HERBICIDE SAFENERS: UPREGULATING DETOXIFICATION MECHANISMS FOR SELECTIVE WEED MANAGEMENT IN GRAIN SORGHUM (SORGHUM BICOLOR L. MOENCH)

BY
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THESIS
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Grain sorghum (*Sorghum bicolor* L. Moench) is one of the world’s most important crops based on area sown and production. Sorghum utilizes C₄ photosynthesis and is one of the most efficient crops for water usage and solar energy conversion. It is grown for human consumption, animal feed, and fuel worldwide. Originating in the northeast Sahel region of Africa, the wide range of sorghum cultivation can be attributed to its tolerance to both high heat and drought conditions. It is the third largest cereal grain grown in the United States with acreage in the U.S. increasing over 20% from 2014.

Controlling weeds selectively is one of the most significant challenges when producing grain sorghum. Growers are restricted to preemergence (PRE) and post emergence (POST) herbicides that typically target broadleaf weeds, but options for controlling grasses are limited. Due to the high potential of gene flow from sorghum to wild and weedy relatives, the use of transgenes to confer herbicide selectivity in grain sorghum is limited. In order to achieve herbicide selectivity, seed-applied herbicide safeners are frequently used with herbicides that normally cause injury in unsafened grain sorghum. The use of herbicide safeners increases the range of herbicides that can be used in grain sorghum to achieve weed control. A current seed safener marketed for sorghum is fluxofenim (Concep® III; Syngenta Crop Protection, LLC.), which was first introduced in 1979. Fluxofenim is typically applied as a seed treatment to avoid safening weedy *Sorghum* relatives. Safeners confer protection to cereal crops by inducing herbicide detoxification and defense systems. This includes massive increases in the expression and activity of glutathione S-transferases (GSTs) and cytochrome P450s, although the precise molecular mechanism of action and signaling pathways remain unknown.
Chapter 1 of this thesis includes a literature review of grain sorghum as a model crop, common PRE herbicides used in grain sorghum with a specific focus on very-long-chain fatty acid (VLCFA)-inhibiting herbicides, a section on the history and use of herbicide safeners in cereals, and safener-mediated herbicide metabolism in plants. Chapter 2 covers a genome-wide association study conducted to identify key molecular-genetic factors involved in the safener-induced detoxification pathway. Diverse sorghum inbred lines (761) were evaluated for their responses to seed-applied safener and PRE herbicide applications. Data analysis revealed that the molecular marker most significantly associated with safener-induced response was located on chromosome 9, where a single nucleotide polymorphism (SNP) was located within a phi-class \(SbGST\) gene and about 15 kb from a different phi-class \(SbGST\). Transcript levels of these two candidate \(SbGSTs\) were quantified in etiolated shoot tissues using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) and gene-specific primers designed from each \(SbGST\) coding region. Basal and safener-induced expression of these \(SbGSTs\) was examined in three sorghum genotypes at 4, 8, and 12 hrs after treatment (HAT) to quantify safener induction of these genes relative to three stably expressed reference genes: \(GTPB\), \(SAND\), and \(EIF4a\). Results indicated expression of each \(SbGST\) gene increased within 12 hr following safener treatment but differed by specific gene and genotype, suggesting these \(SbGSTs\) play a functional role in the safening response from herbicides. Chapter 3 provides applied information on the use of fluxofenim as herbicide safener for hybrid grain sorghum when used with the PRE herbicide pyroxasulfone (Zidua®), a VLCFA inhibitor. A greenhouse study was first conducted to understand how pyroxasulfone affects grain sorghum emergence and seedling growth under controlled conditions, and to determine the efficacy of fluxofenim in protecting sorghum seedlings from pyroxasulfone. Using the data from the greenhouse, a field study was designed to
evaluate the protective ability of seed-applied fluxofenim from pyroxsulfone at single and split application times. Weed control, crop injury, stand count, and yield data were assessed to compare the effects of pyroxsulfone to S-metolachlor (Dual Magnum®; a different VLCFA inhibitor) applied PRE in grain sorghum, with or without fluxofenim. Results indicated that pyroxsulfone provides greater weed control compared to S-metolachlor. However, as weed control increases, crop injury also increases regardless of safener. This finding demonstrates that a herbicide safener tailored towards enhancing the ability of grain sorghum to metabolize pyroxsulfone is needed for future use of this product under field conditions. Chapter 4 summarizes the discussion and conclusions from Chapters 2 and 3 and identifies current limitations and future research goals for utilizing seed-applied safeners in grain sorghum.
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CHAPTER 1

LITERATURE REVIEW

1.1 Grain Sorghum (Sorghum bicolor L. Moench) as a Model Crop

Grain sorghum (Sorghum bicolor L. Moench) is one of the top five cereal crops in the world based on area sown and production. It is the third largest cereal grain grown in the United States with acreage in the U.S. increasing over 20% from 2014 (Anonymous, 2016). Sorghum is used as both food and animal feed, and a staple for the diet of over 500 million of the world’s poorest people. Sorghum originates from the northeast Sahel region of Africa. Due to its domestication in this hot, dry climate, sorghum is well adapted to heat and drought (Wendorf et al., 1992). Sorghum is one of the most versatile plants from the saccharine (C₄) plant family, providing food, fuel, sugar, and cellulosic biofuel to the world’s population (Paterson, 2008). Sorghum has a small diploid genome (~730 Mbp) and ample phenotypic diversity, which makes it an ideal C₄ grass model as a complement to C₃ rice. Sorghum breeding systems have increased during the years due to the high level of inbreeding and genetic variation, two traits desired by breeding programs (Paterson et al., 2009; Mace et al., 2013). Sorghum has become a model system for the study of traits important to perennial cellulosic biomass crops such as sugarcane and Miscanthus (Paterson et al., 2009), as well as for traits directed towards improving stress tolerance in cereal crops (Borrell et al., 2014).

Genetic diversity in sorghum provides multiple genotypes possessing agronomically important traits, including stay-green drought resistance, insect resistance, and increased grain size and quality (Mace et al., 2013). The main trait of interest that sorghum possesses is the ability to tolerate drought conditions, which is especially advantageous in the dry, arid climate found in northeast Africa (Paterson et al., 2009). This trait, including others such as partitioning
of carbon into sugar stores and tillering, is being investigated through whole-genome sequencing of sorghum as well as in related species (Mace et al., 2013, Xu et al. 2014). Sequencing and genome annotation allow for not only strengthened sorghum improvement, but improvement of other cereal crops as well. Increases in drought tolerance will benefit many arid regions where cereal crops like sorghum, rice, and maize are staples. Human populations within regions dependent on sorghum cultivation are increasing by 2.8% per year. Unfortunately, increasing sorghum yield has been difficult, and sorghum yields have lagged behind that of other cereal grains such as maize and rice (Paterson et al., 2009).

One reason for the lack of yield increases in sorghum compared to other crops, such as maize (Zea mays) and soybeans (Glycine max), is the limited availability of genetic improvement through transgenic means. Although bidirectional gene flow is not unique among major field crops (Ellstrand et al., 1999), sorghum is the only major cereal crop where the wild, weedy relatives followed the cultivated species by inadvertent introduction from Africa into the Americas, Asia and Australia. As compared to low risk crops such as maize and rice, sorghum is a high risk crop with gene flow occurring almost everywhere sorghum is cultivated (Paterson et al., 1995). Plant ecologists and population geneticists have investigated problems associated with traditionally improved crops to anticipate possible risks of transgenic crops such as sorghum. The main problems identified include: crop-to-wild relative hybridization, which results in the evolution of increased weediness in wild relatives, evolution of pests that are resistant to new control strategies, and the effects on non-target species in associated ecosystems (Ellstrand, 2001). Sorghum and its weedy relative Johnsongrass (Sorghum halepense) tend to spontaneously hybridize, and unfortunately, due to the high volumes of gene flow between sorghum and Johnsongrass or shattercane (Sorghum bicolor ssp. drummondii), transgenic improvement
approaches are not useful (Morrell et al., 2005; Sagnard et al., 2011).

In the United States, shattercane has developed resistance to acetyl-CoA carboxylase (ACCase) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. Johnsongrass has developed resistance to acetolactate synthase (ALS) inhibitors and the EPSP synthase inhibitor glyphosate. 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It is critical for producers to rethink their weed management strategies to target these tough-to-control weed species in grain sorghum. For the past two decades producers have mainly been focused on controlling weeds with either single or sequential applications of POST herbicides, but this can no longer be the case. Weed management programs need to employ effective soil-applied preemergence (PRE) herbicides with residual activity in sequential applications, as well as POST herbicide tank mixes (Steckel et al., 2002; Evans et al., 2015) to optimize weed control and minimize the risk of continued resistance development and spread.

1.2 Utility of Very-Long-Chain Fatty Acid Inhibitors to Control Weeds in Grain Sorghum

The most commonly used herbicides for PRE weed control in sorghum are (1) photosystem II (PSII) inhibitors, such as atrazine and bromoxynil, (2) very-long-chain fatty acid (VLCFA)-inhibitors, such as S-metolachlor, and (3) protoporphyrinogen oxidase (PPO) inhibitors, such as saflufenacil (Anonymous, 2017). Heavy reliance on atrazine led to widespread development of numerous PSII-resistant weed species (Hess, 2000). Currently, 74 weed species worldwide have developed resistance to PSII-inhibiting herbicides, and ten different weed species have confirmed resistance to the PPO inhibitors. However, only five weed species have confirmed resistance to the VLCFA inhibitors and only one of these, Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), has been reported in the United States (Heap, 2017).

VLCFA are fatty acids with more than 18 carbons, including C20, C22 and C24 VLCFAs. These fatty acids are synthesized in the endoplasmic reticulum through a four-step reaction, resulting in sequential C2 additions to C18 fatty acid substrates, and involve VLCFA elongase enzymes and malonyl-CoA. The elongase activity is critical for the formation of VLCFAs. VLCFA-inhibiting herbicides (K3/Group 15) act within the plant to disrupt the elongase complex by inhibiting the VLCFA synthesis ‘condensing’ enzyme that catalyzes the
first step of VLCFA synthesis (Böger, 2003), where the fatty acyl substrate binds to a critical cysteine residue within the active site. Since VLCFAs are involved in lipid and wax biosynthesis, their inhibition leads to the disruption of plant cuticles and cell membranes needed for actively dividing cells in emerging seedlings (Böger, 2003). Almost 50 years of research contributed to the discovery of the site and mode of action (MoA) of VLCFA herbicides, which was confounded by reports of numerous secondary biochemical and physiological changes downstream to the application of VLCFAs (Jaworski, 1956; Weisshaar and Böger, 1987; Böger, 1997). Early reports linked VLCFA herbicides to lipid biosynthesis in general (Mann and Pu, 1968; Couderchet and Böger, 1993), but were not readdressed until pioneering research led by Dr. Peter Böger, University of Konstanz, Germany (Böger et al., 2000).

One family of VLCFA inhibitors is the chloroacetamides. Herbicides in this family include: S-metholachlor, dimethenamid-p, alachlor, and acetochlor. All are mainly used as soil-applied PRE herbicides and provide residual activity for weed control. Chloroacetamides are effective in controlling annual grass weeds and perennial grass seedlings (grown from seed only), as well as certain small-seeded broadleaves and yellow nutsedge. These herbicides are absorbed through the germinating root and shoot of the weed. Seeds will germinate, but the seedlings either do not emerge from the soil or they emerge but exhibit abnormal growth (Gronwald, 1991). The most common symptom observed is known as “buggy whipping.” Leaves of the emerging seedlings are distorted and fail to emerge from the whorl resulting in a bent, whip-like appearance. Crops such as sorghum and maize avoid injury from these herbicides by detoxifying the active ingredient through rapid glutathione conjugation (Breaux, 1987; Carringer et al., 1987) catalyzed by glutathione S-transferase enzymes (GSTs). To further protect cereal
crop seedlings from injury, herbicide safeners are often applied to the crop that induce GST activity with chloroacetamide herbicide substrates (Hatzios and Hoagland, 1989).

### 1.3 Pyroxasulfone – A Very-Long-Chain Fatty Acid Inhibitor with Unique Properties

A relatively new and novel VLCFA herbicide is pyroxasulfone, with the trade name Zidua® (BASF SE). Pyroxasulfone falls under a different chemical sub-family than the chloroacetamides, and it is considered a pyrazole. However, the MoA of pyroxasulfone is inhibition of the elongation (i.e., condensation step) of VLCFA synthesis occurring in the endoplasmic reticulum, similar to that of the chloroacetamide herbicides. This MoA results in growth deficiencies of the apical meristem and coleoptile, preventing shoot elongation of sensitive species (Tanetani et al., 2009).

Pyroxasulfone was specifically developed for high, stable herbicidal activity, meaning it controls weeds at low application rates and remains active in the soil for a longer period of time with less chance of leaching. For example, pyroxasulfone exhibits herbicidal activity at relatively lower application rates (100–250 g ai ha⁻¹) compared to S-metolachlor (1400 g ai ha⁻¹) (Tanetani et al., 2009). Pyroxasulfone has also demonstrated longer soil residual activity as compared to S-metolachlor, and is less likely to leach through the soil due to its relatively low solubility in water (3.5 mg L⁻¹) and low log P value (2.4) than S-metolachlor (488 mg L⁻¹ and log P 794, respectively). Pyroxasulfone is hydrolytically stable at 25 °C at all pH values, and is therefore less susceptible to decomposition (Shaner, 2014; Nakatani et al., 2016). Several studies demonstrated that PRE applications of pyroxasulfone provide excellent weed control of many broadleaf and grass weeds, often providing effective weed control of a more diverse weed spectrum as compared to S-metolachlor (Steele et al., 2005; Geier et al., 2006; King and Garcia, 2008; Nurse et al., 2011).
Pyroxasulfone is labeled PRE for use in maize (Knezevic et al., 2009), wheat (*Triticum aestivum*) (Walsh et al., 2011), and soybeans (Ulloa and Owen, 2009). Due to the increasing weed pressure from Palmer amaranth and other multiple-resistant weed species, there has been a push for additional crops to be added to the pyroxasulfone label (Doherty et al., 2014). Pyroxasulfone is not currently labeled for use in grain sorghum. However, it is common for herbicides currently registered for use in sorghum to have originally been developed for use in larger cereal crop markets, such as maize or rice (Stahlman and Wicks, 2000).

The need for new, effective herbicides for use in sorghum fields is evident, since current herbicides labeled for sorghum often provide unacceptable weed control. However, field studies using pyroxasulfone PRE in sorghum have yielded positive results for enhanced weed control relative to existing herbicide options, but have also shown greater crop injury compared to S-metolachlor (Geier et al., 2009). It is clear that more testing of pyroxasulfone in sorghum needs to be conducted to determine if crop injury can be overcome through the use of herbicide safeners and/or traditional plant breeding for tolerant varieties.

1.4 History and Use of Herbicide Safeners

Plants contain many different defense and detoxification enzymes and protective phytochemicals that are able to overcome a diverse set of abiotic stresses, including herbicide applications (Kreuz et al., 1996). Many crop lines are chosen based on their ability to naturally metabolize and detoxify herbicides. In addition, safening chemicals may be seed-applied or tanked mixed to increase rates of herbicide metabolism, specifically in cereal crops. This strategy is used frequently with PRE herbicides such as S-metolachlor when applied to grass crops such as maize, wheat, rice, and grain sorghum (Hatzios and Burgos, 2004; Riechers et al., 2010).
In general, herbicide safeners are defined as a chemical treatment, applied either directly to the seed or included in a tank mix, which selectively protects monocot crop plants from herbicide damage without reducing herbicide activity in target weed species (Hatzios and Hoagland, 1989). With environmental and economic pressures to minimize herbicide usage on the rise, the major role of herbicide safeners is to extend the use patterns of currently available herbicides (Yu and Powles, 2014). Herbicide safeners allow for the development of molecules with favorable toxicological profiles, but whose use would otherwise be limited by poor selectivity (Kreuz et al., 1996). In addition, safeners have been used to address difficult weed control problems that are unlikely to be solved by the development of conventional selective herbicides due to technical and economic factors (Davies and Caseley, 1999). For example, herbicide safeners may facilitate the selective control of weeds in genetically related crops (Jablonkai, 2013), such as, controlling Johnsongrass or shattercane in grain sorghum or jointed goatgrass (*Aegilops cylindrica*) in winter wheat.

Herbicide safeners, originally known as adsorbents, were initially used to physically shield the crop seed or plant from contact with a herbicide that would otherwise cause injury. An array of chemicals can be used as herbicide safeners. Initially activated carbon, lignin by-products, ion exchange resins, and various clays were used to achieve crop safety from certain chemicals. However, these applications, expense, and inadequate crop response and weed control resulted in searches for a better alternative (Hatzios and Hoagland, 1989). Low doses of herbicides (Rosinger et al., 2012), insecticides (Anonymous, 2013), or microbial inhibitors (Tam et al., 1988) also act as herbicide safeners in certain situations. Years of pioneering research by Otto Hoffmann led to the introduction of naphthalic anhydride as the first commercial safener in 1969 against thiocarbamate herbicides in maize (Hoffmann, 1969). The research in this field
rapidly progressed during the next two decades and a large number of chemicals were screened and found to be potential safeners (Abu-Qare and Duncan, 2002). It is important in the development of commercial safeners that they safen only the crop and not the weeds the herbicide is targeting; this is especially true when considering herbicide safeners that are applied as POST tank mixes with herbicides. In recent years, newer herbicide safeners have been developed to be more crop-specific rather than herbicide-specific in order to take advantage of unique metabolic interactions within the crop species (Rosinger, 2015).

Many safeners have been commercialized and are used for the protection of large-seeded grass crops, such as maize, grain sorghum, and wet-sown rice, against preplant-incorporated (PPI) or PRE herbicides of the thiocarbamate and chloroacetamide families. Safeners also have been developed to protect winter cereal crops, such as wheat, against POST applications of aryloxyphenoxypropionate (ACCase) and sulfonylurea (ALS) herbicides. The use of safeners for protection of maize and rice against ALS inhibitors, ACCase inhibitors, 4-hydroxyphenylpyruvate oxidase (HPPD) inhibitors, and synthetic auxin herbicides is also well established (Hatzios and Burgos, 2004).

As mentioned above, herbicide safeners are commonly used in sorghum in conjunction with chloroacetamide herbicides to avoid injury to the crop. Safeners developed specifically for sorghum to protect from PRE herbicides include, but are not limited to: cyometrinil (Concep® I), oxabetrinil (Concep® II), fluxofenim (Concep® III), and flurazole (Screen®) (Devlin et al., 1983; Hwang et al., 1998). Safeners developed for maize that have also been tested in sorghum include, but are not limited to: naphthalic anhydride, dichlormid, and benoxacor (Hirase and Molin, 2001). Thus far, fluxofenim has been the only herbicide safener tested for protection of sorghum seedlings from the VLCFA inhibitor pyroxasulfone (Geier et al., 2009), so it is unclear
if other herbicide safeners would provide enhanced protection from this herbicide.

1.5 Safener-Induced Herbicide Metabolism in Plants

It is well documented how safeners work from a whole-plant standpoint because the phenotype of plants treated with safener plus herbicide is evident: a safened plant treated with herbicide will grow unharmed while an unsafened plant treated with herbicide will die. Information regarding how safeners work from a biochemical standpoint is also abundant (Davies and Caseley, 1999; Hatzois and Burgos, 2004; Jablonkai, 2013). However, little is known about the precise mechanisms of how safeners work within the plant to increase metabolic detoxification. Not all safeners act in the same manner, and many theories have been proposed for safener action (Hatzios, 1991; Gaillard et al., 1994; Hatzios and Wu, 1996; Davies and Caseley, 1999; Hatzios and Burgos, 2004; Riechers et al., 2010), as summarized below.

Safeners function by reducing the ability of herbicides to reach and inhibit their target sites. This may be achieved through safener interactions with herbicide target sites or other receptor proteins involved in herbicide activity. Alternatively, safeners may reduce the amount of herbicide reaching its target in an active form, either by the direct chemical reaction of the safener with the herbicide molecule or by safener-induced reductions in herbicide uptake or translocation. Herbicide safeners may also act through safener-enhanced metabolism of herbicides to less active or immobile metabolites (Hatzios, 1991; Hatzios and Wu, 1996; Davies and Caseley, 1999). However, safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action (Hatzios and Burgos, 2004). Safeners increase levels of cellular antioxidants such as reduced glutathione (GSH) and induce the expression/activity of herbicide-detoxifying enzymes such as glutathione S-transferases (GSTs), cytochrome P450 monooxygenases (P450s), and UDP-
dependent glucosyl transferases (uGTs). In addition, safeners enhance the vacuolar transport of GSH-herbicide or glucose-herbicide conjugates (via ABC transporters in the tonoplast) as part of an overall three-phase detoxification system in plants (Pang, 2012). The safener-mediated induction of herbicide-detoxifying enzymes appears to be part of a general abiotic stress response within the plant (Dean et al., 1990; Farago et al., 1994; Hatzios and Burgos, 2004).

Recently, in-depth descriptions and hypotheses for the mechanism(s) behind safener-regulated defense and detoxification reactions have been proposed, including the hypothesis that safeners coordinately induce the expression of numerous genes involved in plant defense and detoxification, such as GSTs and P450s, in a tissue-specific manner in cereal crop seedlings (Riechers et al., 2003, 2010; Cummins et al., 2011; Skipsey et al., 2011). There are five main classes of GSTs found in plants, of which tau- and phi-class GSTs are the most abundant in the plant kingdom (Frova, 2003; Chi et al., 2011). Both classes have been shown to be closely linked to stress responses, and highly responsive to stimuli such as oxidative stress and herbicide applications (Cummins et al., 2011). Studies have also shown that these GSTs play an important role in weed resistance (Cummins et al., 2013; Evans et al., 2017) and detoxification of herbicides (Pang et al., 2012; Yu and Powles, 2014). GSTs catalyze the formation of GSH-conjugates, a key reaction in what is known as Phase II detoxification (Riechers et al., 2010). In sorghum, specific safener-induced phi-class GSTs isozymes have been identified that exhibit activity with the herbicide metolachlor (Gronwald and Plaisance, 1998), and N-terminal sequences were obtained for three distinct GST subunits.

The main location of herbicide uptake is the shoot coleoptile, which corresponds with the large increases in GST enzyme activity detected in the outermost layers of plant coleoptile tissue when a safener is applied (Riechers et al., 2003, 2010). Chloroacetamide herbicides are soil
applied and enter through the developing seedling shoot and coleoptile layers where GSTs are expressed and active (Riechers et al., 2003). Herbicide safeners developed for chloroacetamides then activate herbicide metabolism in the coleoptiles of the shoots by inducing GSTs that rapidly detoxify the herbicide as the developing shoot and leaves emerge from the soil. The localization of this safener-induced detoxifying mechanism to the outermost layers of the coleoptile would logically allow for herbicide detoxification to occur at the site near herbicide uptake in the shoot (Kreuz et al., 1989; Riechers et al., 2003, 2010).

These findings indicate that safeners are tapping into an unidentified, preexisting signaling pathway for detoxification of endogenous toxins or xenobiotics present in cereal crop coleoptiles (Riechers et al., 2005). A new hypothesis resulting from recent research is that safeners may be utilizing an oxidized lipid-mediated (oxylipins) or cyclopentenone-mediated signaling pathway, which subsequently leads to the expression of GSTs and other proteins involved in detoxification and plant defense (Riechers et al., 2010; Skipsey et al., 2011). Oxylipins are chemicals that act as signals regulating plant development and plant stress responses, such as wounding or pathogen infection (Mueller et al., 2008, Mueller and Berger, 2009); thus, the utilization of the oxylipin signaling pathway for safener response is a logical connection. A hypothesis for the safener-induced mechanism of action in the cellular detoxification and signaling pathway was linked to lipase induction (Riechers et al., 2010). In theory, this induction of lipase expression releases free $\alpha$-linoleic acid (Christeller and Galis, 2014), which leads to the subsequent increase in non-enzymatic oxylipin formation through interactions with safener-increased reactive oxygen species in the coleoptile. Specific oxylipins are synthesized in plants in response to stress, such as the jasmonic acid precursor 12-oxo-phytodienoic acid (OPDA) or phytoprostanes (PPA$_1$ for example), from $\alpha$-linoleic acid substrates.
(Eckardt, 2008). When production of oxylipins is reduced within the plant, such as in fad mutants in *Arabidopsis*, a decrease in GST expression was measured in response to herbicide safeners treatment (Skipsey et al., 2011). This finding suggests that there is a direct link between safener-induced defense responses and the oxylipin signaling pathway.

Additional knowledge of the entire safener-induced signaling and detoxification pathway, critical regulatory elements in the promoters or untranslated regions of genes encoding detoxification enzymes, and a comprehensive understanding of how gene expression is up-regulated by safeners might lead to the precise manipulation of transgene expression in plants (Riechers et al., 2010). By understanding which genes to target along the safener-induced detoxification pathway, improvements could be made to certain enzyme activities that these genes control. Increasing the activity of enzymes such as GSTs could result in the development of herbicide-tolerant crops, as well as, crops with increased tolerance to environmental pressures such as drought, temperature, and soil alkalinity.
1.6 Research Objectives

Controlling weedy pests is and will be one of the most significant challenges facing sorghum producers in the future. Unfortunately, sorghum is one of the few major cereal crops where its wild, weedy relatives have followed the cultivated species by inadvertent introduction; therefore, cross species gene flow occurs almost everywhere sorghum is cultivated. The gene flow from cultivated sorghum species to wild, weedy sorghum relatives creates a large problem when considering weed management possibilities (Schmidt and Bothma, 2006). The transgenic approach to improving weed control has brought enhanced attention to the significance of crop-weed hybridization (Dale et al., 2002). Gene flow from crop to weed or from weed to crop can occur in many crop/weed complexes if the crop and the weed have sympatric ranges, are sexually compatible, have flowering times that overlap, and share a common pollinator. These conditions are met in a large number of crop/weed complexes, but none as significant as the crop/weed complexes within the genus Sorghum. Conclusions have been made that any transgene that is either neutral or beneficial to the weedy relatives of sorghum (i.e., Johnsongrass and shattercane) would likely persist in populations growing in agricultural conditions under continued gene flow from the crop (Arriola and Ellstrand, 1997).

Due to this gene flow potential, alternative solutions to generating transgenic, herbicide-tolerant varieties must be considered and developed for sorghum. Achieving enhanced weed control in sorghum requires the use of new herbicide chemistries not currently labeled for the use in sorghum. VLCFA-inhibiting herbicides have few known weed resistance cases (five worldwide) and provide effective management of many hard to control weeds, such as various Amaranthus species and annual grasses (Heap, 2014). Many of these herbicides are labeled for PRE use in sorghum production. Pyroxasulfone is a new, novel VLCFA inhibitor that provides
comparable or greater control of problematic weeds found in grain sorghum relative to S-metolachlor, but is applied at lower rates and with lower impact on the environment (Baker and Mickelson, 1994; Nakatani et al., 2016). Herbicide safeners are used in conjunction with certain VLCFA-inhibiting herbicides in grain sorghum (Hatzios and Hoagland, 1989), which allows for the use of these herbicides that normally cause crop injury without the use of a safener (Yu and Powles, 2014). As a result, it is possible that a seed-applied safener for grain sorghum could be developed to protect against pyroxasulfone applied PRE.

Chapter 2 aims to better understand herbicide safeners through identifying key players of the safener-induced detoxification pathway and/or signaling mechanisms via genome-wide analysis. By correlating phenotypic variability to genotypic variability found in 761 diverse sorghum inbred lines, two key genes associated with the safener response to S-metolachlor with seed-applied fluxofenim were further investigated. Significant genes identified included two tandem SbGSTs located on chromosome 9. Expression of each candidate SbGST was examined in etiolated sorghum shoots with or without fluxofenim treatment, using three stably expressed reference genes, to determine their possible involvement in safener-induced herbicide tolerance in sorghum genotypes differing in phenotypic responses to fluxofenim plus S-metolachlor.

Chapter 3 investigates the possibility of reducing grain sorghum injury to pyroxasulfone through the use of seed-applied safeners in greenhouse and field studies. To date, only one safener (fluxofenim) has been used to safen against pyroxasulfone in sorghum (Geier et al., 2009), but this field study did not compare fluxofenim-treated seed with unsafened seed. As a result, the actual contribution of fluxofenim to safening from pyroxasulfone in this study is unknown. Under greenhouse conditions, five different safeners were tested with a hybrid sorghum line (23012; Advanta US) to protect against pyroxasulfone. Following up on my
findings from the greenhouse, field experiments were conducted to determine if fluxofenim could safen against pyroxasulfone under field conditions. The main objective was to test the hypothesis that a seed treatment of fluxofenim in conjunction with a split application of pyroxasulfone, which did not increase injury in barley but increased weed control in prior research (Boutsalis et al., 2010), could provide similar results in grain sorghum.
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2.1 Abstract

Safeners are frequently used with herbicides that normally cause injury in unsafened grain sorghum (*Sorghum bicolor*), and are typically applied as seed treatments to avoid safening weedy sorghum relatives. Safeners confer protection to cereal crops by inducing herbicide detoxification and defense systems, including massive increases in the expression and activity of glutathione S-transferases (GSTs) and cytochrome P450s, although their precise mechanisms of action remain unknown. Using a genome-wide association study (GWAS), 761 diverse sorghum inbred lines were evaluated to determine key molecular genetic factors in the safener-induced detoxification pathway and/or signaling mechanisms, and to quantify the expression of important genes identified. Greenhouse studies were conducted with the preemergence herbicide S-metolachlor, plus or minus the safener fluxofenim applied as a seed treatment, to determine phenotypes for natural herbicide tolerance and safener-induced responses. GWAS analysis revealed a significant single nucleotide polymorphism (SNP) associated with safener-induced response located on chromosome 9, located within a phi-class *SbGST* gene and about 15 kb from a different phi-class *SbGST*. Transcript levels of these two candidate *SbGSTs* were quantified in etiolated shoot tissues through quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) and gene-specific primers designed from each *SbGST* coding region. Basal and safener-induced expression of the *SbGSTs* was examined in three sorghum genotypes at 4, 8, and 12
hours after treatment (HAT) to quantify safener induction of these genes relative to three stably expressed reference genes: \textit{GTPB}, \textit{SAND}, and \textit{EIF4a}. Results indicated that expression of each \textit{SbGST} gene increased within 12 hr in response to the safener treatment but differed by specific gene and genotype. This approach allowed for the identification of candidate functional genes involved in the safening response from \textit{S}-metolachlor in grain sorghum.

2.2 Introduction

Herbicide safeners allow for the expanded use of certain herbicides, such as \textit{S}-metolachlor, which would normally cause injury to cereal crops without the use of a safener (Yu and Powles, 2014). Safeners provide protection to germinating cereal crop seedlings through enhancing herbicide metabolism mediated by glutathione \textit{S}-transferases (GSTs), cytochrome P450 monooxygenases, and UDP-dependent glycosyl transferases (Hatzios and Hoagland, 1989; Davies and Caseley, 1999; Riechers et al., 2005; Jablonkai, 2013). Specifically, GSTs are phase II detoxification enzymes in plants that are involved in several stress responses (Cummins et al., 2011). Herbicides, herbicide safeners, and other oxidative stresses induce plant specific phi and tau class GSTs (Chi et al., 2011; Cummins et al., 2013; Sharma et al., 2014). Previous studies conducted in sorghum (\textit{Sorghum bicolor}) and other cereal crops demonstrated massive induction of herbicide-metabolizing GSTs in response to several herbicide safeners (Fuerst and Gronwald, 1986; Gronwald et al., 1987; Fuerst et al., 1993; Riechers et al., 1996; Wu et al., 1996; Gronwald and Plaisance, 1998). This biochemical reaction of GST-mediated detoxification through conjugation with reduced glutathione (GSH) is well characterized, but the precise molecular mechanism for the induction of different defense enzymes such as GSTs is poorly understood (Riechers et al., 2010).
Recent studies indicated that safeners induce the expression of GSTs that detoxify xenobiotics mainly in the outermost cell layers of grass seedling coleoptiles (Riechers et al., 2003). These findings suggest that safeners are tapping into an unidentified, pre-existing signaling pathway for detoxification of endogenous toxins, xenobiotics, and ROS in a tissue-specific manner. It has been hypothesized that safeners may be utilizing an oxidized lipid (oxylipin; Mosblech et al., 2009)-mediated signaling pathway in the coleoptile, which subsequently leads to the expression of GSTs and other genes involved in detoxification and plant defense (Riechers et al., 2010; Skipsey et al., 2011). Oxylipins are chemicals that act as signals regulating plant development and plant stress responses, such as wounding or pathogen infection (Mueller et al., 2008; Mueller and Berger, 2009); thus, the utilization of the oxylipin signaling pathway for safener response is a plausible connection. Evaluating the molecular and biochemical effects of safeners provides a useful tool for investigating the early signaling and stress-response genes, regulation of different gene expression patterns, and enzymatic activities of essential components for detoxification in the absence of phytotoxicity.

Utilizing grain sorghum, a genetically diverse, safener-responsive, cereal crop with a fully-sequenced diploid genome, single nucleotide polymorphisms (SNPs) associated with the natural variation and safener responses may be identified in order to further our knowledge of the specific components involved in safener-regulated herbicide detoxification. The genome-wide association study (GWAS) is a statistical analysis used to associate variation found in genotypes with phenotypic variation (Korte and Farlow, 2013). GWAS has been successfully used in sorghum to investigate variation in yield and agroclimatic traits such as plant height and inflorescence (Morris et al., 2013; Zhang et al., 2015), disease response (Adeyanju et al., 2015), and grain composition (Rhodes et al., 2014; Rhodes et al., 2017). In our research, GWAS was
utilized to associate the natural phenotypic variation of stress responses caused by herbicide treatment with the genotypic data in a sorghum diversity panel determined through genotyping by sequencing (GBS) (Thurber et al., 2013). The overall goal of this study was to quantify the natural variation of safener-response in sorghum and to detect associated SNPs to identify new signaling and defense genes for enhancing abiotic stress tolerance of cereal crops, and ultimately gain a comprehensive understanding of the safener-induced, tissue-specific detoxification pathway in grain sorghum. Specific research objectives were to quantify expression of candidate target genes identified from GWAS through RT-qPCR, using suitable reference genes identified and characterized in etiolated sorghum shoot tissue for normalization of candidate gene expression in response to safener treatment.

2.3 Materials and Methods

2.3.1 Phenotyping and Genotyping Sorghum Inbred Lines

Sorghum inbred lines used in this study were obtained from the Sorghum Conservation Program (Thurber et al., 2013). Three trials were completed using a different randomization in each trial. Sorghum plants were grown in soil flats in the greenhouse. Flats for trials one and two were planted in a soil, peat, sand mix at a ratio of 1:1:1. Flats in trial three were planted in Metro Mix 900 (BFG Supply Co., USA) series soil. Flats contained 24 cells of randomized sorghum genotypes in unsafened/safened pairs plus BTx623 as a control check in each flat. Safened seeds were treated with fluxofenim (Sigma-Aldrich, St. Louis, MO, USA) at the rate 0.4 g kg\(^{-1}\) seed. Unsafened seeds were not subjected to treatment. Sorghum seeds were planted in the flats 3.5 cm deep and 1 cm apart, bottom watered, and allowed to sit under greenhouse conditions for 24 hours before the herbicide treatment was applied. Flats were planted in pairs, one to receive a herbicide treatment and the other to serve as the control (sprayed with water only). For trials one
and two, the herbicide S-metolachlor was applied at 2.52 kg ha\(^{-1}\) to the soil of the herbicide flats. Herbicide treatments were applied using a Generation III Research Sprayer (DeVries Manufacturing, USA) with a moving-nozzle, compressed air research spray chamber with an adjustable platform and equipped with a TeeJet 80015EVS even flat-spray nozzle. The nozzle was maintained at approximately 35 cm above the flat and the sprayer was calibrated to deliver 185 L ha\(^{-1}\) at 275 kPa. For trial three, S-metolachlor was applied at 37 µM in a 40 mL solution as a drench treatment to individual flat cells using a 50 mL syringe. Herbicide treatment methods were switched between trails 2 and 3 to ensure that an optimal herbicide response was achieved. Greenhouse conditions for trials one and three were set at 28 °C/22 °C day/night with a 16/8-hour photoperiod. Greenhouse conditions for trial two were set at 24 °C/22 °C day/night with a 16/8-hour photoperiod. Sorghum seedlings were overhead watered daily. After two weeks seedlings were harvested at the soil level, and seedling counts and height were taken along with fresh weight to quantitatively evaluate herbicide injury.

Genotyping-by-sequencing (GBS) was used to generate genome-wide SNP data for the 761 inbred sorghum genotypes evaluated in this study, which was conducted by Dr. Patrick Brown at the University of Illinois (Thurber et al., 2013). There were 100,610 SNPs overall, but SNPs with a minor allele frequency below 0.026 and identical SNPs within 64 bp of each other were excluded, leaving 60,167 SNPs for GWAS.

2.3.2 Genome-wide Association Analysis

Genome-wide association was performed using the genomic association and prediction integrated tool (GAPIT) in R (Lipka et al., 2012). GAPIT settings included the following programs in R: efficient mixed model association (EMMA), mixed linear model (MLM), and population parameters previously determined (P3D). Mean measurements for plant height and
weight of the grain sorghum control line BTx623, used to create the sorghum reference genome (Paterson et al., 2009), were collected from each tray and the data from BTx623 was used as a covariate in the GWAS analysis. From the analysis, candidate genes were selected based on a subset of SNPs with the lowest p-values after false discovery rate (FDR)-correction (0.1) through GAPIT. SNPs were then compared to sorghum reference genome and paired with closely associated genes. Candidate genes of interest identified through GWAS were selected for further expression analysis.

2.3.3 Plant Materials and Tissue Collection for Gene Expression Analysis

Samples were collected from etiolated sorghum shoots grown in a growth chamber at 28 °C in the dark. Three sorghum genotypes were chosen for analysis: BTx623, SC0037, and SC0087. Each genotype represents a diverse phenotypic response to the S-metolachlor and fluxofenim treatment (Figure 2.1). Seeds from the three genotypes were planted 3 cm deep in vermiculite in 648 cm$^3$ plastic pots, and were watered with 150 mL ddH$_2$O. Pots were covered in aluminum foil and placed in the growth chamber for 72 hours. After 72 hours, pots were removed and treated with either 0.02% DMSO in 50 mL total as a control treatment or 10 µM fluxofenim in 50 mL ddH$_2$O and placed back into the growth chamber. Samples were then harvested at 4, 8, and 12 hours after treatment (HAT). Etiolated seedlings were harvested above the seed, and the shoot was dissected away from the new leaf by excising the top 1 cm portion of the seedling. Samples were frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The experiment was conducted independently 3 times, and each treatment had three replicates.
2.3.4 RNA Isolation, cDNA Synthesis, and Semi-quantitative RT-PCR to Test Primer Specificity

Semi-quantitative RT-PCR was used to determine target gene and candidate reference gene primer specificity and amplicon size. Total RNA was isolated from 500 mg of shoot material using previously described methods (Xu et al., 2002), and stored at -80 °C. RNA concentration and purity was determined with a NanoDrop1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit 2.0 fluorometer (Invitrogen, USA). Samples with concentrations more than 100 ng/µL and absorption ratio A_{260}/A_{280} more than 1.8 and an A_{260}/A_{230} ratio between 2.0 and 2.3 were utilized for cDNA synthesis and semi-quantitative RT-PCR analysis. To evaluate rRNA integrity, total RNA was denatured at 55 °C in the presence of formamide and formaldehyde and visualized on 1% ethidium bromide (EtBr) stained agarose gels containing 0.4 M formaldehyde (Riechers et al., 2003). The Maxima H-minus cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) was used to perform first-strand cDNA synthesis following the manufacturer’s protocol using 500 ng total RNA. Semi-quantitative RT-PCR was performed using PTC-200 Pellier Thermal Cycler (MJ Research Inc., USA). The following amplification program was used for semi-quantitative RT-PCR, with 1 µL first-strand cDNA reaction: initial denaturation at 95 °C for 4.5 minutes, then 30 amplification cycles of 95 °C for 30 seconds and 62 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes.

RT-PCR products were separated and visualized on 1.8% agarose gels stained with EtBr.

To test the primer specificity of the two candidate SbGST genes, synthetic gene plasmids were synthesized using GeneArt Gene Synthesis (Invitrogen, USA). Each plasmid was synthesized using the entire coding region of the corresponding candidate gene. Semi-quantitative RT-PCR was conducted as above using the 1 µL of the 10 ng/µL plasmid solution.
with both sets of primers to test for specificity of amplification at varying annealing temperatures.

2.3.5 Analysis of Candidate Reference Gene Stability

Semi-quantitative RT-PCR (described above) was used to determine the candidate reference gene primer specificity and amplicon size. Standard curves were determined by qPCR using ten-fold dilution series over five dilution points of pooled cDNA from the sorghum genotype BTx623 as a template using the linear regression model (Pfaffl et al., 2004). Primer efficacy for the standard curves was calculated in the SDS 2.3 software (Applied Biosystems, USA). Gene expression stability of the seven candidate reference genes selected was estimated using four statistical algorithms: geNorm (Vandesompele et al., 2002), NormFinder (Anderson et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative ΔCt method (Silver et al., 2006). RefFinder (http://fulxie.0fees.us/) was used to compare and integrate the ranking of the tested candidate reference genes. The RefFinder analysis tool yields ranking orders and stability values for a set of reference genes through four separate statistical algorithms: ΔCt, geNorm, NormFinder, and BestKeeper.

GeNorm software was used to determine the gene expression stability value (M) for the seven candidate reference genes. In this analysis, the lower the M value the more stable the gene. It will also estimate the optimal number of reference genes, recommending a cutoff value for M of 1.5; values lower than 1.5 are considered stable (Vandesompele et al., 2002). NormFinder software uses an ANOVA-based model to determine stability based on intra- and inter-group variation (Anderson et al., 2004). BestKeeper will calculate a pairwise correlation coefficient for each gene and the candidate reference gene with the highest coefficient of correlation will be the most stable (Pfaffl et al., 2004). The comparative ΔCt method compares
the relative expression of pairs of genes to identify reference genes with low variability among samples, which are considered more stable (Silver et al., 2006).

2.3.6 Primer Design and RT-qPCR Conditions for Candidate Reference Genes

Sequences of the two SbGST candidate genes identified through GWAS were analyzed for gene-specific primer design using the software Primer3 and BLAST. Sequences of candidate reference gene primers that previously displayed stable expression in various sorghum tissues and organs (Zhang et al., 2013; Reddy et al., 2016) were redesigned to meet specific criteria for RT-qPCR analysis in etiolated sorghum shoot tissue. Candidate gene-specific primers and reference gene primers were required to meet the following stringent parameters (Table 2.2, 2.3): melting temperature (T_m) of 60–63 °C, primer lengths of 20–25 base pairs (bp), guanine-cytosine content 45–55%, amplicon length of 100–250 bp, and the absence of stable hairpins and dimers, determined using the OligoAnalyzer 3.1 tool (Integrated DNA Technologies, USA). To test the specificity as a result of the parameters, primers designed for the candidate genes identified through GWAS were analyzed using semi-quantitative RT-PCR and synthetic candidate gene plasmids as template (described above).

RT-qPCR was conducted using a 7900 HT Sequence Detection System (Applied Biosystems, USA) and reactions performed in 20 μL volumes following the manufacturer’s protocol (Power Syber® Green RNA-to-C_T™ 1-Step Kit; Applied Biosystems, USA). The following program was used for qRT-PCR: 48 °C for 30 minutes, 95 °C for 10 minutes, then 40 cycles at 95 °C for 15 seconds, 62 °C for 1 minute, and a melting curve at 95 °C for 15 seconds and 62 °C for 15 seconds. Each sample was analyzed in three technical replicates, and mean Cq values were calculated. Reverse-transcription negative controls were included to ensure the absence of genomic DNA in the template. Dissociation curves for each reaction were analyzed to
ensure only one replicon was amplified. SAF induced gene expression for each \textit{SbGST} gene was calculated relative to transcript levels in the unsafened control samples (per genotype and time after treatment) and normalized using three reference genes (\textit{GTPB}, \textit{SAND}, and \textit{EIF4a}; described below) using the $2^{-\Delta\Delta Ct}$ method. For quantitative analysis of \textit{SbGSTF1} and \textit{SbGSTF2} expression in BTx623, SC0037, and SC0087, expression data represents the combined results from three independent experiments (i.e. biological replicates), with three technical replicates per sample. ANOVA followed by Tukey’s multiple comparison test and LSD ($\alpha = 0.05$) using PROC GLM in SAS (Release 9.2) was conducted to determine significant differences in gene expression among sorghum genotypes at each harvest time point.

\textbf{2.4 Results and Discussion}

\textbf{2.4.1 Genome-wide Association Mapping Identifies Potential Candidate Genes Involved in Safener Response}

Sorghum inbred lines exhibited various phenotypes in response to the different combinations of safener and herbicide applications (Figure 2.1). The specific phenotypic response of most interest was the safener-induced response (Table 2.1). SAF induced response was chosen in order to identify genes induced by fluxofenim when sorghum was under an abiotic stress (S-metolachlor). Using the phenotypic measurement for seedling fresh weight per cell and covariate check, GWAS analysis was conducted combining results from trials 1-3. Association analysis with the 60,167 SNPs allowed identification of a peak of a strongly associated SNP ($P= 0.11$) with safener-induced response located at 4.128 Mbp on chromosome 9 (Figure 2.2). Associations with the phenotypic measurement for height were not significant. The SNP on chromosome 9 associated with safener-induced response was located within the 5’ UTR of a phi-class glutathione \textit{S}-transferase gene (Sobic.009G043600) and about 15 kbp from a
second phi-class *SbGST* gene (Sobic.009G043700) (Figure 2.3). These two *SbGSTs* were renamed *SbGSTF1* and *SbGSTF2*, respectively, according to the proposed nomenclature system for plant GSTs (Edwards et al., 2000; Pearson, 2005). In order to validate the GWAS predicted SNP and associated genes, expression analyses were conducted using RT-qPCR and reference genes verified from among several tested previously in various sorghum tissues and organs, as described below.

### 2.4.2 Selection of Candidate Reference Genes in *S. bicolor*

Gene expression studies have not been reported in young, etiolated sorghum seedling shoots (with and without safener), which necessitated finding suitable reference genes to determine *SbGST* expression. The candidate reference genes selected for this study represent genes stably expressed in sorghum shoots under both abiotic and biotic stresses (Zhang et al., 2013; Reddy et al., 2016) (Table 2.2). A total of eight candidate genes were tested for their suitability for the use as reference genes in etiolated sorghum shoot tissue treated with or without fluxofenim at each time point. These eight candidates included reference genes encoding protein phosphatase 2A-1 (*PP2A.1*), protein phosphatase 2A-4 (*PP2A.4*), GTP binding protein (*GTPB*), uridylate kinase (*UK*), eukaryotic initiation factor 4a (*EIF4a*), peptidylprolyl isomerase (*CYP*), SAND family protein (*SAND*), and actin-1 (*ACT1*) (Table 2.2). All RT-PCR products ranged from 117 to 172 bp (Table 2.3).

Primer pairs for the eight candidate reference genes were used for semi-quantitative RT-PCR amplification of pooled sorghum cDNA. Agarose gel electrophoresis displayed single RT-PCR amplification products of the expected lengths expect for the *CYP* gene (Figure 2.4). The *CYP* gene was then omitted from further analysis due to the inability to design gene-specific primers for that specific gene that met established criteria. Primer dimers or non-specific
amplicons were not evident, and products were not detected in the minus template negative controls. Standard curve analysis of the seven advanced candidate reference genes using qPCR with SYBER Green staining yielded single melting curve peaks for each gene (data not shown). PCR amplification efficiencies of the candidate reference genes ranged from 102.2% (EIF4a) to 125.6% (ACT1), and the regression coefficient values ($R^2$) ranged from 0.976 (UK) to 0.999 (GTPB) (Table 2.3).

2.4.3 Expression Profiling, Stability Analysis, and Comprehensive Ranking of Candidate Reference Genes

Seven candidate reference genes were selected for qPCR transcriptional profiling. Quantification cycle (Cq) values were obtained from each reaction with the seven primer pairs. The genes varied in their transcript abundance. The differences in transcript level between the candidate reference genes were calculated by averaging the Cq value for each gene across all samples (Figure 2.5). The mean Cq value for the seven candidate reference genes ranged from 17.2 to 21.0 cycles, with most falling between 19 and 21. GTPB had the lowest mean Cq value of 17.2, indicating that it had the most abundant transcript level followed by UK at 18.8. Most of the candidate reference genes were expressed at intermediate levels with a mean Cq value of about 20. None of the Cq values were above 25 for any of the candidate reference genes, indicating that all of the genes were expressed at relatively high levels.

The comprehensive rankings of the candidate reference genes as calculated through RefFinder are shown in Table 2.4. The top three candidate reference genes from the RefFinder comprehensive ranking were: (1) GTPB, (2) SAND, and (3) PP2A.4. The RefFinder comprehensive ranking data was checked by separately evaluating gene stability of the candidate reference genes in each statistical program. Stability values given for each candidate reference
gene were consistent between the individual statistical programs and RefFinder, therefore it can be concluded that RefFinder is a valid tool to evaluate candidate reference gene stability over multiple statistical algorithms.

All genes in the geNorm analysis had an M value of less than 1.5, which verified that all reference genes are stable under these conditions. The expression stability rankings for each of the tested candidate reference genes were calculated (Figure 2.6 A). The lowest M value was calculated for the GTPB and SAND gene pair (M=0.30) and corresponded to the most stable expression among all candidate reference genes with PP2A.4 and EIF4a being the next most stable genes. GeNorm analysis also determines the optimal number of reference genes through pairwise variation. A large variation value indicates that the addition of another reference gene is necessary for reliable normalization. A threshold of 0.15 is used to determine when the addition of another gene is not necessary. The optimal number of reference genes required for gene expression normalization in this study was determined to be three (Figure 2.7). NormFinder rankings resulted in a similar gene stability ranking order to geNorm (Figure 2.6 B). Both sets of software ranked GTPB and SAND as being the most stable genes while ACT1 and UK are the least stable (Figure 2.6 A and B). BestKeeper was used to determine the most stable genes based on Pearson’s correlation coefficient (r). BestKeeper determined that the best correlations were for GTPB (0.985), PP2A.4 (0.982), and SAND (0.962) (Figure 2.6 C).

Three reference genes were chosen based on the determined optimal number of reference genes for this study by geNorm (Table 2.6). SAND (SAND family protein), GTPB (GTP binding protein), and EIF4a (Eukaryotic Initiation Factor 4a), were determined to be the most suitable stably expressed candidate reference genes for etiolated sorghum seedling shoot tissue when taking to account the comprehensive stability values from the statistical analysis software (Table
2.4; Figure 2.6), as well as, data for PCR amplification efficiency and R² (Table 2.3). The use of multiple and robust reference genes for RT-qPCR analyses (Vandesompele et al., 2002) results in a more accurate and reliable expression profile for the two potential safener-induced SbGSTs identified through GWAS.

2.4.4 Expression Analysis of SbGSTs Identified through GWAS using Stably Expressed Reference Genes

Gene-specific primers were designed for the two potential safener-induced SbGST genes, and specificity and amplification efficiency was checked using semi-quantitative RT-PCR. Primer specificity for analyzing the two SbGST genes was paramount due to the high nucleotide sequence identity between the two coding regions (81%). Agarose gel electrophoresis displayed single bands at the expected sizes for both SbGSTs only when matching primer-template were used. Primer dimers or non-specific amplicons were not evident, and products were not detected in the minus template negative controls. For example, a single band 245 bp band was present when using the gene-specific SbGSTF1 primers with SbGSTF1 template, but a product was not present when using gene-specific primers for SbGSTF2 with SbGSTF1 template and vice-versa (Figure 2.8).

Expression levels of both SbGST genes in each sorghum genotype and time point revealed different responses to fluxofenim. The calculated fold induction of SbGSTF1 and SbGSTF2 (Figure 2.9) increased in all three genotypes as time increased ranging from 1-fold at 4HAT to 8-fold at 12HAT. A difference in the scale of fold induction in response to fluxofenim treatment was noted between the two SbGST genes, with SbGSTF1 induced at higher levels during the time course (1.1–8-fold) than SbGSTF2 (1–4.5-fold). This indicates that SbGSTF1 is initially more responsive to fluxofenim treatment than SbGSTF2. A significant difference was
determined in the expression of both SbGSTs in the sorghum genotype SC0037 at each harvest time point compared to the genotypes BTx623 and SC0087 (Figure 2.9). Safener-induced SbGSTF1 expression ranged from 1.1–2.6-fold in BTx623 and from 1.1–3-fold in SC0087, but ranged from 2–8-fold in SC0037. Similarly, safener-induced SbGSTF2 expression ranged from 1–1.6-fold in BTx623 and from 1–1.1-fold in SC0087, but ranged from 1.4–4.5-fold in SC0037. SC0037, the sorghum genotype that displays natural tolerance to S-metolachlor relative to the other two sorghum genotypes tested (Figure 2.1), displayed the highest safener-induced expression of both SbGSTs during the time course examined. This finding implies that these SbGSTs may play a role in determining phenotypic response to herbicide plus safener application and is in accord with the GWAS results (Figure 2.2).

2.4.5 Discussion

In this study, 761 sorghum inbred lines obtained from the Sorghum Conservation Program were evaluated for their responses to herbicide and safener applications. GWAS analysis identified a SNP highly associated with the safener-induced response in sorghum seedlings. A single genomic region on chromosome 9 strongly associated with safener-induced response was identified. This 20 kb interval on chromosome 9 contains two sorghum phi-class glutathione S-transferase genes, SbGSTF1 and SbGSTF2.

Quantitative expression analysis investigated these two phi-class GSTs further. Since gene expression analysis has not been reported in etiolated sorghum seedling shoot tissues, a primary goal of this research was to identify and verify suitable reference genes. The most stable reference genes for one experiment may not be the most suitable reference genes for another experiment, and a single gene has not exhibited stable expression across all conditions and plant types (Olsvik et al., 2008; Cruz et al., 2009). Therefore, the reference genes used for
normalization in this study required validation in etiolated sorghum shoot tissue exposed to fluxofenim. Traditional housekeeping genes such as 18S rRNA, actins, tubulins, and GADPH are commonly used for normalization, but recent findings indicated that there are many other genes expressed more stably than these housekeeping genes (Guenin et al., 2009). Thus, eight candidate reference genes were chosen from the literature displaying stable expression in the shoot tissue and young tissue of sorghum plants under normal and abiotic stress conditions (Zhang et al., 2013; Reddy et al., 2016). Of the seven of those genes that were evaluated, GTPB and SAND were the highest ranked reference genes while ACT1, UK, and PP2A.1 were consistently ranked the lowest. GTPB was consistently ranked the top reference gene. GTPB and SAND were the most stably expressed genes in BMV-infected sorghum plants (Zhang et al., 2013) and chosen for normalization. SAND is a suitable reference gene under many conditions in monocots such as sorghum, barley, and buckwheat (Demidenko et al., 2011; Zhang et al., 2013), as well as in dicots such as carrot, tomato, and eggplant (Expósito-Rodriguez et al., 2008; Tian et al., 2015; Kanakachari et al., 2016). The third reference gene was chosen from EIF4a and PP2A.4. EIF4a, which both displayed stable expression. However, EIF4a was ultimately selected due to stable expression in RT-qPCR normalization in other monocots under abiotic stress (Huang et al., 2014; Reddy et al., 2015; Reddy et al., 2016). Therefore, the multiple reference genes (GTPB, SAND, and EIF4a) identified in this study could be used in combination for the normalization of gene expression patterns in fluxofenim treated etiolated sorghum shoots.

There are five main classes of GSTs found in plants, of which tau- and phi-class GSTs are the most abundant in the plant kingdom (Chi et al., 2011; Labrou et al., 2015). A total of 99 GSTs have been identified in the Sorghum bicolor genome with a distribution of 64% tau-class and 22% phi-class genes. This proportion of phi-class GSTs is typical of cultivated cereal crops.
Both classes are closely linked to stress responses, and highly responsive to stimuli such as herbicides (Cummins et al., 2011). Phi-class GSTs play an important role in weed resistance (Cummins et al., 2013; Evans et al., 2017) and detoxification of herbicides (Pang et al., 2012; Yu and Powles, 2014). Phi-class GSTs are characterized by having two introns and three exons at conserved positions (Dixon et al., 2002) and tend to be found in tandem gene clusters, since tandem duplication is a major mechanism for GST expansion in grasses (Chi et al., 2011). A previous study identified safener-responsive phi-class GST isozymes in sorghum (Gronwald and Plaisance, 1998), but only obtained the N-terminal amino acid sequences. Using the sorghum reference genome, the amino acid sequences of \(SbGSTF1\) and \(SbGSTF2\) were compared to the N-terminal amino acid sequence of the phi-class GST isozymes identified previously (Gronwald and Plaisance, 1998). \(SbGSTF1\) and \(SbGSTF2\) are different safener-responsive phi-class \(SbGSTs\) than what were previously discovered. The coding region nucleotides of \(SbGSTF2\) are 81% identical with the coding region of \(SbGSTF1\). We hypothesize that \(SbGSTF2\) is most likely a parologue of \(SbGST1\) (Lynch, 2013) resulting from a tandem duplication event (Chi et al., 2011). Gene expression was investigated in order determine if these two \(SbGST\) genes are safener-induced and to determine if their expression patterns differed among sorghum genotypes.

An increase in fold induction for both \(SbGSTF1\) and \(SbGSTF2\) was quantified following safener treatment in all three genotypes as time increased (Figure 2.9), particularly in the naturally tolerant genotype SC0037. This indicates that both \(SbGST\) genes are safener-inducible and may play a role in safener-induced herbicide detoxification in sorghum, although enzymatic activity with \(S\)-metolachlor needs to be examined with each encoded protein. The difference in the scale of gene expression in response to a safener treatment between \(SbGSTF1\) and \(SbGSTF2\) likely results from their difference functions as paralogues (Lynch, 2013). As a result of this
duplication, one of the GSTs may have acquired additional functions that the other lacks, or it could have accumulated degenerative mutations (Innan and Kondrashov, 2010). Following the neofunctionalisation model proposed by Lynch, one GST may preserve the ancestral function of phi-class SbGSTs while the other is free to evolve different functions and diverge away from specific xenobiotic detoxification responses or expression patterns.

Each SbGST gene showed a significant increase in expression at all three time points in genotype SC0037 compared to BTx623 and SC0087. SC0037 exhibits natural tolerance to S-metolachlor, which is enhanced further by fluxofenim treatment; seedlings will survive and germinate in the presence of S-metolachlor without fluxofenim. Relatively high expression of the two SbGSTs in SC0037 may aid in rapid herbicide detoxification, thus allowing SC0037 to survive without a safener treatment. In order to determine this experimentally, however, GST activity would need to be tested with S-metolachlor as substrate. Fold induction differing by genotype suggests these SbGSTs play a functional role in the phenotypic response of each genotype to fluxofenim. An ongoing RNAseq study is investigating this hypothesis further to determine up- and down-regulated genes involved in safener-response in the coleoptile of different grain sorghum genotypes. The presence of highly expressed SbGSTs in the naturally tolerant phenotype through RNAseq would support the significant increase in SbGST expression in SC0037 found in this study. Pairwise comparisons of transcriptomes among the three genotypes showed no differences in the coding regions of each SbGST, leading to the speculation that differences exist in the GST promoter or untranslated regions leading to the expression differences. Comparative analysis of each SbGST promoter, 5’ and 3’ UTR, and introns may identify key regulatory elements for safener response, as well as differences among genotypes.
An increase in GST expression results in an increased tolerance to certain herbicides (Hu, 2014; Sharma et al., 2014). This study allowed us to focus on specific phi-class GSTs associated with a safener-induced response in sorghum. By understanding which genes to target, an increase of certain enzyme activities encoded by these genes could result in the development of crops that are tolerant to a wider range of herbicides, as well as abiotic stress, and environmental pressures such as drought, temperature, and soil alkalinity.
2.5 Tables and Figures

Table 2.1 Traits tested through the genome-wide association study (GWAS) of the sorghum inbred lines.

<table>
<thead>
<tr>
<th></th>
<th>Herbicide (+)</th>
<th>No herbicide (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated with safener (+)</td>
<td>HT</td>
<td>NT</td>
</tr>
<tr>
<td>Untreated with safener (-)</td>
<td>HU</td>
<td>NU</td>
</tr>
</tbody>
</table>

HT-HU: Safener-induced response in the presence of herbicide  
NT-NU: Safener effect without herbicide  
NT-HT: Ability to maintain optimal growth in the presence of the herbicide  
NU-HU: Natural tolerance to the herbicide
Figure 2.1 Phenotypic variability observed among sorghum lines. Sorghum seeds were treated with 0.4 g kg⁻¹ seed fluxofenim. Trays were treated with 2.52 kg ha⁻¹ S-metolachlor preemergence. BTx623, the normal, fluxofenim-induced tolerance (N) phenotype is shown in the two cells at the top; here sorghum seedlings will only grow in the presence of herbicide with a treatment of fluxofenim. SC0037, shown in the middle two cells, exhibits the natural tolerance (T) phenotype; here sorghum seedlings will grow in the presence of herbicide without a treatment of fluxofenim. SC0087, the sensitive (S) phenotype, is shown in the bottom two cells; here sorghum seedlings will not grow in the presence of herbicide even with a treatment of fluxofenim.
Figure 2.2 Manhattan plot of the marker-trait associations for plant weight (g pot⁻¹) for the safener-induced response (HT-HU) of grain sorghum across the sorghum reference genome (BTx623). The most significant hit is located in Chromosome 9 at 4.128 Mb. The closest gene to the top hit is a glutathione S-transferase gene (Sobic.009G043600). Significance thresholds were determined using a false discovery rate of 0.1 (green horizontal line).
Figure 2.3 Grain sorghum safener-response associated SNP. The SNP on sorghum chromosome 9 located at 4.128 Mb falls in the 5’ UTR of a phi-class glutathione S-transferase gene, *SbGSTF1*. *SbGSTF1* is about 15 kb from *SbGSTF2*, which is also a phi-class *SbGST*. 
Table 2.2 Candidate reference genes selected for gene expression normalization of target *SbGST* genes in *S. bicolor*.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Accession number</th>
<th>Location</th>
<th>Cellular Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REFERENCE GENES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP2A.1</td>
<td>Serine/threonine Protein Phosphatase 2A-1</td>
<td>KXG37326</td>
<td>Chr 1</td>
<td>Control specific dephosphorylation</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td>PP2A.4</td>
<td>Serine/threonine Protein Phosphatase 2A-4</td>
<td>XM_002453490</td>
<td>Chr 4</td>
<td>Control specific dephosphorylation</td>
<td>Reddy et al. 2016</td>
</tr>
<tr>
<td>GTPB</td>
<td>GTP binding protein</td>
<td>XM_002441511</td>
<td>Chr 9</td>
<td>Signal transduction</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td>UK</td>
<td>Uridylate Kinase</td>
<td>XM_002452867</td>
<td>Chr 4</td>
<td>Pyrimidine metabolism</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td>EIF4a</td>
<td>Eukaryotic Initiation Factor 4a</td>
<td>XM_002451491</td>
<td>Chr 4</td>
<td>Eukaryotic translation</td>
<td>Reddy et al. 2016</td>
</tr>
<tr>
<td>CYP</td>
<td>Peptidylprolyl Isomerase</td>
<td>XM_002453800</td>
<td>Chr 4</td>
<td>Cis-trans isomerization of prolineimidic peptide bonds</td>
<td>Reddy et al. 2016</td>
</tr>
<tr>
<td>SAND</td>
<td>SAND family protein</td>
<td>XM_002459139</td>
<td>Chr 3</td>
<td>Vesicular transport</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td>ACT1</td>
<td>Actin-1 protein</td>
<td>P53504</td>
<td>Chr 1</td>
<td>ATP binding</td>
<td>Zhang et al. 2013</td>
</tr>
<tr>
<td><strong>TARGET GENES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SbGSTF1</td>
<td>Glutathione S-transferase</td>
<td>XM_002439233</td>
<td>Chr 9</td>
<td>Herbicide/xenobiotic metabolism</td>
<td></td>
</tr>
<tr>
<td>SbGSTF2</td>
<td>Glutathione S-transferase</td>
<td>XM_002439234</td>
<td>Chr 9</td>
<td>Herbicide/xenobiotic metabolism</td>
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</tbody>
</table>
Table 2.3 Candidate reference gene and target gene primer details and performance at optimum concentration in *S. bicolor*.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primers sequences (5’-3’) (forward/reverse)</th>
<th>Amplicon length (bp)</th>
<th>Tm (°C)</th>
<th>PCR Efficiency (%)</th>
<th>Regression coefficient ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REFERENCE GENES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP2A.1</td>
<td>ATGCGGTGATATTCTATGGACAA GGTAGTAAACCACGGGTCAACATAA</td>
<td>117</td>
<td>62</td>
<td>106.8</td>
<td>0.988</td>
</tr>
<tr>
<td>PP2A.4</td>
<td>GTGGCCCTCTTCCGTACTTG TGTAAACTCTCTCTTTGGTG</td>
<td>153</td>
<td>61.5</td>
<td>107.7</td>
<td>0.997</td>
</tr>
<tr>
<td>GTPB</td>
<td>ACACTGCTGGGCAAGAGAAG TTACGGCAGGGACAAATGGG</td>
<td>172</td>
<td>62</td>
<td>105.8</td>
<td>0.999</td>
</tr>
<tr>
<td>UK</td>
<td>CACAGTTGTTGATGGCGCT TGTGCCCTTCTACTCCAG</td>
<td>118</td>
<td>63</td>
<td>108.2</td>
<td>0.976</td>
</tr>
<tr>
<td>EIF4a</td>
<td>CTGTCCGTGAGGACAAAGG CTGTAATCCAGGGGAGGACAA</td>
<td>161</td>
<td>63.5</td>
<td>102.2</td>
<td>0.998</td>
</tr>
<tr>
<td>CYP</td>
<td>TTGTTCTCGACCCTCTGACTCG GATGTCCGGGACGGAAAAGG</td>
<td>137</td>
<td>65.5</td>
<td>NA*</td>
<td>NA*</td>
</tr>
<tr>
<td>SAND</td>
<td>GAAAAGCCCTTTCTCTGAT GACAAACCCGACCCCTCATA</td>
<td>151</td>
<td>62</td>
<td>106</td>
<td>0.996</td>
</tr>
<tr>
<td>ACT1</td>
<td>CTAGCAACCATGAAGATCAAGGTT GCCAGACTCGTGTACTCAG</td>
<td>134</td>
<td>60</td>
<td>125.6</td>
<td>0.992</td>
</tr>
<tr>
<td><strong>TARGET GENES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SbGSTF1</td>
<td>ACGTTTCCGCAAGCAACAA GTACTGCTCTCGTCAGCC</td>
<td>245</td>
<td>62</td>
<td>98.4</td>
<td>0.997</td>
</tr>
<tr>
<td>SbGSTF2</td>
<td>GAAGCTCAAGAAGTGGTCTTG ACATGAGGGAGTGGCTTGG</td>
<td>159</td>
<td>62.4</td>
<td>104.5</td>
<td>0.998</td>
</tr>
</tbody>
</table>

*CYP reference gene primers did not produce a PCR product. The CYP gene was not used in future experiments.*
Figure 2.4 Confirmation of amplicon size and primer specificity for the eight candidate reference genes. Reverse transcriptase-PCR fragments were separated by a 1.8% EtBr-stained agarose gel. A 50 bp molecular ladder (M) was used as a size comparison. Gel image shows fragments of the expected size for each gene.

*CYP primers did not produce a PCR product. The CYP gene was not used in future experiments.
Figure 2.5 Expression levels of the seven candidate reference genes. Values are given as real-time PCR quantification cycle (Cq) values for individual reference genes. Boxes indicate the interquartile range and the line across the box represents the median. Bars represent the standard deviations.
Table 2.4 RefFinder ranking order of candidate reference genes. Table shows the ranking of candidate reference genes by four separate methods. Ranking is from most recommended (1) to least recommended (7).

<table>
<thead>
<tr>
<th>Method</th>
<th>Reffinder Ranking Order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Delta Ct</td>
<td>GTPB</td>
</tr>
<tr>
<td>BestKeeper</td>
<td>GTPB</td>
</tr>
<tr>
<td>Normfinder</td>
<td>GTPB</td>
</tr>
<tr>
<td>GeNorm</td>
<td>GTPB</td>
</tr>
<tr>
<td>Comprehensive Ranking</td>
<td>GTPB</td>
</tr>
</tbody>
</table>
Figure 2.6 Expression stability and ranking of the seven candidate reference genes as calculated by three different evaluation methods. (A) GeNorm stability rankings of candidate reference genes based on each gene’s average expression stability (M) value. A lower M value indicates more stable expression. The least stable genes are on the left and the most stable are on the right. (B) NormFinder gene stability rankings of candidate reference genes based on their stability value calculated by combining intra- and inter-group variations for each reference gene. (C) Reference gene ranking by gene stability in BestKeeper. Statistic calculations of gene stability based on the correlations between reference genes and the BestKeeper Index. Values given as Pearson’s correlation coefficients (r) are shown in the figure.
Figure 2.7  Determination of the optimal number of control genes for normalization in qRT-PCR. Pairwise variation is calculated through geNorm to determine the minimum number of reference genes for accurate normalization of qRT-PCR data. Using the threshold of 0.15, the optimal number of reference genes was determined to be 3. The * denotes the optimal number of reference genes.
Figure 2.8 Glutathione S-transferase gene primer specificity. (A) the *SbGSTF1* gene template shows a single band at 245 bp present when amplified by the *SbGSTF1* gene-specific primers (GSP). No band was present when amplifying the *SbGSTF1* DNA with the *SbGSTF2* GSP, indicating gene specificity had been achieved. (B) the *SbGSTF2* gene template shows a single band at 159 bp present when amplified by the *SbGSTF2* GSP. No band was present when amplifying the *SbGSTF2* DNA with the *SbGSTF1* GSP, indicating gene specificity had been achieved. Signals were not detected in the minus template controls. DNA templates were separated on a 1.8% agarose gel stained with ethidium bromide (EtBr).
Figure 2.9 Fold induction of the sorghum glutathione S-transferase genes. (A) Fold induction of the SbGSTF1 gene relative to unsafened control for each sample at each time point. (B) Fold induction of the SbGSTF2 gene relative to the unsafened control for each sample at each time point. Fold induction for each gene at each time point was calculated by $2^{(\Delta \Delta C_t)}$. * indicate a significant fold induction difference between genotypes at each time point at alpha 0.05. The sorghum genotype SC0037 shows a significantly higher fold induction of the SbGSTF1 gene and the SbGSTF2 gene at each time point as compared to sorghum genotypes BTx623 and SC0087.
2.6 Literature Cited


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doi:10.1104/pp.110.153601


doi:10.1074/jbc.M111.252726

doi:10.1186/1471-2199-7-33


CHAPTER 3
THE SAFENER FLUXOFENIM AND SEQUENTIAL APPLICATIONS OF
PYROXASULFONE REDUCE CROP INJURY AND INCREASE PREEMERGENCE
WEED CONTROL IN GRAIN SORGHUM (SORGHUM BICOLOR L. MOENCH)

3.1 Abstract

Controlling weeds selectively is one of the most significant challenges when producing grain sorghum. A relatively new herbicide, pyroxasulfone, has demonstrated potential for improving weed control in sorghum. However, crop injury remains a limitation to overcome. Five different herbicide safeners were evaluated in the greenhouse with the objective of determining their ability to protect hybrid sorghum from soil-applied pyroxasulfone. Quantitative phenotypic data indicated that the seed-applied safener fluxofenim provided the best protection to the sorghum seedlings from pyroxasulfone. Following from results in the greenhouse, a field study was conducted in 2015 and 2016 to evaluate the protective ability of fluxofenim from soil-applied pyroxasulfone at single and sequential application times. A randomized complete block plot design, split by a seed treatment of fluxofenim, was used to evaluate six different rates of pyroxasulfone. A preemergence (PRE) treatment of S-metolachlor, an untreated-weedy control, and a weed-free control were included for comparison with pyroxasulfone to assess broadleaf weed control, crop injury and stand count, and final grain yield. Results indicated that pyroxasulfone provided greater weed control compared to S-metolachlor, but as weed control increased, crop injury also increased regardless of safener. However, sequential applications of pyroxasulfone did not elicit as much crop injury as single applications. In spite of enhanced crop tolerance, particularly in sequential applications of pyroxasulfone, these results indicate that a
more effective herbicide safener tailored toward enhancing the ability of grain sorghum to metabolize pyroxasulfone is needed.

3.2 Introduction

Grain sorghum (*Sorghum bicolor* L. Moench) is one of the top five cereal crops in the world based on area sown and production. In the United States, sorghum is the third largest cereal grain in cultivation, with acreage increasing more than 20% since 2014 (Anonymous, 2016). Sorghum is used for both food and animal feed, and is a staple for the diet of over 500 million of the world’s poorest people (Wendorf et al., 1992). Unfortunately, increasing sorghum yield has been difficult through traditional breeding, and sorghum yields have lagged behind that of other cereal grains such as maize and rice (Paterson et al., 2009). One reason for the lack of yield increases in sorghum compared to other crops, such as maize and soybeans, is the limited availability of genetic improvement through transgenic means. Although bidirectional gene flow is not unique among major field crops (Paterson et al., 1995; Ellstrand et al., 1999), sorghum is the only major cereal crop where the wild, weedy relatives have followed the cultivated species by inadvertent introduction from Africa into the Americas, Asia and Australia.

Controlling weeds selectively is one of the most significant challenges when producing grain sorghum. Growers can utilize several preemergence (PRE) and post-emergence (POST) herbicides for broadleaf weed control, such as atrazine, pyrasulfotole, synthetic auxins, and saflufenacil, but have relatively few options for selective grass weed control (Anonymous, 2017). Seed-applied herbicide safeners are frequently used with herbicides that normally cause injury in unsafened grain sorghum to achieve selectivity and increase the range of potential herbicides for grass weed control (Hatzios and Hoagland, 1989). For example, very-long-chain fatty acid (VLCFA)-inhibiting herbicides, such as S-metolachlor, are commonly used in grain sorghum along with a herbicide safener for selective weed control (Devlin et al., 1983; Hwang et
VLCFA-inhibiting herbicides have few reported weed resistance cases and control small-seeded dicot weeds, such as various *Amaranthus* and *Chenopodium* species, as well as annual grasses and yellow nutsedge (*Cyperus esculentus*) (Heap, 2014).

Pyroxasulfone is a new, novel VLCFA inhibitor that provides comparable or greater control of problematic weeds found in sorghum fields over that of *S*-metolachlor. Weed control is achieved using lower rates and with lower impact on the environment as compared to *S*-metolachlor (Baker and Mickelson, 1994; Nakatani et al., 2016). Pyroxasulfone was specifically developed for high, stable herbicidal activity, exhibiting excellent weed control at relatively lower application rates and demonstrating longer residual activity as compared to *S*-metolachlor (Tanetani et al., 2009). Pyroxasulfone is less likely to leach through the soil due to its relatively low water solubility, low log *P* value, and hydrolytic stability at 25 °C (regardless of pH), and is therefore less susceptible to decomposition compared to *S*-metolachlor (Shaner, 2014; Nakatani et al., 2016). PRE applications of pyroxasulfone provide excellent weed control of many broadleaf and grass weeds, often providing better weed control over a more diverse weed spectrum than *S*-metolachlor (Steele et al., 2005; Geier et al., 2006; King and Garcia, 2008; Nurse et al., 2011).

Only one study has examined crop tolerance and weed control with pyroxasulfone in grain sorghum, which evaluated only two single application treatments at rates labeled for maize (Geier et al., 2009). However, several studies have shown that sequential applications of soil-applied herbicides with residual activity are more effective than a single application at the same total rate (Steckel et al., 2002; Mathiassen and Kudsk, 2016). Sequential applications of PRE herbicides provide an advantage over single PRE or POST herbicide applications by increasing and extending control of troublesome *Amaranthus* species (Steckel et al., 2002; Duzy et al.,
Previous research demonstrated that split applications of pyroxasulfone did not increase crop injury in barley but provided increased weed control relative to a single application PRE (Boutsalis et al., 2010).

To date, only one safener (fluxofenim) has been used to protect grain sorghum from pyroxasulfone (Geier et al., 2009), but this field study did not compare fluxofenim-treated seed with unsafened seed. As a result, the actual contribution of fluxofenim to safening from pyroxasulfone in this study is unknown. The objectives of this study were to evaluate several seed-applied safeners used in maize or grain sorghum for their efficacy in safening grain sorghum from pyroxasulfone in the greenhouse. Using the results of the growth response study, a field study was designed to test the hypothesis that a safener treatment of fluxofenim along with split applications of pyroxasulfone would improve weed control (relative to a single PRE application of S-metolachlor) while decreasing the amount of crop injury normally caused by pyroxasulfone in grain sorghum.

3.3 Materials and Methods

3.3.1 Interactions between Pyroxasulfone and Various Herbicide Safeners in Greenhouse Studies

The effects of pyroxasulfone applied alone or in combination with the safeners benoxacor, cyprosulfamide, fluxofenim, naphthalic anhydride, and oxabetrinil on the growth of the commercial grain sorghum hybrid 23012 (Advanta Seeds, USA) were studied under greenhouse conditions. Formulated pyroxasulfone (Zidua®) was provided by Kumiai, Japan. Analytical grade fluxofenim (Sigma-Aldrich, USA), benoxacor (Sigma-Aldrich, USA), cyprosulfamide (Chem Service, USA), naphthalic anhydride (Sigma-Aldrich, USA), and formulated oxabetrinil (Concep® II: 700g kg\(^{-1}\) a.i., Syngenta Crop Protection, LLC., USA) were evaluated as seed-applied safeners.
Sorghum seeds were surface sterilized with a 5% bleach solution for 5 minutes, then rinsed three times in ddH$_2$O for 5 minutes to improve and ensure even germination. Batches of sorghum seed (50 g) were treated with either 3 mL of 80% MeOH for the unsafened control, or with one of the following safeners applied as a seed treatment in 3 mL of 80% MeOH (adapted from Riechers et al., 1996): benoxacor (1.5 g ai kg$^{-1}$ seed), cyprosulfamide (1.5 g ai kg$^{-1}$ seed), fluxofenim (0.4 g ai kg$^{-1}$ seed), naphthalic anhydride (2.5 g ai kg$^{-1}$ seed), and oxabetrinil (1.5 g ai kg$^{-1}$ seed) (Table 3.1). Seeds were continually stirred with a metal spatula for 30 s in the aqueous methanol solution under a stream of air to ensure even coverage, then allowed to air dry on filter paper at room temperature overnight. Unsafened and safened seeds were planted 3 cm deep in 648 cm$^3$ plastic pots containing vermiculite (twelve seeds per pot), and watered with 150 mL of ddH$_2$O. Pots were placed in the greenhouse for 24 hrs. Greenhouse conditions were set at 28 °C/22 °C day/night with a 16/8-hour photoperiod. After 24 hrs, the pots were treated with a 40 mL aqueous soil drench of pyroxasulfone (0, 1, 2, or 3 µM), which also included 0.02% DMSO. After one week, a 50 mL fertilizer solution of 4 mM KNO$_3$ and 1 mM K$_2$HPO$_4$ (pH 7.0) was applied to each pot. Sorghum seedlings were left to grow in the greenhouse in the pots for two weeks with daily overhead watering.

Treated pots were arranged on greenhouse benches in a randomized complete block design. Each herbicide x safener combination was replicated three times (pots) with twelve plants per pot. Three separate experiments (runs) were conducted over a period of five weeks. After 14 days after treatment, the vermiculite was cleaned from the sorghum seedlings and the following measurements were taken: plant count, total plant length, total plant weight, above ground plant height, above ground plant weight, below ground plant length, and below ground plant weight. Plant height and weight were taken as an average per pot.
3.3.2 Experimental Field Site and Design

A field study was conducted at the Crop Sciences Research and Education Center, Urbana, Illinois in the 2015 and 2016 field seasons. Research plots were located on a Flanagan silt loam (fine, smectitic, mesic Aquic Argiudolls) soil with a pH of 6.5 and an organic matter content of 4.9%. Preplant tillage was performed each spring to prepare the seedbed for planting and to control any emerged weed seedlings. In 2015, a broadcast application of 12-12-12 NPK (123 kg N ha⁻¹) was applied in row of V8 sorghum on August 10th. In 2016, both fields received a fall application of diammonium phosphate (DAP) and potash (560 and 224 kg ha⁻¹, respectively) followed by a liquid application of nitrogen (135 kg N ha⁻¹) applied on May 16th.

Plots were arranged in a randomized complete block design, split by safener treatment, with three replications per herbicide treatment in 2015 and four replications per herbicide treatment in 2016. The study included one field site in 2015 and two field sites in 2016. Three experimental factors were examined in this study: herbicide treatment (pyroxasulfone or S-metolachlor), pyroxasulfone application rates (single rates and split rates), and safener application (present or absent). An untreated, weedy control and a manually maintained weed-free control were included in the study. Plot sizes measured 3 m wide by 8.2 m long.

3.3.3 Experimental Procedure

The commercial grain sorghum hybrid 23012 was obtained as a gift from Advanta Seeds, TX, and is a medium-early maturity line. Grain sorghum seeds had either a full seed treatment (Concep® III, Apron® XL LS, NipsIt®, and Maxim® 4FS) or a base treatment (Apron® XL LS, NipsIt®, and Maxim® 4FS) without Concep® III.

Grain sorghum seeds were planted at a density of 168,000 plants ha⁻¹ using a four-row test plot planter. The planting dates were June 7, 2015 and June 8, 2016. Single application preemergence (PRE) herbicide treatments were applied the following day and the split early
postemergence (EPOST) treatments were applied two weeks following the initial herbicide treatment. Treatments consisted of single PRE herbicide applications of pyroxasulfone at 90, 120, 180, and 210 g ai ha\(^{-1}\) and split PRE/EPOST applications of pyroxasulfone at 90 followed by 120 or 120 followed by 90 g ai ha\(^{-1}\). These rates are approximately one half the 1X and 2X rates used by Geier et al. (2009). A single application of S-metolachlor at 1.4 kg ai ha\(^{-1}\), an untreated, weedy control, and a weed-free control (maintained by hand weeding) were used for comparison with pyroxasulfone. Herbicides were applied using a pressurized CO\(_2\) backpack sprayer equipped with TeeJet AI110025 nozzles spaced 51 cm apart on a 3 meter boom calibrated to deliver 187 L ha\(^{-1}\) at 276 kPa.

### 3.3.4 Field Environmental Conditions

During the 2015 and 2016 field seasons the amount of precipitation differed drastically (Figure 3.2). The cumulative precipitation both years was above the 23-year average for the area, with 2015 being about 20 cm above average and 2016 being 10 cm above average. Precipitation in May–June 2015 were far above normal, with over a 10 cm difference in precipitation from the average in June alone. The majority of precipitation in June occurred directly after planting and herbicide application. Contrastingly, there was only a 4 cm increase in rainfall in June 2016 compared to the 23-year average, and the majority of precipitation occurred much later in the month with a two-week dry spell occurring after planting and herbicide application. The amounts and timings of the precipitation likely influenced the results for weed control and crop injury during these field seasons.

### 3.3.5 Data Collection

Field sites had natural populations of waterhemp (Amaranthus tuberculatus), as well as green foxtail (Setaria viridis), velvetleaf (Abutilon theophrasti), and tall morningglory (Ipomoea purpurea). Overall weed control was evaluated by visual estimation using a scale of 0 (no
control) to 100% (no weeds present) at 14, 21, 28, and 42 days after treatment (DAT); however, only ratings from 28 and 42 DAT were used for analysis. Weed control by species was estimated by taking weed counts from a one m² area randomly selected in the middle of the plot between rows 2 and 3. Weed counts for the weed species mentioned above were taken at 14, 21, 28, and 42 DAT and harvested for dry weight at 42 DAT. Weed dry weights taken from the above ground plant mass from each plot were also combined and total weed weight from each plot was analyzed. Crop density was estimated using stand counts averaged from a meter length in the center of rows 2 and 3 recorded at 14 and 21 DAT. Visual estimates of crop injury (stunting and buggy-whip symptoms) were taken using a scale of 0 (no injury) to 100% (plant death) at 14, 21, 28, and 42 DAT; however only ratings from 28 and 42 DAT were used for analysis. Grain yields were estimated by hand harvesting panicles from 3 m in the middle of rows 2 and 3 from each plot on October 20-22, 2015 and October 5-7, 2016. Panicles from rows 2 and 3 were combined and threshed. Final yield weight was adjusted for 15% moisture and weight was converted into kg ha⁻¹ for each plot.

3.3.6 Statistical Analysis

All data were subjected to ANOVA and treatment means were separated using Fisher’s Protected LSD test at alpha = 0.05. For the greenhouse trial, residuals were tested for normality and homogeneity of variance with PROC UNIVARIATE and PROC GLM respectively (SAS 9.4). The data was analyzed using PROC MIXED (SAS 9.4). The interaction between herbicide treatments and safener treatments was considered a fixed effect in the model, while replication (nested within run) was considered a random effect, and the interactions between replication and the fixed effects were treated as random effects.
For the field trial, weed counts and visual estimates of weed control and sorghum injury were subjected to a $\log_{10} +10$ transformation. The transformation did not affect results, so untransformed data for visual estimates are presented and both transformed and untransformed means are given for weed counts. For sorghum grain yield and stand count data, residuals were tested for normality and homogeneity of variance with PROC UNIVARIATE and PROC GLM respectively (SAS 9.4). The data was analyzed using PROC MIXED (SAS 9.4). Due to the differences in field number and replication number between 2015 and 2016, year was a significant factor when the combined data was analyzed for crop injury, weed control, stand count, and final grain yield. Since year was a significant factor in all analyses, the data were separated by year and reanalyzed. As noted in Table 3.3, the significant sources of variation in the data from the field experiments differed depending on the year. The interaction between herbicide treatments and safener treatments was considered a fixed effect in the model, while replication was a random effect, and the interaction between replication and the fixed effects were also considered random effects. In 2016 there were two fields, so field and replication (nested within field) were considered random effects.

3.4 Results and Discussion

3.4.1 Safening of Grain Sorghum against Pyroxasulfone Injury under Greenhouse Conditions

Five different herbicide safeners were tested for their ability to protect the grain sorghum hybrid 23012 against injury from pyroxasulfone (Table 3.1). Grain sorghum is inherently sensitive to pyroxasulfone at rates typically used in maize, particularly without safener treatment. Under greenhouse conditions, pyroxasulfone is a strong inhibitor of shoot growth in grain sorghum. Total length (shoots plus roots) and weight of unsafened grain sorghum seedlings
treated with pyroxasulfone decreased significantly compared with the unsafened, untreated control (Table 3.2).

Herbicide treatments had the most significant effect on the above ground height of the grain sorghum seedlings. Fluxofenim was the only safener that significantly increased total plant length and above ground height as compared to the unsafened control at all three concentrations of pyroxasulfone (Table 3.2 A). Treatments with fluxofenim displayed a positive effect on the growth of the sorghum seedlings, particularly the above ground growth, in the absence of herbicide (Cedergreen et al., 2007). Conversely, safener treatments of cyprosulfamide and naphthalic anhydride stunted untreated seedlings, providing a minor safening effect only at the higher concentrations of pyroxasulfone.

Similar to the height measurements, data analysis of the total weight, above ground weight, and below ground weight of the grain sorghum seedlings revealed that herbicide treatments of pyroxasulfone had the most significant effect on above ground weight. When considering the mean weight values for both total and above ground weight, fluxofenim, was the only safener to provide a significant increase in total plant length and above ground height as compared to the unsafened control (Table 3.2 B). The increase in seedling weight was most significant at the higher concentrations of pyroxasulfone. Fluxofenim displayed a positive effect on the weight of the sorghum seedlings, even those not treated with pyroxasulfone. The total weight of the untreated fluxofenim seedlings is significantly greater than that of the untreated, unsafened sorghum seedlings. As described for total seedling length, treatments with cyprosulfamide and naphthalic anhydride reduced the weight of the untreated seedlings as compared to the unsafened control at the lower concentrations of pyroxasulfone.
The shoot stunting on safened and unsafened grain sorghum seedlings was most prominent at the highest concentration of pyroxasulfone (3 µM; Figure 3.1). When compared to unsafened control seedling tissue treated with 3 µM pyroxasulfone (Figure 3.1 B and F), significant shoot and root stunting was observed. Similar injury was observed in seedling tissue safened with naphthalic anhydride treated with 3 µM pyroxasulfone (Figure 3.1 D and H). Severe stunting of the above ground tissue was the most constant and evident sign of injury in the sorghum seedlings in response to increasing concentrations of pyroxasulfone. However, sorghum seedling tissues safened with fluxofenim and treated with 3 µM pyroxasulfone (Figure 3.1 C and G) showed similar growth to the untreated, unsafened control seedlings. Therefore, fluxofenim was most effective among the five safeners tested to safen grain sorghum from pyroxasulfone under greenhouse conditions.

3.4.2 Weed Control in the Field

Target weeds evaluated for control at 28 and 42 DAT in the field study were waterhemp (Amaranthus tuberculatus), green foxtail (Setaria viridis), velvetleaf (Abutilon theophrasti), and tall morningglory (Ipomoea purpurea) (Table 3.4 and 3.5). S-metolachlor provided the least control among the herbicide treatments, and was significantly different than the weed free control at both 28 and 42 DAT in 2015 and 2016 (Table 3.4). Weed control did not differ between rates of pyroxasulfone and the weed free control in 2015 at 28 DAT. At 42 DAT, pyroxasulfone at 90 and 120 g ai ha\(^{-1}\) provided significantly less weed control than the weed free control. In 2016 at 28 DAT, the lowest rate of pyroxasulfone (90 g ai ha\(^{-1}\)) exhibited significantly less control than the weed free control. At 42 DAT, the two lowest rates of pyroxasulfone (90 and 120 g ai ha\(^{-1}\)) showed significantly less control as compared to the weed free control. At all herbicide rates, weed control was significantly greater as compared to the untreated control.
Weed control at each herbicide rate was also determined quantitatively in the 2016 field season. There was no significant difference between target weeds collected between the two field sites, so data were combined for analysis and means (Table 3.5). Based on the mean total weed dry weight, there was no significant difference between the weed-free control and the higher rates of pyroxasulfone. However, significant differences in weed control were measured between the single rate of pyroxasulfone at 90 g ai ha\(^{-1}\) and the weed free control (Table 3.5). S-metolachlor was also significantly different than the weed free control and all rates of pyroxasulfone, similar to the visual estimation rating results (Table 3.4). Total weed dry weight means for all herbicide rates were significantly different than the untreated control. Overall, results indicate that weed control was significantly greater with the higher single rates and split application rates of pyroxasulfone than with the lower single rates of pyroxasulfone or S-metolachlor.

### 3.4.3 Crop Injury in the Field

Sorghum plant density was reduced in both years due to herbicide injury (Table 3.6). A significant interaction between herbicide treatment and stand count in both the 2015 and 2016 field seasons. In the 2015 field season, treatments of pyroxasulfone reduced the stand count from 60 to 90% of the weed free control plots, even when fluxofenim was applied. Treatments of S-metolachlor reduced the stand count less than 10% with fluxofenim and 20% without fluxofenim (Table 3.6), illustrating the need for a herbicide safener in grain sorghum. However, an interaction between safener application and stand count was not measured, and an interaction was not determined between herbicide treatment and safener application on stand count for the 2015 season (Table 3.3). This can be attributed to the lack of difference in stand count between the safened and the unsafened plots. In the 2016 season a significant interaction existed between safener application and stand count, as well as a significant interaction between herbicide
treatment and safener application on stand count (Table 3.3). In the 2016 season, treatment with the highest rate of pyroxasulfone (210 g ai ha\(^{-1}\)) resulted in a 63% stand count reduction with fluxofenim; however, stand count was reduced over 90% without fluxofenim (Table 3.6). All herbicide treatments, except S-metolachlor, resulted in a significant difference in stand count between safened and unsafened plots. Notably, the split application of pyroxasulfone (90/120 g ai ha\(^{-1}\)) resulted in less than 10% reduction of stand count with fluxofenim (similar to the stand count of S-metolachlor), but without fluxofenim the stand count was reduced by 25% (Table 3.6).

Significant interactions were determined between herbicide treatment and fluxofenim on sorghum injury in both the 2015 (Table 3.7 A) and 2016 (Table 3.7 B) seasons. Crop injury resulting from the pyroxasulfone treatments was much higher (over 60%) in some cases in the 2015 season than those measured in the 2016 season (Table 3.7 A). S-metolachlor was the only herbicide treatment that recorded consistent crop injury ratings between the two seasons, which is most likely related to the chemical properties of S-metolachlor (Shaner, 2014; Nakatani et al., 2016). Rainfall in 2015 and 2016 may have caused S-metolachlor to leach out of the soil profile reducing the observed injury. Both sequential applications of pyroxasulfone (120/90 and 90/120 g ai ha\(^{-1}\)) resulted in less crop injury than the single application of pyroxasulfone (210 g ai ha\(^{-1}\)) in 2015 and 2016 (Table 3.7 A and B), indicating the effectiveness of sequential herbicide applications in sensitive cereal crops. The importance of a safener application with herbicide treatments in grain sorghum was documented in the 2016 field season, with over a 20% difference in crop injury detected between the safened and unsafened plots (Table 3.7 B).

Grain sorghum yield was affected by herbicide injury in both the 2015 and 2016 field seasons (Table 3.8). A significant interaction was determined between treatment and yield for
both years. Overall grain yield in the 2015 field season was lower than the grain yield in 2016. This decrease in yield may be in part to the differences in fertilizer application timings between years, as well as environmental conditions. Safened plots treated with S-metolachlor showed consistent yields between the two field seasons. Yield generally decreased as rates of single pyroxasulfone applications increased, which was especially pronounced in 2015. However, exceptions to this yield decrease by high rates of pyroxasulfone were measured in response to the sequential applications (Table 3.8). In 2015, the yield of the safened, split herbicide plots was within 20% of the weed free control, and within 10% in 2016. During the 2015 field season, a significant interaction between safener application and yield was determined that was not detected in 2016. In 2016, there was no significant difference between any of the unsafened plot yields and safened plot yields. This finding is contradictory to the stand count results (Table 3.6), suggesting that the individual plants were able to compensate for the reduced stand count by producing larger panicles with more seeds, or seeds with greater test weights.

3.4.4 Discussion

Overall, these results demonstrated that the seed-applied safener fluxofenim improved the tolerance of grain sorghum to pyroxasulfone, and that pyroxasulfone provided greater weed control relative to S-metolachlor under field conditions. The differing environment between field seasons did not affect the degree of weed control measured with either pyroxasulfone or S-metolachlor. However, the degree of crop injury was environment and dose dependent. The 2015 field season was consistently wet; as a result, more grain sorghum injury was evident, less germination was measured leading to a reduced stand count in the herbicide treated plots, and the presence of more buggy whipping and stunting symptoms. This crop injury resulted in a reduction in yield, with a significant difference between safened and unsafened plots depending on the herbicide treatment. The 2016 field season was drier and closer to the 23-year average for
the area. Less crop injury, higher germination and stand count in the herbicide treated plots, and less stunting and buggy whipping symptoms were measured, resulting in higher yields with no significant differences between the safened and unsafened plots in 2016.

The need for a herbicide safener that allows grain sorghum to more rapidly metabolize soil-applied herbicides, such as pyroxasulfone, was most evident in a wet year like 2015. Fluxofenim was an effective safener, but still requires improvement and optimization combined with improved crop tolerance to protect grain sorghum against pyroxasulfone. Crop injury was greater with pyroxasulfone than with S-metolachlor, resulting in higher yields for plots treated with S-metolachlor despite the decrease in weed control. However, grain yields from plots with split applications of pyroxasulfone were within 15% of grain yields from plots treated with S-metolachlor during 2015, and actually produced higher yields than plots treated with S-metolachlor in 2016. These results suggest that sequential applications of PRE residual herbicides in combination with effective safeners for sensitive cereal crops provide a potential alternative to generating transgenic, herbicide tolerant crops. Sequential applications of pyroxasulfone provided the same amount of weed control as the highest single rate similar to other residual PRE herbicides (Steckel et al., 2002), possibly resulting from longer residual activity for extended weed control. In addition, when the high rate of pyroxasulfone was split into two separate applications (90/120 and 120/90 g ai ha⁻¹), crop injury was reduced when used in conjunction with seed-applied fluxofenim. Further research on sequential soil-applied herbicide applications in cereal crops exhibiting herbicide sensitivity and few available herbicide options for weed control should be pursued to improve selective weed management systems.
### 3.5 Tables and Figures

#### Table 3.1 Safeners evaluated as seed-treatments on sorghum for use in conjunction with the herbicide pyroxasulfone.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical name</th>
<th>Target herbicide</th>
<th>Target crop</th>
<th>Application method in target crop</th>
<th>Rate seed-applied in Sorghum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benoxacor</td>
<td>(R,S)-4-dichlooroacetyl-3,4-dihydro-3-methyl-2H-1,4-benzoxazine</td>
<td>Metolachlor</td>
<td>Maize</td>
<td>Mixture with preemergence herbicide</td>
<td>1.5 g ai kg⁻¹ seed</td>
</tr>
<tr>
<td>Cyprosulfamide</td>
<td>(N)-4-(cyclopropylcarbamoyl)phenylsulfonyl-2-methoxybenzamide</td>
<td>Isoxaflutole</td>
<td>Maize</td>
<td>Mixture with either preemergence or post emergence herbicide</td>
<td>1.5 g ai kg⁻¹ seed</td>
</tr>
<tr>
<td>Fluxofenim</td>
<td>4-Chloro-2,2,2-trifluoroacetophenone O-1,3-dioxolan-2-ylmethylxime</td>
<td>Metolachlor</td>
<td>Sorghum</td>
<td>Seed-treated</td>
<td>0.4 g ai kg⁻¹ seed</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>Naphthalene-1,8-dicarboxylic anhydride</td>
<td>Thiocarbamates</td>
<td>Maize, Sorghum</td>
<td>Seed-treated</td>
<td>2.5 g ai kg⁻¹ seed</td>
</tr>
<tr>
<td>Oxabetrinil</td>
<td>(Z)-1,3-dioxolan-2-ylmethxyimino-(phenyl)acetonitrile</td>
<td>Metolachlor</td>
<td>Sorghum</td>
<td>Seed-treated</td>
<td>1.5 g ai kg⁻¹ seed</td>
</tr>
</tbody>
</table>
Table 3.2 Effect of pyroxasulfone concentration on sorghum seedling length (A) and fresh weight (B). Values represent treatments means ± standard errors; values marked with an asterisk (*) are significantly different from their respective unsafened control at a 95% confidence interval. Sorghum seeds were treated with 80% methanol (control) or seed applied safener treatments as listed in Table 3.1. Seeds were planted in vermiculite and treated with a 40 mL preemergence aqueous drench of pyroxasulfone (0, 1, 2, and 3 µM), then seedlings were harvested 14 days after planting.

<table>
<thead>
<tr>
<th>Pyroxasulfone Concentration (µM)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean seedling length (cm)</td>
<td>Mean above ground height (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>----------------------------------</td>
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</tr>
<tr>
<td>Unsafened control</td>
<td>34.2 (±1.7)</td>
<td>30.9 (±1.1)</td>
<td>25.1 (±1.4)</td>
<td>20.9 (±1.0)</td>
<td>11.8 (±1.2)</td>
<td>9.5 (±0.5)</td>
<td>6.9 (±0.7)</td>
<td>4.8 (±0.6)</td>
</tr>
<tr>
<td>Benoxacor</td>
<td>34.3 (±1.6)</td>
<td>29.3 (±0.9)</td>
<td>24.8 (±1.1)</td>
<td>21.3 (±1.1)</td>
<td>12.2 (±0.9)</td>
<td>9.4 (±0.7)</td>
<td>7.2 (±0.6)</td>
<td>5.8 (±0.7)</td>
</tr>
<tr>
<td>Cyprosulfamide</td>
<td>30.6 (±2.8)</td>
<td>27.4 (±1.3)</td>
<td>23.1 (±1.1)</td>
<td>21.4 (±1.1)</td>
<td>11.9 (±1.3)</td>
<td>9.0 (±0.6)</td>
<td>6.0 (±0.6)</td>
<td>5.8 (±0.5)</td>
</tr>
<tr>
<td>Fluxofenim</td>
<td>36.9 (±1.1)*</td>
<td>34.0 (±1.2)*</td>
<td>31.9 (±1.4)*</td>
<td>29.5 (±0.9)*</td>
<td>13.0 (±0.7)</td>
<td>11.0 (±0.4)*</td>
<td>10.2 (±0.6)*</td>
<td>7.9 (±0.5)*</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>30.4 (±1.4)</td>
<td>23.6 (±0.9)</td>
<td>22.80 (±1.1)</td>
<td>20.0 (±0.8)</td>
<td>11.4 (±0.8)</td>
<td>8.5 (±0.4)</td>
<td>8.4 (±0.5)</td>
<td>7.3 (±0.4)*</td>
</tr>
<tr>
<td>Oxabetrinil</td>
<td>34.3 (±1.7)</td>
<td>31.5 (±1.4)</td>
<td>27.1 (±1.3)</td>
<td>23.7 (±1.3)</td>
<td>12.1 (±0.8)</td>
<td>10.1 (±0.7)</td>
<td>7.3 (±0.7)</td>
<td>6.2 (±1.0)</td>
</tr>
<tr>
<td></td>
<td>Pyroxasulfone Concentration (µM)</td>
<td></td>
<td>Mean above ground fresh weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------</td>
<td>----------------</td>
<td>------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Unsafened control</td>
<td>3.7 (±0.2)</td>
<td>3.3 (±0.2)</td>
<td>2.6 (±0.2)</td>
<td>2.5 (±0.2)</td>
<td>1.3 (±0.1)</td>
<td>1.1 (±0.1)</td>
<td>0.7 (±0.1)</td>
<td>0.6 (±0.1)</td>
</tr>
<tr>
<td>Benoxacor</td>
<td>3.7 (±0.2)</td>
<td>3.0 (±0.2)</td>
<td>2.7 (±0.2)</td>
<td>2.3 (±0.2)</td>
<td>1.2 (±0.1)</td>
<td>1.0 (±0.1)</td>
<td>0.8 (±0.1)</td>
<td>0.6 (±0.1)</td>
</tr>
<tr>
<td>Cyprosulfamide</td>
<td>3.4 (±0.3)</td>
<td>2.7 (±0.2)</td>
<td>2.7 (±0.1)</td>
<td>2.4 (±0.2)</td>
<td>1.2 (±0.2)</td>
<td>0.9 (±0.1)</td>
<td>0.9 (±0.1)</td>
<td>0.8 (±0.1)</td>
</tr>
<tr>
<td>Fluxofenim</td>
<td>4.3 (±0.2)*</td>
<td>3.6 (±0.1)</td>
<td>3.7 (±0.2)*</td>
<td>3.5 (±0.3)*</td>
<td>1.4 (±0.1)</td>
<td>1.2 (±0.1)</td>
<td>1.2 (±0.1)*</td>
<td>1.0 (±0.1)*</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>3.5 (±0.2)</td>
<td>3.2 (±0.2)</td>
<td>3.1 (±0.2)</td>
<td>2.7 (±0.2)</td>
<td>1.0 (±0.10)</td>
<td>0.9 (±0.1)</td>
<td>0.87 (±0.1)</td>
<td>0.7 (±0.1)</td>
</tr>
<tr>
<td>Oxabetrinil</td>
<td>3.5 (±0.3)</td>
<td>3.5 (±0.2)</td>
<td>2.9 (±0.2)</td>
<td>2.6 (±0.1)</td>
<td>1.3 (±0.1)</td>
<td>1.1 (±0.1)</td>
<td>0.8 (±0.1)</td>
<td>0.7 (±0.1)</td>
</tr>
</tbody>
</table>
Figure 3.1 Effects of pyroxasulfone treatment on safened and unsafened grain sorghum seedlings. Unsafened control sorghum seedlings (without pyroxasulfone) above ground (A) and below ground (E), compared to unsafened control seedlings treated with 3 µM of pyroxasulfone (B, F), fluxofenim-treated seedlings plus 3 µM pyroxasulfone (C, G), and naphthalic anhydride (NA)-treated seedlings plus 3 µM pyroxasulfone (D, H). Severe stunting is evident in the unsafened control and in the NA-treated seedlings plus 3 µM pyroxasulfone, while the fluxofenim-treated seedlings plus the same rate of pyroxasulfone are comparable to the untreated-unsafened control.
Figure 3.2 Precipitation at the Champaign ICN station. Values are for the total rainfall for the months of May–August in centimeters.
Table 3.3 Analysis of variance significance level for the main effects and interactions of herbicide treatment (H) and safener application (S). Significance level was determined for sorghum injury 28 and 42 days after treatment (DAT), reductions in stand count and yield, and weed control 28 and 42 DAT.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>2015</th>
<th>2016</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stand Count 28 DAT 42 DAT 28 DAT 42 DAT Yield</td>
<td>Stand Count 28 DAT 42 DAT 28 DAT 42 DAT Yield</td>
</tr>
<tr>
<td>Herbicide treatment (H)</td>
<td>* * * * * * *</td>
<td>* * * * * * *</td>
</tr>
<tr>
<td>Safener application (S)</td>
<td>NS† * NS NS NS *</td>
<td>* * * NS NS NS</td>
</tr>
<tr>
<td>H x S</td>
<td>NS * * NS NS *</td>
<td>* * * NS NS NS</td>
</tr>
</tbody>
</table>

* Significance at α = 0.05.
† NS – not significant.
Table 3.4 Weed control ratings at 28 and 42 days after herbicide treatment (DAT) for the 2015 and 2016 field seasons. Preemergence (PRE) applications occurred the day of planting and early post-emergence (EPOST) applications occurred two weeks after the PRE application treatments. Split application treatment ratings began after the PRE application treatment. Visual estimates of weed control are presented as a percentage of the untreated plot.

<table>
<thead>
<tr>
<th>Herbicide Treatment</th>
<th>Rate</th>
<th>Timing</th>
<th>2015 28 DAT</th>
<th>2015 42 DAT</th>
<th>2016 28 DAT</th>
<th>2016 42 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g ai ha(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>78</td>
<td>73</td>
<td>86</td>
<td>76</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>91</td>
<td>87</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>94</td>
<td>88</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>98</td>
<td>96</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>98</td>
<td>97</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>96</td>
<td>94</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>98</td>
<td>96</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).
Table 3.5 Influence of the herbicide treatment on the control of target weeds. Target weeds included *Amaranthus tuberculatus*, *Abutilon theophrasti*, *Setaria viridis*, and *Ipomoea purpurea*. Target weeds were collected at 56 days after treatment (DAT) from a m² square placed randomly in the middle of rows two and three of each plot. Total target weed dry weight was measured for each plot.

<table>
<thead>
<tr>
<th>Herbicide Treatment</th>
<th>Rate g ai ha⁻¹</th>
<th>Timing</th>
<th>(\log_{10} + 10 \text{ g dry weight}^*)</th>
<th>g dry weight</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
<td>39.3</td>
<td>D</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>1.52</td>
<td>22.7</td>
<td>C</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>1.11</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>1.06</td>
<td>1.5</td>
<td>AB</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>1.01</td>
<td>0.3</td>
<td>A</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>1</td>
<td>0</td>
<td>A</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>1.03</td>
<td>0.6</td>
<td>A</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>1.02</td>
<td>0.4</td>
<td>A</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>A</td>
</tr>
</tbody>
</table>

\*Fisher's protected LSD = 0.07 (\(\alpha = 0.05\)) (\(\log_{10} + 10 \text{ g dry weight}\))

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).
Table 3.6 The interaction of herbicide treatments and seed-applied safener (fluxofenim) on sorghum stand count (plants ha\(^{-1}\)) in 2015 and 2016.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate</th>
<th>Timing</th>
<th>Y(^a)</th>
<th>N(^b)</th>
<th>Difference</th>
<th>p-value</th>
<th>Y</th>
<th>N</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>-</td>
<td>166263</td>
<td>157512</td>
<td>8751</td>
<td>0.58</td>
<td>166536</td>
<td>165716</td>
<td>820</td>
<td>0.88</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>153137</td>
<td>131260</td>
<td>21877</td>
<td>0.17</td>
<td>161614</td>
<td>158332</td>
<td>3282</td>
<td>0.55</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>52504</td>
<td>39378</td>
<td>13126</td>
<td>0.40</td>
<td>143566</td>
<td>130440</td>
<td>13126</td>
<td>0.02*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>26252</td>
<td>21877</td>
<td>4375</td>
<td>0.78</td>
<td>136182</td>
<td>106649</td>
<td>29534</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>17501</td>
<td>8751</td>
<td>8751</td>
<td>0.58</td>
<td>104084</td>
<td>41019</td>
<td>63065</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>17501</td>
<td>0</td>
<td>17501</td>
<td>0.27</td>
<td>63169</td>
<td>19689</td>
<td>43480</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>61255</td>
<td>26252</td>
<td>35003</td>
<td>0.03*</td>
<td>129619</td>
<td>103073</td>
<td>26547</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>65630</td>
<td>30627</td>
<td>35003</td>
<td>0.03*</td>
<td>157512</td>
<td>126338</td>
<td>31174</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>-</td>
<td>170638</td>
<td>131260</td>
<td>39378</td>
<td>0.02*</td>
<td>168997</td>
<td>168177</td>
<td>820</td>
<td>0.88</td>
</tr>
</tbody>
</table>

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).

\(a\) Sorghum plots with seed-applied fluxofenim (Y)

\(b\) Sorghum plots without seed-applied fluxofenim (N)

* p-values that represent a significant difference (\(\alpha = 0.05\)) in stand count between safened and unsafened plots
Table 3.7 Crop injury ratings at 28 and 42 days after treatment (DAT) for the 2015 (A) and 2016 (B) field seasons. Preemergence (PRE) applications occurred the day of planting and early post-emergence (EPOST) applications occurred two weeks after the PRE application. Split application treatment ratings began after the PRE application treatment. Visual estimates of crop injury are presented as a percentage of the untreated plot.

<table>
<thead>
<tr>
<th>Herbicide Treatment</th>
<th>Rate</th>
<th>Timing</th>
<th>28 DAT</th>
<th>42 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g ai ha$^{-1}$</td>
<td>Safened</td>
<td>Unsafened</td>
<td>p-value</td>
</tr>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>85</td>
<td>87</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>60</td>
<td>77</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).
* p-values that represent a significant difference ($\alpha = 0.05$) in crop injury between safened and unsafened plots.
Table 3.7 (cont.)

<table>
<thead>
<tr>
<th>Herbicide Treatment</th>
<th>Rate</th>
<th>Timing</th>
<th>Safened</th>
<th>Unsafen</th>
<th>p-value</th>
<th>Safened</th>
<th>Unsafen</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g ai ha⁻¹</td>
<td></td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>1</td>
<td>2</td>
<td>0.97</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>5</td>
<td>18</td>
<td>0.04*</td>
<td>1</td>
<td>8</td>
<td>0.04*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>6</td>
<td>46</td>
<td>&lt;.0001*</td>
<td>5</td>
<td>23</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>24</td>
<td>63</td>
<td>&lt;.0001*</td>
<td>9</td>
<td>34</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>43</td>
<td>81</td>
<td>&lt;.0001*</td>
<td>20</td>
<td>56</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>18</td>
<td>34</td>
<td>0.01*</td>
<td>9</td>
<td>17</td>
<td>0.04*</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>3</td>
<td>20</td>
<td>0.01*</td>
<td>2</td>
<td>10</td>
<td>0.07</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).

* p-values that represent a significant difference (α = 0.05) in crop injury between safened and unsafened plots.
Table 3.8 The interaction of herbicide treatment and seed-applied fluxofenim on grain sorghum yield (kg ha\(^{-1}\)) for the 2015 and 2016 field seasons.

<table>
<thead>
<tr>
<th>Herbicide Treatment</th>
<th>Rate</th>
<th>Timing</th>
<th>Y&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Difference</th>
<th>p-value</th>
<th>Y&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g ai ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
<td>kg ha(^{-1})</td>
</tr>
<tr>
<td>Untreated†</td>
<td>-</td>
<td>-</td>
<td>5755</td>
<td>4238</td>
<td>1517</td>
<td>0.14</td>
<td>5565</td>
<td>6078</td>
<td>-513</td>
<td>0.48</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>1430</td>
<td>PRE</td>
<td>6331</td>
<td>2723</td>
<td>3608</td>
<td>0.001*</td>
<td>7747</td>
<td>6478</td>
<td>1269</td>
<td>0.08</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90</td>
<td>PRE</td>
<td>3212</td>
<td>2028</td>
<td>1184</td>
<td>0.24</td>
<td>7622</td>
<td>6573</td>
<td>1049</td>
<td>0.15</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120</td>
<td>PRE</td>
<td>2055</td>
<td>1094</td>
<td>961</td>
<td>0.34</td>
<td>7241</td>
<td>6823</td>
<td>418</td>
<td>0.56</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>180</td>
<td>PRE</td>
<td>1374</td>
<td>654</td>
<td>720</td>
<td>0.47</td>
<td>7298</td>
<td>7111</td>
<td>187</td>
<td>0.80</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>210</td>
<td>PRE</td>
<td>1744</td>
<td>442</td>
<td>1302</td>
<td>0.19</td>
<td>7202</td>
<td>6366</td>
<td>836</td>
<td>0.25</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>120 + 90</td>
<td>PRE + EPOST</td>
<td>5281</td>
<td>653</td>
<td>4628</td>
<td>&lt;.0001*</td>
<td>7754</td>
<td>7003</td>
<td>751</td>
<td>0.29</td>
</tr>
<tr>
<td>Pyroxasulfone</td>
<td>90 + 120</td>
<td>PRE + EPOST</td>
<td>5411</td>
<td>1639</td>
<td>3772</td>
<td>0.0006*</td>
<td>8205</td>
<td>7133</td>
<td>1072</td>
<td>0.14</td>
</tr>
<tr>
<td>Weed free†</td>
<td>-</td>
<td>-</td>
<td>7321</td>
<td>6272</td>
<td>1049</td>
<td>0.15</td>
<td>8188</td>
<td>7398</td>
<td>790</td>
<td>0.27</td>
</tr>
</tbody>
</table>

† Two control treatments included weekly hand weeding (Weed free) or no weeding (Untreated).

<sup>a</sup> Sorghum plots with seed-applied fluxofenim (Y)

<sup>b</sup> Sorghum plots without seed-applied fluxofenim (N)

* p-values that represent a significant difference (α = 0.05) in yield between safened and unsafened plots
3.6 Literature Cited


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CHAPTER 4
SYNOPSIS OF RESEARCH AND FUTURE IMPACTS

4.1 Synopsis and Impacts

Since their discovery and development in the 1960s, countless studies have been conducted to understand how and why herbicide safeners work within plants (Hatzios, 1991; Gaillard et al., 1994; Davies and Caseley, 1999; Abu-Qare and Duncan, 2002; Hatzois and Burgos, 2004; Riechers et al., 2010; Jablonkai, 2013). Herbicide safeners play an important role in selective weed control for many crops. Selective control of grass weeds is particularly difficult to achieve in grain sorghum due to its sensitivity to most commonly used chloroacetamide herbicides, as well as the risk in using transgenics crops in conjunction with glyphosate or glufosinate (Paterson et al., 2009; Sagnard et al., 2011). Thus, herbicide safeners are required to achieve herbicide selectivity in grain sorghum. Despite the decades of research on herbicide safeners, there is still a lot we do not understand. The pathway(s) herbicide safeners induce are still being investigated (Riechers et al., 2005; Riechers et al., 2010; Skipsey et al., 2011). If the pathways safeners utilize for inducing herbicide metabolism could be understood more thoroughly, it may be possible to develop crops that tolerate abiotic stresses such as herbicide application more efficiently and provide effective safeners tailored for specific crops and herbicides.

This research aimed to identify key players of the safener-induced detoxification pathway and/or signaling mechanisms. Through a genome-wide association study (GWAS) of 761 diverse grain sorghum inbred lines, phenotypic variability was correlated to genotypic variability in order to identify single nucleotide polymorphisms (SNPs) associated with plant responses involved in safener application. GWAS analysis identified a SNP highly associated with the
safener-induced response in sorghum seedlings. A single genomic region on chromosome 9 strongly associated with safener-induced response was identified. A 20 kb interval on chromosome 9 contained two sorghum phi-class glutathione S-transferase (GST) genes, Sobic.009G043600 (SbGSTF1) and Sobic.009G043700 (SbGSTF2).

To better understand the extent of their involvement in the safener-induced response in sorghum, gene expression of these candidate SbGSTs genes was determined by quantitative real-time polymerase chain reaction (RT-qPCR). In order to normalize gene expression of these two genes, three stably expressed reference genes needed to be identified in etiolated grain sorghum shoot tissue. Previous RT-qPCR analyses in sorghum had not studied etiolated shoot tissue. Since a single reference gene, thus far, has not been shown to be stable across all conditions and plant types (Olsvik et al., 2008; Cruz et al., 2009), RT-qPCR analysis was conducted to determine the most suitable reference genes for etiolated sorghum shoot tissue. SAND, EIF4a, and GTPB were the most suitable reference genes and used for normalization of the SbGST gene expression. An increase in fold-induction in both SbGSTF1 and SbGSTF2 in response to safener application in all three genotypes as time increases was identified. This finding indicated that both SbGSTs are safener-inducible and likely play a role in safener-induced herbicide detoxification in sorghum. Phi-class GSTs were the first plant GSTs identified as having herbicide detoxifying capabilities (Dixon et al., 2002a, b) and are typically found in tandem gene clusters (Chi et al., 2011), just like the two identified SbGSTs. However, a difference in the scale of gene expression in response to a safener treatment was noted between the two SbGST genes. SbGSTF1 was expressed higher over time than SbGSTF2. Both SbGST genes showed a significant increase in gene expression in genotype SC0037 compared to BTx623 and SC0087 at all three harvest time points. With GWAS identification of these two phi-class SbGSTs and
subsequent expression study showing a difference by genotype, we concluded that these two SbGSTs might play a functional role in the phenotypic response of each genotype to safener application. However, comparative analysis of each SbGST promoter, 5’ and 3’ UTR, and introns may identify key regulatory elements for safener response, as well as explain differences among genotypes.

There were limitations to this research. We did not directly sequence the coding region of the two SbGST genes in the sorghum genotypes SC0037 and SC0087 for additional comparisons to the reference genome (BTx623), which is available since it was the sorghum line used for genome sequencing. The GST gene sequence information from these three genotypes may have allowed us to investigate deeper into why the two SbGSTs were expressed at higher levels in SC0037 than the other two genotypes, or if amino acid changes were present in the coding regions that might affect substrate specificity or activity. As of now, we have not investigated the promoter regions interactions of these two SbGSTs, so we do not know how those regions may affect the expression of the GST genes. From an RNA sequencing project in our laboratory, we were able to compare the transcriptomes of the three sorghum genotypes. The transcriptome comparison showed no major differences in the coding region of the two genes between the three genotypes, leading us to speculate there must be differences in the GST promoter regions between the three genotypes leading to the expression differences observed.

This research only investigated one plant trait interaction type. Three different trait interactions were possible besides the safener-induced response including the effect of the safener on sorghum in the absence of a herbicide, the ability of sorghum to maintain normal growth in the presence of a herbicide with a safener application, and the natural tolerance of sorghum to a herbicide. The GWAS analysis of these trait interactions identified two GST genes
correlated to significant SNPs for these interactions. One additional gene that stood out was a sulfite oxidase gene located on chromosome 9 near the two identified SbGSTs. The sulfite oxidase gene was associated with the ability of sorghum to maintain normal growth in the presence of a herbicide with a safener application. Since this gene has not been further investigated under these conditions, it is unclear how it may be connected to the phi-class SbGSTs and what role(s) they play in the safener-induced detoxification pathway. However, one possibility is that GSTs require GSH (derived from cysteine) as a co-substrate for herbicide conjugation-detoxification reactions, as well as cellular reductant. GSH levels may increase by different mechanisms; stimulation of sulfate assimilation linked to cysteine biosynthesis (Farago et al., 1994) or repression of sulfite oxidase activity (Hänsch and Mendel, 2005; Brychkova et al., 2015), resulting in enhanced herbicide detoxification.

Increases in GST expression results in an increased tolerance to certain herbicides (Cummins et al., 2011; Yu and Powles, 2014). This study allowed us to focus on specific SbGSTs associated with a safener-induced response in sorghum; however, there are still more facets of this response pathway to be discovered. By increasing our understanding of the pathway as a whole and which genes to target, improvement to certain enzymes activities that these genes control could result in the development of crops that are tolerant to a wider range of herbicides, as well as abiotic stresses and, environmental pressures such as drought, temperature, and soil alkalinity.

A separate study was conducted under greenhouse and field conditions to investigate the possibility of reducing crop injury in grain sorghum from a relatively new herbicide, pyroxasulfone, through the use of seed-applied herbicide safeners. Under greenhouse conditions, five different safeners were tested in a hybrid sorghum line to protect against pyroxasulfone. To
date, only one safener, fluxofenim, has been used to safen against pyroxasulfone in sorghum (Geier et al., 2009). Fluxofenim, benoxacor, oxabetrinil, cyprosulfamide, and naphthalic anhydride were evaluated for their ability to safen against pyroxasulfone in grain sorghum. Benoxacor and cyprosulfamide are traditionally applied to maize as safeners in tank mixes with the herbicide, while oxabetrinil and naphthalic anhydride have been used in sorghum as seed-applications. These diverse safeners were chosen because a safener has not yet been determined for use against pyroxasulfone in any crop, so a range of safener activity needed to be considered. After analyzing height and weight measurements, the herbicide safener fluxofenim proved to provide the most enhanced level of protection to grain sorghum from pyroxasulfone. Taking our findings from the greenhouse, field experiments were conducted to determine if fluxofenim could safen against pyroxasulfone under field conditions.

The main objective of my research was to determine if a seed treatment of fluxofenim in conjunction with a sequential application of pyroxasulfone could prevent or reduce injury in hybrid sorghum. A split-application study conducted in barley showed an increase in weed control with no increase in crop injury when sequential applications of pyroxasulfone were made (Boutsalis et al., 2010), but would the result be the same in sorghum? Our results from the field confirmed what we documented in the greenhouse. The herbicide safener fluxofenim improved the tolerance of grain sorghum to pyroxasulfone relative to unsafened grain sorghum, and pyroxasulfone provided enhanced weed relative to over S-metolachlor. Specifically, the split applications of pyroxasulfone in conjunction with fluxofenim improved crop tolerance over that of single applications of the same total rate while providing the same high level of weed control.

While the difference in environment between the two field seasons did not affect the degree of weed control measured with either pyroxasulfone or S-metolachlor, the degree of crop
injury was environment and dose dependent. The 2015 field season was consistently wet and, as a result, less germination (leading to a smaller stand count in the herbicide-treated plots) and more herbicide injury symptoms (buggy whipping and stunting) were present. This crop injury resulted in a significant reduction in yield in unsafened plots relative to safened plots, depending on the herbicide treatment and rate. Increasing rates of pyroxasulfone led to higher degrees of crop injury, with even the lowest rates of pyroxasulfone causing significantly more injury than S-metolachlor. The 2016 field season was drier and closer to the 23-year average for precipitation in the area. Under these environmental conditions, less crop injury, higher germination and stand count in the herbicide-treated plots, and less stunting and buggy whipping symptoms were measured. The reduction in crop injury was associated with a higher yield with no significant differences between the safened and unsafened plots for any of the herbicide treatments.

This research also had its limitations. For the greenhouse experiment, we had a limited number of herbicide safeners available to be tested for their ability to safen against pyroxasulfone. Some had been tested in small, pilot studies (such as dichlormid) and were determined to be ineffective. Others could not be obtained in our timeframe or due to monetary or proprietary restrictions. The herbicide safeners chosen for this study covered a broad range of safening ability and were the best available candidates to safen against pyroxasulfone. For both the greenhouse and field study, only one grain sorghum hybrid line was evaluated. In a broader study covering additional years, it would be advantageous to determine if other diverse grain sorghum lines, hybrid or inbred, have higher pyroxasulfone tolerance or better ability to respond to fluxofenim than Advanta hybrid 23012. Conducting a preliminary GWAS experiment with numerous grain sorghum inbreds and hybrids would allow for rapid and high throughput evaluation of grain sorghum responses to pyroxasulfone. The highest responding lines could then
be further analyzed under field conditions. Extending the field trials for more than two years and over multiple locations would also allow for a more robust data collection.

The need for a herbicide safener that allows grain sorghum to more rapidly metabolize soil-applied herbicides such as pyroxasulfone was most evident in a wet year like 2015, but also in a year with more normal rainfall like 2016. Fluxofenim is an effective safener but is still not an optimal safener to protect grain sorghum against pyroxasulfone. Crop injury is still greater with pyroxasulfone than S-metolachlor resulting in higher yields for plots treated with S-metolachlor despite the decrease in weed control. As our knowledge of how herbicide safeners work within the plant increases, the ability to develop safeners tailored to induce crop metabolism of specific herbicides more efficiently should expand. Alternatively, manipulation of the natural ability of a specific crop to metabolize any herbicide may offer new selective herbicide options in cereal crops (Kraehmer et al., 2014).

Until then, it is promising to see that grain sorghum yields from plots with sequential applications of pyroxasulfone were within 15% of grain yields from plots sprayed with S-metolachlor during the 2015 field season and produced higher yields than plots sprayed with S-metolachlor in the 2016 season. These results indicate that sequential applications of PRE herbicides are worth considering and further developing, especially in VLCFA-inhibitor-sensitive cereal crops. Here, sequential applications provided the same amount of weed control as the highest single rate, but by splitting the high rate into two separate applications (90/120 and 120/90 g ai ha\(^{-1}\)) injury to the crop was reduced when used in conjunction with a herbicide safener. As a result, further research investigating split herbicide applications in cereal crops with high herbicide sensitivity and few herbicide options for weed control should be pursued to
improve selective weed management strategies without generating transgenic, herbicide-tolerant varieties.
4.2 Literature Cited


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