IMPROVING TOLERANCE TO DINITROANILINE HERBICIDES IN TALL FESCUE, *SCHEDONORUS ARUNDINACEUS*

by

BRODY TAYLOR DEATON

(Under the Direction of Paul L. Raymer)

ABSTRACT

Reported cases of dinitroaniline resistance are credited to mutations within the α-tubulin protein: Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Met-268-Thr, Arg-243-Met/Lys. There are no reports of dinitroaniline resistance within tall fescue (*Schedonorus arundinaceus)* or other turfgrass species. The research goal was to find and identify a mutation in tall fescue that confers resistance to pendimethalin and possibly other dinitroaniline herbicides. Calli were generated and exposed to selection cycles on 3 µM pendimethalin medium. Putative tolerant genotypes were regenerated and hydroponically screened at 2 µM pendimethalin. Promising genotypes PR#39, PR#49, PR#74 were confirmed as tolerant. DNA extraction and sequence analysis failed to identify the presence of reported mutations. Metabolism as a possible mechanism of tolerance was tested using malathion, a P450 metabolic pathway inhibitor, to inhibit expression of pendimethalin tolerance. When PR#74 was treated with malathion prior to exposure to pendimethalin, tolerance was suppressed indicating the mode-of-action was associated with P450s herbicide metabolism.

INDEX WORDS: Tall fescue, Dinitroaniline, Herbicide resistance, Pre-emerge herbicide, Cool-season, Group 3, Mitotic inhibitor, Microtubule, Mutation, Target-site, Point-mutation, Hydroponics, Tissue culture, Callus induction, Plant regeneration, P450, Plant metabolism, Metabolic resistance, Malathion, DNA extraction, Dinitroaniline resistance, α-tubulin, Turfgrass, Pendimethalin, Herbicide metabolism

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DEDICATION

I dedicate this work to everyone who believed in me and my ability to succeed in graduate school and as a scientist. I would like to especially thank all of my friends and family for helping me through the many challenging times of my graduate studies. Without them, I would not have been able to make it through this journey. A special thanks to my girlfriend Kaylee Shaver, who helped ground me when I doubted myself and pushed me to be a better person. My dedication cannot be complete without the recognition of my parents, Michael and Tena Deaton, who worked tirelessly to help shape me into the man I am today and who were there for me every single step of the way throughout my life. Without them, I would not be where I am today nor the man I am today.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The genus name for tall fescue has been under serious debate among plant systematists. Tall fescue was formerly known as *Festuca arundinacea*, with *Festuca* being comprised of several related lineages (Fribourg et al., 2009). Some plant systematists argue that tall fescue should be in the *Lolium* genus while others say it should be in the *Schedonorus* genus (Fribourg et al., 2009). In the *Catalogue of New World Grasses* and *Flora of North America* grass volume, tall fescue is placed in the *Schedonorus* genus (Soreng et al., 2009). While there remains some confusion and debate about what genus tall fescue should be placed in, the classification of tall fescue on the USDA database recognizes it as *Schedonorus arundinaceus*. To avoid confusion, the scientific name of tall fescue will be referred to as *Schedonorus arundinaceus* in this thesis.

Tall fescue (*Schedonorus arundinaceus)* is a deep rooted, cool season perennial grass (Duble, 1983). The popularity of tall fescue has quickly expanded in the United States since 1940, going from 39,500 acres to 37.1 million acres today, making it the most widely grown introduced grass (Rogers and Locke, 2013). Tall fescue was initially used as a forage grass but with the rise of the turf industry during the late 1940s, began to be used in turf applications. The release of cultivars with enhanced turf quality during the 1960's gave rise to increased use as turf in landscape and lawn plantings. The rise in popularity is attributed to ease of establishment and retention of color through the winter months. Tall fescue is a durable grass species even under

drought and low light conditions (Duble, 1983). Additionally, it is adapted to a wide range of climatic and soil pH conditions (Rogers and Locke, 2013).

Currently, there are no reports indicating tall fescue has resistance to any pre-emergent herbicides, and specifically dinitroanilines. The dinitroaniline herbicides have been used for decades around the world in many crops and lawns for pre-emergent weed control (Chu et al., 2018). There are reports of multiple weed species such as goosegrass (*Eleusine indica*), annual ryegrass (*Lolium rigidum*), and annual bluegrass (*Poa annua*) with resistance to pre-emerge herbicides (Chen et al., 2021). It could be economically beneficial to obtain and deploy improved tolerance to dinitroanilines herbicides in tall fescue cultivars used for turfgrass applications.

The overwhelming majority of tall fescue seed is produced in the Pacific Northwest and utilizes carbon seeding whereby a band of activated charcoal is sprayed over the seeded row. The activated charcoal absorbs any previous phytotoxic herbicides and allows growers to surface apply a strong pre-emergence herbicide improving rapid and weed free establishment of tall fescue seed production fields.

Improved tolerance to dinitroaniline herbicides would be useful at both the seed production and the homeowner/consumer level. Seed producers' need for carbon seeding would decrease since the seed they are attempting to establish would have improved crop tolerance to one or more pre-emerge herbicides, thus eliminating the need to protect seedlings from pre-emerge herbicide applications at planting as well as from residues from previous applications of pre-emerge herbicides. For the consumer, it would increase their efficiency of establishing their yards and enhance opportunities for overseeding in established lawns. Homeowners would be able to apply a pre-emerge herbicide for weed control at the time of seeding and thereby reduce

weed competition during establishment and reduce the need to use post-emergence herbicides later to eliminate weeds arising during establishment of the tall fescue.

The dinitroaniline resistance has been previously reported in several weed species and has been attributed to target-site point mutations found within genes coding for components of microtubules. One component of microtubules is α-tubulin. The genes coding for α-tubulins are highly conserved across plant species (Chen et al., 2020a). There are several mutations known to occur in α-tubulin that can confer resistance to dinitroanilines. Finding a mutation that confers improved tolerance to dinitroaniline herbicides in tall fescue and the subsequent development of dinitroaniline tolerant cultivars could lead to decreased use of post-emergence herbicides globally, provide for more efficient production of tall fescue seed, and provide a product easier for homeowners to establish and maintain reliably. Dinitroaniline resistant cultivars would decrease the use of carbon seeding in tall fescue production fields and minimize the risk of seedling injury from residual dinitroaniline herbicides in both production and landscape applications.

Literature Review

Tall Fescue

Tall fescue is a cool season perennial grass. It is adapted to grow in a wide range of climatic conditions, tolerates a wide range of soil pH from 4 to 8.5 (Harivandi, 1987), and has good drought resistance (Rodgers and Locke, 2013). It has a coarse leaf texture and a bunch-type growth habit (Stier et al., 2013). Tall fescue is found from the Pacific Northwest to the southern states and is well adapted to the “transition zone” states of Oklahoma, Arkansas, Missouri, Tennessee, Kentucky, Virginia, North Carolina, Georgia, and Texas (Duble, 1983). In the

transition zone, tall fescue is mainly used as a low maintenance turf (Stier et al., 2013). Its mowing height requirement limits its use to areas such as lawns, parks, golf course roughs, and other areas where it is mowed at a height above 1.5 inches (Duble, 1983). Tall fescue is not advised where desired mowing height requirements fall below 1.5 inches during the summer months.

Tall fescue has deeper root systems than other cool season grasses, which provides improved drought tolerance (Stier et al., 2013). It is predominately cross-pollinated, with a high degree of self-incompatibility, which allows population improvements and phenotypic and genotypic selection to be effective breeding practices (Stier et al., 2013). The oldest varieties of tall fescue are Kentucky 31 and Alta (Harivandi, 1987). These cultivars have been widely used as a turfgrass for many decades and are still used today. Those varieties produce weak turf and are widely used for forage in the transition zone (Lacefield and Evans, 1984). The first turf-type cultivars were Rebel, Houndog, Olympic, and Falcon (Duble, 1983). These turf-type cultivars provide a finer leaf texture and produce denser turf compared to the forage-type tall fescue cultivars (Duble, 1983). Today there are around 300 tall fescue cultivars commercially available for turf use in the United States (Stier et al., 2013).

Microtubules

The primary component of microtubules is a single type of globular protein called tubulin (Blume et al., 2003), composed of α and β heterodimers, which polymerize to form microtubules (Bronson et al., 2014). Microtubules are ubiquitous cytoskeleton structures that form by self-assembly of α- and β-tubulin heterodimers and are around thirteen parallel protofilaments in size (Downing and Nogales, 1998). The α- and β-tubulins heterodimers bind head to tail to form linear protofilaments (Délye et al., 2004) assembled around a hallow core (Cooper, 2000). Alpha

and β tubulin are proteins approximately 450 amino acids each (Downing and Nogales, 1998). Microtubules are polar structures with a fast growing (+) end and a slow-disassembling (-) end (Cooper, 2000). Each α- and β-monomer bind a guanine nucleotide which is nonexchangeable in α-tubulin and exchangeable in β-tubulin; binding of the guanine nucleotide in α-tubulin occurs at the non-exchangeable N-site and binding in the β-tubulin occurs at the exchangeable E-site (Downing and Nogales, 1998). Microtubule assembly requires that guanosine triphosphate (GTP) is present at the E-site, the binding site for β-tubulin, and hydrolysis follows the addition of a dimer to the microtubule end, which makes it non-exchangeable (Downing and Nogales, 1998). Both α- and β-tubulin bind GTP to regulate polymerization, but GTP bound to β-tubulin is hydrolyzed to guanosine diphosphate (GDP) shortly after polymerization. The GTP hydrolysis weakens binding affinity of tubulin for adjacent molecules leading to favoring de-polymerization (Cooper, 2000). Tubulin molecules bound to GDP are lost from the minus end and replaced by the addition of tubulin molecules bound to GTP at the plus end (Cooper, 2000). The GTP hydrolysis is known as dynamic instability in that the individual microtubules alternate between cycles of growth and shrinkage. The rate at which the microtubules grow or shrink is determined by the rate of tubulin addition compared to the rate of GTP hydrolysis. Microtubules are essential for survival of all eukaryotes and the α- and β-tubulins are among the most conserved proteins (Délye et al., 2004). Microtubules meditate multiple cellular processes such as mitosis, cell transport, and cell motility (Nogales, 1999).

As cells enter mitosis the features of microtubule assembly and disassembly change significantly. The rate of microtubule disassembly increases tenfold, which results in overall de-polymerization and shrinkage of microtubules. The number of microtubules emanating from the centrosomes increase five to tenfold (Cooper, 2000). In interphase of mitosis the microtubules

extend outward towards the cells' periphery and the minus ends are anchored. The array of microtubules present in cell interphase disassembles and tubulin subunits are reassembled to form mitotic spindles which allows for the separation of daughter chromosomes (Cooper, 2000). During prophase as the mitotic spindle starts to develop and centromeres begin moving to opposite poles, the centrosome serves as the initiation site for microtubule assembly in which they grow outwards from the centrosome towards the periphery of the cell. The restructuring of the microtubule cytoskeleton directed by duplication of centrosomes forms two separate microtubule-organizing centers at opposite poles of the mitotic spindle (Cooper, 2000). Microtubules form the mitotic spindle which correctly positions chromosomes within the cell plane during metaphase. The mitotic spindle, composed of microtubules, will then guide the separated chromatids to opposite ends of the daughter cell during anaphase (Chen et al., 2020b).

The mitotic spindle involves selective stabilization of some microtubules radiating from centrosomes, these microtubules are of three types. Kinetochore microtubules attach to condensed chromosomes of mitotic cells at the centrosomes and the attachment stabilizes these microtubules (Cooper, 2000). Polar microtubules are not attached to chromosomes but emanate from two centrosomes and are stabilized by overlapping each other in the center of the cell (Cooper, 2000). Astral microtubules extend outward from centrosomes to the cell periphery and have exposed plus ends (Cooper, 2000).

Dinitroanilines

Dinitroanilines are a group of pre-emergent herbicides and are one of the five classes of microtubule inhibitors used to control grass and broadleaf weeds (Chen et al., 2021). The commercialized dinitroaniline herbicides include trifluralin, pendimethalin, ethalfluralin, oryzalin, butralin, benefin/benfluralin, and prodiamine (Chen et al., 2021). Their base structure

consists of a benzene ring with an NH_2 group attached with a nitro group (NO_2) attached to the 2- and 6 – C's, and a substitution at the 4-C. It is believed dinitroanilines, when bound to tubulin, cause significant redistribution of energy within tubulin due to reorganization of amino acid side chains/R-groups of α-tubulin (Blume et al., 2003).

Dinitroanilines are prone to decomposition due to photodegradation (Chen et al., 2020b), and their effectiveness depends on its soil incorporation depth (Chen et al., 2020b). Dinitroanilines should be incorporated into surface soil to minimize volatilization and photodegradation. When applied correctly, a barrier near the top of the soil is formed in which seedlings will pass through to emerge from the soil. Low solubility and strong soil-binding allow dinitroanilines to remain near the site of application on or near the soil surface. Dinitroanilines enter germinating seedlings primarily by gaseous absorption through roots, coleoptile node, or hypocotyl upon contact with the herbicide (Congreve and Cameron, 2014). Their translocation within the plant is limited because of their site of action. This class of herbicides inhibits lateral root and secondary root development is a more characteristic growth response (Parka and Soper, 1976). These herbicides affect root and shoot elongation and development, leading to stunting and swollen tips of both roots and shoots (Délye et al., 2004). The result is seedling death or highly reduced fitness.

Herbicides representing the dinitroaniline family injure plants by binding to tubulin. The exact tubulin binding sites for dinitroanilines have not yet been determined (Chu et al., 2018). Structural modeling along with analysis of tubulin resistance mutations demonstrated dinitroanilines likely interact with α-tubulin, but β-tubulin may also be a target (Chu et al., 2018). Dinitroanilines prevent tubulin from polymerizing into microtubules (Appleby and Valverde, 1988) by binding with polymerized tubulin heterodimers (Stokkermans et al., 1996) leading to

arrested cell division and elongation and resulting in death of the plant (Chu et al., 2018). Dinitroanilines bind to non-ligand α/β-tubulin heterodimers forming an herbicide-tubulin complex (Hugdahl and Morejohn, 1998). The herbicide-tubulin complex inhibits the polymerization at the assembly end (+) of the tubulin but does not stop de-polymerization at the disassembly end (-). The inhibited polymerization at the (+) end prevents further elongation of the microtubule. The de-polymerization at the (-) end continues, causing the microtubule to become progressively shorter until its complete dissociation (Cleary and Hardham, 1998). By preventing tubulin polymerization, the spindle apparatus does not form, and chromosomes are unable to move into the metaphase configuration, daughter chromosomes fail to migrate to their poles, chromosomes coalesce in the middle of the cell, and cell division ceases. This causes disorientation of microfibrils, spherical cells, and cells with more than the normal complement of chromosomes (Appleby and Valverde, 1988). Therefore, mitosis in emerging seedlings becomes disrupted and the two daughter cells do not separate (Chen et al., 2020b). The cessation of cell division causes treated seedlings to show swollen and stunted root symptoms, leading to the seedlings either not emerging from the soil or halted growth after emergence. (Lignowski and Scott, 1971).

Known Mutations

Due to widespread and long-term use of repeated applications of these herbicides, there are reports of target-site point mutations to dinitroaniline herbicides in 12 different weed species (Wang et al., 2021). However, resistance to dinitroanilines is rare when compared to other major herbicides. There have been reports of 158 cases of resistance to ALS-inhibiting herbicides, 47 to ACCase-inhibiting herbicides, and 34 to glyphosate (Chu et al., 2018). Field evolved resistance to dinitroanilines is known in goosegrass (*Eleusine indica*), green foxtail (*Setaria viridis*) Palmer

amaranth (*Amaranthus palmeri*), annual ryegrass (*Lolium rigidum*), annual bluegrass (*Poa annua*), blackgrass (*Alopecurus myosuroides*), and water foxtail (*Alopecurus aequalis*) (Wang et al., 2021). The known target-site point mutations include Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Arg-243-Met/Lys, and Met-268-Thr. Various fitness studies of α-tubulin mutations have shown a moderate fitness penalty is associated with some tubulin mutations and may explain the low frequency of dinitroaniline herbicide resistance (Chu et al., 2018). *E. indica* populations from South Carolina were some of the first reports of dinitroaniline resistance and sometime later, populations of *A. palmeri* and *P. annua* from the Carolinas and Georgia were reported. (Chen et al., 2021). Other main reports of dinitroaniline resistance were in *Setaria viridis* in Canada and *Lolium rigidum* populations in Australia (Chen et al., 2021).

There have been several reported cases of dinitroaniline resistance in rigid ryegrass populations around Australia. One study found that two biotypes of rigid ryegrass exhibited an approximately 10-fold reduction in sensitivity to trifluralin and anywhere from 32-fold to 2.5-fold reduction in sensitivity to five other dinitroaniline herbicides (McAlister et al., 1995). More recent studies have found that the Val-202-Phe (Chen et al., 2020a) and Thr-239-Ile (Fleet et al., 2017) mutations confer dinitroaniline resistance within rigid ryegrass populations. The resistance levels varied when compared to the susceptible population and the type of dinitroaniline applied. In goosegrass populations, some studies have found that the Thr-239-Ile mutation confers resistance (Blume et al., 2003) with 17- to 60-fold levels of resistance (Russel et al., 2021). The Leu-136-Phe mutation was also studied within goosegrass populations. Researchers found that when this mutation was present there was also a Val-202-Phe mutation with a 5.7-fold resistance with a low survival rate following treatment with trifluralin (Russel et al., 2021). Chu and others (2018) found that rice calli transformed with the Arg-243-Met/Lys mutation from resistant

Lolium rigidum populations were 4- to 8-fold more resistant to trifluralin and other dinitroaniline herbicides. *Eleusine indica* and *Setaria viridis* have been thoroughly investigated for resistance to trifluralin. Resistance was found in *Eleusine indica* and attributed to target site α-tubulin mutations of Thr-239-Ile and Met-268-Thr. In *Setaria viridis,* resistance was attributed to mutations at Leu-136-Phe and Thr-239-Ile (Chu et al., 2018). Resistance conferring mutations, Val-202-Phe, Leu-125-Met, and Leu-136-Phe, have also been found in *Alopecurus aequalis* plants (Chu et al., 2018). The target site resistance (TSR) of dinitroaniline herbicides have been reported to be a recessive traits (Chen et al., 2021).

CHAPTER 2

SELECTION FOR PENDIMETHALIN TOLERANCE IN TALL FESCUE USING TISSUE CULTURE

Introduction

Plant tissue culture is a valuable tool in applied studies and with commercial applications. It is defined as the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions in vitro (Thorpe, 2007). The basis of plant tissue culture was proposed by Gottlieb Haberlandt in 1902 (Thorpe, 2007) and since then it has grown in popularity, efficiency, and application. The 1940s – 1960s was the period that many of the in-vitro techniques used today were developed (Thorpe, 2007). Tissue culture, over the years, has played an important role in plant modification and improvement. The first attempts to utilize these systems for plant improvement occurred in the 1970s with attempts to exploit somaclonal variation. However, that variation is dependent upon the natural variation in a population of cells and is observed in regenerated plantlets (Thorpe, 2007). Only a few cases have shown that it is possible to regenerate plants with the desired traits (Hughes, 1983; Ranch et al., 1983).

A major activity involved with the development of new cultivars is creating or manipulating genetic diversity within a plant species. Genetic diversity is an important prerequisite for the success of plant breeding. Tissue culture offers a path to overcome limitations presented by lack of genetic diversity. The genetic variability detected within callus tissue can be due to epigenetic changes and represents a possibility for recovering somaclonal

variants or mutants that possess specific agronomic characteristics (Larkin and Scowcroft, 1981). A piece of callus can consist of thousands or millions of cells, and those cells can be subjected to selective pressures of various kinds to isolate resistant cells under a controlled condition (Loyola-Vargas and Ochoa-Alejo 2018). Tissue culture offers a way to introduce and screen for new traits within a plant species in a controlled environment.

Like many other crops, turfgrass mainly relies on conventional breeding methods. This limits the assessable genetic material to those capable of sexual reproduction (Qian et al., 2006). Tall fescue cultivars are typically developed by polycrosses of ten or more parent plants. As a result, the cultivars developed are genetically heterogeneous with high levels of genetic variation occurring within cultivars (Bai and Qu, 2000). Tissue culture provides methods that afford opportunities to shorten the cultivar development process by bypassing many of the required crosses, introduce new genetic variations and traits, and select for specific traits. The development and incorporation of new traits often requires multiple crosses and backcrosses to introduce and stabilize the trait of interest within the plant genome. This takes multiple years and requires large inputs of resources to screen, select, and reach the desired outcome. Tissue culture offers a way to shorten that process by allowing plants to be efficiently screened for desired and perhaps novel traits by applying controlled levels of selection pressure at the cellular level. It offers innovative tools that can supplement conventional methods of plant improvement and provide the possibility for genetic and physiological investigations at the tissue and cell level (Lowe and Conger, 1979). Plantlet formation from callus of somatic tissue has been reported for Italian ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*) (Ahloowali 1975), orchardgrass (*Dactylis glomerata*) (Chin and Scott, 1977), and big bluestem (*Andropogon geradii*) (Chen et al., 2021). To have a successful system, it is necessary to have competent

explant material and an in-vitro culture system that allows for high frequency regeneration (Qian et al., 2006).

Currently, tissue culture techniques are used in many different plant species. Breeders of cereals and grasses, legumes, vegetable crops, and tuber crops have successfully utilized forms of tissue culture to aid in the development of new cultivars (Thorpe, 2007). However, limited success has been achieved in the realm of somoclonal variation and the regeneration of useful plantlets from mutant cells (Thorpe, 2007). Tissue culture has also achieved progress in extending cryopreservation technology, artificial seed technology, and the study of primary metabolism (Thorpe, 2007). The various techniques and uses of tissue culture have expanded over the years and has helped speed up the incorporation of new traits and cultivar development.

Seeds of a University of Georgia tall fescue breeding population from a polycross of 25 superior single plants were utilized as the tissue culture explant donor for induction, maintenance, and regeneration of tall fescue callus. Once successful protocols were established, callus was increased and exposed to a targeted concentration of pendimethalin, a widely used dinitroaniline pre-emergence herbicide, in attempts to select for enhanced pendimethalin tolerance.

Materials & Methods

Callus Induction from Tall Fescue

Research presented in this thesis utilized tall fescue callus generated using seeds as well as basal nodes as explant tissue. Tall fescue seeds from a tall fescue polycross population, 17G5Z, were sanded with 100 grit sand paper to break the seed coats before sterilizing. Sanded seeds were soaked in 75% ethanol for 2 minutes and rinsed with sterilized water, the seeds were

then sterilized in 10% bleach for 30 minutes with gentle shaking of the sterilizing container every 5 minutes to ensure all the seeds were sterilized uniformly. After the bleach treatments, seeds were washed with sterilized water 4 times for 2 minutes each time followed by 7 washes in sterilized water for 10 minutes each. Sterilized seeds (about 50 seeds) were spread on induction medium in 15 cm Petri dishes. Induction media was MS medium supplemented with 2 µg /L 2.4-D, 2.25 µg/L BAP, 30 g/L of sucrose, and 1 mL/L of PPM solution (Pant Cell Technology, EPA Reg.71806-1). Calli were placed in the dark in a growth chamber at 27 ^{o}C. Callus growth was observed after 2 to 3 weeks on induction media. Five weeks later healthy and solid calli were separated into 3-5 mm diameter pieces and transferred to new induction medium in 3 x 3 format for propagation in 15 cm Petri dishes. Calli were separated and transferred every 5 weeks. At each transfer one callus was separated and transferred onto regeneration medium to check regeneration ability.

Callus generated from basal nodes was also used in portions of this research and utilized tillers of a plant regenerated during early protocol development UGA 17G5Z seed-derived callus as the explant donor for callus generation. This plant was designated as the tissue culture parental control (TCPC) and used in later experiments in this research. Plant tillers were collected and soaked in 75% ethanal for 2 minutes and rinsed with sterilized water, tillers were then sterilized in 10% bleach for 30 minutes with gentle shaking of the sterilizing container every 5 minutes to ensure all the tillers were sterilized uniformly. After the bleach treatments, tillers were washed with sterilized water 4 times, 2 minutes each time, followed by 7 washes with sterilized water for 10 minutes each. Sterilized tillers were trimmed at the crown. Leaf sheaths were removed to expose the stem nodes. Stem internodes were cut and trimmed close to the nodes. Each node was cut in half laterally to expose the meristem cells. These cut stem basal nodes were used as callus

induction materials and placed face down in 3 x 3 format on induction medium in 15 cm Petri dishes.

Callus Maintenance

The medium used for tissue culture maintenance was the same as the induction media. The medium was adjusted to a volume of one liter and the pH adjusted to 5.8 before addition of 2.3 g/L Gelrite (Sigma-Aldrich, St. Louis, MO). After autoclaving 2.25 µg/L BAP and 1 mL/L of PPM solution (Pant Cell Technology, EPA Reg.71806-1) was added to the medium. Media was then poured into 100 x 15 mm Petri dishes as needed. Calli were placed in the dark in a growth chamber at 27 ^{o}C. Calli were sub-cultured in a grid pattern onto the maintenance media and sealed with Nescofil (Karlan Research Products Co., Cottonwood AZ) every 30 days to ensure no nutrient deficiency occurred and to allow for propagation of callus.

Callus Regeneration

The medium originally used for the induction of callus was modified by increasing BAP from 2.25 µg/L to 5 µg/L and replacing the 2 µg/L of 2,4,D with 0.1 µg/L of NAA, thus allowing it to serve as the regeneration medium. Established calli were placed on regeneration medium in a 3 x 3 grid, using as many plates as necessary. Calli were placed in a growth chamber at 27 ^{o}C with a 1-h dark, 23-h light photoperiod, and a light intensity of 66 to 95 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes (Heckart et al., 2010). All plates were evaluated for regeneration at the end of a 30-d period. If green shoots appeared, the cell lines were sub-cultured for an additional month on regeneration media and sealed with Nescofil (Karlan Research Products Co., Cottonwood AZ) to allow roots to develop. When adequate roots were formed, the plants were removed from the medium and transplanted to soilless media in pots.

The potted plants were maintained in domed trays in a growth chamber for 1 wk, then were transferred to a greenhouse at 20/10 ºC day/night temperature.

Tall Fescue Callus Response to Pendimethalin Dosage

Two experiments were conducted to model the response of tall fescue callus tissue to pendimethalin concentration and to determine an appropriate pendimethalin dosage for selection. Both experiments used the same research protocols and differed only in the source of callus tissue used. The first dose-response was performed on callus generated from seeds of the UGA 17G5Z breeding population. This dose response experiment was repeated using callus from a single plant regenerated during protocol development. This plant denoted as UGA 17G5Z TCPC (tissue culture parental control) was regenerated from callus induced with seed as the explant source and without selection for tolerance to pendimethalin. Laboratory grade pendimethalin obtained from Fischer Scientific (Waltham, MA) and dissolved in methanol was used to create a stock solution with a concentration of 100 µg pendimethalin per 1 mL methanol. Pendimethalin at concentrations of 0, 0.2, 0.3. 0.5, 1, 2, 3, 6, 12, and 24 µM was added to callus induction/maintenance media after the autoclaved medium had cooled to approximately 55 ºC (Heckart et al., 2010). After 5 to 6 cycles of callus increase on maintenance media, calli were separated into 3-5 mm size pieces and 9 pieces were placed on 15 cm Petri plates containing the pendimethalin concentrations in 3 x 3 format. Two plates at each pendimethalin concentration, each containing 9 calli, served as replications for the experiment. Plates were photographed by placing each petri dish into a 20 cm x 25 cm box with one side cut out for ambient light and a hole cut out in the top for the camera lens. Images of each plate were captured on days 1 and 24 for basal note induced calli and on days 1, 24, and 32 for seed induced calli. Digital images of the calli were analyzed using Adobe Photoshop to determine the area by calculating the number

of pixels occupied by each callus piece as a proportional estimate of callus mass. Treatment means from each pendimethalin concentration were used to develop a dose-response curve to determine an appropriate pendimethalin dosage for selection.

Callus Selection

Callus tissue approximately 5-months old (5 to 6 cycles of increase) was sub-cultured onto the induction/maintenance modified MS/B5D2 media without PPM and supplemented with pendimethalin in a staged selection process. The appropriate pendimethalin dosage was added after the autoclaved medium had cooled to approximately 55 ºC.

For the initial selection cycle, 1 µM pendimethalin was added to the medium described above. Medium was then poured onto 100 x 15 cm Petri dishes as needed. Calli were placed in the dark in a growth chamber at 27 ºC. After 30 days on the first selection medium, only calli showing distinct and healthy growth at 1 µM of pendimethalin were selected and transferred onto fresh selection medium containing 3 µM of pendimethalin. At the end of the second 30-day selection cycle, calli that exhibited continued growth in the presence of pendimethalin at 3 µM were selected and transferred for increase onto fresh selection medium containing 3 µM of pendimethalin. Following two cycles of increase, selected calli were transferred to regeneration media and incubated under light (405 µmol/m^2/s) in the culture chamber with 23-hr light period at 27 ºC for four weeks.

When green shoots appeared, the cell lines were sub-cultured for an additional month on MS/B5D2 modified regeneration media to allow roots to develop. When adequate roots were formed, individual plants were removed from the medium and transplanted into pots containing

soilless media. The potted plants were maintained in domed trays in a growth chamber for 1 wk, before being transferred to the greenhouse at 20/10 ^{o}C day/night temperature.

Results

Tissue Culture Protocol Development

Successful callus induction, maintenance, and regeneration protocols were developed using both seed and basal nodes as explant tissues. Calli were induced using a modified MS/B5D2 media previously used on seashore paspalum, and were maintained using this same media to allow for callus increase (Fig. 2.1). For plant regeneration, the same medium used for induction and maintenance was modified by increasing BAP and replacing 2,4-D with NAA to serve as the regeneration medium. Green shoots were developed after 30 days on regeneration medium (Fig. 2.2A, 2.2B) and underwent sub-culturing on MS/B5N1 media for an additional 30 days for root development prior to transplanting (Fig. 2.2C, 2.2D). In total, it took approximately 3 months for tissue explants to be induced and regenerated into new plant material. The induction and regeneration of new plant material further demonstrated that a successful callus induction and regeneration protocol were developed for tall fescue.

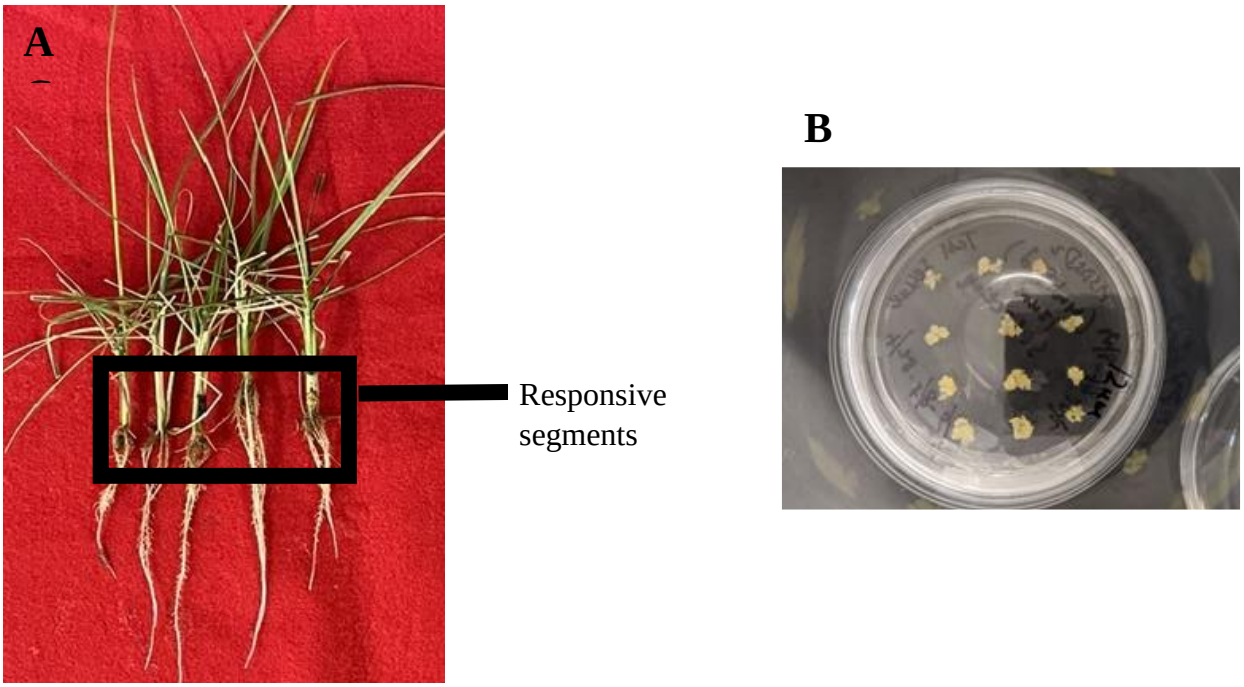

Figure 2.1: Induction of callus using basal node segments as explants. (A) Responsive segments (enclosed in black square) from individual tall fescue tillers. (B) Callus induction after 4 weeks on MS/B5N1 induction medium.

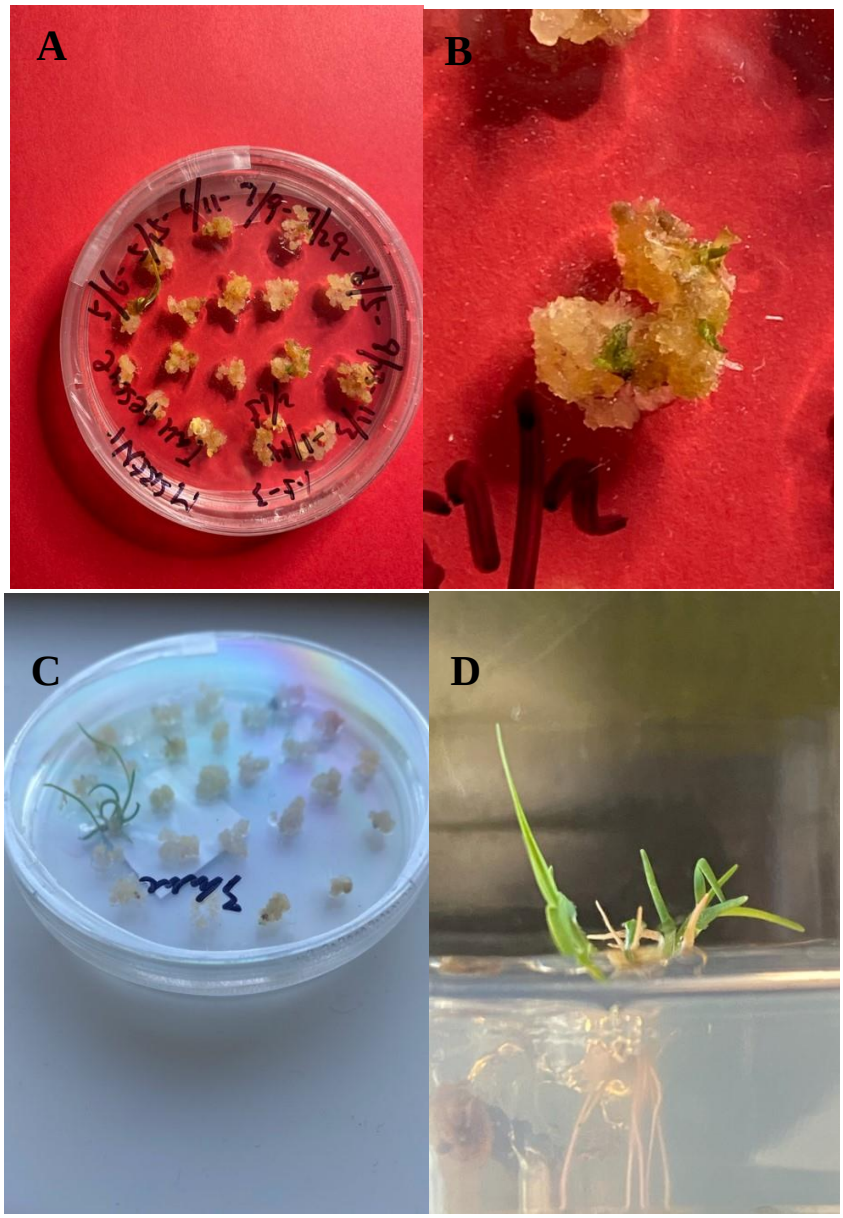

Figure 2.2: Selection stages of plant regeneration of calli. (A) Bulked calli initiating green shoots. (B) Close-up image of green shoots beginning to develop on a callus. (C) Regenerating plants cultured for an additional month for root development. (D) Image of shoot and root formation of regenerated plant(s) ready for transplant into soilless media.

Callus Dose-Response

The callus dose-response experiment was repeated twice over time with callus from different explant sources. The first dose-response was performed on callus generated from seed of the UGA 17G5Z breeding population (Fig. 2.3). The second dose-response was performed six months later with calli generated from basal nodes of a single plant regenerated from callus induced from seed and denoted as UGA 17G5Z-TCPC (tissue culture parental control) (Fig. 2.4) to test if different explant source of calli have any difference to pendimethalin response.

The dose-response curves generated from each pendimethalin dose response experiment are presented in Fig. 2.5 and 2.6. Both dose-response curves were very similar and indicated tall fescue calli were very sensitive to even very low concentrations. Dramatic reductions in calli growth were observed at the lowest pendimethalin concentration of 0.2 µM. Calli exposed to 3 µM of pendimethalin or more showed no to little growth.

These dose response experiments showed similar response to pendimethalin concentration regardless of explant tissue used to derive callus. These experiments indicate that selection at 1 to 3 µM should provide adequate selection pressure to inhibit growth of cells without tolerance to pendimethalin and thereby allow identification and multiplication of any cells expressing tolerance.

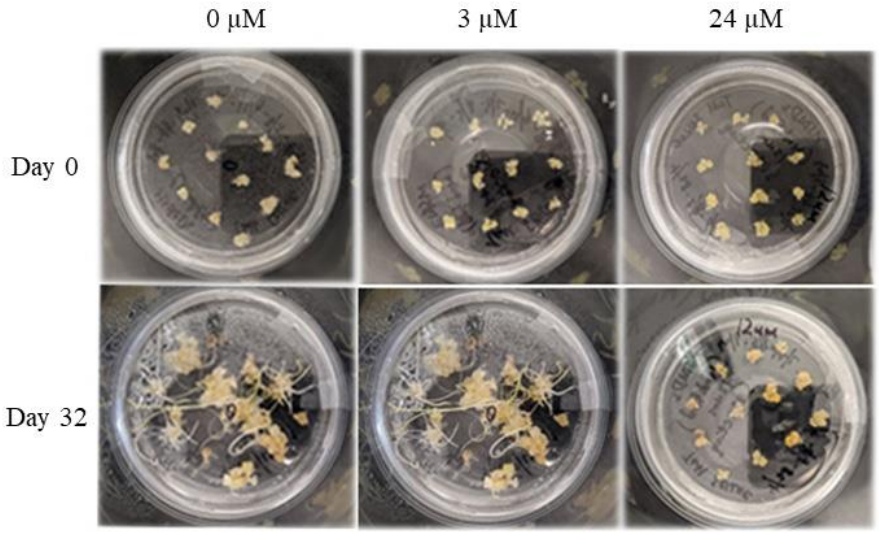

Figure 2.3: Plate images depicting seed derived callus at day 0 and 32 in response to pendimethalin concentrations of 0 µM, 3 µM, and 24 µM. Callus was generated from seeds of UGA 17G5Z tall fescue. The top row shows three plates at the lowest, highest, and chosen selection concentration of pendimethalin at day 0. The bottom row shows the same three plates at the 24-day mark. Callus growth and health is indicated by size and color, respectively.

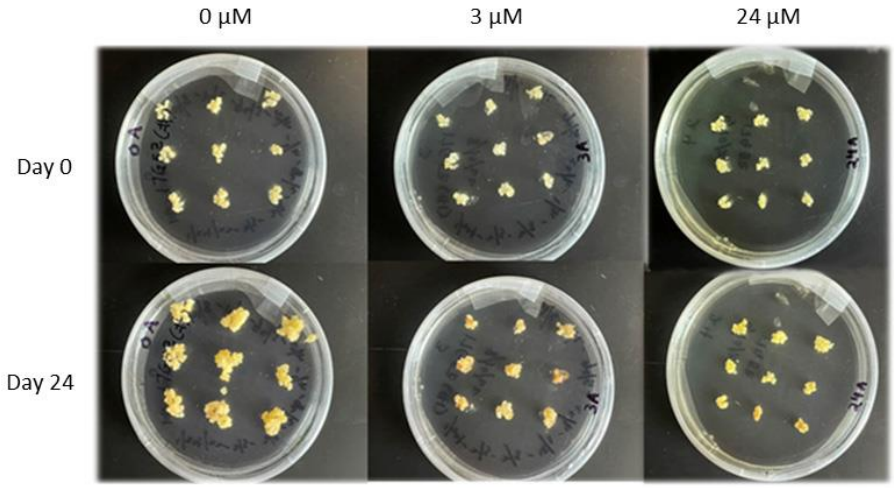

Figure 2.4: Plate images depicting basal node derived callus at day 0 and 24 in response to pendimethalin concentrations of 0 µM, 3 µM, and 24 µM. Callus was generated from tiller basal nodes of UGA 17G5Z TCPC. The top row shows three plates at the lowest, highest, and chosen selection concentration of pendimethalin at day 0. The bottom row shows the same three plates at the 24-day mark. Callus growth and health is indicated by size and color, respectively.

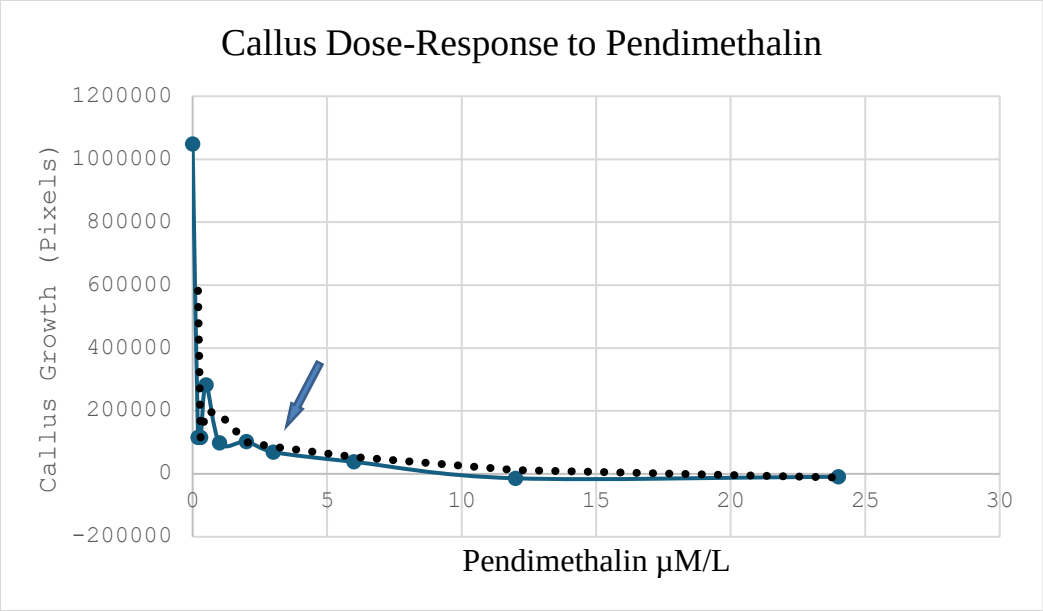

Figure 2.5 First dose response curve generated from seed-derived callus growth after 32 days at 10 pendimethalin concentrations. The blue arrow indicates the final selection point of 3 µM pendimethalin. Fitting trendline shown in black dotted line.

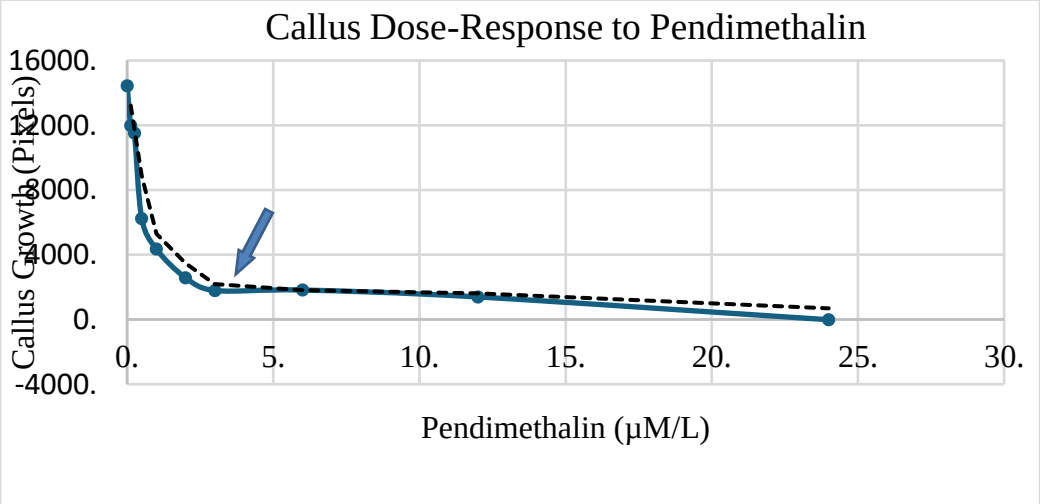

Figure 2.6 Second dose response curve generated from basal node callus growth after 32 days at 10 pendimethalin concentrations. The blue arrow indicates the final selection point of 3 µM pendimethalin. Fitting trendline shown in black dashed line.

Selection of Callus for Pendimethalin Tolerance

Over 12,000 calli were produced from culture on maintenance medium after 5 to 6 cycles of calli increase. They were selected for pendimethalin tolerance by first culturing them for 30 days on selection medium (induction/maintenance medium) supplemented with 1 µM of pendimethalin. A total of 35 calli showed growth after one month on the selection medium. They were separated into smaller pieces and sub-cultured for the second round of selection medium with increased selection pressure at 3 µM of pendimethalin. Each callus was sub-cultured into one plate for the second selection cycle requiring 35 plates. After 30 days on 3 µM selection medium, only 3 calli still showed healthy growth and were sub-cultured on selection medium with 3 µM of pendimethalin for two more cycles of selection. At the end, 65 calli were produced. Calli that exhibited a yellow to brown color and were slightly firm to the touch were selected for plant regeneration having exhibited a visible increase in size (Fig. 2.7).

These 65 calli were transferred into regeneration medium. Six of them turned green and only 4 calli regenerated into plants. When green shoots appeared, the cell lines were sub-cultured for an additional month on MS/B5D2 modified regeneration media to allow roots to develop. When adequate roots were formed, individual plants were removed from the medium and transplanted into pots containing soilless media. A total of 163 plants were regenerated but 23 of them did not survive transplanting/transfer from the growth chamber to the greenhouse. The potted plants were maintained in domed trays in a growth chamber for 1 wk, before being transferred to pots with soil in the cool-season greenhouse at 10 - 21 °C night/day. Approximately 140 putative tolerant plants were regenerated and moved into the cool-season greenhouse.

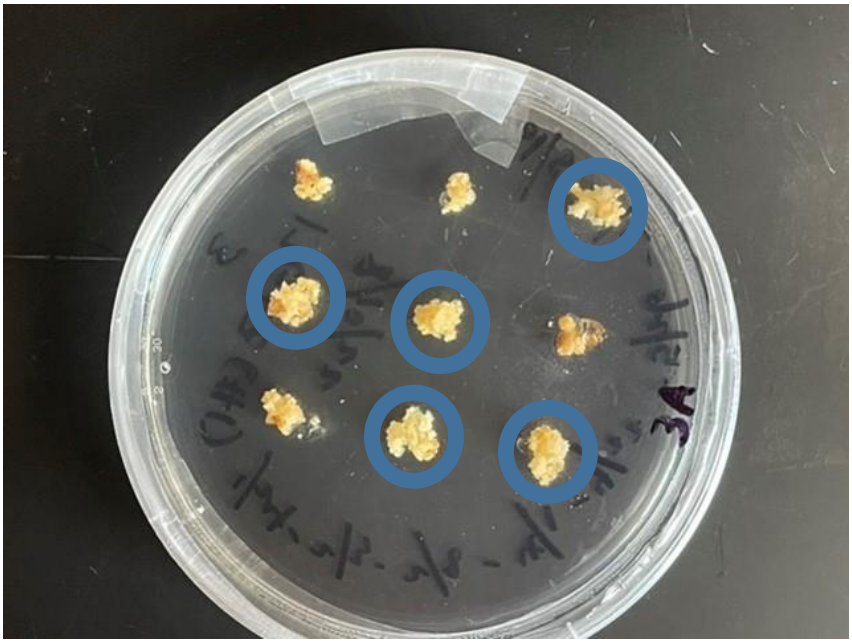

Figure 2.7: Callus growth after multiple cycles of selection and one cycle of increase on media containing 3 uM pendimethalin. The blue circles indicate calli selected for an additional cycle of increase prior to attempted regeneration.

CHAPTER 3

VERIFICATION OF PENDIMETHALIN TOLERANCE USING HYDROPONICS

Introduction

Healthy roots play a crucial role in plant and turfgrass survivability. The primary functions of the root are anchoring the plant, absorption of water and dissolved minerals, and storage of reserve foods. The root itself can be split into four zones: root cap, apical meristem, elongation zone, and the hair. Each of which play a vital role in overall plant health and sustainability. Characteristics of healthy roots are white in color, branching, and hairy. When roots encounter dinitroanilines its morphology starts to become affected.

Pre-emerge herbicides do not prevent seed germination, but rather root and/or shoot elongation that occurs after germination (McElroy & Martins, 2013). Dinitroanilines are mitotic inhibitors that arrest cell division through inhibition of spindle fiber formation (Parka & Soper, 1977). The inhibition of mitosis in the roots can lead to a decline in plant health and often results in plant death before it emerges from the soil. Visual characteristics of dinitroaniline damage include inhibition of lateral or secondary root development (Parka & Soper, 1977), stunted or swollen roots, and roots that appear club-shaped.

Multiple studies have been performed to screen for resistance to dinitroaniline herbicides in weeds using hydroponics systems. Identified resistant weeds were screened using a deep-water culture hydroponics system where various dinitroaniline herbicides were added directly into the nutrient solution. These studies (Brosnan et al., 2014; McAlister et al., 1995) were able

to process material in hydroponics systems to screen for dinitroaniline resistance. Their results demonstrated that hydroponics was an effective and efficient way to screen for resistance. From literature and published studies, it was determined that screening for dinitroaniline tolerance using hydroponics would allow for high throughput of plant materials and was an effective way to identify tolerant plants.

Hydroponics is a method used for growing plants using mineral nutrients solutions without soil (Pandey et al., 2009). There are two primary types of hydroponics, solution culture and medium culture. A solution culture consists of no solid medium for root support, just a nutrient solution in which they are suspended (Pandey et al., 2009). A deep-water culture (DWC) is one example, where plants are suspended above the reservoir containing nutrient solutions and the roots grow down into the solution and remain submerged (Wootton-Beard, 2019). The screens used in these experiments are DWC screens. There are several advantages and disadvantages associated with this type of system. The advantages of this system include: 1) no solid media used, 2) simple to implement, and 3) high capacity for throughput of plant material (Wootton-Beard 2019). While the main disadvantages include 1) low circulation, 2) prone to water-borne diseases, and 3) wasteful of water and nutrients (Wootton-Beard, 2019). The overall aim of hydroponic systems is to enhance environmental control and efficiency. (Wootton-Beard, 2019).

The primary objectives of this portion of the research were to: 1) adapt and develop greenhouse hydroponic protocols specifically for the verification of pendimethalin tolerance in tall fescue plants; 2) conduct a hydroponic pendimethalin dose response curve experiment for tall fescue to determine the appropriate concentration of pendimethalin necessary to reliably identify

pendimethalin tolerant plants; and 3) utilize the adapted hydroponic protocol to efficiently screen all putative pendimethalin tolerant plants regenerated following tissue culture selection.

Materials and Methods

Protocols used commonly by weed scientists for studying herbicide resistance in weedy species (McCullough et al., 2017) were adapted specifically for use in determining pendimethalin tolerance in tall fescue plants and the suitability of these protocols verified in two preliminary experiments. The two preliminary experiments, experiment 1-a and 1-b, provided proof of concept and an opportunity for protocol refinement and confirmation.

Tillers from putative tolerant plants were compared to parental controls through screens to confirm pendimethalin tolerance using a modification of existing hydroponic protocols. The protocol used 5.4 L plastic tubs filled with half-strength Hoagland Basal Salt Solution H353 (PhytoTech Labs, Lenexa, KS). Twenty holes 6.35 mm in diameter spaced 25 mm apart in a 4 x 5 grid pattern were drilled into each tub lid (McCullough et al., 2017). The hydroponic solution was aerated using an electric aquarium pump. Mature plants with 10 or more tillers were used and individual tillers were separated from plants growing in the greenhouse. Roots were rinsed with a solution containing sterilized water and hydrogen peroxide to remove soil and bacteria present. The tillers/roots were then placed in the base of trimmed pipet tips for support before being placed into holes drilled into container lids to suspend them in the aerated hydroponic solution. Tillers were allowed to recover in the hydroponic solution for one to two weeks before undergoing herbicide treatments.

Prior to initiation of herbicide treatments, roots of all tillers were uniformly trimmed to a length of 12.7 mm. Tubs containing specific pendimethalin concentrations were placed in the

cool-season greenhouse at 10 °C night / 24 °C day temperatures and aquarium pumps were used to provide oxygen to the solution (McCullough et al., 2017). Sterilized water was added to the tubs as needed to maintain the 5.4 L volume.

After one month in the hydroponic solutions, tillers were removed, and the roots were rinsed under tap water and blotted dry with a paper towel (McCullough et al., 2017). The maximum length of roots was then measured, and each root system was given a visual rating and recorded on a scale of 1 to 9 with 9 representing a vigorous, extensive, and healthy root system. Scissors were used to cut the roots of individual tillers back to the initial length of 12.7 mm. Trimmed roots were placed into labeled seed envelopes and oven dried for 48 hours at 65 ºC. After drying root weights were recorded.

Figures 3.1 shows the general greenhouse setup for the hydroponic experiments and Fig. 3.2 provides a visual image of rooting responses observed using this protocol.

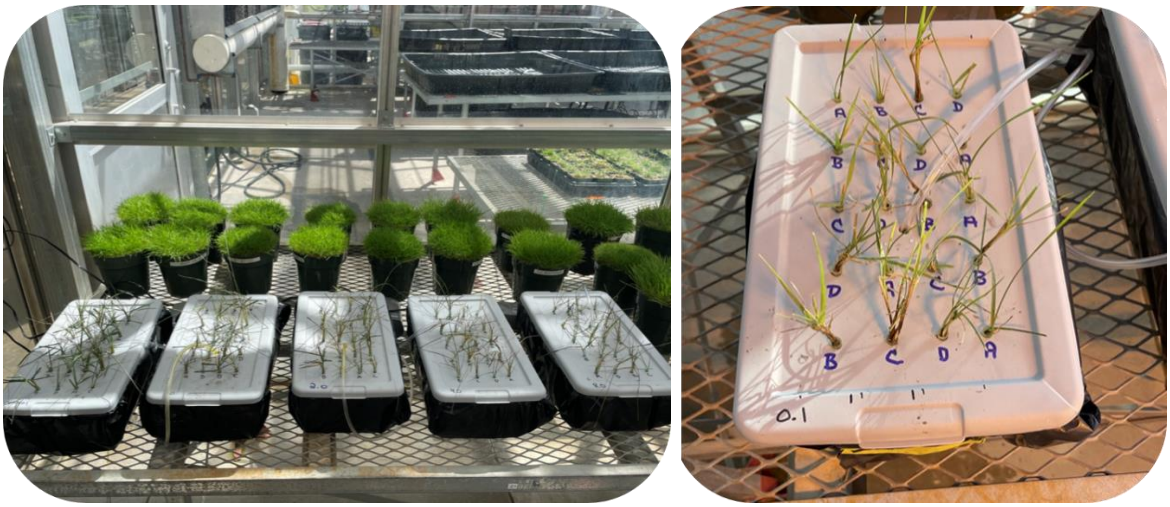

Figure 3.1: Photographs of the general setup for each pendimethalin hydroponics screen. The photograph on the left shows the arrangement and construction of the tubs used in screening. The photograph on the right illustrates how the tillers are arranged and placed into their respective tubs.

Figure 3.2: A close-up view of the tiller's roots at 2 µM 30 days after being in the pendimethalin Hoagland solution. The resistant genotypes demonstrate good rooting ability and healthy roots. The parent genotype shows little to no root growth over the same 30-day period.

Preliminary Experiments 1-a and 1-b

Two preliminary experiments were conducted to verify the utility of the above protocols in identifying pendimethalin tolerant tall fescue plants. All hydroponic experiments utilized tillers taken from callus tissue-regenerated plants from the experimental line UGA 17G5Z without selection for herbicide tolerance and are hereafter referred to as the tissue culture parental control or TCPC. In preliminary experiment 1-a, the parental control, TCPC, was compared to three putative pendimethalin tolerant lines PR#14, PR#49, PR#79 at pendimethalin

concentrations of 0 µM, 0.1 µM, and 1 µM. The experimental design was a split plot with herbicide concentration as the whole plot and genotype as the subplot. Each genotype was replicated using five tillers randomized within each herbicide concentration (single tub). Herbicide concentration was not replicated. The variables root length and dry root weight were measured as described above after 30 days exposure to herbicide treatments.

Preliminary experiment 1-b was conducted in a similar fashion to 1-a using different putative pendimethalin genotypes and the addition of a higher herbicide concentration. Genotypes were the parental control, TCPC, in comparison with the putative tolerant genotypes PR#18, PR#39, and PR#74. Herbicide concentrations were 0 µM, 0.1 µM, 1 µM, and 2 µM. The variables measured following 30 days exposure to pendimethalin herbicide were maximum root length and total root weight, as well as a visual rating of each root system on a 1 to 9 scale. Data from each of the herbicide concentration treatments was analyzed separately using JMP Pro 16.0.0 (2021 SAS Institute Inc., Cary, NC). The effects of genotype within each herbicide concentration (tub) were tested for significance using ANOVA. Genotype means for each of the measured variables were separated using Student's t-test.

Hydroponic Pendimethalin Dosage Response

The response of tall fescue tillers to pendimethalin concentration while in hydroponic culture was determined using the protocols described above. Laboratory grade pendimethalin obtained from Fischer Scientific (Waltham, MA) was dissolved in methanol and was used to create a stock solution with a concentration of 100 µg pendimethalin per 1 mL methanol. This stock solution was added to the hydroponic solutions to create pendimethalin solution concentrations of 0, 1, 2, 4 and 8 µM. Genotypes tested were the parental control, TCPC, and putative tolerant lines, PR#39, PR#49, and PR#74. The experimental design was a split plot with

herbicide concentration as the whole plot and genotype as subplots. Individual tubs were used for each pendimethalin concentration and a minimum of five tillers from each tall fescue genotype was randomized within each herbicide concentration. The experiment was repeated in time (runs). Four weeks after the initiation of the dose response experiment individual tillers were evaluated for root growth by measuring maximum root length, root dry weight, and a visual root rating. Data from the combined data was analyzed using a mixed model with herbicide concentration and genotype as fixed effects and run and run*herbicide as random effects. These data were used to generate a hydroponic dose response curve and thereby determine a suitable pendimethalin concentration to confirm pendimethalin tolerance among putative pendimethalin tolerant plants produced by tissue culture selection.

Hydroponic Screen to Verify Pendimethalin Tolerance in Putative Tolerant Lines

Once an appropriate pendimethalin concentration for screening putative pendimethalin tolerant plants was established, the hydroponics screen was used to evaluate phenotypic expression of pendimethalin tolerance of all previously untested genotypes regenerated following tissue culture selection. A total of 51 genotypes were screened in four sets designated A-D. All screens included a parental control, TCPC, for comparison and four tillers of each genotype served as replicates. Measured variables included maximum root length and visual root rating. Data from each of the sets were analyzed separately using JMP Pro 16.0.0 (2021 SAS Institute Inc., Cary, NC). The effects of genotype within each set (A-D) were tested for significance using ANOVA. Genotype means for each of the measured variables were separated using Student's t-test. Genotypes with variable means statistically greater than the parental control were considered pendimethalin tolerant and candidates for future research.

Results

Preliminary experiment 1-a consisted of putative tolerant genotypes PR#14, PR#49, PR#74 and TCPC with dosage concentrations of 0, 0.1, and 1 µM. The root length and root weight response of tillers from the four tested genotypes are presented in Table 3.1 and illustrated in Fig. 3.3. PR#14, PR#49, and PR#74 were not different from TCPC for root length and root weight when no herbicide was present. At 0.1 µM, root length and root weight of TCPC was dramatically reduced as compared to when no herbicide was present confirming the sensitivity of the tall fescue parent to pendimethalin. In contrast, 0.1 µM of pendimethalin had no effect on root length of PR#49 and PR#79 which produced longer roots than TCPC indicating that these lines may have tolerance to low concentrations of pendimethalin. PR#14 responded similar to TCPC at 0.1 µM indicating a similar level of sensitivity. At the 1 µM concentration, root length and weight was reduced in all genotypes. Comparisons among the genotypes tested at 1 µM showed a similar trend as observed at 0.1 µM with PR#14 performing similar to TCPC and PR#49 and PR#79 producing longer roots than TCPC. Tiller root dry weights were very small and despite being weighed on a four-place scale were highly variable and showed few statistically significant differences in comparisons among genotypes. A root visual rating was added to all subsequent experiments to provide additional data to document root responses.

Preliminary experiment 1-a showed that tolerant genotypes were able to demonstrate root growth at 1 µM. Preliminary experiment 1-b increased the dosage concentration to see if rooting ability would be inhibited at a higher concentration. Preliminary experiment 1-b tested three different putative tolerant genotypes PR#18, PR#39, PR#74 and TCPC at pendimethalin concentrations of 0, 0.1, 1, and 2 µM. Summarized data for this experiment are presented in Table 3.2. In the absence of the herbicide, all genotypes produced healthy and vigorous roots

and no differences were observed for maximum root length among the genotypes. PR#39 did have higher root weight and visual rating than TCPC but was not different from the other PR lines tested. At the herbicide concentration of 0.1 µM, all putative tolerant genotypes exhibited higher visual ratings than TCPC, but no differences occurred among the genotypes for root length or weight. The herbicide concentration of 1 µM showed PR#39 and PR#74 had significantly higher root lengths and visual ratings than TCPC and no genotypic differences were observed for root weight (Table 3.2). At the concentration of 2 µM, all three of the putative resistant genotypes had higher root lengths and visual ratings than TCPC with only PR#39 and PR#74 having higher root weights (Table 3.2). The pendimethalin concentration of 2 µM provided clear visual separation between TCPC and putative tolerant lines arising through tissue culture selection (Fig. 3.4).

Both preliminary experiments established that an effective hydroponics protocol was in place and the results demonstrated that our tissue culture selections were effective in enhancing pendimethalin tolerance levels in tall fescue (Tables 3.1 and 3.2). The regenerated putative tolerant tall fescue plants were able to outperform TCPC and demonstrated significantly higher rooting ability in the presence of pendimethalin.

Table 3.1: Summary of data from preliminary experiment 1-a comparing rooting performance of the parental control, TCPC, to three putative tolerant genotypes following 30 days of exposure to three pendimethalin concentrations. Statistical comparisons of genotype performance are made within pendimethalin concentration treatments.

Genotype	Pendimethalin Concentration (µM)	Root Length (cm)	Dry Root Weight (g)
TCPC	0	12.42a[1]	0.0089a,b
PR#14	0	10.68a	0.0034b
PR#49	0	13.88a	0.0076a,b
PR#79	0	10.28a	0.0105a
TCPC	0.1	6.72b	0.0024b
PR#14	0.1	7.12b	0.0040b
PR#49	0.1	**15.46a**[2]	**0.0087a**
PR#79	0.1	**11.74a**	0.0050a,b
TCPC	1	1.86b	0.0007a
PR#14	1	1.70b	0.0016a
PR#49	1	**5.20a**	0.0033a
PR#79	1	**3.90a**	0.0019a

1. Genotypic variable means followed by the same letter within a pendimethalin concentration treatment are not significantly different according to Student's t-test.
2. Bolded means indicate statistical difference from TCPC.

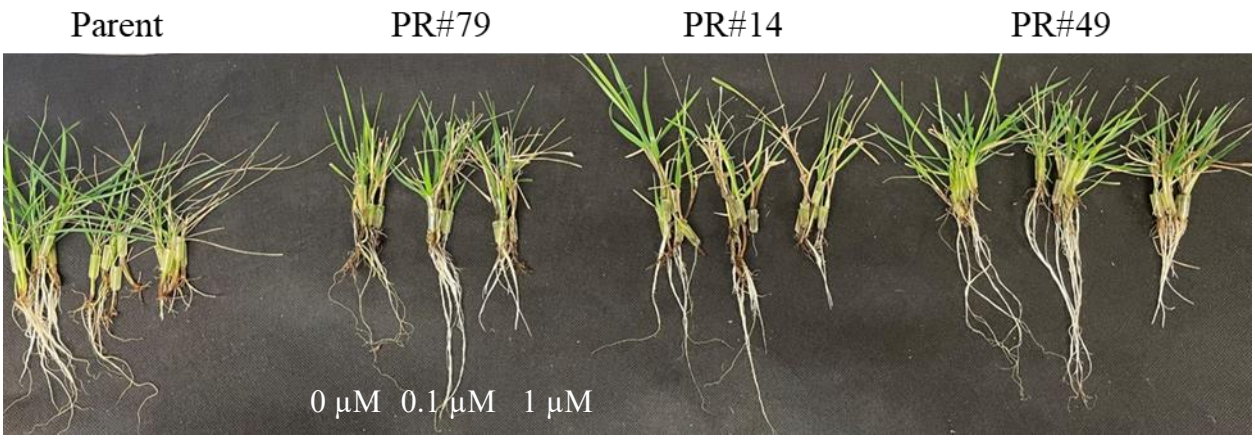

Figure 3.3: Tillers from three resistant genotypes and the parent genotype (TCPT) from preliminary experiment 1-a. Tiller replicates are grouped from left to right by herbicide dosage within genotype groups. The resistant genotypes showed root growth across all pendimethalin concentrations while TCPT did not demonstrate root growth at the highest concentration.

Table 3.2: Summary of data from preliminary experiment 1-b comparing rooting performance of the parental control, TCPC, to three putative tolerant genotypes following 30 days of exposure to three pendimethalin concentrations. Statistical comparisons of genotype performance are made within pendimethalin concentration treatments.

Genotype	Pendimethalin Concentration (µM)	Root Length (cm)	Dry Root Weight (g)	Visual Rating
TCPC	0	14.05a	0.0028b	2.2b
PR#18	0	13.66a	0.0115a,b	4.6a,b
PR#39	0	13.66a	**0.0116a**	**4.6a**
PR#74	0	13.46a	0.0091a,b	4.6a,b
TCPC	0.1	18.56a	0.0034a	2.6b
PR#18	0.1	20.18a,b	0.0170a	**6.4a**
PR#39	0.1	20.18a,b	0.0170a	**6.4a**
PR#74	0.1	22.64a	0.0217a	**7.2a**
TCPC	1	5.70b	0.0005a	0.4b
PR#18	1	12.02a,b	0.0048a	3.4a,b
PR#39	1	**15.86a**	0.0048a	**3.4a**
PR#74	1	**14.15a**	0.0081a	**5.2a**
TCPC	2	3.70b	0.0002b	0.8b
PR#18	2	**11.240a**	0.0095b	**5.8a**
PR#39	2	**13.70a**	**0.0095a**	**5.8a**
PR#74	2	**13.20a**	**0.0146a**	**5.6a**

1. Genotypic variable means followed by the same letter within a pendimethalin concentration treatment are not significantly different according to Student's t-test.
2. Bolded means indicate statistical difference from TCPC.

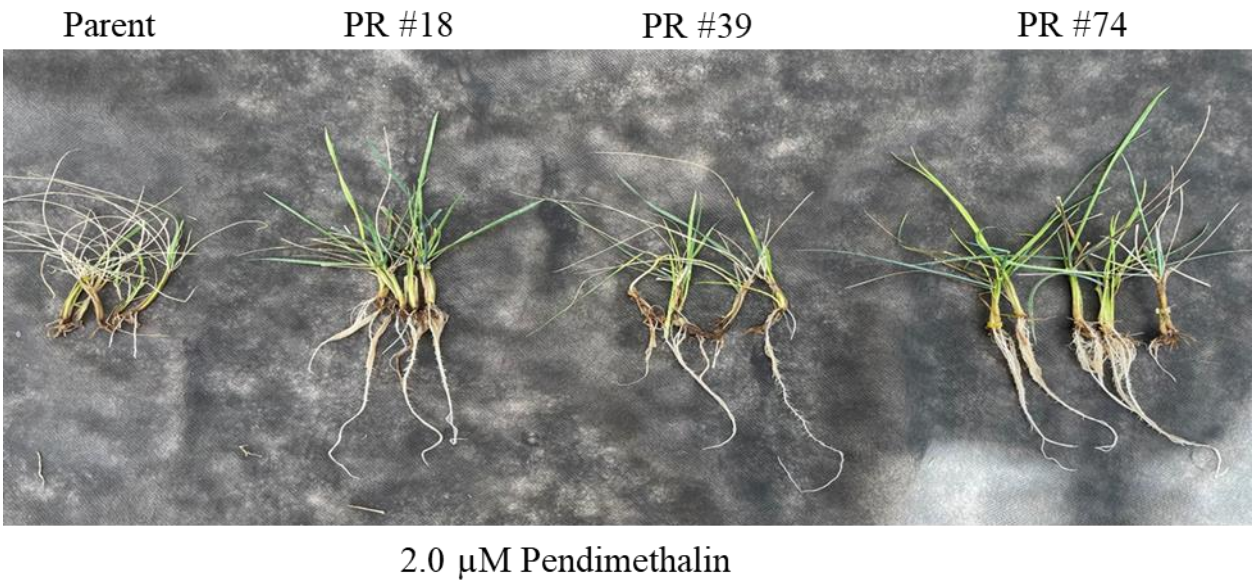

Figure 3.4: Photograph of plants from preliminary experiment 1-b showing rooting performance of the parental control (TCPC) in comparison with three pendimethalin tolerant genotypes following 30 days of exposure to pendimethalin at 2 µM. The tolerant genotypes demonstrated sufficient root growth and health at this concentration, where as the parental control plants produce little root growth at 2 µM.

Hydroponic Pendimethalin Dosage Response

Hydroponic-based pendimethalin dose response experiments compared rooting characteristics of TCPC to three lines identified as pendimethalin tolerant in preliminary experiments 1-a and 1-b at five pendimethalin dosage rates. The results are summarized in Table 3.3. TCPC root length and visual ratings were severely reduced (80-90%) when herbicide was present. Measured rooting variables of PR#39, PR#49, and PR#74 indicated significantly better tolerance than TCPC to pendimethalin at dosages of 1 and 2 µM. Among these, PR#74 appeared to be the most tolerant expressing tolerance at 4 µM and to a lesser extent at 8 µM. Interestingly, the previously identified tolerant lines PR#39, PR#49, and PR#74 all displayed better rooting than TCPC even in the absence of herbicide (0 µM treatment).

Figure 3.5 provides a graphic illustration of root length response to pendimethalin dosage for the parental line, TCPC, and the most tolerant line, PR#74. TCPC root length was dramatically reduced by pendimethalin rates of 1 µM or greater. PR#74 root length was also reduced by these pendimethalin dosages but to a much lesser extent. PR#74 root length was significantly greater than TCPC at dosages of 1, 2 and 4 µM (Fig. 3.5 and Table 3.3). Based on comparisons of these dose response curves, 2 µM was chosen as the standard dosage to be used to confirm the expression of pendimethalin tolerance when screening of all putative tolerant lines arising from tissue culture selection. Fig. 3.6 provides a visual comparison of rooting characteristics at 2 µM between TCPC and tolerant lines.

Table 3.3. Summary of data from pendimethalin dosage response experiment comparing rooting performance of the parental control, TCPC, to three putative tolerant genotypes following 30 days of exposure to five pendimethalin concentrations. Statistical comparisons of genotype performance are made within pendimethalin concentration treatments.

Genotype	Pendimethalin Concentration (µM)	Root Length (cm)	Root Weight (g)	Visual Rating
TCPC	0	12.81b	0.0084b	5.6b
PR#39	0	**26.59a**	0.0177a,b	6.6a,b
PR#49	0	**26.74a**	**0.0223a**	6.4b
PR#74	0	**23.21a**	**0.0205a**	**7.7a**
TCPC	1	1.52c	0.0083a	1.8b
PR#39	1	4.59b,c	0.0066a	**4.5a**
PR#49	1	**6.07a,b**	0.0092a	**4.5a**
PR#74	1	**8.80a**	0.0138a	**6.1a**
TCPC	2	2.53a	0.0004b	1.8c
PR#39	2	**5.71b**	**0.0100a**	**3.7b**
PR#49	2	**7.63b**	**0.0087a**	3.3bc
PR#74	2	**8.04b**	**0.0138a**	**6.0a**
TCPC	4	2.10b	0.0022a	1.5b
PR#39	4	2.07b	0.0042a	2.9a,b
PR#49	4	1.81b	0.0024a	2.9b
PR#74	4	**4.15a**	0.0070a	**4.8a**
TCPC	8	1.39a	0.0015a	1.1c
PR#39	8	1.85a	0.0016a	**2.0b**
PR#49	8	2.10a	0.0008a	1.7b,c
PR#74	8	2.15a	0.0044a	**3.2a**

1. Genotypic variable means followed by the same letter within a pendimethalin concentration treatment are not significantly different according to Student's t-test.
2. Bolded means indicate statistical difference from TCPC.

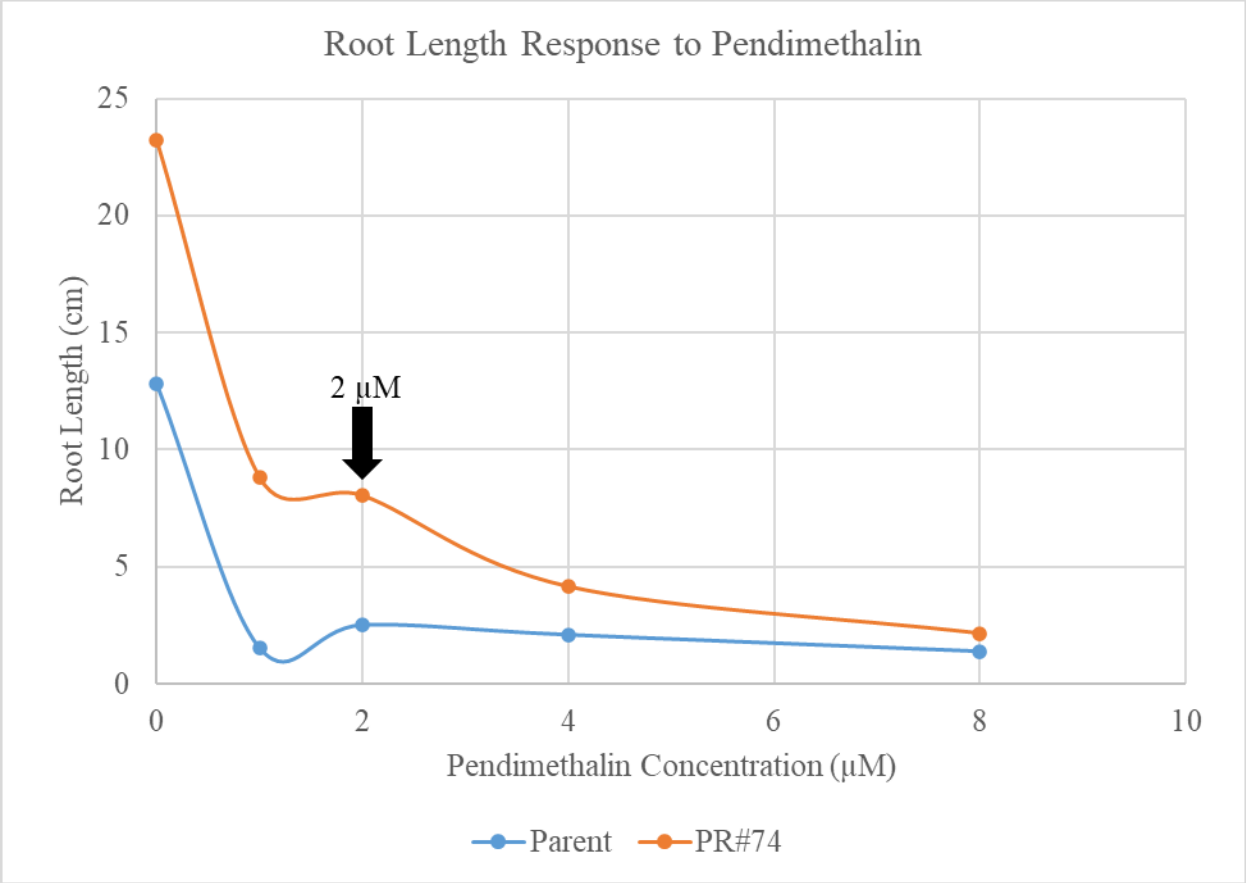

Figure 3.5. Dose-response curves comparing the sensitive parent with a tolerant selection for root length when exposed for 30 days to a wide range of pendimethalin concentrations. The black arrow indicates the pendimethalin dosage selected for further screening efforts. Rooting ability of both parent and putative resistant genotypes were greatly inhibited at concentrations above 2 µM.

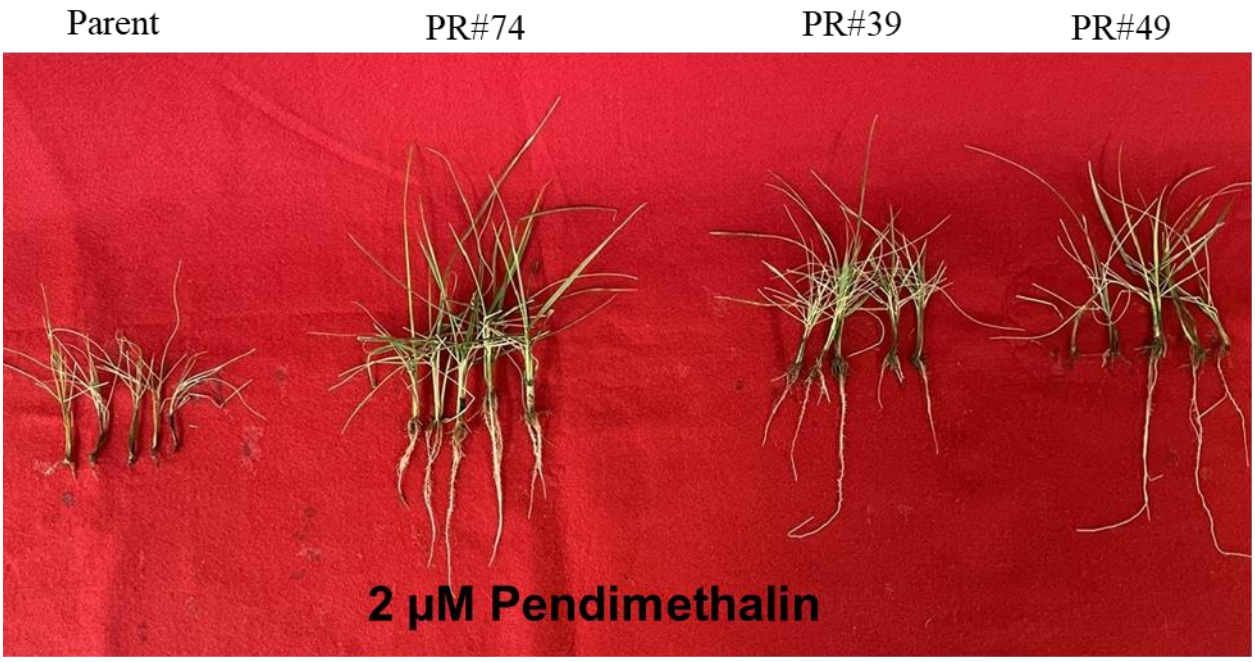

Figure 3.6. Photograph of plants from pendimethalin dosage response experiment showing rooting performance of the parental control (TCPC) in comparison with three pendimethalin tolerant genotypes after 30 days of exposure to pendimethalin at 2 µM. The tolerant genotypes demonstrated sufficient root growth and health at this concentration, whereas the parental control plants produced little root growth at 2 µM.

Verification of Pendimethalin Tolerance for Putative Tolerant Lines

The third aspect of this research was the verification of pendimethalin tolerance in putative tolerant lines arising from tissue culture selection. The affore discussed pendimethalin dose response experiments indicated 2 µM was an appropriate pendimethalin dosage for verification of expression of pendimethalin tolerance among 51 putative tolerant lines in 4 separate screens designated as A-D. Maximum root length and root visual ratings were used to comparing rooting performance of putative tolerant lines to TCPC and the resulting data shown in Table 3.4.

In hydroponics screen ‘A’, 21 putative tolerant lines were compared to TCPC using hydroponic protocols at a pendimethalin dosage of 2 µM. The experiment was discarded at 3 weeks due bacterial contamination within the tubs that led to the death of multiple tillers and decreased overall plant survival. No significant differences from the parental control were found for any of the 21 putative tolerant lines for either root length or visual rating (Table 3.4). Hydroponics screen B consisted of 16 putative tolerant genotypes and five genotypes were identified with both improved root length and visual rating when compared to TCPC (Table 3.4). Screen C consisted of 19 putative tolerant lines and seven putative tolerant genotypes were identified with differences in root length in visual ratings. Finally, hydroponics screen D consisted of 17 putative resistant genotypes of which five genotypes were found to demonstrate better root length and higher visual ratings than the parental control. (Table 3.4).

Due to issues with bacterial contamination, genotypes evaluated in screen A should be re-evaluated. Among the four screens conducted, 17 of the 51 putative tolerant lines evaluated demonstrated better rooting than the parental control and should be retained for further evaluation.

Table 3.4: Summary of data from pendimethalin screens A-D comparing rooting performance of the parental control, TCPC, to putative tolerant genotypes following 30 days of exposure to 2 µM pendimethalin.

Screen	Genotype	Pendimethalin Concentration (µM)	Root Length (cm)	Visual Rating
A	TCPC	2	1.67abcde	2.0bcde
A	PR#105	2	1.20bcde	2.0abcde
A	PR#19	2	1.71abcde	2.3abcde
A	PR#24	2	2.10abcde	2.5abcde
A	PR#30	2	2.93abcd	2.8 abcd
A	PR#34	2	2.30abcde	2.6abcd
A	PR#36	2	1.92abcde	2.4abcde
A	PR#41	2	3.3abc	3.3ab
A	PR#42	2	2.15abcde	2.5abcde
A	PR#43	2	1.87abcde	2.3abcde
A	PR#48	2	4.15a	3.5ab
A	PR#53	2	2.27abcde	2.5abcd
A	PR#55	2	1.59bcde	2.0bcde
A	PR#59	2	1.00bcde	2.0abcde
A	PR#63	2	1.27cde	2.0bcde
A	PR#70	2	2.98abc	3.0abcd
A	PR#76	2	2.33abcde	2.7 abcde
A	PR#78	2	2.8abcd	2.8abcd
A	PR#84	2	0.83de	1.6bcde

A	PR#86	2	0.62e	1.5de
A	PR#98	2	1.60abcde	2.0bcde
A	PR#105	2	1.20bcde	2.0abcde
B	TCPC	2	1.67b	1.5e
B	PR#19	2	2.26b	2.0cde
B	PR#24	2	2.62b	2.5cde
B	PR#30	2	**2.93a**	2.7bcde
B	PR#34	2	3.62ab	**3.0abcd**
B	PR#36	2	1.92b	1.6de
B	PR#41	2	**3.30a**	**3.0abcd**
B	PR#42	2	**3.32a**	**3.0abcd**
B	PR#43	2	1.87b	1.6de
B	PR#48	2	**4.15a**	**4.0ab**
B	PR#53	2	**3.70a**	**3.5abc**
B	PR#55	2	2.05b	2.0cde
B	PR#63	2	1.85b	1.6de
B	PR#70	2	**5.15a**	**4.2a**
B	PR#76	2	2.33b	2.3cde
B	PR#78	2	2.80b	2.7bcde
B	TCPC	2	1.67b	1.5e
C	TCPC	2	2.32h	1.7ef
C	PR#2	2	2.80h	2.7def
C	PR#32	2	**13.72a**	**6.2a**

C	PR#46	2	**8.62bcde**	**5.2ab**
C	PR#50	2	4.27fgh	**3.7bcd**
C	PR#54	2	**8.72bcd**	**5.2ab**
C	PR#59	2	**10.67ab**	**6.0a**
C	PR#62	2	4.00fgh	3.5bcde
C	PR#67	2	3.62gh	3.2cdef
C	PR#69	2	**7.30bcdef**	**5.0abc**
C	PR#75	2	5.50defgh	**4.7abc**
C	PR#84	2	4.50fgh	**3.7bcd**
C	PR#86	2	3.77fgh	3.2cdef
C	PR#94	2	**6.97cdefg**	**4.7abc**
C	PR#96	2	5.35defgh	**4.3abcd**
C	PR#98	2	2.17h	1.0f
C	PR#131	2	**9.57bc**	**5.2ab**
C	PR#120	2	3.65gh	3.2cdef
C	PR#109	2	5.17efgh	**3.7bcd**
C	PR#107	2	3.02h	3.2cdef
D	TCPC	2	1.67e	1.5e
D	PR#12	2	2.57de	**3.5bcd**
D	PR#18	2	**4.32abc**	**4.3bc**
D	PR#21	2	3.12bcde	**3.7bcd**
D	PR#31	2	2.20de	1.6de
D	PR#71	2	**5.22a**	**6.7a**

D	PR#92	2	**4.65ab**	**6.5a**
D	PR#95	2	2.62de	**3.5bcd**
D	PR#99	2	2.83cde	**3.6 bcd**
D	PR#100	2	2.50de	**3.5bcd**
D	PR#101	2	2.60de	**3.5bcd**
D	PR#105	2	2.33de	3.0cde
D	PR#116	2	2.30de	2.7cde
D	PR#124	2	2.90cde	**3.7bcd**
D	PR#128	2	**4.35abc**	**5.3ab**
D	PR#130	2	2.42de	3.0cde
D	PR#135	2	2.30de	2.3cde
D	PR#141	2	**3.50bcd**	**4.0bc**

CHAPTER 4

CHARACTERIZATION OF THE MODE OF ACTION OF DNA TOLERANT GENOTYPES

Introduction

Unwanted plants that are out of place have been termed as weeds. Management of weeds in the past mostly consisted of hand weeding and tillage, but in recent times, the application of synthetic herbicides has taken its place. The adoption of synthetic herbicides has decreased the time used to manually remove the weed and the cost of paying someone to do so, in turn it has increased the efficiency and efficacy of weeding. However, over time the increased application of synthetic herbicides has led to widespread evolution of resistance to many chemical classes of herbicides (Dayan et al., 2019). The intensive selection pressure applied by repeated application of synthetic herbicides has led to a vast variety of resistance mechanisms (Gaines et al., 2020). Resistance to herbicides can be endowed by target site (TSR) alterations or by non-target mechanisms (NTSR) (Matzrafi et al., 2014). Target site resistance (TSR) involves mutations in genes that encode the protein target of herbicides thus affecting the binding of the herbicide and are mostly constituted of nonsynonymous single nucleotide polymorphisms (SNPs) (Gaines et al,. 2020). Non-target site resistance mechanisms are complex and involve genes that are a part of many large gene families (Gaines et al., 2014).

TSR mechanism have led to the evolution of resistance to 167 herbicides with 512 weed species evolving resistance to one or multiple herbicides (Gaines et al., 2020). A single change in a nucleotide in a gene that encodes a protein that is bound by an herbicide can lead to a singular

amino acid change that disrupts the herbicide's ability to bind the protein without disrupting the enzyme function. Many of the same molecular mechanisms of SNPs constitute the foundation of resistance for many of the different herbicide site of actions. TSRs are specific to a single site of action and the mechanism involved can be associated with SNPs, multiple nucleotide polymorphisms, receptor/co-receptor interaction, codon deletion, or increased expression of target site genes. The most common TSR are nonsynonymous SNPs that provide resistance to the herbicide target site. Dinitroaniline herbicides such as trifluralin and oryzalin bind to plant tubulin and inhibit meristem development by depolymerizing microtubules (Dayan et al., 2019). The Thr-239-Ile found in a-tubulin was first reported in goosegrass (*Eleusine indicia*) and conferred resistance to dinitroaniline herbicides. When this mutation was transformed into maize resistance to trifluralin and oryzalin was expressed, confirming the mutation was the molecular basis of dinitroaniline resistance (Anthony et al., 1998). Since that mutation was reported other mutations such as Val-202-Phe (Chen et al., 2020) and Arg-243-Met/Lys (Chu et al., 2018) in annual ryegrass (*Lolium rigidum*), Met-268-Thr in *E. indica*, and both Thr-239-Ile and Leu-136-Phe in green foxtail (*Setaria viridis*) (Délye et al., 2004) have also been reported. This shows that substitutions at different amino acid positions in a target site gene have the potential to confer resistance (Gaines et al., 2020).

Widespread and long-term repeated applications of dinitroanilines have led to target-site point mutations to occur in 12 different weed species (Wang et al., 2021). Field evolved resistance is known to occur in goosegrass (*Eleusine indica*), green foxtail (*Setaria viridis*) (Delye et al. 2004), Palmer amaranth (*Amaranthus palmeri*), annual ryegrass (*Lolium rigidum*), annual bluegrass (*Poa annua*), blackgrass (*Alopecurus myosuroides*), and water foxtail (*Alopecurus aequalis*) (Wang et al., 2021). These target-site point mutations that confer

resistance occur in microtubules. The primary component of microtubules is a globular protein called tubulin (Blume et al., 2003), composed of α and β heterodimers, which polymerize to form microtubules (Bronson et al., 2014). The α-tubulin gene is 1500 - 1600 bp in length (Chen et al., 2017), and the mutations that confer resistance occur in a section of 300 amino acids (about 900 nucleotides). The reported target-site point mutations including Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Met-268-Thr, and Arg-243-Met/Lys mostly occur in the α-tubulin1 gene.

NTSR includes all the mechanisms that reduce the active herbicide available to interact with target site proteins, in addition to mechanisms that allow the plant to adapt with inhibition of target sites (Gaines et al., 2020). These mechanisms are ways such as reduced herbicide uptake, reduced translocation, increased herbicide sequestration, and enhanced degradation or metabolism of the herbicide to a less toxic substance. Plants contain a vast number of genes that encode enzymes for biochemical reactions for synthesis of secondary metabolites and for detoxifying xenobiotic compounds (Yuan et al., 2007). Some members of these gene families can detoxify herbicides, and the selective action of several herbicides can depend on rapid metabolism of the active ingredient into harmless products (Gaines et al., 2020). Herbicide detoxification is split into three phases: Phase 1) addition of a functional group to the herbicide that is often mediated by cytochrome P450 monooxygenases through oxidation, reduction, or hydrolysis; Phase 2) a conjugation to GSH mediated by GSH S-transferases or to glucose mediated by glucosyltransferases; and Phase 3) compartmentalization of herbicide metabolites in the vacuole or cell wall (Gaines et al., 2020). Enhanced metabolism such as P450 and GST can confer broad-spectrum resistance and the addition of TSR can reduce translocation that leads to higher levels of resistance.

Cytochrome P450 monooxygenases are proteins that are membrane bound and localized in the endoplasmic reticulum and constitute one of the largest gene families in all organisms (Werck-Reichhart et al., 2000 a). The diversity of P450s in plants is vast with weeds having been found to have 917, 323, and 277 in barnyard grass (*Echinochloa crus-galli*), *C. canadensis*, and *L. rigidum* (Gaines et al., 2020). This vast array of diversity of P450s likely was a result of the evolution of defenses used for degradation of exogenous chemicals and by chance, due to substrate promiscuity, the P450 genes gained the ability to detoxify herbicides (Werck-Reichhart et al., 2000). P450 genes catalyze the aryl or alkyl hydroxylation, which is the first step in herbicide detoxification (Powles and Yu, 2010). They insert molecular oxygen onto an herbicide molecule that allows it to be more reactive and soluble using an electron from NADPH P450 reductase (Gaines et al., 2020). This leads to the herbicide being metabolized into products that have reduced phytotoxicity in plants with metabolism-based resistance.

When NTSR is due to enhanced metabolism by P450s the inhibition of P450 activity allows the herbicide to reach its target site in a concentration high enough to cause phytotoxicity (Gaines et al., 2020). The addition of a P450 inhibitor prior to herbicide treatment will block P450 activity. P450 inhibitors, like the organophosphate insecticide malathion, enhance injury to resistant biotypes when applied in addition to the herbicide (Christopher et al., 1991, 1994; Yasuor et al., 2009). The inhibition by malathion occurs when atomic sulfur released from the oxygenated organophosphate inhibits the P450 by forming a sulfur protein adduct (Werck-Reichhart et al., 2000 a).

Literature has shown that DNA extraction and analysis of the α-tubulin gene is an efficient way to confirm if a known resistance conferring mutation is present (Délye et al. 2014; Chu et al., 2018; Lyons-Abbott et al., 2010). We generated 140 tall fescue plants with putative

tolerance to pendimethalin by screening tall fescue callus tissue on media having normally lethal doses of pendimethalin and regenerating plants from callus that were able to grow. Phenotypic expression of pendimethalin tolerance was confirmed in three of these plants (PR#39, PR#49, PR#74) using hydroponics screens with rates of pendimethalin sufficient to halt root growth of the reference material (UGA 17G5Z TCPC). In an attempt to characterize the mechanism of pendimethalin tolerance in these plants, we sequenced the α-tubulin gene to determine if one or more known target-site point mutations were present. We also conducted experiments to determine of if the mechanism of tolerance in PR#74 could be due to P450 metabolism by using malathion, a known inhibitor of P450 metabolism, to determine if expression of pendimethalin tolerance in PR#74 was suppressed when treated with malathion.

Materials and Methods

Plant α-tubulin Gene Alinement and Primer Design

We searched the NCBI Genebank of National Library of Medicine (NCBI) for plant α-tubulin genes. We selected α-tubulin genes from five distinct species, *Eleusine indica*, *Panicum hallii*, *Setaria viridis*, *Sorghum bicolor*, and *Zea mays*. Gene sequence alignment was performed using Geneious, Biomatters software (New York, NY) (Fig. 4.2) and two pairs of primers, Tub1F (5”ATGAGGGAGTGCATCTCG3”), Tub1R (5’GATGCGGTCAAGGCACAGGT3’), Tub2F (5’TTGGCAAGGAGATTGTTGAC3’), and Tub2R (5’AGCATGAAGTGGATCCTCGGGTA3’) covering the previously reported mutation region of the 700-800 bp range.

mRNA Extraction and α-tubulin Gene Sequencing.

In efforts to characterize the mechanism of resistance, RNA was extracted from the tissue culture donor plant UGA 17G5Z TC1, and five dinitroaniline tolerant plants (PR# 17, 18, 39, 49, 74) following a protocol of Invitrogen PureLink™ RNA Mini Kit by Thermofisher Scientific. cDNA generated with a High-Capacity cDNA Reverse Transcription Kit by Applied Biosystems, ThermoFisher Scientific. cDNA was used for PCR with two pairs of primers, atub1F/1R and atub2F/2R to generate two fragments, αtub1 and αtub2 respectively, trying to cover the reported meaningful mutation sites for dinitroaniline tolerant in α-tubulin gene. PCR products were confirmed using electrophoresis and the expected fragments were cleaned with a gel extraction kit (Qiagen Sciences, MA, USA). PCR products were sequenced by MCLAB, San Francisco, CA, USA.

Gene Sequencing and Alignment

Two PCR products generated with aTub1F/1R and aTub2F/2R were extracted and cleaned with a gel extraction kit, Qiagen Sciences. For PCR amplification, 10 µL DNA was added to a standard 25 µL PCR GoTaq Master Mix (Promega, Madison, WI) [Consisting of DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer]. Amplification was carried out using primer with The PCR amplification reactions were performed in a 20-µl volume having 20 ng of gDNA, 1× Taq buffer, 2 mM of MgCl2, 0.2 mM dNTP, 0.5 mM each of the forward and reverse primers, and 1 unit of Taq polymerase. The PCR procedure was as follows: one cycle of 3 min denaturing at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C and a final extension of 5 min at 72 °C. The PCR products were analyzed on 1 % agrose gel stained with ethidium bromide.

DNA sequencing was carried out by MCLAB. Both fragments were successfully sequenced with their correspondent primers. Sequences of tissue culture sensitive plant UGA 17G5Z TCPC, and five dinitroaniline tolerant mutant plants (PR# 17, 18, 39, 49, 74) were aligned with the consensus α-tubulin1 gene from *Eleusine indica* as the reference sequence. Standard PCR conditions and primers designed for the goosegrass α- tubulin gene sequence were used to amplify a 750-bp fragment (Fleet et al., 2017). The primers amplify a 746-bp fragment in rigid ryegrass and 112-360 bp equivalent in goosegrass (Fleet et al., 2017). The AWO and TUB primers have successfully sequenced the α-tubulin gene in rigid ryegrass and goosegrass species (Fleet et al., 2017). The relatedness between ryegrass and tall fescue species suggests that the AWO and TUB primers should allow isolation of the α-tubulin gene for sequencing in tall fescue. To prepare the AWO and TUB primers 189 µL and 187 µL of water was added to AWO-F and AWO-R primers, 152 µL and 179 µL of water was added to the TUB-F and TUB-R primers to create a 100 µM solution; this is based on the oligonucleotide data sheet. Each primer was vortexed and centrifuged at 15,000 rpm for 3 minutes. A 5 µM concentration of primer was used by adding 450 µL of water and 25 µL each for the forward and reverse primers in a 1.5 mL centrifuge tube. For PCR amplification, 10 µL DNA was added to a standard 25 µL PCR GoTaq Master Mix (Promega, Madison, WI). Consisting of DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer. Amplification was carried out using AWO primer (Fleet et al., 2017) with PCR conditions of 3-min denaturing at 94 C, 37 cycles of 30-s denaturation at 94 C, 30-s annealing at 55 C, and 45-s elongation at 68 C, and final extension for 7 min at 68 C (Fleet et al., 2017).

PCR products were visualized on ethidium bromide-stained (1 mg ml^{-1}) 0.85% agrose gel. A 100-bp marker was added to help visualize the base pair lengths of each band. The samples were electrophoresed in TBE buffer (10.8 g Tris, 55 g Boric acid, 900 mL, 40 mL 0.5 M

Na_2EDTA [pH 8.0]) at 100 V for approximately one hour. The samples were then photographed under UV light (ThermoFisher Scientific, Waltham, MA). DNA fragment sizes were compared to the 100-bp marker and bands in the 700- to 800-bp range were cut out. RNA extraction and purification were done using a TRIzol Plus RNA Purification Kit and protocol (ThermoFisher, Waltham, MA). Once the RNA was isolated and purified a cDNA synthesis procedure occurred next. A cDNA synthesis reagent was made using ReadyScript cDNA synthesis mix with a final concentration of 1X, RNA template at 2 µg total RNA, and Rnase/Dnase-free water at 12 uL to have a total volume of 20 µL. The reagents were combined on ice then mixed and briefly centrifuged to collect contents at the bottom of the tube. The reagents were then combined in a 96-well plate sitting on ice. It was then briefly centrifuged to collect components at the bottom of the plate and then incubated for 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and held at 4 °C. Once cDNA synthesis occurred, the first strand of cDNA was diluted in RNA-free water at a 1:20 ratio. A 1 µL dilution was used for PCR and 10 µL dilution for PCR amplification. Once the cDNA was amplified, the 700 – 800 bp band was cut out and sent off for sequence analyzation.

P450 Metabolism Experiments

Tall fescue genotypes with confirmed expression of tolerance to pendimethalin herbicides were further evaluated to determine if tolerance could be associated with enhanced metabolism due to P450 activity. The top performing tolerant genotype PR#74 (DNA tolerant) and TCPC (DNA sensitive) were evaluated at two dosage concentrations of pendimethalin 0 and 2 µM and treated and untreated levels of malathion, a known inhibitor of P450 metabolism. Experiment 1 was a split plot design with herbicide as a whole plot factor and genotype and malathion as subplot factors. Experiment 2 was split plot design with tub as a whole plot factor, genotype as a

subplot factor, 6 replications with tub designations 1-4 to give the 2 x 2 factorial of herbicide:malathion effect. Tub 1 is + malathion / + pendimethalin, tub 2 – malathion / + pendimethalin, tub 3 + malathion / - pendimethalin, tub 4 – malathion / - pendimethalin.

The greenhouse hydroponic protocol used 5.4 L plastic tubs filled with half-strength Hoagland basal salt solution H353 (PhytoTech Labs, Lenexa, KS). Twenty holes 6.35 mm in diameter spaced 25 mm apart in a 4 x 5 grid pattern were drilled into each tub lid (McCullough et al., 2017). The hydroponic solution was aerated using an electric aquarium pump. Individual tillers were separated from mature plants with 10 or more tillers growing in the greenhouse. Roots were rinsed with a solution that contained sterilized water and hydrogen peroxide to remove soil and any bacteria present. The tillers/roots were then placed in pipet tips for support before being placed into holes to suspend them in the aerated hydroponic solution. Tillers were allowed to recover in the hydroponic solution for one to two weeks before beginning herbicide treatments. Roots of all tillers were uniformly trimmed to a length of 1.3 cm. Prior to initiation of treatments, individual tillers were submerged in a malathion (Spectracide malathion insect spray concentrate, Middleton, WI) solution at a concentration of 2.4 mL / L and arranged in a split plot design. Once the tillers were treated with malathion, tubs containing either 0 µM or 2 µM pendimethalin concentrations were placed in the cool-season greenhouse at 23 °C and aquarium pumps were used to provide oxygen to the solution (McCullough et al., 2017). The tillers treated with malathion were placed in their own individual tubs containing either 0 µM or 2 µM pendimethalin to decrease malathion carryover to untreated tillers, the same was done to tillers not treated with malathion. Sterilized water was added to the tubs as needed to maintain the 5.4 L volume. After one month in the hydroponic solutions, tillers were removed, and the roots were rinsed under tap water and blotted dry with a paper towel (McCullough et al., 2017). The

maximum length of roots was then measured and each root system given a visual rating and recorded on a scale of 1 to 9 with 9 representing a vigorous, extensive, and healthy root system. Scissors were used to cut the roots of individual tillers back to the initial length of 1.3 cm. Trimmed roots were placed into labeled seed envelopes. The root samples were oven dried for 48 hours at 65 ^{o}C. After drying, root weights were taken and recorded.

P450 Metabolism Experiment Data Analysis

The data was analyzed in R (R Core Team 2024) using a linear mixed affects model using the lmer function from the *lme4* package (Bates et al. 2015). Experiment 1 had fixed effects of genotype, herbicide, and malathion with random effects rep(herbicide). Experiment 2 had fixed effects of genotype:tub (herbicide/malathion treatments) and random effects of run, rep(run), tub(rep(run)). Model assumptions of normally distributed residuals and homoscedasticity were tested and confirmed. We report the results of the models in the ANOVA tables (Table 4.3 and 4.4) with the Kenward-Roger denominator degrees of freedom correction. Eight different contrasts were analyzed to test the following hypotheses 1) herbicide effect for the parent genotype; 2) herbicide effect for PR#74; 3) malathion effect for the parent genotype; 4) malathion effect for PR#74; 5) compares malathion effect with herbicide present for the parent genotype; 6) compares malathion effect with herbicide present for PR#74; 7) compares genotypes when both malathion and herbicide are present; 8) compares genotypes when only herbicide is present. Refer to Table 4.1 and Table 4.2 to see the 8 orthogonal contrasts.

Results

Tall Fescue α-tubulin Gene Amplification

We designed two pairs of primers based on the conserved regions in five grass species α-tubulin genes. These primers were used to amply the fragments with genomic DNA extracted from sensitive line UGA 17G5Z TCPC, and five herbicide tolerant plants PR#17, PR#18, PR#39, PR#49, and PR#74. No PCR fragments were amplified with these primers (Fig. 4.1). Then we extracted mRNA from these plants and generated cDNA from them. These cDNA were used as templates (Fig. 4.2) for PCR and two DNA fragments were amplified (Fig. 4.3). These two fragments were successfully sequenced with good quality sequence readings. Total nucleotides readings were 1053 bp, covering all the previously reported meaningful herbicide resistant mutations (Fig. 4.3).

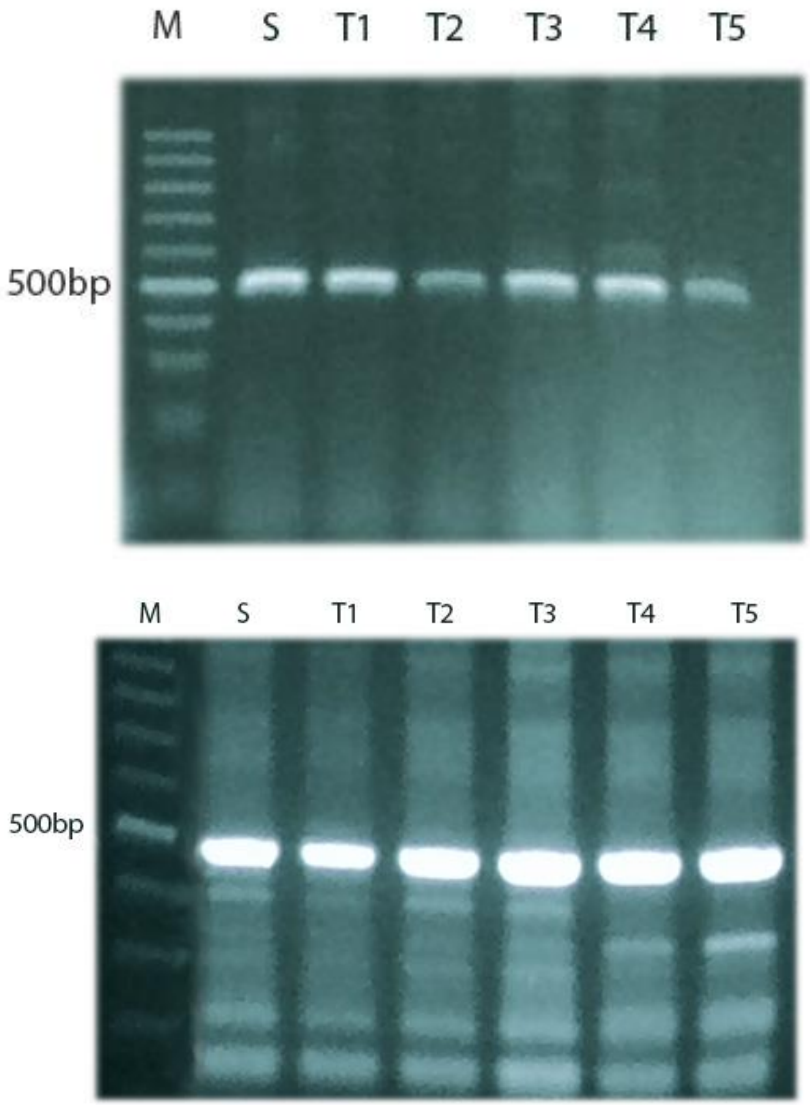

Figure 4.1 PCR Amplification Results from cDNA generated from mRNA. M is A 100 bp ladder, S is herbicide sensitive plant, T1 to T5 are herbicide tolerant plants.

Figure 4.2: Reference Gene Alignment. The reference gene sequence for the α-tubulin gene of five different species. This was used to design primers for generating two DNA fragments that cover the targeted mutation sites in α-tubulin gene. Primer sites are shown with directional arrows.

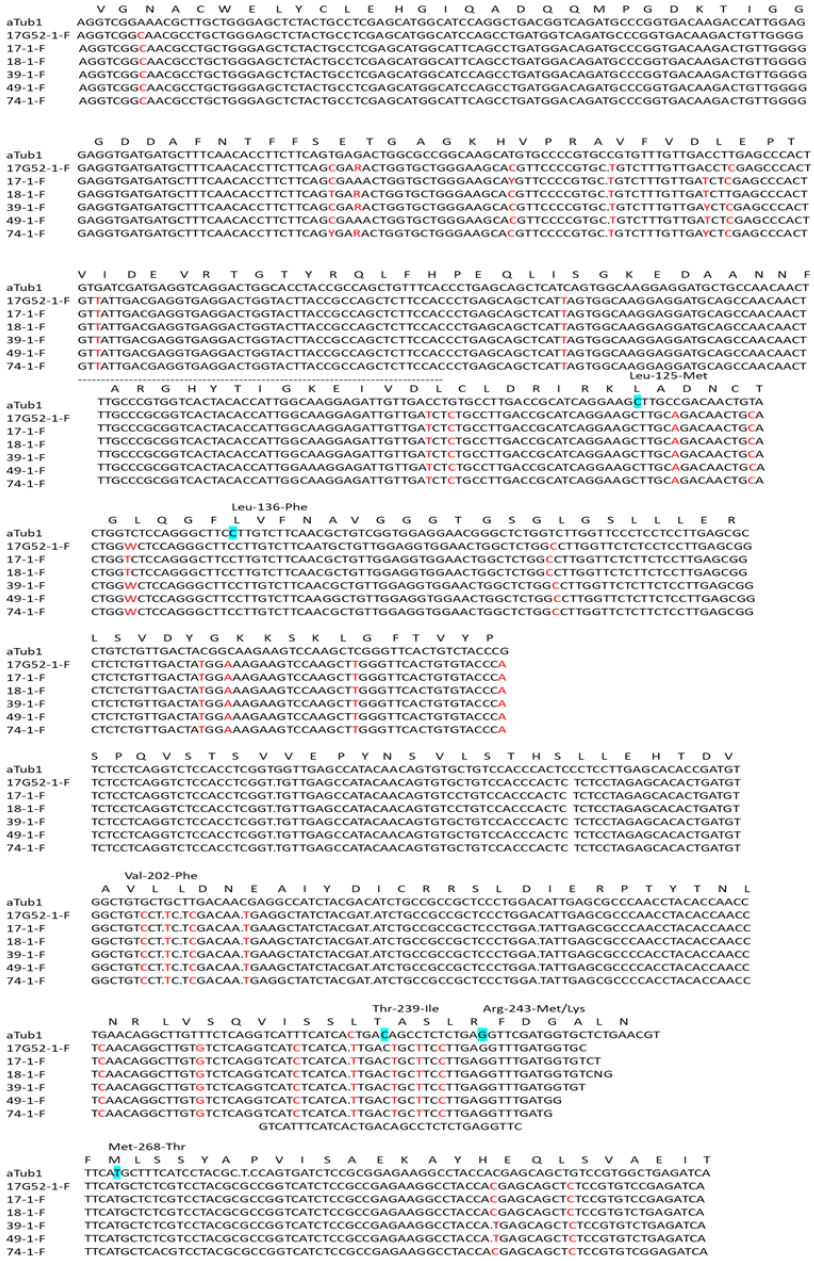

Figure 4.3 cDNA Sequence Data for Mutation Region with known mutations. The top line is the consensus wildtype strand. The second line from the top is the control mRNA strand and the subsequent lines below are designated by their genotype numbers with 17G5Z being the parent. The mutations present in the sequences are indicated in red. The locations of reported mutations are indicated in blue. No known mutations are present within the sequence data.

DNA Alignment

DNA sequence data of 6 tall fescue plants including one tissue culture donor plant, and 5 herbicide tolerant plants were first pre-aligned using Geneious alignment software checking the sequence quality and the alignment among the 6 plants using the default criteria setting (70% similarity, 12 nucleotide gap open penalty, 3 nucleotides gap extension penalty, global alignment with free end gaps). After the pre-alignment, chromatogram files were used to trim the sequence ends and adjust the undefined nucleotides based on the majority sequence information and the chromatogram. Two amplified fragments from cDNA were both sequenced successfully and consistent among the 6 plants with their correspondent forward primers. However, the sequence data with the reverse primers had too many unidentified nucleotides or no sequence data for some plants. So, they are not usable for alignment for further analysis. We used the sequence data from the forward primers only for this investigation since the data quality was high and alignment was consistent for all 6 investigating plants (Fig. 4.3). Sequence alignments for two fragments were then aligned to goosegrass α-tubulin gene sequence as the reference gene sequence. The sequences of the two fragments aligned well with sequences from the goosegrass α-tubulin gene, covering all the previously reported nonsynonymous mutations for tubulin-inhibiting herbicides.

Identifying nonsynonymous sequence mutations in our dinitroaniline resistance tall fescue plants.

Among the 6 tall fescue plants between the tissue culture sensitive plant and 5 tissue culture mutant plants, there were 8 synonymous nucleotides variations. There were 35 synonymous nucleotides mutations between tall fescue and goosegrass α-tubulin genes. We did not find any nonsynonymous mutations between the α-tubulin genes of the tissue culture donor

plant and dinitroaniline tolerant plants, or between tall fescue and goosegrass α-tubulin genes. All the single nucleotides mutations identified were synonymous mutations (Fig. 4.3).

The problems that occurred with DNA extraction forced us to resort to RNA extraction and cDNA synthesis. The success rate for this process was efficient and we were able to synthesize cDNA suitable for sequencing. The sequence results were analyzed using the consensus band for that region of the tall fescue genome with the computer program Genesis. We focused our sequence examination on the 100-codon region of the α-tubulin gene where the known mutations occur. Figure 4.4 shows the sequence alignment that contains the most common mutation Thr-239-Ile. In that mutation the ACA codon is mutated to TCA which causes Ile rather than Thr to be encoded, conferring dinitroaniline resistance. The αTub1mRNA sequence when aligned to the consensus strand showed that there were no changes in the amino acid coding at this position (Fig 4.4). The same region of the sequences for PR#17, 17G5Z, PR#18, PR#39, PR#49, and PR#74 when aligned with the consensus strand showed a change in the amino acid sequence from ACA to ACT. This single nucleotide change is a synonymous mutation and does not change the amino acid encoded since both ACA and ACT encode Thr. Since the amino acid did not change, the protein was also unaffected. Figure 4.4 shows the chromatogram file for the 239-ACT codon, which further supports what the sequence data showed. This further confirmed that a synonymous mutation occurred and that none of the reported target-site point mutations were present in the region of interest. Thus, demonstrating that the resistance was not due to a known mutation in α-tubulin.

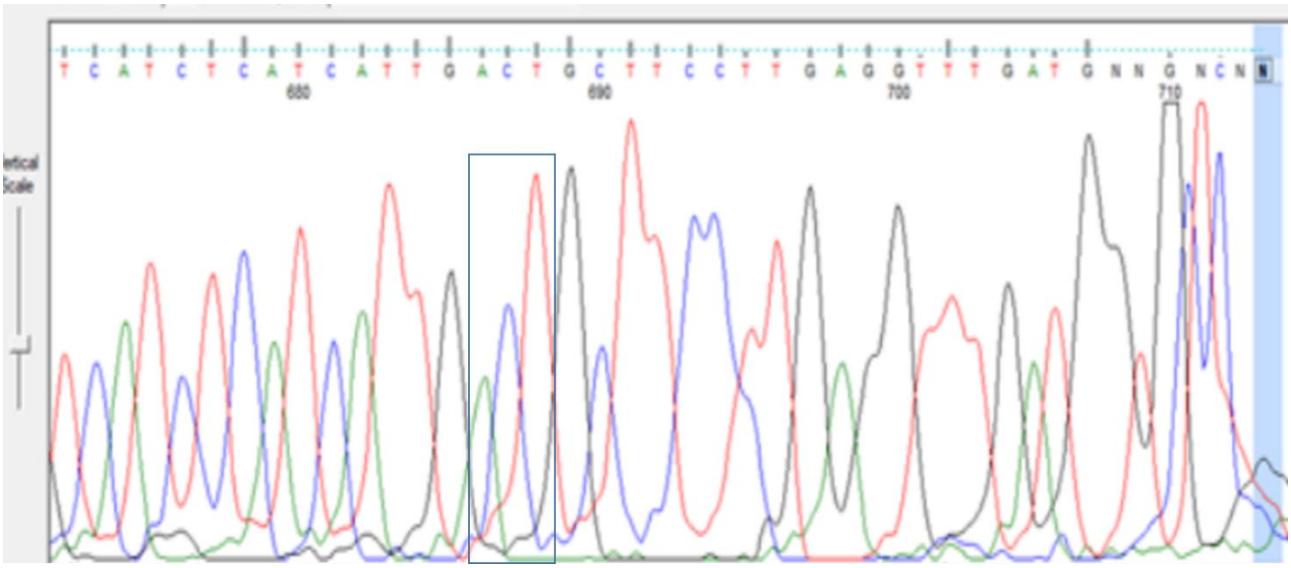

Figure 4.4 Chromatogram of Thr-239-Ile Sequence Region PR#74 with aTub1-2F Primer. The black box shows the codon of ACT for codon Thr.

P450 Metabolism Experiment

In the first experiment conducted, PR#74 without malathion treatment failed to express tolerance to pendimethalin when compared to TCPC (data not shown). Since this design called for both malathion treated and untreated plants to be placed in the same tub we concluded that malathion from treated plants likely was transferred to untreated tillers within the same tub. The experiment was therefore redesigned to minimize the potential for malathion carryover.

The second experiment separated malathion treated and untreated plants into different tubs and showed expression of tolerance in PR#74 consistent with previous pendimethalin experiments performed. Table 4.3 shows that experimental factors genotype, and tub (herbicide/malathion treatment combinations) are statistically significant while genotype:tub showed no statistical significance for root length. The first and second contrast showed the herbicide treatment significantly reduced root length for both the TCPC and PR#74 (to a lesser

extent) indicating an herbicide affect for both genotypes tested (Table 4.1, Fig. 4.5). Both contrasts three and four were not statistically significant indicating malathion in the absence of herbicide had no effect on root length of either PR#74 or TCPC (Table 4.1, Fig. 4.5). Contrast five showed no difference in TCPC root length when both herbicide and malathion were present. Contrast six indicated PR#74 had lower root length when both herbicide and malathion were present compared to when only herbicide was present (Table 4.1, Fig. 4.5) indicating suppression of tolerance expression when plants were treated with the P450 inhibitor, malathion, prior to herbicide exposure. Contrast seven showed no statistical difference in root length between TCPC and PR#74 when both herbicide and malathion were present indicating P450 metabolism as the mechanism for tolerance expressed by PR#74. Contrast eight showed that PR#74 had significantly higher root length than TCPC in the absence of malathion (Table 4.1, Fig. 4.5) and confirmed the tolerance of PR#74 to pendimethalin herbicide.

Table 4.2 shows that experimental factors genotype, tub (herbicide/malathion treatment combinations), and genotype:tub were all statistically significant for visual rating. The first and second contrast showed herbicide treatment significantly reduced visual ratings for both the TCPC and PR#74 (to a lesser extent) indicating an herbicide affect for both genotypes tested (Table 4.2, Fig. 4.6). Both contrasts three and four were not statistically significant indicating malathion in the absence of herbicide had no effect on visual root ratings of either PR#74 or TCPC (Table 4.2, Fig. 4.6). Contrast five showed no difference in TCPC root visual ratings when plants were treated with malathion prior to exposure to herbicide. In contrast six, visual ratings of PR#74 decreased numerically, but not significantly, when treated with malathion prior to exposure to herbicide (Table 4.2, Fig. 4.6).

Contrast seven showed no statistical difference in root visual ratings between TCPC and PR#74 when both herbicide and malathion were present indicating P450 metabolism as the mechanism for tolerance expressed by PR#74. Contrast eight showed that PR#74 had a significantly higher root visual rating than TCPC in the absence of malathion (Table 4.2, Fig. 4.6) and confirmed the improved tolerance of PR#74 to pendimethalin herbicide.

Table 4.1: Eight Orthogonal Contrast for Experiment 2 Root Length.

Contrast	Hypothesis	Orthogonal Contrast	estimate	SE	df	t.ratio	p.value
1	Herbicide: Parent	0.5, 0, 0.5, 0, -0.5, 0, -0.5, 0	-19.322	1.37	28	-14.107	**<0.0001**
2	Herbicide: Resistant	0, 0.5, 0, 0.5, 0, -0.5,0,-0.5	-22.097	1.37	27.9	-16.11	**<0.0001**
3	Malathion: Parent	0.5, 0, -0.5, 0, 0.5, 0, -0.5, 0	0.792	1.37	27.9	0.578	0.5678
4	Malathion: Resistant	0, 0.5, 0, -0.5, 0, 0.5, 0, -0.5	-1.184	1.37	27.7	-0.864	0.395
5	Tub 1-2: Parent	1, 0, -1, 0, 0, 0, 0, 0	-1.706	1.97	29.6	-0.867	0.3927
6	Tub 1-2: Resistant	0, 1, 0, -1, 0, 0, 0, 0	-4.813	1.96	28.8	-2.45	**0.0206**
7	Tub 1: Genotype	1, -1, 0, 0, 0, 0, 0, 0	-0.457	1.46	328.7	-0.312	0.7551
8	Tub 2: Genotype	0, 0, 1, -1, 0, 0, 0, 0	-3.564	0.146	327.1	-2.442	**0.0151**

1. Bolded numbers indicate statistical difference.

Table 4.2: Eight Orthogonal Contrast for Experiment 2 Visual Rating.

Contrast	Hypothesis	Orthogonal Contrast	estimate	SE	df	t.ratio	p.value
1	Herbicide: Parent	0.5, 0, 0.5, 0, -0.5, 0, -0.5, 0	-2.2201	0.481	19.4	-4.616	**0.0002**
2	Herbicide: Resistant	0, 0.5, 0, 0.5, 0, -0.5,0,-0.5	-1.2743	0.481	19.5	-2.647	**0.0157**
3	Malathion: Parent	0.5, 0, -0.5, 0, 0.5, 0, -0.5, 0	0.0946	0.481	19.4	0.197	0.8461
4	Malathion: Resistant	0, 0.5, 0, -0.5, 0, 0.5, 0, -0.5	-0.3535	0.481	19.5	-0.734	0.4714
5	Tub 1-2: Parent	1, 0, -1, 0, 0, 0, 0, 0	-0.4244	0.685	20	-0.62	0.5426
6	Tub 1-2: Resistant	0, 1, 0, -1, 0, 0, 0, 0	-0.9678	0.685	19.9	-1.412	0.1733
7	Tub 1: Genotype	1, -1, 0, 0, 0, 0, 0, 0	-0.6239	0.347	326.7	-1.799	0.0729
8	Tub 2: Genotype	0, 0, 1, -1, 0, 0, 0, 0	-1.1673	0.3461	325.9	-3.376	**0.0008**

1. Bolded numbers indicate statistical difference.

Table 4.3: Summary of visual ratings from P450 metabolism Experiment 2 showing Type III analysis of variance table with Kenward Roger's method.

Root Length	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif.
Genotype	1009.5	1009.5	1	326.55	22.7299	2.8127E-06	***
Tub	14205.1	4735	3	14.94	106.617	2.589E-10	***
Genotype:Tub	276	92	3	326.74	2.0717	0.1038	

1. Significance codes are : '***'=0.001, '**'=0.01, and '*'=0.05.

Table 4.4: Summary of visual ratings from P450 metabolism Experiment 2 showing Type III analysis of variance table with Kenward Roger's method.

Visual Data	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	Signif.
Genotype	15.614	15.6139	1	325.67	6.2749	0.012734	*
Tub	41.534	13.8447	3	14.99	5.5639	0.009061	**
Genotype:Tub	24.205	8.0683	3	325.78	3.2425	0.022294	*

1. Significance codes are : '***'=0.001, '**'=0.01, and '*'=0.05

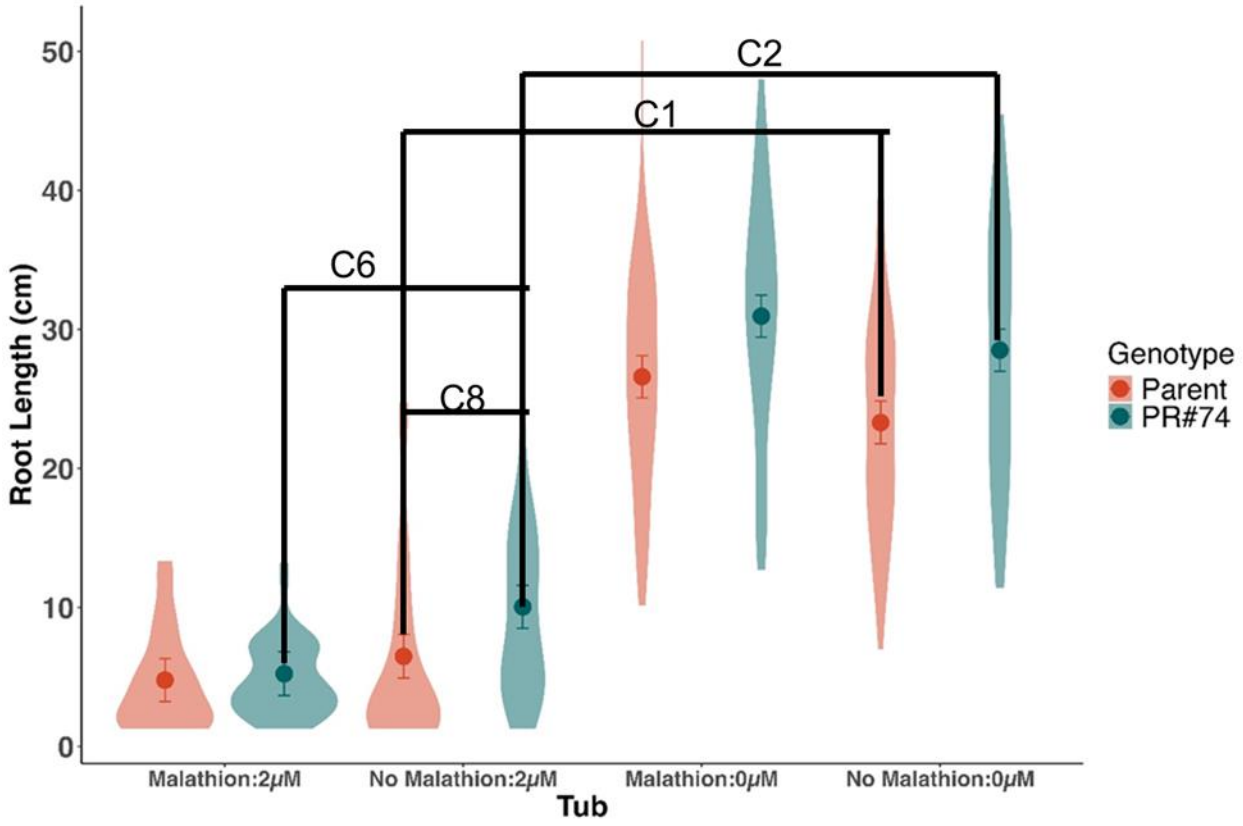

Figure 4.5: P450 Metabolism Experiment 2 results of Eight Orthogonal Contrast in a Violin Plot Design Showing Root Length. Refer to Table 4.1 in combination to determine the level of significance.

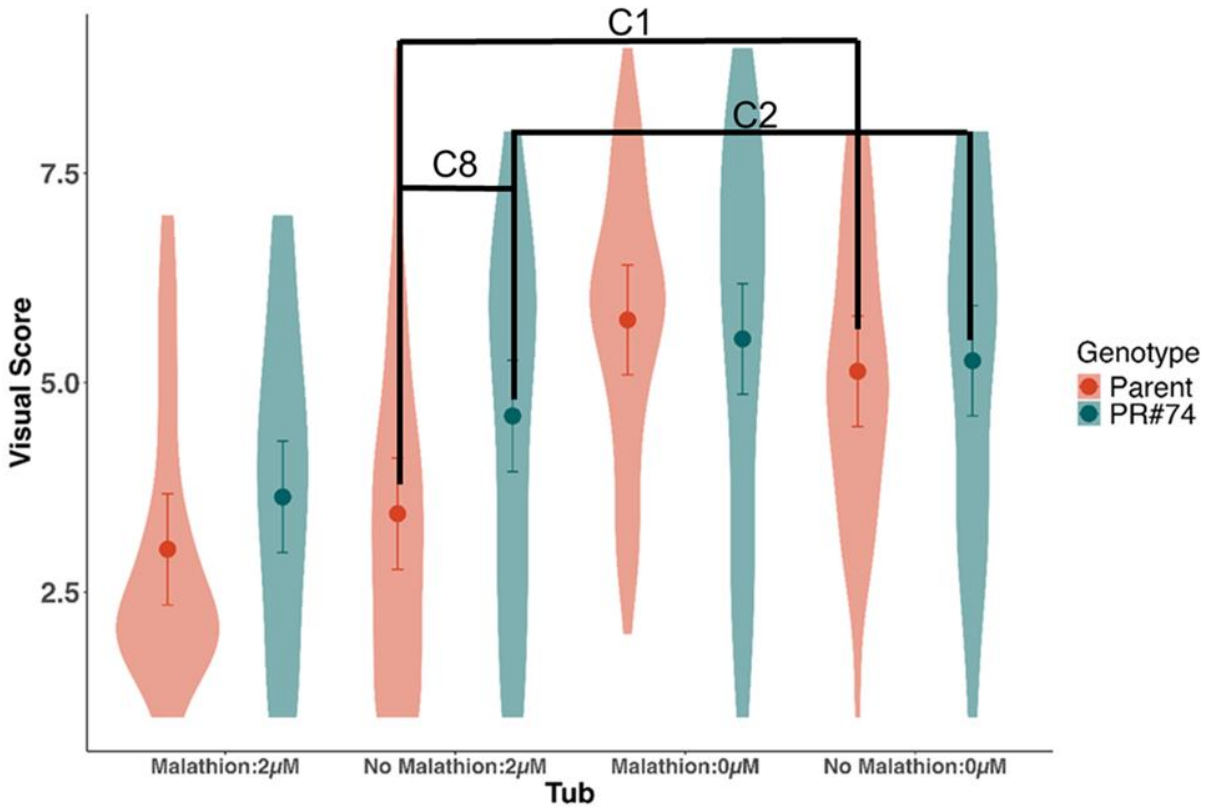

Figure 4.6: P450 Metabolism Experiment 2 of Eight Orthogonal Contrast in a Violin Plot Design Showing Visual Rating. Refer to Table 4.2 to combination to determine significance.

CHAPTER 5

SUMMARY AND DISUCCSION

Tissue culture can alter cell developmental processes and stimulate cell growth and division increasing the chance of genetic changes or mutations. Mistakes occur when DNA duplicates itself during cell divisions even with a self-repairing system. If we let cells keep growing during tissue culture, random mutations accumulate and improves the chances that the trait of interest will occur. Similar research utilized tissue culture to generate and successfully selected for a mutation conferring sethoxydom resistance in seashore paspalum (Heckart et al., 2009).

In this research, tall fescue calli were generated and exposed to selection cycles on 3 µM pendimethalin medium. A total of 140 plants were regenerated from pendimethalin tolerant callus and hydroponically screened at 2 µM pendimethalin to verify expression of improved tolerance to pendimethalin. Seventeen of the 51 putative tolerant lines evaluated demonstrated better rooting than the parental control. Five genotypes exhibiting high levels of pendimethalin tolerance during the hydroponics experiments were selected for α-tubulin gene sequencing. There were no nonsynonymous mutations identified in the targeted sites in 1054bp region we sequenced. No consistent mutations were identified in this region in 5 tolerant plants we sequenced. The α-tubulin gene has about 1400bp, and we covered 1054bp. Further sequencing studies should to be done to determine if there are any consistent mutations residing in the remaining unsequenced 400bp region in these 5 tolerant plants.

Herbicide tolerance in the tall fescue line investigated in this experiment could have been hiding in this genetically diverse population. The seeds we used in the project were from a polycross where each seed can be genetically unique and as a result, calli induced from these seeds likely will be genetically diverse. After five or more rounds of callus propagation, the genetic composition for each cell line consists of the original genetic makeup from the original seed plus any mutations accumulated during tissue culture. When these calli were placed on herbicide selection medium, the majority of calli could not grow and the tolerant recombinant genotypes were exposed and stood out. The tolerance trait observed within regenerated plants could be due to a mutation arising during tissue culture or could have resided unnoticed within the original seeded population due to its low frequency. Research has shown that resistant individuals are naturally present at extremely low frequencies in a given population of a weed species (Murphy, 1996). Continued application of the same herbicide over years controls the susceptible biotypes but allows the population of resistant biotypes to increase. The selection pressure exerted by the herbicide is analogous to a plant breeder selecting biotypes that are resistant (or more commonly tolerant) to various types of imposed selection stresses (drought, mowing height, diseases, insects, etc.). A common end result of continued herbicide use is an herbicide-resistant population of weeds if resistant individuals are naturally present on site.

A 1054 bp region of the α-tubulin gene of pendimethalin tolerant plants and one sensitive plant was examined. The sequenced region covered all the 6 previously reported mutation sites related to pendimethalin tolerance. Gene sequencing revealed substantial differences among the herbicide sensitive plant and tolerant plants. There are 6 single nucleotide polymorphisms in the 1054bp region, which counts for 0.57% genetic diversity in exonic region. In mungbean, 6.26 %

SNPs resided within the exonic region and most of the SNPs were located in intergenic and intronic regions (45.30 and 48.43 % respectively) (Keyuan Jiao at al., 2016).

Pendimethalin tolerance in plants can also be associated with enhanced herbicide metabolism catalyzed by glutathione transferases and cytochromes P450 in plants such as black-grass (*Alopecurus myosuroides*). In black-grass, herbicides were metabolized and detoxified by cytochromes P450 and glutathione transferases. Black-grass showed increased levels of *Am*GSTF1, a protein functionally linked to enhanced herbicide metabolism, in herbicide tolerant plants compared to herbicide sensitive plants. In wild grasses, CYPs are involved in the initial metabolism and inactivation of herbicides acting on ACCase (Kreuz and Fonné-Pfister, 1992; Ahmad-Hamdani et al., 2013), ALS (Iwakami et al., 2014a, 2019), and other modes of action (Evans et al., 2017).

Cytochrome P450 monooxygenases play a major role in phase 1 herbicide metabolism (Siminszky, 2006). P450 enyzmes have been implicated in herbicide tolerance to herbicides that have different structures and modes of action (Hall et al., 1994). Metabolic herbicide resistance has been detected by using malathion to suppress P450 metabolism. The result is enhanced injury to resistant biotypes when herbicide is applied. In some studies that examined chlorsulfuron resistant *L. rigidum*, it was found that resistant plants treated with malathion in combination with chlorsulfuron had increased mortality rates when compared to the herbicide treatment only (Yu et al., 2009). These results indicate that malathion can reverse chlorsulfuron resistance (Yu et al., 2009).

Since none of the reported dinitroaniline resistance mutations were found and no consistent mutations were found in the sequence data, the next step was to determine if the tolerance was possibly metabolism based. The best performing tolerant line, PR#74, and TCPC

were compared in a metabolism experiment to determine if malathion treatment altered the expression of pendimethalin tolerance in PR#74. Pre-treatment of PR#74 with malathion, a known inhibitor of P450 metabolism, inhibited the expression of tolerance previously demonstrated by PR#74 in prior experiments (Table 4.1, Fig. 4.5). These data indicate the pendimethalin tolerance observed in PR#74 is likely due to P450 metabolism. While the exact metabolic pathway of P450 resistance in this case is unknown, the data supports the claim that the tolerance shown by PR#74 is metabolism based.

Further evaluation and research are needed to determine what P450 pathway is leading to this metabolic resistance of pendimethalin. Additional research should also evaluate PR#74's cross resistance to other DNA herbicides along with evaluating if malathion can inhibit any tolerance that might be shown. Inferences can be made based on literature that PR#74 could show cross-resistance to other DNA herbicides and that malathion could inhibit any tolerance that might be displayed (Hall et al., 1994; Kaspar et al., 2011). Based on the results from our metabolism experiment, it was determined that PR#74's tolerance was not due to a mutation near the active binding site but rather was endowed by metabolic resistance via the P450 pathway.

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