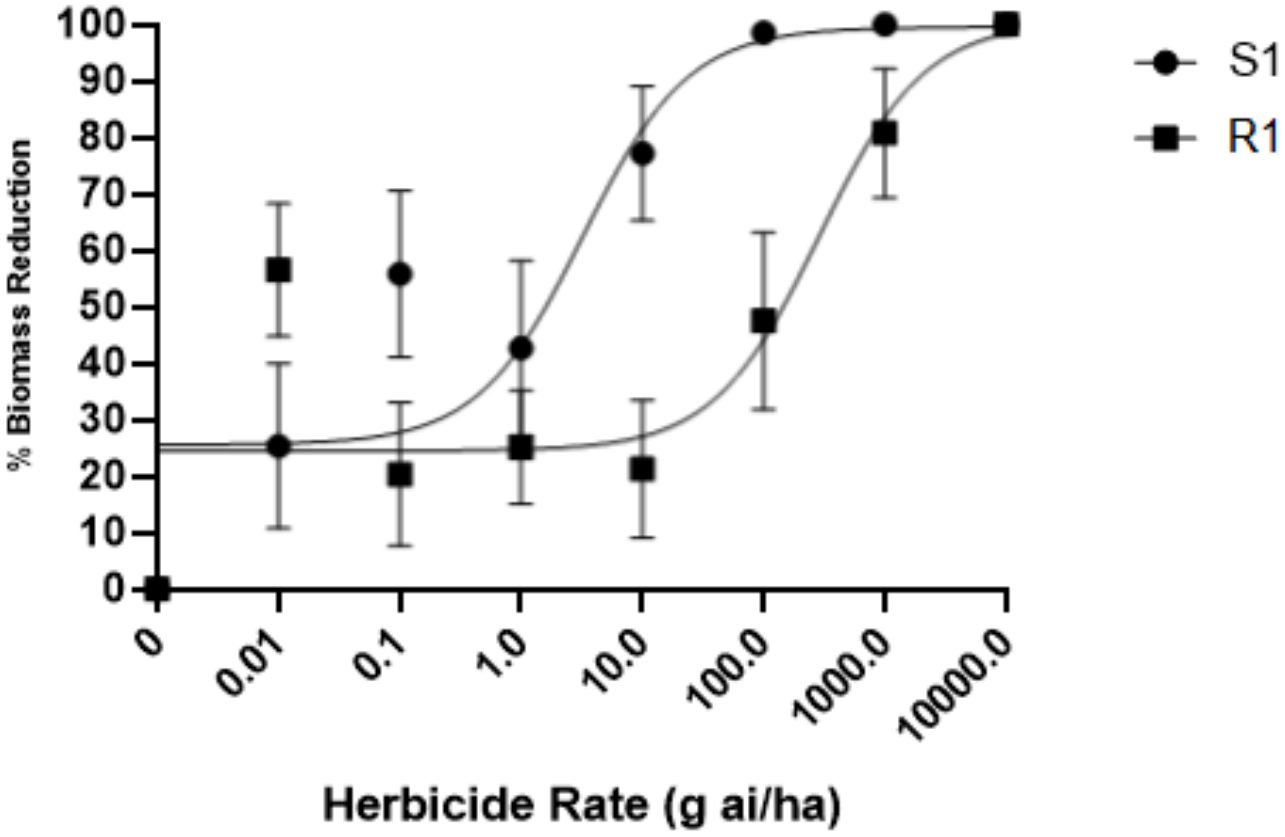
Herbicide	% Seedling Emergence										
Dithiopyr	Population	I50	R squared	Top	Bottom	Population	I50	R squared	Top	Bottom	
	R1	919.2	0.9351	87.46	-6.187	S1	16.99	0.8493	91.88	-2.255	
Prodiamine		I50	R squared	Top	Bottom		I50	R squared	Top	Bottom	
	R1	73.60	0.9779	97.49	0.6891	S1	15.57	0.7211	61.39	-2.577	
Pendimethalin		I50	R squared	Top	Bottom		I50	R squared	Top	Bottom	
	R1	7640	0.8397	93.17	-24.32	S1	1.347	0.7595	66.37	-1.855	
Oryzalin		I50	R squared	Slope	YIntercept		I50	R squared	Slope	YIntercept	
	R1	$1.32*10^{17}$	0.1061	-2.24	88.40	S1	0.79	0.3198	-5.942	49.38	
	% Biomass Reduction										
Dithiopyr		I50	R squared	Top	Bottom		I50	R squared	Top	Bottom	
	R1	285.9	0.7887	100.5	24.5	S1	3.234	0.8541	99.55	25.58	
Prodiamine		I50	R squared	Top	Bottom		I50	R squared	YM	Yo	k
	R1	25.25	0.9560	103.0	11.43	S1	3.22	0.8413	89.37	85.79	1.063
Pendimethalin		I50	R squared	Top	Bottom		I50	R squared	Top	Bottom	
	R1	8885	0.7458	146.5	24.24	S1	0.000002	0.7706	85.06	-42778	
Oryzalin		I50	R squared	Slope	YIntercept		I50	R squared	YM	Yo	k
	R1	5907	0.4028	4.863	31.66	S1	1.47	0.9067	81.69	81.67	2.932

Figure 4.

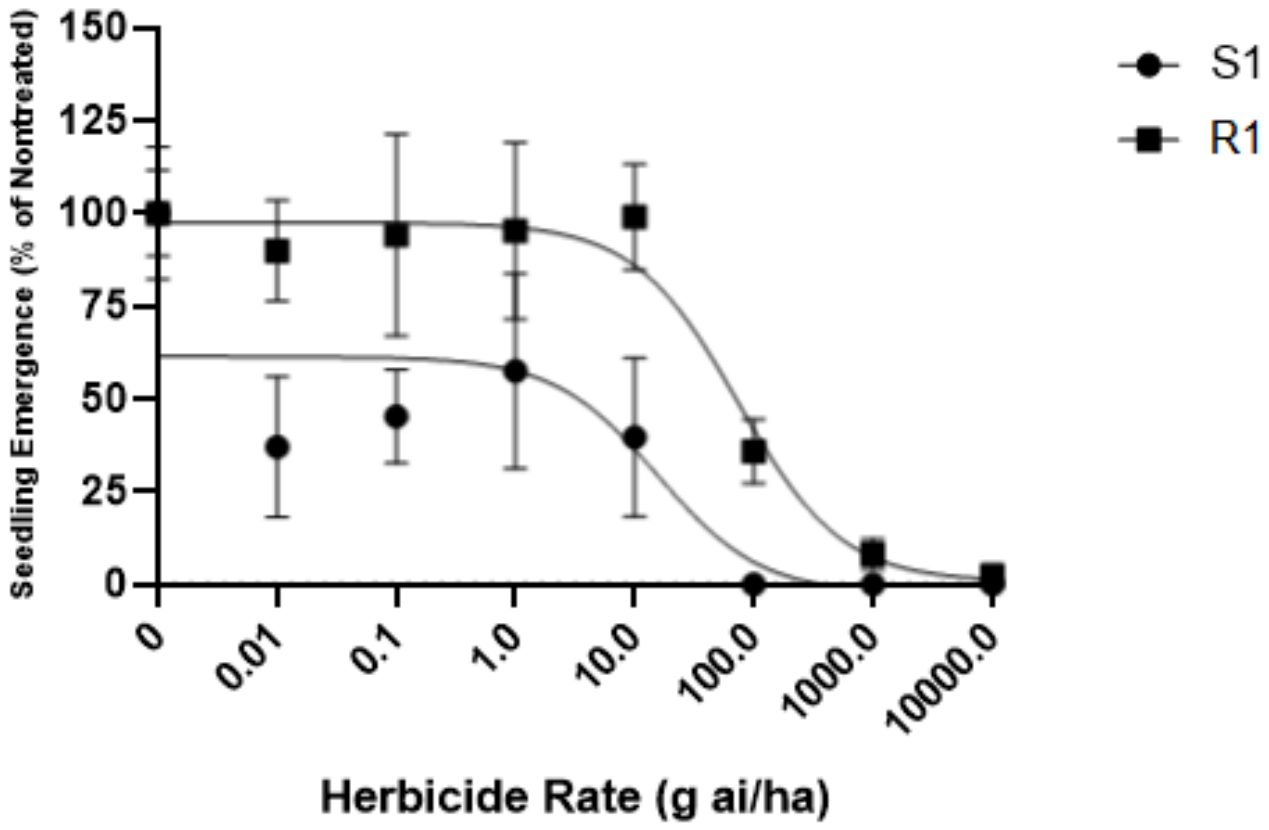
A. Seedling emergence response of R and S populations to increasing rates of dithiopyr. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



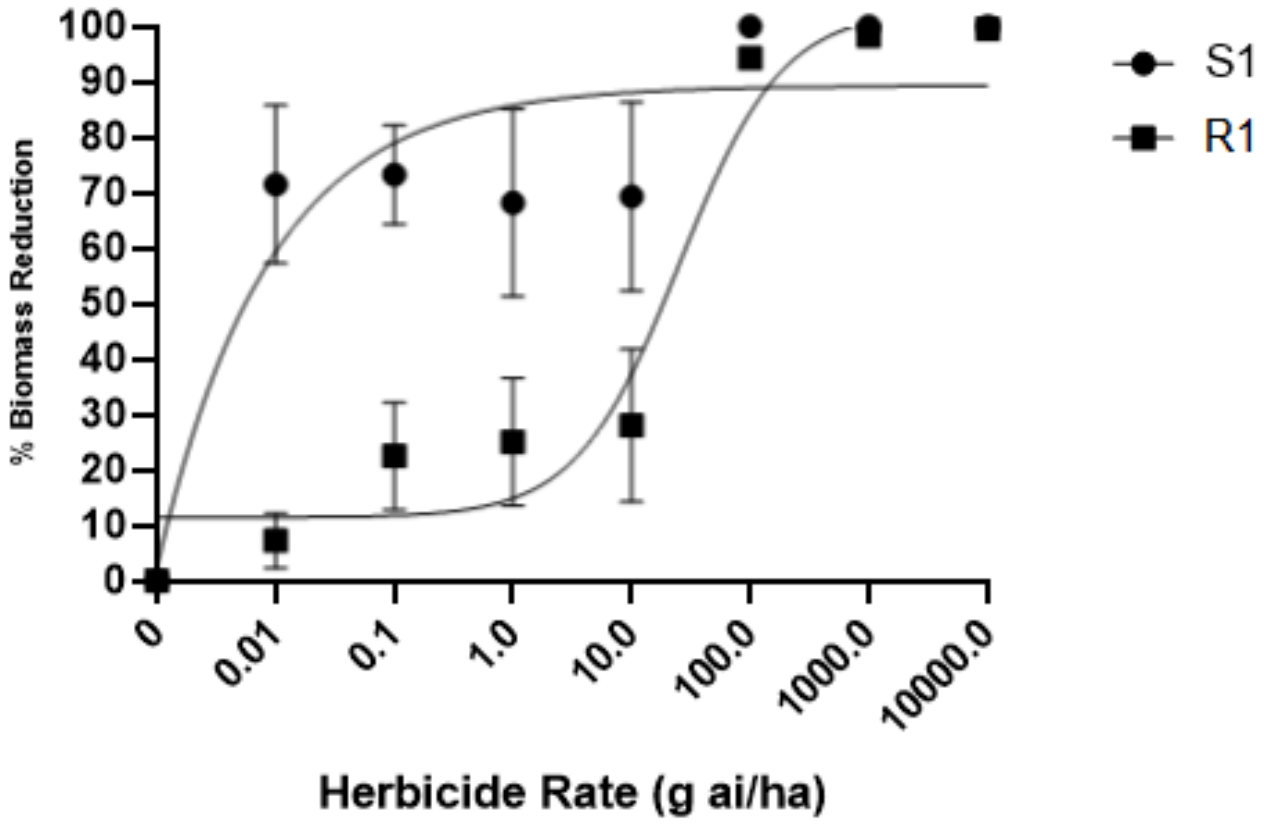
B. Biomass reduction response of R and S populations to increasing rates of dithiopyr. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.



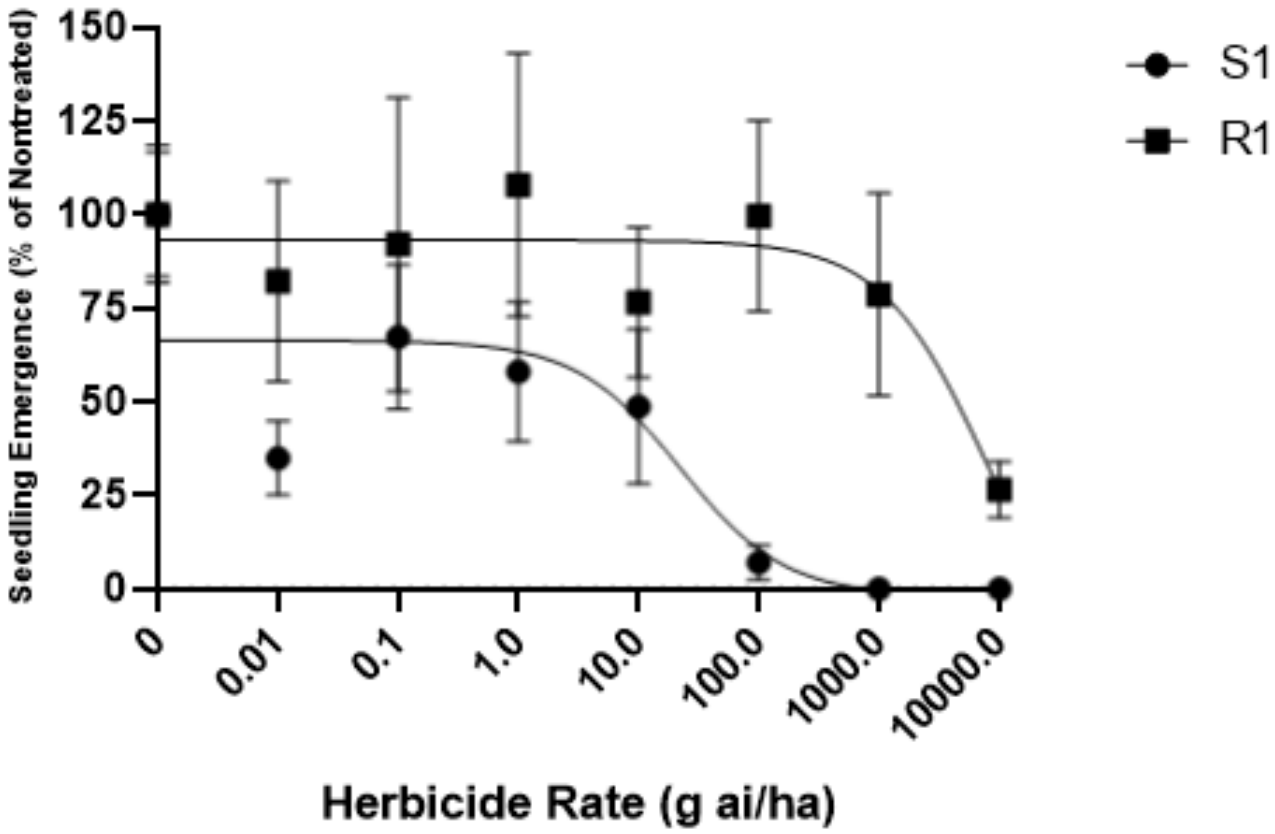
C. Seedling emergence response of R and S populations to increasing rates of prodiamine. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



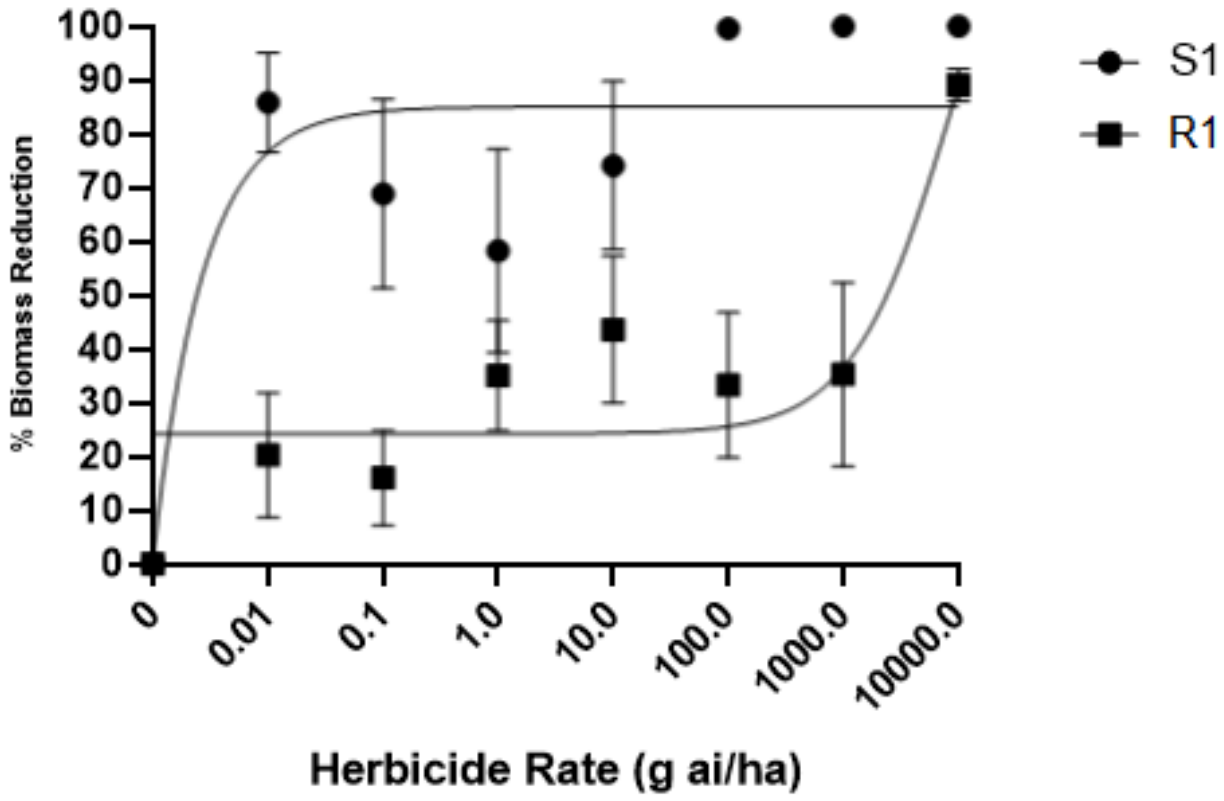
D. Biomass reduction response of R and S populations to increasing rates of prodiamine. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.



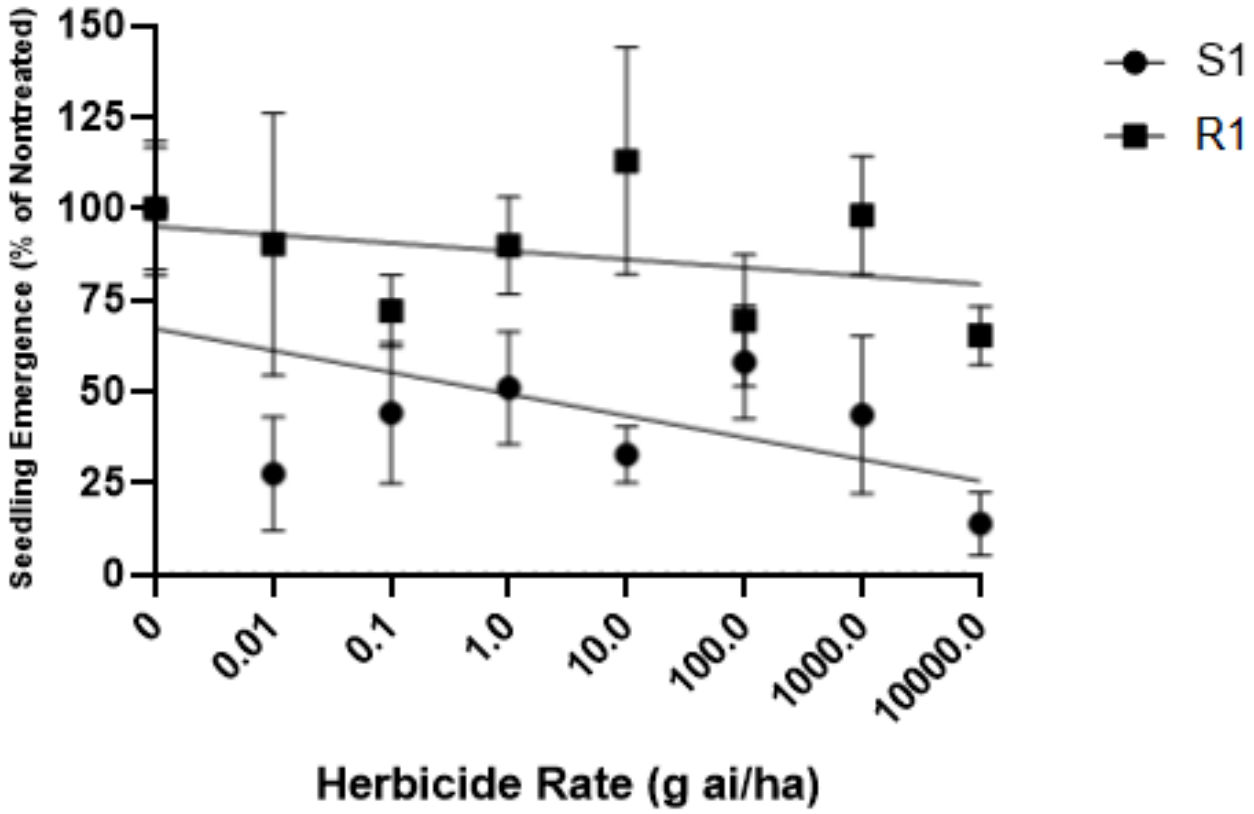
E. Seedling emergence response of R and S populations to increasing rates of pendimethalin. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



F. Biomass reduction response of R and S populations to increasing rates of pendimethalin. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.



G. Seedling emergence response of R and S populations to increasing rates of oryzalin. Seedling emergence is relative to the nontreated. Vertical bars are standard errors of individual means.



H. Biomass reduction response of R and S populations to increasing rates of oryzalin. Biomass reduction is relative to the nontreated. Vertical bars are standard errors of individual means.

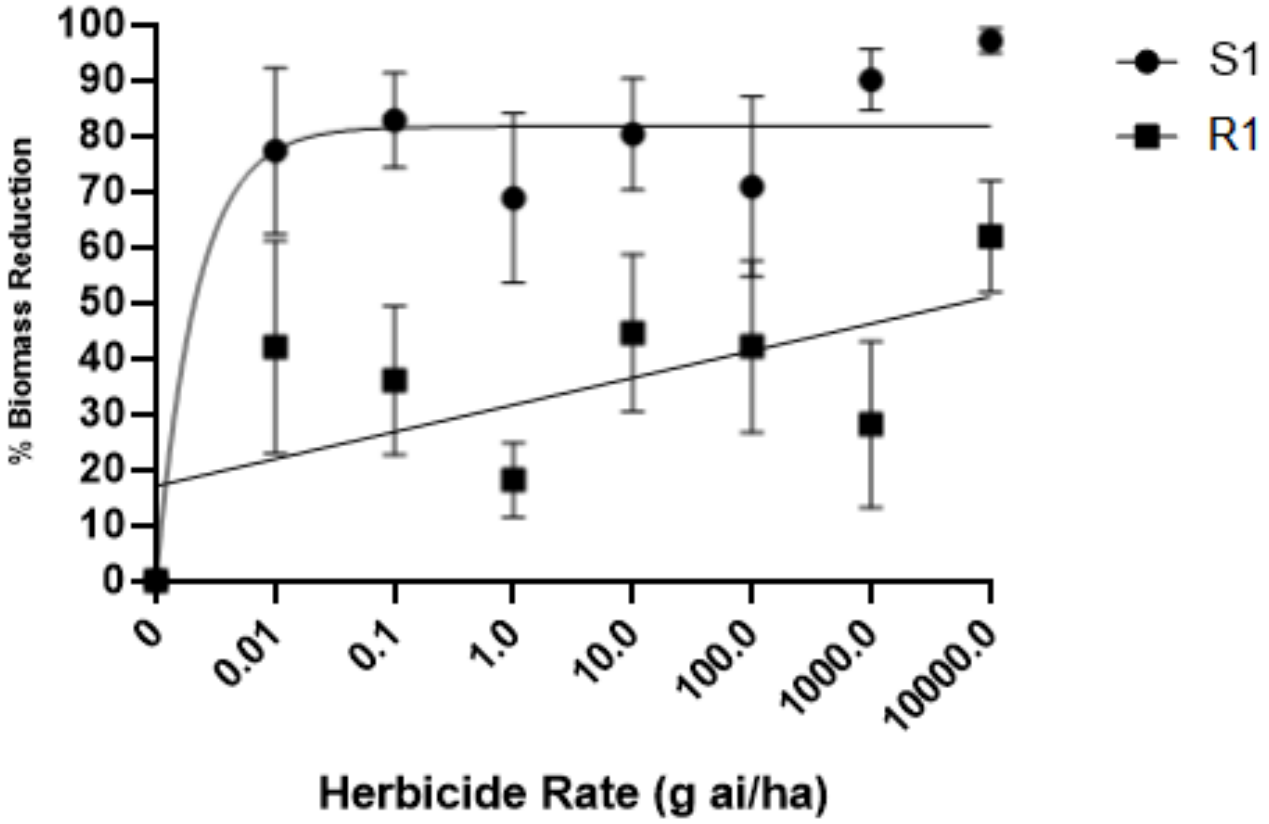
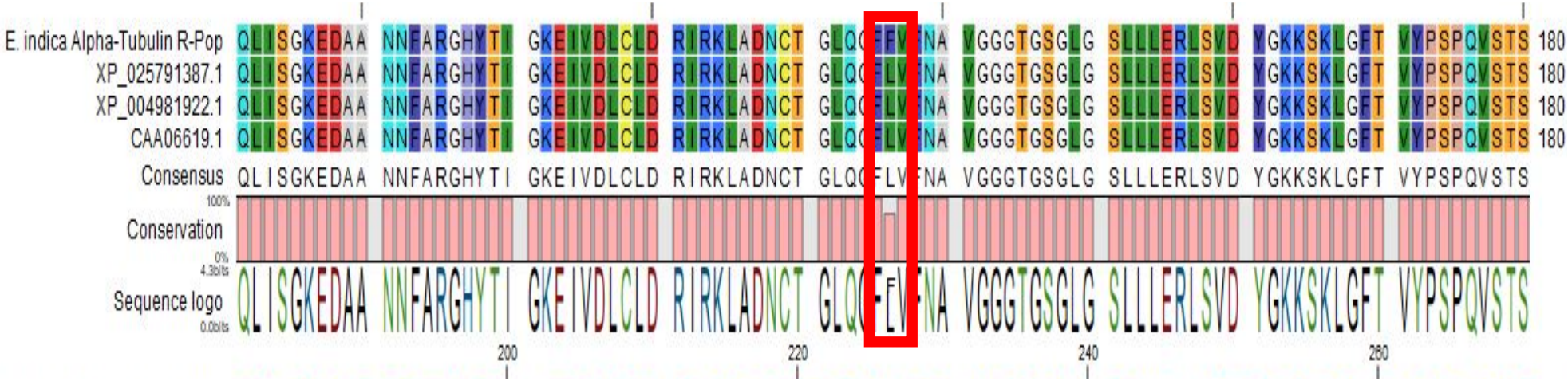


Figure 5. α-tubulin contig alignment for the R population with other *Eleusine indica* sequences. R population had an amino acid substitution Leu136-Phe.



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