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Herbicide Safening to Aid in the Establishment of Three Native Warm Season Grass Species

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By

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Herbicide safening to aid in the establishment of three native warm season grass species

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Difficulties with stand establishment are a major factor limiting further agronomic use of native warm season grasses. One significant cause of stand failure is competition with rapidly growing annual weed species during the early development of the perennial native grass. Broad spectrum preemergent herbicides can provide the needed weed control, but only if tolerance exists in the desired grass. Herbicide safeners, synthetic compounds that protect crops from herbicide injury, applied as seed treatments offer a potential strategy to achieving the needed herbicide tolerance where it does not naturally occur. This study tested the efficacy of five herbicide safeners (benoxacor, fenclorim, fluxofenin, naphthalic anhydride, and oxabetrinil) in protecting three native warm season grass species (big bluestem, *Andropogon gerardii* Vitman; little bluestem, *Schizachyrium scoparium* (Michx.) Nash; indiangrass, *Sorghastrum nutans* (L.) Nash) from herbicidal injury caused by preemergent application of S-metolachlor and quantifies this establishment method's impact on early stand performance.

DEDICATION

I would like to dedicate my research to Erin for your love, support, patience, encouragement, and confidence in me.

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CHAPTER I

INTRODUCTION

Native warm season grasses (NWSGs) are of agronomic interest as forages (Moser and Vogel, 1995), biofuel feedstock (Parrish and Fike, 2005; Gonzalez-Hernandez et al., 2009), and as aids in soil and water conservation (Sanderson et al., 2004). In addition to these agronomic roles, NWSGs are also important plant materials for wildlife habitat (Harper et al., 2007) and in ecological restoration projects (Wiygul et al., 2003; Jones et al., 2007). However, difficulties with stand establishment are significant obstacles to their wider agronomic use and can impede restoration efforts. Establishment can be limited by site and cultivar selection, planting time and depth, seed dormancy, and soil moisture (Masters et al., 2004). Competition with weed species is another major cause of stand failure, since most NWSGs do not compete well with weeds at the establishment stage (Mitchell and Britton, 2000). Toward this end, selective herbicides can play an important role in stand establishment (Martin et al., 1981). Tolerance to the desired herbicides in the chosen grass cannot always be found, though, and when planting mixtures of grasses a uniform response between different grasses to a single herbicide is often lacking (Harper et al., 2004).

The use of herbicide safeners, synthetic compounds that protect crops from herbicide injury, as seed treatments offers a potential strategy to achieving the needed herbicide tolerance where it does not naturally occur. However, a single safener does not

protect a single crop against all herbicides or all crops against a single herbicide. Empirical studies are therefore necessary to determine a proper crop-herbicide-safener relationship. This research aimed to determine the efficacy of five herbicide safeners (benoxacor, fenclorim, fluxofenin, naphthalic anhydride, and oxabetrinil) in protecting three NWSGs (big bluestem, *Andropogon gerardii* Vitman; little bluestem, *Schizachyrium scoparium* (Michx.) Nash; and indiangrass, *Sorghastrum nutans* (L.) Nash) from herbicidal injury due to preemergent application of S-metolachlor, a member of the chloroacetamide class of herbicides.

Of the safeners included in this study, oxabetrinil and fluxofenin are commonly used in sorghum (*Sorghum bicolor* L. Moench) (Fuerst and Gronwald 1986) and benoxacor in maize (*Zea mays* L.) (Kreuz et al., 1989) to protect against metolachlor injury. Fenclorim is commonly used to safen rice (*Oryza sativa* L.) from pretilachlor, another member of the chloroacetamide class of herbicides (Wu et al., 1996). Naphthalic anhydride is less widely used today, but is included as it is the first commercially available safener (Abu-Quare and Duncan, 2002) and is previously reported to be effective in safening the NWSGs indiangrass and switchgrass (*Panicum virgatum* L.) (Griffin et al., 1988).

A safener effective in protecting big bluestem, indiangrass, and little bluestem from S-metolachlor injury would allow stands to be established with the use of this broad spectrum preemergent herbicide. The most significant benefit would be the suppression of annual grassy weeds while the perennial NWSGs are establishing. This could aid establishment in a nursery setting where crossing blocks are needed to improve varieties and foundation fields are needed for seed increase. More importantly though, would be

increased success of in-field establishment, whether pasture, field margins, or restored prairie.

CHAPTER II

REVIEW OF LITERATURE

Botanical Description

Native Warm Season Grasses

Native warm-season grasses are grasses "historically indigenous" to a given region whose active growth occurs during that region's warm seasons (Harper et al., 2007). In the southeastern United States, "historically indigenous" is generally understood to mean: "present prior to European settlement" (Harper et al., 2007). Native species must be clearly distinguished from naturalized species. A naturalized species is one that persists without cultivation, and is in that sense naturally occurring, but originated outside of the specified region and is therefore not native to it. Many of the grasses most readily found in the Southeast are naturalized species introduced to the region to serve as forages: tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) and orchardgrass (*Dactylis glomerata* L.) from Europe, bermudagrass (*Cynodon dactylon* (L.) Pers.) and crabgrass (*Digitaria sanguinalis* (L.) Scop.) from Africa, and bahiagrass (*Paspalum notatum* Fluegge) and dallisgrass (*Paspalum dilatatum* Poir.) from South America (Harper et al., 2007).

Grasses can be further and more precisely distinguished as warm or cool season according to the pathway they utilize in photosynthesis. Warm season grasses store energy by fixing carbon into four-carbon units and are therefore known as "C_4 grasses"

while cool season grasses fix carbon into three-carbon units and are known as "C_3 grasses" (Moser et al., 2004). C_4 grasses are better adapted to high temperature conditions than C_3 grasses (Moser et al., 2004). C_4 plants differ from C_3 plants both anatomically and enzymatically. These differences allow C_4 plants to fix carbon more efficiently under higher temperatures by avoiding photorespiration, a process that occurs at the expense of photosynthesis and increases in rate as temperature increases (Taiz and Zeiger, 2010).

Southeastern NWSGs are, in sum, C_4 grasses present in the southeastern United States prior to European settlement. While many species fit this description, this study focuses specifically on big bluestem, little bluestem, and indiangrass. These three species, along with switchgrass, are the dominant components of many southeastern grassland ecosystems (Deselm and Murdoch, 1993) as well as the tallgrass prairies of the Great Plains (Sims and Risser, 2000). They, along with eastern gamagrass (*Tripsacum dactyloides* L.), are also the most extensively used NWSG for forage production and wildlife habitat (Harper et al., 2007).

The Bluestems

Grass species considered 'bluestems' belong to the genera *Andropogon* and *Schizachyrium*, which worldwide contain 100 and 60 species, respectively, with big and little bluestem the most economically important species in North America (Boe et al., 2004). *Andropogon* contains 13 species and *Schizachyrium* nine species native to North America (Barkworth et al., 2007). Little bluestem was previously classified as a *Andropogon scoparius* (Hitchcock, 1971), but contemporary taxonomies classify it as

Schizachyrium scoparius (Michx.) Nash (Barkworth et al., 2007). Little bluestem has an extensive range from Canada to Mexico and occurs across the conterminous United States with the exceptions of: Nevada, California, and Oregon (Barkworth et al., 2007). Across this range it exhibits significant clinal variation and is further divided into three subspecies: *divergins*, *scoparium*, and *stoloniferum* (Barkworth et al., 2007). Big bluestem, *Andropogon gerardii* Vitman, has a similarly expansive range (from Canada to Mexico, across the conterminous United States with the exceptions of: Nevada, California, Oregon, Washington, and Idaho) but is less prominent along its margins than little bluestem, shows less clinal variation, and is not divided into subspecies (Barkworth et al., 2007). Big bluestem does hybridize with sand bluestem, *Andropogon hallii* Hack., and so the two are considered conspecific subspecies by some (Barkworth et al., 2007).

Big bluestem and little bluestem are both perennial, but big bluestem has a more rhizomatous growth habit and forms larger 'clumps' whereas little bluestem has no or very short rhizomes and forms smaller 'bunches' (Harper et al., 2007). Both bluestems begin growth in early spring and reach their maximum height in July, but big bluestem can reach up to three meters while little bluestem just over one meter tall (Harper et al., 2007). Big bluestem flowers from late June to October and sets seed in late September through October (Kaeser and Kirkman, 2010). Little bluestem flowers from July to October and seeds mature from late November to early December (Kaeser and Kirkman, 2010). Big and little bluestem are both outcrossing species but, selfing reduces vigor in big bluestem more significantly than in little bluestem (Boe et al., 2004). The base chromosome number for the genera *Andropogon* and *Schizachyrium* is x=10, with big

bluestem most often occurring as a hexaploid (2n=6x=60) and little bluestem as a tetraploid (2n=4x=40) (Boe et al., 2004).

Indiangrass

Indiangrass, *Sorghastrum nutans* (L.) Nash, is the most widely distributed species in North America of the genus *Sorghastrum* which consists of 20 species worldwide (Mitchell and Vogel, 2004). Three *Sorghastrum* species occur in North America and indiangrass has a range similar to that of big bluestem described above (Barkworth et al., 2007). Indiangrass is perennial and, like big bluestem, forms clumps due to its short rhizomes (Harper et al., 2007). Sod forming populations with longer rhizomes do occur, though, in native prairie stands of the Great Plains (Mitchell and Vogel, 2004). Indiangrass begins growth in early spring and reaches one to two meters tall, setting seed in a pronounced, gold, panicle seed head (Hitchcock, 1971). Indiangrass flowers from August to September and its seeds mature throughout October (Kaeser and Kirkman, 2010). Indiangrass is largely self incompatible, does not perform apomixis, and is cross pollinated by wind dispersed pollen (Mithcell and Vogel, 2004). Indiangrass is an allopolyploid most often occurring as a tetraploid (2n=4x=40), although diploids and hexaploids have been reported (Mitchell and Vogel, 2004).

Mississippi Prairie Natural History, Restoration, and Conservation

Attempts to restore native Mississippi grasslands must be guided by an understanding of their natural history and the interacting influences that establish and maintain them. The southeastern United States is home to a great variety of grasslands which vary according to climatic and edaphic conditions and disturbance regime (Deselm

and Murdock, 1993). The prairies of Mississippi are blackland prairies. Blackland prairies are calcareous grasslands occurring on alkaline soils formed from underlying limestone formations created by a shallow sea that covered the southeastern United States 100-30 mya (Wilson, 1981; Peacock and Schauwecker, 2003). The chalk and marl formed from the marine sediment weather to soils high in shrink-swell, "self-mulching" clays with deep A horizons rich in organic matter (Moran et al., 1997; Campbell and Seymour, 2011a). It is this high organic matter content which gives southeastern blacklands their name. Mississippi's blackland prairies are confined to two physiographic regions: central Mississippi's Jackson Prairie Belt (Jones, 1971; Moran et al., 2003; Barone, 2005b) and northeast Mississippi and central Alabama's Black Belt (Barone, 2005a; Barone and Hill, 2007; Campbell and Seymour, 2011a).

The Black Belt region begins in McNairy County, Tennessee and stretches south, in a crescent, through northeast Mississippi and central Alabama ending in Russell County, Alabama (Hill et al., 2009). The Black Belt, defined as a physiographic region, is the area underlain by Cretaceous sediment forming the Selma Group with Demopolis Chalk predominating (Campbell and Seymour, 2011a). Although the Black Belt is often referred to as the "Prairie Belt" (Webster and Samson, 1992), it is important to note that the region was never continuous prairie, but instead contained "prairie islands" scattered within the broader physiographic region (Rostlund, 1957; Jones and Patton, 1966; Rankin and Davis, 1971; Barone, 2005a; Barone and Hill, 2007). The distribution of prairies within the region is correlated with these soil types. Broadly, prairies occurred on alkaline clays and woodlands on acidic loams (Jones and Patton, 1966; Rankin and Davis, 1971; Campbell and Seymour, 2011a). The most conservative estimate is that the

Black Belt contained at least 145,000 ha of prairie as recently as 1830 (Barone, 2005a). The Jackson Prairie Belt extends across central Mississippi from the eastern edge of the Loess Hills Region east to the Mississippi-Alabama border, continuing a short distance into Washington County, Alabama (Moran et al., 1997). The Jackson Prairie Belt is underlain by the Yazoo Clay Formation of the Jackson Group which is composed of Eocene-aged marine sediment deposited by the shallow coastal sea that receded 30 mya (Moran et al., 1997). The Jackson Prairie Belt, defined as a physiographic region, is the eastern portion of the broader Yazoo Clay formation not covered by the Loess Hills or the sandy Jackson Hills and covers nearly 250,000 ha (Moran, et al., 1997; Elsen and Weiland, 2003). Within the Jackson Prairie Belt, prairies occur on soils which are 'alkaline islands' surrounded by acidic hardwood and pine forests (Jones, 1971; Moran et al., 2003). The most conservative estimate is that the Jackson Prairies Belt contained 12,000 ha of blackland prairie as recently as 1830 (Barone, 2005b).

Less than two percent of Mississippi and Alabama's blackland prairie ecosystem exists today (Noss et al., 1995). These remnant prairies range from 1 to 65 ha in the Jackson Prairie Belt (Moran et al., 2003) and up to 200 ha in the Black Belt (Campbell and Seymour, 2011b). Road side right-of-ways and power-line clearings serve as refugia to prairie species but not as intact prairie ecosystems (Peacock and Shauwecker, 2003). The fertile, alkaline prairie soils of the Black Belt were converted to plantation-based farming as large-scale planters moved to the Deep South from Virginia and the Carolinas (Webster and Samson, 1992; Peacock and Shauwecker, 2003). Although agriculture was not practiced on the same scale in the Jackson Prairie Belt, the prairie soils were the region's most productive and the prairies were plowed in the 1800's (Elsen and Wieland,

2003). In both the Black Belt and the Jackson Prairie Belt many of the remnant prairies occur on especially thin soils since prairies on deeper soils were lost to agriculture (Barone and Hill, 2007).

The conservation of southeastern prairies has practical consequences beyond the region. Remnant prairies are valuable reserves of grassland species germplasm (Moser and Vogel, 1995). Grasslands were established in the Black Belt 7-5 mya, and the region served as refuge for grassland species during the subsequent glaciations (Brown, 2003). The Southeast is the "source region" of the big bluestem, indiangrass, little bluestem and switchgrass composing the prairies of the Great Plains (Brown and Gersmehl, 1985). As a center of origin for these species, one would expect the Southeast to also serve as an important center of diversity. In fact, grasslands of the Southeast have been established as "hot spots" of genetic diversity for switchgrass (Zhang et al., 2011). As native grasses are developed for agronomic use, the remnant prairies of the Southeast stand as potentially important gene pools. Ironically, the vast majority of these grasslands have already been lost to past agricultural practices leaving them unable to contribute to future agricultural development.

Agronomic Uses

NWSG as Forages

When used as forage, NWSGs serve as complements to cool-season grasses and alternatives to introduced warm season grasses. Historically in the Southeast, animal agriculture based on native grasses went undeveloped. European settlers to the region found the big and little bluestem, indiangrass, and switchgrass composing the native grasslands to be both nutritious and highly favored by livestock, but these grasses were

soon overgrazed and once healthy stands were replaced by less desirable native grasses, notably broomsedge bluestem (*Andropogon virginicus* L.) (Ball et al., 2007). Improvement of pastures was, then, slower to develop in the Southeast than in the North due to the South's cash-crop system (Ball et al., 2007). Livestock production eventually increased in the Southeast, beginning in the 1930's, with pasture development based largely on introduced species: bermudagrass, johnsongrass (*Sorghum halepense* (L.) Pers.), and bahiagrass as warm season grasses; tall fescue and annual ryegrass (*Lolium perenne* L.) as cool-season grasses (Ball et al., 2007). Grazing systems utilizing southern NWSGs are now being developed, though, due to their nutritional quality and yield potential (Harper et al., 2007).

Warm season grasses, whether native or exotic, are important additions to grazing systems because they offer high quality, actively growing forage while cool season grasses are dormant or less productive and, thereby, reduce the need to harvest and store hay for summer feed (Anderson, 2000). Average daily weight gain of cattle grazing cool season grasses in spring and fall and warm season grasses in summer is superior to average daily weight gain of cattle grazing cool season grasses year round (Anderson, 2000). There are significant quality differences between warm and cool season forages, though, with these differences due largely to anatomical differences in their C_3 and C_4 photosynthetic pathways (Anderson, 2000; Coleman et al., 2004). C_4 grasses achieve photosynthetic efficiency under high temperatures by enclosing the primary CO_2 acceptor, the protein Rubisco (Ribulose-1,5-biphosphate carboxylase oxygenase), in gas impermeable bundle sheath cells (Taiz and Zeiger, 2010). These cells are less digestible

and, therefore, yield less crude protein and soluble carbohydrates to the livestock grazing C_4 grasses (Coleman et al., 2004). Intake of C_4 grasses is also less than C_3 grasses, but this is thought to be due to the difference in digestibility (Coleman et al., 2004). Despite these differences in quality, warm season C_4 grasses still serve to complement cool season C_3 grasses when grazed during C_3 grasses' dormant season.

The advantages of including warm season grasses in grazing systems are well known, but rarely are NWSGs chosen in the South. The simplest argument in favor of using NWSGs for forage is low-input yield. Stands of switchgrass, eastern gamagrass, big and little bluestem and indiangrass can yield greater than or comparably to exotic grasses with less nitrogen fertilizer (Harper et al., 2007). Some NWSG also offer unique advantages distinct to their species. Big and little bluestem are both drought tolerant and yield well even with low soil moisture (Anderson, 2000; Boe et al., 2004). Indiangrass is less drought tolerant, but has an atypical response to drought stress which can be valuable in forage production. Most grasses respond to drought by setting seed earlier, resulting in lignification and diminished quality, but indiangrass responds by temporarily entering quiescence (Mitchell and Vogel, 2004). Indiangrass resumes growth once rains return, and can therefore produce quality forage after drought stress while other forages have converted to lower quality reproductive growth (Mitchell and Vogel, 2004). Indiangrass also initiates flowering later than other grasses, extending the season of warm season forage production by two to four weeks (Mitchell and Vogel, 2004).

Management of NWSG for forages differs from management of exotic grasses. The main difference is the emphasis on height of grazing and the need for rest between

grazings (Anderson, 2000). One reason the South's NWSGs were easily overgrazed was that these grasses, unlike exotic cool season grasses, evolved under intermittent grazing instead of continuous defoliation (Anderson, 2000). Multiple defoliations in a season can be especially detrimental to big and little bluestem (Boe et al., 2004). Length of rest between grazings is a major focus of management strategies which must allow adequate time for carbohydrate accumulation in the underground crown (Anderson, 2000). Without proper rest, stands of NWSG will decline. If harvested for hay, cutting once dormant will allow carbohydrates to accumulate below ground. This practice is helpful for future yields, but not quality (Boe et al., 2004). Timing of grazing is also more important for NWSGs than cool season grasses because NWSGs' rate of lignification is greater and nutritional value deteriorates faster as a consequence (Harper et al., 2007).

Erect, bunching, stiff-stemmed NWSGs offer wildlife habitat not provided by exotic, sod forming warm season grasses. Grassland birds, such as bobwhite quail (*Colinus virginianus*), utilize these erect NWSG, but not the sod forming bermudagrass and bahiagrass (Harper et al., 2007). Managing a stand of NWSG for the dual purpose of wildlife habitat and forage production requires different management techniques than if managed for just one of these goals alone. Pastures should only hayed or grazed once a year and timed so that winter cover is available, whereas the pasture would normally be grazed twice if forage was the only consideration (Harper et al., 2007).

Buffers for Soil and Water Conservation and Wildlife Habitat

Native warm season grasses are important elements in many soil and water conservation programs. These efforts take on their largest scale in the Great Plains where NWSGs (chiefly big bluestem, little bluestem, indiangrass, and switchgrass),

disseminated by the Natural Resource Conservation Service's (NRCS) Plant Material Centers (PMC), are the primary component of planted grasslands. Conservation Reserve Program (CRP) planted grasslands have been established on 17 million ha of land designated as highly erodible (Sanderson et al., 2004). These same NWSGs are also used for phytoremediation on severely contaminated or disturbed industrial sites (Parrish and Fike, 2005). NWSGs are important in managing soil erosion and water quality along agricultural fields, serving as riparian buffers, filter strips, and windbreaks along field margins. Perennial NWSGs such as big bluestem, little bluestem, indiangrass, and switchgrass can serve to slow soil erosion and agrochemical runoff if planted along field margins.

In 1997, the NRCS began the National Conservation Buffer Initiative which emphasized using warm season grasses as filter strips within fields, riparian buffers on field margins, grassed waterways, and windbreaks through the CP33 program (Sanderson et al., 2004). Field margins often have lower yields than the field on average due to increased competition and shade from surrounding woody vegetation, resulting in diminishing returns on agricultural inputs. These marginal areas can be converted to grass buffers to serve in water and soil conservation and the lost revenue largely made up by a combination of savings on inputs previously yielding a diminishing return plus CRP incentives (Harper et al., 2007). Grass buffers do not result in the same competition as the woody vegetation along unbuffered fields.

Due to its stiff stems and substantial root system, switchgrass is effective as a grass hedge reducing soil erosion and can support natural soil terraces (Gilley et al., 2000). Switchgrass filter strips have proven effective in reducing atrazine and

metolachlor runoff into streams by slowing runoff velocity and increasing soil absorption (Mersie et al., 1999) through intercepting soil particles (Rankins et al., 2001). Switchgrass serves as filter strips for agrochemicals and grass hedges for soil erosion more effectively than cool season grasses because it possesses stiffer stems, a stronger root system, and more erect growth (Lee et al., 1999). Big bluestem, switchgrass, and eastern gamagrass are equally effective filter strips in reducing herbicide runoff (Rankins et al., 2001). Other bunch forming, perennial NWSG such as indiangrass and little bluestem possess similar morphological traits and could potentially serve the same ends. Narrow buffers planted in these stiff stemmed NWSG reduce soil erosion and chemical runoff as effectively as wider buffers planted in cool season exotic grasses (Lee et al., 1999). However, if a producer wants the buffers to serve the dual goal of wildlife habitat a wider buffer is preferable. Narrow borders are disproportionally less preferred by wildlife and can serve as sinks when they do serve as habitat (Harper et al., 2007). In addition to vertebrate wildlife, field buffers also house pollinators and insect predators that can perform biological control of insect herbivores that otherwise go unchecked in unbuffered agricultural fields (Olson and Wackers, 2007).

Field buffers utilizing NWSGs integrate semi-natural grasslands into row crop production. While this is well short of grassland restoration, the practice can result in less chemical runoff reaching waterways, less soil lost from agricultural fields via erosion, provide increased habitat to grassland birds and insects, and possibly a measure of refugia to some grassland plant species. These buffers can allow for a less destructive meeting between our agricultural working lands and the natural environment.

Potential Contribution in Switchgrass Biomass Production

Early research into biofuels led to the conclusion that cropping systems producing dedicated bioenergy feedstock would be necessary to support large scale renewable fuel production based on biomass conversion (McLaughlin and Kszos, 2005). In contrast, 'first generation' biofuels are based on preexisting agricultural production of grain and oilseed food crops: ethanol from maize and sugarcane (*Saccharum officinarum* L.), biodiesel from soybean (*Glycine max* (L.) Merr.) and rapeseed (*Brassica napus* L.) (Jessup, 2009). Development of dedicated bioenergy crops began in earnest in 1978 when the Department of Energy (DOE) sponsored the creation of the Bioenergy Feedstock Development Program (BFDP) at Oak Ridge National Laboratory (ORNL), initially concentrating on short rotation forestry (McLaughlin and Kszos, 2005). In 1992, DOE, still working through BFDP at ORNL, initiated a new research program on herbaceous energy crops (HEC) emphasizing perennial grasses as feedstock in the production of ligno-cellulosic ethanol (Sanderson et al., 1996).

Through its research on HEC, the DOE designated switchgrass a 'model' species, and six project sites were established in Tennessee, Texas, Virginia, Alabama, and Oklahoma to develop cultural practices, conduct variety trials, assess germplasm, begin breeding work, develop tissue culture protocols, and advance basic biological research (Sanderson et al., 1996). Switchgrass was not the only perennial grass to be endorsed by the DOE's research on HEC, as giant miscanthus (*Miscanthus x giganteous*) and energycane (*Saccharum* spp.) are also the focus of ongoing studies (Jessup, 2009). Switchgrass was, though, the only native perennial grass granted the status of 'model' species for HEC development, which has led to what some researches see as a "distinctly

switchgrass flavor" to much of the research on herbaceous feedstock (Gonzalez-Hernandez et al., 2009). Despite this 20 year focus on switchgrass, there are reasons to expand research so as to include in the development of HEC other NWSGs, including big bluestem, indiangrass, and little bluestem.

An important attribute of switchgrass as a biomass crop is its ability to yield well on marginal and degraded lands (Parrish and Fike, 2005). If an HEC can maintain yields on marginal sites, then it can better avoid the tradeoffs that plague first generation biofuel crops: i.e., "the food versus fuel debate" (Cassman and Liska, 2007; Sanderson and Adler, 2008). However, Gonzalez-Hernandez et al. (2009), reviewing research on biomass production on marginal lands in the Upper Midwest, argue that there are both significant differences between and many kinds of marginal lands. They found that across these sites other native grasses out yielded switchgrass on certain sites: little bluestem on dry semiarid sites and prairie cordgrass (*Spartina petinata* Bosc ex Link) in low areas with poorly drained soils. They concluded that given the range of climatic and edaphic zones across the United States, multiple stress tolerant species must be evaluated for their potential biomass production and specific species matched to specific conditions in an "integrated multispecies approach" (Gonzalez-Hernandez et al., 2009). Switchgrass would still be treated as a 'model' species, but with its extensive body of research serving as a model for how to evaluate and develop additional species.

A second line of argument for the development of multiple native species as HEC is based on the prospect of creating multi-species mixtures. Tillman et al. (2006) argue that low-input high-diversity (LIHD) stands of multiple species, both grasses and legumes, can outperform monocultures when grown on degraded lands with little to no

agricultural inputs. Increased soil cover reducing the need for herbicides, nitrogen fixation by legumes reducing the need for nitrogen fertilizers, and decreased disease pressure through host diversity were all cited as explanations for the greater yields recorded for LIHD stands over switchgrass monocultures on degraded sites in the absence of agricultural inputs (Tilman et al., 2006). However, Tilman's results have received much criticism: biomass was burned on site potentially restoring many of the minerals which would normally only be replaced by fertilizer application, skewing the results concerning low-inputs; establishment difficulties of many of the species included in the mixtures were ignored; and results from a limited sample were extrapolated too broadly (Russelle et al., 2007). It may also be the case that the study's focus on nearly eliminating agricultural inputs is too extreme. While it is true that petroleum based synthetic fertilizers impact the net balance of biofuels' ability to reduce consumption of petroleum fuels, this balance must be carefully considered. The return on limited inputs may well exceed the cost.

Regardless of the validity of Tilman's specific study, the potential of mixtures to limit disease pressure in agricultural crops is well established (Mundt, 2002). This potential could prove valuable in switchgrass biomass production as disease pressure in HEC will likely be exacerbated by their being grown under sub-optimal conditions on marginal and degraded lands (Stewart and Cromey, 2011). Disease pressure may be further increased as native grasses are bred to improve their performance as dedicated bioenergy crops. Switchgrass populations improved as biofuel feedstock (selected for greater growth rate and more efficient conversion through reduced recalcitrance to digestion) have shown increased susceptibility to the insect-vectored barely yellow dwarf

virus (BYDV) and cereal yellow dwarf virus (CYDV) (Schrotenboer et al., 2011). Natural populations of switchgrass, big bluestem, and little bluestem are known to be hosts for BYDV, though not for the race most commonly affecting wheat (*Triticum* spp.), a concern for cereal producers in the Great Plains given the dominance of these grasses in the region's remaining grasslands (Garrett et al., 2004). The phytopathological consequences of growing 'improved' (more BYDV susceptible) varieties of switchgrass on marginal lands on increasing acreage is, therefore, also a concern to producers outside the biomass market.

There is agreement, though, that the most significant source of increased disease pressure will result from switchgrass being grown as a monoculture on large scales (Parrish and Fike, 2005; Gonzalez-Hernandez et al., 2009). One of the largest switchgrass production projects to date, over 1,600 ha planted with the cultivar Cave-in-Rock, was sponsored by the USDA in the Chariton Valley of Iowa, an area less suitable to row cropping than most of the state. Over multiple years of production, yield declined in many fields involved in the project (Thomsen et al., 2008). The decline in yield coincided with an outbreak of switchgrass smut caused by the fungus *Tilletia maclaganii* (Gravert et al., 2000). The disease cycle of *T. maclaganii* is not well studied, but it is thought to be similar to *T. controversa*, the pathogen causing dwarf bunt in cereals, which spreads by wind dispersed spores, overwinters in soil, infests plants through roots and emerging tillers, and spreads systemically eventually infesting the inflorescence (Thomsen et al., 2008). In a study to assess its impact on yield, *T. maclaganii* was found in 15 of 17 fields surveyed, was estimated to be present in 50% to 82% of the sampled area, and was calculated to result in yield losses ranging from 0.6% to 40.1% in different

fields (Thomsen et al., 2008). Yield losses of 40% are not economically acceptable in any crop, but even less so in biomass production where the profit margins are especially tight. Furthermore, these same tight margins make the use of chemical disease control prohibitive (Stewart and Cromey, 2011).

In the absence of chemical control, cultural practices and host genetics are the primary tools left to combat disease, but options for cultural practices are limited by the persistence of perennial crops. Crop rotation, tillage, and properly timing one's seeding date, strategies effective in managing disease in annual crops, simply are not possible for perennial crops beyond the establishment year (Cox et al., 2005). Plus, perennial crops can be subject to diseases that annual crops are not, since they can play host to pathogens that must overwinter on live tissue (Cox et al., 2005). Disease management is further confounded by perenniality due to the fact that a perennial stand infected by a disease has the potential to harbor the pathogen from season to season resulting in increased duration of epidemics and continuously declining yields. This was observed in the example from Iowa's Chariton Valley cited above.

The impracticality of chemical and cultural controls highlights the importance of host genetics in managing disease in HEC. Host genetics can play this role by way of host resistance as well through host diversity. In addition to the smut and viruses already cited, switchgrass has also been shown to be susceptible to bunt caused by *T. pullcherrima* and rust caused by *Puccinia emaculata*, with instances identified in Texas, Arkansas, and Tennessee (Carris et al., 2008; Zale et al., 2008; Hirsch et al., 2010). Genetic variation for susceptibility to *P. emaculata* has been found within and between switchgrass populations suggesting that breeding efforts could potentially lead to

cultivars with increased rust resistance (Gustafson et al., 2003). Switchgrass is also susceptible to spot blotch caused by *Helminthosporium sativum*, and genetic variation has been found for susceptibility, again suggesting that breeding efforts could increase resistance within populations (Zeiders, 1984). Both examples show that breeding for increased disease resistance will play an important role in switchgrass production.

If disease pressure has been increased, though, due to growing switchgrass as a monoculture we should also look at managing disease through increasing host genetic diversity not just improving host resistance. It is important to note that switchgrass, and the three NWSGs included in this study, are all outcrossing species so that a single species NWSG 'monoculture' is in fact a true population. A single species NWSG monoculture contains more genetic diversity than a multiline cultivar of wheat, for instance. 'Monoculture' is a relative term. However, that does not preclude the possibility of increasing genetic diversity within a switchgrass monoculture or increasing genetic diversity within a switchgrass for biofuel stand by intercropping with different species.

The genetic diversity of a switchgrass cultivar (i.e., population) can be increased by creating cultivars through "multiple origin poly-crosses" within the limits of outbreeding depression (Booth and Jones, 2001). The genetic structure of switchgrass and other outcrossing NWSG populations (i.e., significant variation existing within populations but not between populations) suggests that outbreeding depression is very unlikely to occur in these multiple origin poly-crosses (Casler et al., 2007). However, greater increases in genetic diversity in an HEC stand can be achieved by intercropping multiple native perennial grasses, than can be achieved by any effort to widen the genetic

base of a switchgrass population. The strategy of intercropping perennial grasses is supported by ecological studies of grasslands where pathogen loads of foliar fungal diseases increase as species diversity decreases (Mitchell et al., 2002). Disease management through host genetics could then be maximized by deploying switchgrass populations with improved resistance and widened genetic bases plus intercropping those populations with different species of NWSGs, such as big bluestem, little bluestem, and indiangrass.

Intercropping is not guaranteed to provide decreased disease pressure *a priori*, since not all diversity results in "functional diversity," the ability of host diversity to lead to disease reduction (Mundt, 2002). Garret and Mundt (1999) suggest circumstances wherein such functional diversity is likely (host genotype unit area is small, host-pathogen specialization is strong, the pathogen possess a shallow dispersal gradient, and the number of pathogen generations over an epidemic is large) and note that much depends on the life cycle of the pathogen (wind dispersal versus splash dispersal and overwintering requirements). One requirement for functional diversity is that the diversity includes differential responses to the disease in question, especially race specific differences (Mundt, 2002). Exactly such race specific differences in response were recorded between big bluestem, little bluestem, and switchgrass for BYDV (Garrett et al., 2004). Indiangrass was found to be resistant to all of the races of BYDV infecting the other grasses, suggesting it would be an important component of a mixture aiming to impede the spread of BYDV, a potentially important goal in cereal producing regions.

Whether mixtures of these grasses provide functional diversity and serve to suppress disease within switchgrass for biofuel stands in the Southeast will depend upon

the specific pathogens that prove most problematic in the region and their life cycle, as outlined above. However, there is reason to expect host diversity to be effective in the region. Host diversity is known to be most effective in controlling wind dispersed foliar pathogens (Cox et al., 2005). Given the incidence of rust reported in Arkansas and Tennessee (Zale et al., 2008; Hirsch et al., 2010), one may expect diversity strategies to be important in managing disease in the Southeast.

Important questions will be the effect intercropping has on yield in the absence of the pathogen, the expected yield loss due to the pathogen, how effectively intercropping mitigates that yield loss, and how intercropping compares to widening the genetic base of a switchgrass monoculture with regard to the first three questions. Empirical studies will be necessary to answer each. While those questions await answers, the possibility of disease management through intercropping, coupled with the need to appropriately match different species to the range of marginal sites on which HEC could potentially be grown (Gonzalez-Hernandez et al., 2009), together serve as powerful arguments to expand HEC research to include other NWSGs, including big bluestem, indiangrass, and little bluestem in the Southeast. If we are going to attempt to utilize these other grasses in HEC production, we cannot repeat the mistakes of Tilman et al. (2006) and ignore the difficulties of multi-species stand establishment (Russelle et al., 2007).

Establishment with Metolachlor and Safeners

Overview of Establishment Methods

Methods for establishing NWSGs occur across a spectrum of site disturbance. On one extreme, exotic grasses can be controlled with herbicide application and the naturally occurring seed bank allowed to revegetate the site with no seeding (Harper et al., 2007).

If a specific grass composition is desired seeding will be necessary. Seeding can be accomplished by drilling into preexisting sod that has been suppressed by herbicide application or by tilling and creating a seed bed (Masters et al., 2004). Creation of a seed bed represents the extreme, opposite reliance on the existing seed bank.

Whether drilling into sod or creating a tilled seed bed, the seeding rate will be determined by end use objectives and must be based on a calculation of pure live seed (PLS) (Harper et al., 2007). Pure live seed is calculated as the percent of the bulk material that is pure seed, multiplied by the seed's germination rate, divided by 100 (Harper et al., 2007). When planting a recommended rate of seed, one must plant according to PLS of the seed and not bulk weight. Seeding rates are heavier if the stand is intended for forage than if intended for wildlife habitat. Recommended seeding rates for big bluestem, indiangrass, and little bluestem are 3.36-5.60 kg PLS ha^{-1} (3-5 lbs PLS $acre^{-1}$) for wildlife habitat and 11.21-13.45 kg PLS ha^{-1} (10-12 lbs PLS $acre^{-1}$) for forage stands (Harper et al., 2007).

When seeding NWSGs, common causes of establishment failure are drilling seed too deeply, planting too late into the season, failing to control weeds and using equipment that is inappropriate for planting the fluffy seed (Harper et al., 2007). The latter is especially problematic when seeding big bluestem, indiangrass, and little bluestem given the awns and seed hairs present on seed. NWSGs can be surface broadcasted or planted with no-till drills, but when planting bluestems or indiangrass with a drill it is necessary to use specialized seed boxes to reliably feed the fluffy seed into the drill (Harper et al., 2007). Seeding should be completed in April through May as soil temperatures approach

14.5°C (58° F), and seed planted no more than 0.6 cm (¼") deep with 30% of applied seed visible on the surface (Harper et al, 2007).

Methods to control weed competition prior to seeding will vary according to the previous use of the site. If the site was previously pasture, then seed may be sewn directly into sod using no till drills after a prescribed burn followed by herbicide application (Masters et al., 2004). If converting pasture previously planted in fescue or other cool-season exotic perennial grasses an application of glyphosate in October or November prior to spring seeding is recommended (Harper et al., 2007). Imazapyr has proven most effective in controlling bermudagrass when converting warm season pasture (Bond et al., 2005). If seeding big bluestem, indiangrass, and little bluestem, then johnsongrass can be controlled using preemergent applications of imazapic or with spot application of glyphosate for postemergent control (Harper et al., 2007). Under each of these scenarios, it is recommended that herbicide application follow haying or burning in order to remove litter that can intercept herbicide application and to stimulate active growth in the grass being controlled (Bond et al., 2005; Harper et al., 2007). If big bluestem, indiangrass, or little bluestem are being established on a site previously used in row crop production, then glyphosate is recommended to control winter annual weeds and a preemergent application of imazapic should be applied after seeding to control broad leaf warm season annuals (Harper et al., 2007). While preemergent application of imazapic is effective in establishing big bluestem, indiangrass, and little bluestem, switchgrass is not tolerant to imazapic (Harper et al., 2004). Preemergent application of atrazine has been used to increase establishment of switchgrass, but with less success in big bluestem and indiangrass (Martin et al., 1981). Metolachlor has proven more

effective as a preemergent herbicide in NWSG establishment than atrazine due to controlling a broader spectrum of annual grasses (Mitchell and Britton, 2000).

If, instead of drilling into the sod, a seed bed is to be prepared, several factors should be considered. An ideal seed bed possesses a pulverized, friable surface, but is firm below the planting surface, has been cleared of established vegetation, and avoids clodding and puddling (Masters et al., 2004). A firm seed bed is important because it discourages seed from being planted too deeply, provides support to young roots, and encourages water to move up to the planting surface by capillary action as the surface dries out (Masters et al., 2004). A seed bed that is too 'fluffy' contains pore spaces that are too large for capillary water movement and therefore inadequate moisture near the germinating seed. Seed bed preparation through tillage allows for incorporation into the soil profile of plant residue, disturbance of germinating weed seeds and emerged weeds, reduction of soil compaction, and breaking apart of clods, but excessive tillage can increase soil erosion, destroy soil structure, and encourage ungerminated weed seeds (Masters et al., 2004). Many of these problems can be avoided by conservation tillage practices. However, the plant residue left on the soil surface in conservation tillage can interfere with herbicide efficacy and emerging grass seedlings if too thick (Masters et al., 2004).

During establishment in a tilled seed bed, the primary focus of weed control is on controlling annual warm season grassy and broad-leaved weeds, but this focus shifts to controlling cool-season grasses once the stand is established (Mitchell and Britton, 2000). Currently, the most important tool for weed management in established stands of big bluestem, indiangrass and little bluestem is imazapic, which can be applied

postemergence without harming these grasses (Harper et al., 2004). A broader spectrum herbicide, such as metolachlor, could potentially serve, though, as a more effective preemergent control of annual grassy weeds than imazapic and further serve to improve stand establishment. Once the stand is established, the NWSGs can compete well with the weeds, which can be further controlled with postemergent application of imazapic when necessary.

Metolachlor

Metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide] is an extensively used, preemergent herbicide in the chloroacetamide class (Lebaron et al., 1988). Metolachlor does not prevent germination, but instead distorts seedling growth to such an extent as to prevent emergence and establishment (Fuerst, 1987). Seedling emergence is impeded by inhibiting growth of the coleoptile in monocots and the hypocotyl node in dicots through decreased rates of both cell division and cell elongation, and the herbicidal effect is most pronounced when absorbed at these sites (Deal and Hess, 1980; Ebert 1980). If the coleoptile does grow enough to emerge it is malformed, hindering the unfolding leaves (Ebert, 1980). Establishment is further decreased by reduced root growth (Deal and Hess, 1980). Early cellular observations suggested impaired membrane formation as the cause of growth inhibition (Ebert, 1980).

Several physiological processes have been shown to be inhibited by metolachlor and other chloroacetamide herbicides including: the synthesis of lipids, isoprenoids, flavonoids, and gibberellic acids and acetyl-CoA metabolism (Fuerst, 1987; Lebaron et al., 1988). Chloracetamide herbicides, form covalent bonds with biological molecules containing sulfhydryl functional groups and are therefore considered alkylating agents

(Fuerst, 1987). Despite having many physiological effects, the effectiveness of metolachlor at sub-micromolar levels led investigators to believe that it functions primarily through a single, highly specific target site as opposed to being a general inhibitor of sulfhydryl containing enzymes (Couderchet and Boger, 1993). An enzyme was suspected as the target site due to the stereospecificity of chloroacetamides: for example, only the S-enantiomer of metolachlor is phytotoxic (Boger 1997). Metolachlor's primary mode of action has now been established as the inhibition of very long chain fatty acids (VLCFA) synthesis, fatty acids longer than C18 (Boger et al., 2000; Wu et al., 2000), and this is how metolachlor is classified for purposes of managing weed resistance (Mallory-Smith, 2003). Inhibition of VLCFA synthesis results in cell death by disrupting plasma and organelle membrane function and cell wall formation (Boger et al., 2000; Boger 2003). The range of effects cited above - inhibition of lipids, proteins, and gibberellic acids - is consistent with this more specific function if the more diverse effects occur due to unspecific binding under higher concentrations or if they are secondary effects resulting from the inhibition of VLCFA synthesis (Boger et al., 2000).

The synthesis of VLCFA is catalyzed by the enzyme system 'elongase,' four enzymes catalyzing four elongation reactions (Boger et al., 2000). The first step is a condensation reaction that is specific to VLCFA synthesis whereas the other three steps are shared by all fatty acid elongations (Boger et al., 2000). The enzyme catalyzing the first step was hypothesized as the specific chloroacetamide target site (Boger et al., 2000). A reaction mechanism proposed by Boger et al. (2000) posits the inhibition of elongase occurring through a covalent bond between chloroacetamides and the cysteine site of the condensation enzyme. This bond would be formed in competition with the

enzyme's substrate, acetyl-CoA. Subsequent studies have demonstrated metolachlor's ability to inhibit enzyme function by bonding to active cysteine sites (Eckerman et al., 2003). A specific fatty acid elongase enzyme (FAE1) encoded by a gene cloned from *Arabidopsis* (*FAE1*) (Millar and Kunst, 1997) has now been confirmed as the condensing enzyme targeted by chloroacetamides (Boger et al., 2003).

Just as chloroacetamides' status as alkylating agents is important in understanding their herbicidal mode of action, it is also a key to understanding their detoxification. Chloroacetamides are conjugated by glutathione (GSH), a sulfhydryl compound, and the conjugated form is non-phytotoxic (Shimabukuro, 1985). The conjugation occurs both non-enzymatically and enzymatically by way of glutathione-S-transferase (GST) (Gronwald et al., 1987), an enzyme involved in many plant stress responses (Marrs, 1996). Pang et al. (2012) explain the metabolism and subsequent detoxification of metolachlor in maize as proceeding through a three phase process: 1) oxidation to reveal metolachlor's chloride group, 2) conjugation to GSH by GST (encoded by *ZmGST27*), and 3) transport of the metolachlor-GSH conjugate to the vacuole for degradation by GSH-transporter (encoded by *ZmGT1*) or by an ATP-binding-cassette (ABC) transporter (encoded by *ZmMRP1*).

Pang et al. (2012) also demonstrate that metolachlor's stereospcificity is at least as important to its detoxification as to its mode of action. Pang et al. treated maize with both racemic and S-metolachlor and, using RT-PCR, quantified the expression of *ZmGST27*, *ZmGT1*, and *ZmMRP1*. They found no significant differences in the expression of *ZmGST27* or *ZmGT1* when exposed to racemic or S-metolachlor, but they did find significantly higher expression of *ZmMRP1* when exposed to racemic

metolachlor than when exposed to S-metolachlor. This suggests the presence of a stereospecific interaction between the metolachlor-GSH conjugate and the ABC-transporter, but not GST or GSH-transporter, which could potentially result in greater detoxification of racemic metolachlor than S-metolachlor, possibly explaining the increased toxicity of S-metolachlor compared to racemic metolachlor. To further test this hypothesis Pang et al. generated dose response curves by treatment of maize with racemic and S-metolachlor and found racemic metholachlor to have an EC_{50} 2.1 times that of S-metolachlor. They repeated this test using maize treated with vanadate, an ABC-transporter inhibitor, and found that the EC_{50} of racemic metolachlor was reduced from 2.1 times to 1.3 times that of S-metolachlor. This provided evidence for concluding that the difference in phytotoxicity between racemic and S-metolachlor lies largely in the differences between the rates at which they are detoxified, not the rate at which they bind to their target site, and that the difference lies specifically with the role of ABC-transporters in the detoxification process. The results of Pang et al. are surprising given that the difference in phytotoxity between racemic and S-metolachlor has generally been assumed to be due to stereospecificity of the target site within the VLCFA synthesis pathway, not differences within the detoxification process. The "quasi-equal growth inhibition effect between [racemic] and S-metolachlor in the presence of an ABC transporter inhibitor (vanadate)," strongly argues against this assumption, though (Pang et al., 2012).

Variation in levels of GSH has been correlated with the selectivity of metolachlor and other chloroacetamide herbicides with tolerance resulting from more rapid metabolism (Breaux et al., 1987). Naturally occurring variation of susceptibility to

metolachlor has been correlated with variation in GSH levels and GST activity in maize (Sari-Gorla et al., 1993) and in the weedy species blackgrass (*Alopecurus myosuroides* Huds.) (Cummins et al., 1997). The naturally occurring detoxification of chloroacetanilides by the GSH-GST system, makes it a natural place to begin when trying to determine the mode of action of herbicide safeners in protecting crops from metolachlor injury. It should be noted, though, that there are instances where naturally occurring variation within a species to susceptibility to metolachlor was not correlated with variation in levels of GSH or GST activity (Wang and Dekker, 1995) and factors other than detoxification by GST, such as differential absorption and translocation, have been show to contribute to the selectivity of metolachlor (Dixon and Stoller, 1982). We should expect, therefore, to find differences between safeners in how they protect crops from metolachlor just as we find differences between species in how they protect themselves from metolachlor.

Herbicide Safeners

The concept of herbicide safeners began with Otto Hoffman's observation of an "antagonistic interaction" between the herbicides 2,4-D and 2,4,6-T (Hoffman, 1953; 1978). Herbicide safeners act through three general mechanisms: competition between the herbicide and the safener at the active site of the herbicide's target enzyme, reductions in herbicide uptake and translocation, and safener-enhanced metabolism of herbicides to detoxified forms (Abu-Quare and Duncan, 2002), with enhanced herbicide metabolism the predominant mechanism (Siminszky, 2006). Safeners are 'botanically specific,' with results varying between crops (Abu-Quare and Duncan, 2002). Four of the five safeners included in this study demonstrate this botanical specificity. Oxabetrinil

and fluxofenin protect sorghum (Fuerst and Gronwald 1986; Anonymous, 2008) and benoxacor, maize (Kreuz et al., 1989) from metolachlor injury. Fenclorim safens rice from pretilachlor, a chloroacetamide herbicide (Wu et al, 1996). Naphthalic anhydride shows less botanical specificity than the others, protecting a range of grasses from several herbicides of different classes (Hatzios, 1989).

All five of these herbicide safeners - oxabetrinil, fluxofenin, benoxacor, fenclorim, and naphthalic anhydride - have had their safening action correlated with the GSH-GST system. In particular, these safeners have been shown to increase GST activity more so than increasing levels of GSH. Sorghum seed treated with either naphthalic anhydride or oxabetrinil was safened from metolachlor and showed increased GST activity, but no increase in GSH content (Gronwald et al., 1987). The increase in GST activity resulted in rapid metabolism of the herbicide to the non-toxic GSH conjugate. In a study by Fuerst and Gronwald (1986), three safeners not discussed in this study, cyometrinil, dichlormid, and flurazole, were also shown to protect sorghum from metolachlor, in addition to naphthalic anhydride and oxabetrinil. The relative degree of protection between the five safeners was correlated with their relative ability to increase the plant's metabolism of metolachlor through enhanced GST activity (Fuerst and Gronwald, 1986). The safening of corn by benoxacor has been correlated with increased metabolism of metolachlor to the GSH conjugate (Rowe et al., 1991) and this increase in metabolism has been linked to enhanced GST activity (Kreuz et al., 1989). The safening of rice from pretilachlor injury by low concentrations of fenclorim has likewise been linked to increased GST activity resulting in enhanced metabolism of the herbicide to a GSH conjugate (Wu et al., 1996).

Studies of benoxacor, fenclorim, and fluxofenin in *Arabidopsis* indicate that these safeners increase GST activity by inducing increased expression of specific genes through multiple signal transduction pathways (Smith et al., 2004; DeRidder and Goldsbrough, 2006). Increased levels of RNA, transcribed from five genes known to function in the glutathione-GST system, were observed in *Arabidopsis* treated with benoxacor, fenclorim, and fluxofenin (DeRidder and Goldsbrough, 2006). Although GST activity was increased, these safeners failed to protect *Arabidopsis* from metolachlor injury. It was found that the increased levels of GST activity were expressed in the roots of *Arabidopsis* as opposed to the shoots. Since the primary anatomical site of metolachlor activity is the coleoptilar node of the shoot, increased GST activity in the roots failed to provide protection from injury in *Arabidopsis* (DeRidder and Goldsbrough, 2006). The authors hypothesized that this might explain the selectivity of these safeners toward monocots, since *Arabidopsis* is a eudicot and was not protected despite increasing its GST activity. As a practical consequence, this selectivity allows safeners to be applied simultaneously with herbicides, as opposed to being used exclusively as seed treatments. When applied with herbicides, these safeners selectivity safen grasses while excluding eudicots, which succumb to the herbicide.

Cytochrome P450, a membrane-bound oxidative enzyme involved in detoxification of xenobiotics including herbicides, is also believed to be a target of herbicide safeners (Ohkawa et al., 1999). Cytochrome P450-mediated metabolism has been established as effective in detoxifying chloroacetamide herbicides including metolachlor (Barrett, 1995; Frear, 1995). Naphthalic anhydride and benoxacor have both been demonstrated to induce enhanced P450-mediated metabolism of metolachlor

(Barrett, 1995). Due to the same safeners stimulating both GSH-GST mediated and cytochrome P450 mediated herbicide metabolism, one model posits a general signal transduction pathway that initiates both systems (Hatzios and Burgos, 2004), but there is also evidence against this model (Siminszky, 2006). Cytochrome P450 transcripts induced by naphthalic anhydride application are tissue specific (Persans, 2001). Similar to what was observed for GSH-GST induction (DeRidder and Goldsbrough, 2006), naphthalic anhydride application specifically induces P450 transcripts in young shoots, and not in roots or older tissue (Persans, 2001).

At least one safener, R-29148, not included in this study, counteracts chloroacetamide herbicides through competition with the herbicide for binding to the active site of the target enzyme, not metabolic detoxification (Walton and Casida, 1995). Under this theory the safener is an inactive analog acting as a receptor antagonist of the herbicide (Walton and Casida, 1995). It is important to note the dramatic difference between this mode of action and the increase in GST outlined above. Here, the safener is inert and functions by binding to the herbicide's target site due to structural similarities. Above, the safener is active, shares no structural similarity to the herbicide, and works by enhancing the activity of enzymes that metabolically detoxify the herbicide while interact with the herbicide's target site in any way.

Prospects for Safeners in Native Warm Season Grasses

Studies on the efficacy of safeners toward native grasses are limited compared to the major crops corn, sorghum, and rice. However, the studies that have been conducted observed successful protection from metolachlor, but also botanical specificity as to which safeners were effective toward which grasses. Big bluestem was protected from

metolachlor by oxabetrinil and R-29148 (Griffin et al., 1988). Indiangrass was protected by R-29148 and naphthalic anhydride (Griffin et al., 1988). Little bluestem was only protected by cyometrinil (Roder et al., 1987). Cyometrinil and R-29148 are not commercially available. Fluxofenin is effective in protecting the NWSG switchgrass from metolachlor injury and is commercially available (Rushing et al., 2013).

Unfortunately the botanical specificity of these safeners is still not understood. We, therefore lack the information necessary to make predictions as to which safeners will work in which grasses. The organ specific expression of enhanced GST activity in the roots of *Arabidopsis*, as opposed to the coleoptilar region of the shoot, cited above, provides a possible explanation for why these safeners are selective between monocots and eudicots, but does not help us to understand the botanical specificity observed between grass species. The impressive similarity in mode of action of the safeners discussed above, only makes the question more confounding. If each safener works to enhance the expression of an enzyme system common to all plants, why do some compounds achieve this effect in one species and not another, especially if that latter species is amendable to safening from another compound? Without an answer to this question, specific crop-safener relationships will have to be determined purely empirically. An understanding of the mode of action of the herbicide under question, the physiology of its detoxification, and the mode of action of the safener can allow us to carry out these empirical studies with an informed understanding of the herbicide-safener interaction. This can, at least, allow us to limit our lab and field tests to safeners with modes of action relevant to the herbicide. All five safeners included in this study are known to increase GST activity leading to enhanced detoxification of metolachlor

through GSH conjugation. We have, therefore, limited our study to safeners with the potential to be effective, but whether that potential is actual must be determined in the field.

CHAPTER III

MATERIALS AND METHODS

Plant Materials and Chemicals

Seed safener trials were conducted on big bluestem, little bluestem, and indiangrass. Field and lab tests were both performed on the cultivars, Kaw (big bluestem), Aldous (little bluestem), and Holt (indiangrass). Big bluestem and indiangrass seed were obtained from Roundstone Native Seeds, LLC (Upton, KY). Little bluestem seed was obtained from Ernst Conservation Seed (Meadville, PA). In the field and lab tests seed were treated with five safeners: oxabetrinil (Sigma-Aldrich; St Louis, MO), fluxofenin (Concep III®; Syngenta; Greensboro, NC), benoxacor (Sigma-Aldrich), fenclorim (VWR; Secaucus, NJ), and naphthalic anhydride (Sigma-Aldrich). Safener chemical names are given in Table 1. Field trials were conducted using S-metolachlor (Dual Magnum®; Syngenta; Greensboro, NC).

Table 3.1 Safener chemical names used in lab and field testing.

Safener	Chemical name
Oxabetrinil	(*Z*)-1,3-dioxolan-2-ylmethoxyimino-(phenyl)acetonitrile
Benoxacor	(*RS*)-4-Dichloroacetyl-3,4-dihydro-3-methyl-2H-1,4-benzoxanine
Fluxofenin	4-Chloro-2,2,2-trifluoroacetophenone *O*-1,3-dioxolan-2-ylmethyloxime
Fenclorim	4,6-Dichloro-2-phenylpyrimidine
Naphthalic Anhydride	Napthalene-1,8-dicarboxylic anhydride

(Abu-Quare and Duncan, 2002)

Laboratory Study: Dose Response and Germination Rates

Seed Treatments

Dose response curves were generated for each species x safener combination (5 safeners x 3 species = 15 combinations) to determine the optimal application rate. All three species were tested in the spring of 2012 and the test was repeated for indiangrass in the spring of 2013, due to poor indiangrass field performance in 2012. Seed of each species was treated with five concentrations (25%, 50%, 100%, 150%, and 200%) of a published rate of each of five safeners and a control (0%). Published rates (100%) were: 1.5 g a.i. / kg seed for oxabetrinil (Griffin et al., 1988); 0.4 g a.i. / kg seed for fluxofenin (Anonymous, 2008); 100µM solution for benoxacor (Smith et al., 2004); 100µM solution for fenclorim (Wu et al., 1996); 5.0 g a.i. / kg seed for naphthalic anhydride (Griffin et al., 1988). Sources of published rates were based on a previous safener screening in NWSG by Rushing et al. (2013).

For oxabetrinil and naphthalic anhydride, the dry weight of compound, necessary to yield the percentage of the published rate of active ingredient (Table 3.2), was dissolved in enough water to thoroughly wet and coat the seed without any excess liquid. Safener solution was applied to seed by stirring on a Vortex-Genie 2 Mixer (Scientific Industries; Bohemia, NY) until evenly distributed. The wet seed was dried in an open Lucite box in a ventilation hood under continuous air flow for 48 hrs.

Table 3.2 Applied rate (100%) of oxabetrinil and NA.

Safener	Published Safener Rate	Mg a.i. g^{-1} seed	Water
Oxabetrinil	1.5 g a.i. kg^{-1} seed (Griffin et al., 1988)	1.5 mg g^{-1} seed	10 ml H_20 g^{-1} seed
Naphthalic Anhydride	5.0 g a.i. kg^{-1} seed (Griffin et al., 1988)	5 mg g^{-1} seed	10 ml H_20 g^{-1} seed

Stock solutions (100,000x) of benoxacor and fenclorim were created by dissolving the appropriate amount of compound in 1 ml methanol (Table 3.3). Stock solution was diluted to the appropriate percentage of the 100µM concentration in enough water to wet seed thoroughly without excess liquid. Safener solution was applied to seed by stirring on Vortex-Genie 2 Mixer until evenly distributed. The wet seed was dried in an open Lucite box in a ventilation hood under continuous air flow for 48 hrs.

Table 3.3 Applied rate (100%) of fenclorim and benoxacor.

Safener	Published Safener Rate	Stock Solution (100,000x)	Dilution (100%)	Solution
Fenclorim	100 µM (Smith et al., 2004)	22.5 mg a.i. ml^{-1} methanol	1µL: 1 ml H_20	10 ml g^{-1} seed
Benoxacor	100 µM (Wu et al., 1996)	26 mg a.i. ml^{-1} methanol	1µL: 1 ml H_20	10 ml g^{-1} seed

Recommended rate of a.i. of fluxofenin was suspended in solution by creating an emulsion of Concep III® (Syngenta) and water (Table 3.4). The emulsion was created by mixing Concep III® and water in a 1:25 ratio (2000µL Concep III® : 5 ml water) (Anonymous, 2008). The emulsion was diluted in water and mixed using a stir bar and stir plate. The volume of emulsion to add to water was determined by the percentage of recommended rate being applied and the amount of seed being treated, and the volume of

water was enough to thoroughly wet and coat the seed without excess liquid. The solution was applied to seed by stirring on Vortex-Genie 2 Mixer. Wet seed was dried in an open Lucite box in a ventilation hood under continuous air flow for 48 hrs.

Table 3.4 Applied rate (100%) of fluxofenin.

Safener	Published Safener Rate	Emulsion	Final Ratio
Fluxofenin	0.4 g a.i. kg^{-1} seed (Anonymous, 2008)	200 µL Concep III® 5 ml^{-1} H_20	10.85 µL emulsion : 10 ml H_20 : 1 gram of seed

Germination Test

One-hundred seed of each treatment x species combination were spaced in a Petri dish lined with moist (5 ml H_2O) filter paper. Each species was treated with each of five safeners at each of the five concentrations plus a control for a total of 26 treatments per species. Seed with no safener application served as the control. Each treatment was replicated six times (AOSA, 1992). Petri dishes containing seed were placed in a growth chamber (Percival Scientific® GR-36VL; Perry, IA) set on alternating light and dark temperatures (30/25ºC) with 16 hrs of light under cool white fluorescent tubes (50-60 µmol/m^2/s illumination) (AOSA, 1992). Germinated seed in each dish were counted and recorded every other day for 22 days. Analysis of variance was conducted in Proc GLM with means separated by LSMeans ($p \leq 0.05$) in SAS E.G. 5.1 (SAS Institute, Cary, NC 2012). Optimal rates of safener were defined to be the greatest dose of the safener that could be applied while causing either no significant decline in total mean germination compared to the control, or the highest application rate in the mean grouping below the control. Optimal rates were relative to both species and safener: i.e., within a species

there could be differences between safeners; within a safener there could be differences between species.

Field Study: Safening Trial

Planting

Seed of each species were treated with its optimal rate of each safener, as determined by dose response curve, prior to an in-field safening trial. The amount of seed to treat and plant was determined by a planting rate of 12.33 kg ha^{-1} (11 lbs $acre^{-1}$) PLS (Harper et al., 2007). Germination rate of the seed, for calculating PLS, was based on the total mean germination of the control (no safener application) for each species in the lab study. Safener was applied at the optimal rate and seed was allowed to dry in a ventilation hood for 48 hours.

Field trials were conducted at the H.H. Leveck Animal Research Unit of Mississippi State University near Starkville, MS (33º27'45", -88º49'12") on a Catalpa silty clay loam (fine, smectitic, thermic, Fluvaquentic Hapludolls) in the spring of 2012 and 2013. A second field location was added at Mississippi State University's Black Belt Branch Experiment Station near Brooksville, MS (33 º26'02.86", -88 º55'34.9") on a Brooksville silty clay (fine, smectic, thermic, Aquic Hapladerts), in the spring of 2013.

Safened seed were planted in a randomized complete block design. Each block consisted of seven plots (one species with five treatments and a positive and negative control). Each plot contained one treatment. Treatments consisted of one species, treated with one of five safeners and the positive and negative controls. The two controls were: no safener with herbicide applied (negative control) and no safener with no herbicide applied (positive control). Each species was assigned four blocks, with each of the seven

treatments repeated and randomized in each block. Plots were 1.83 x 3.35 m (6'x11') with eight rows per plot set on 25.4cm (10") centers. Seed was drilled using an Almaco® 8-row planter. Seed was drilled 0.635 cm deep (¼") (Harper et al., 2007). Planting dates were May 17, 2012 and May 21, 2013 in Starkville, MS and May 20, 2013 in Brooksville, MS.

After planting, the control plots receiving no herbicide and no safener were covered with 4 mil polyethylene plastic. The entire test field was then sprayed with S-metolachlor (Dual Magnum®; Syngenta) at a rate of 1.07 kg a.i. ha^{-1} (0.95 lb a.i. $acre^{-1}$). In 2012, S-metolachlor was allowed to remain undisturbed on the surface for 24 hours and after 24 hours the field received overhead irrigation sufficient to incorporate the herbicide. In 2013, both the Starkville and Brooksville trials received rain on the morning of May, 22: 1.68 cm (0.66”) in Starkville; 1.12 cm (0.44”) in Brooksville.

Establishment Phase Observations

Seedling emergence counts and weed control ratings were taken two weeks after planting and then every other week for ten weeks. Biweekly emergence counts were based on the mean number of seedlings within 30 cm (1') of row. From each eight row plot, four rows were chosen at random. Within each of those rows 30 cm were chosen at random. The emergence count for each plot was the mean of these four counts. Weed control ratings were based on a visual rating on a scale of 1 to 5; 1 describing very poor weed control and 5 describing excellent weed control. Seedling emergence counts and weed control ratings were analyzed using PROC GLM in SAS E.G. 5.1 (SAS Institute, Cary, NC, 2012). The biweekly observations were analyzed as repeated measures. For seedling emergence, data were analyzed with safener treatment as the only fixed effect

and the study (year x location), block within a study, and the study x treatment interaction as random effects. Weed control ratings were analyzed across species because they were thought to be very likely independent of species and, were species to have an effect on weed control, this unexpected result would need to be verified statistically. Species was treated as a fixed effect with study (year x location), block within a study, and the study x treatment interaction as random effects.

End of Season Measurements

End of season measurements were taken in late October before harvest. Harvests were conducted on October 25, 2012 and October 31, 2013 in Starkville, MS and on October 30, 2013 in Brooksville, MS. End of season measurements included: leaf canopy height, seed head height, crown counts, coverage ratings, and plot yield. Canopy height and seed head height were both based on the mean height (cm) of five randomly chosen plants within each plot. Analysis of variance and mean separations were analyzed using PROC GLIMMIX in SAS E.G. 5.1 (SAS Institute, Cary, NC, 2012) to allow for missing data. End of season crown counts were conducted by the same method as seedling emergence counts described above and analyzed using PROC GLM (SAS Institute, Cary, NC, 2012). End of season coverage ratings were based on a scale of 1 to 10, with the rating number corresponding to the estimated percentage ground coverage provided by desired grass: 1 corresponding to 10% or less coverage and 2 to 20% to 10% coverage, and so forth. End of season coverage ratings were analyzed using PROC GLM (SAS Institute, Cary, NC, 2012).

Harvests of first year growth were split between weeds and desired grass. In Starkville 2012 weeds in each plot were harvested by hand, dried, and weighed. Desired

grass was then harvested with a mower set on 7.6 cm (3") height, wet weight recorded, percentage moisture estimated from a dried grab sample, and total dry weight of each plot calculated. Due to substantially poorer grass yield in Starkville and Brooksville 2013, desired grass was harvested by hand and weeds were harvested by mower, and the entire plot's yield was dried and weighed. Treatment effect on yield of dry harvest of weeds and desired grass was analyzed using PROC GLM (SAS Institute, Cary, NC, 2012). Weed yield was analyzed across species. Species were treated as a fixed effect with study (year x location), block within a study, and the study x treatment interaction as random effects.

The Starkville 2012 planting provided second year data in 2013. The entire field received an application of imazapic (Plateau®, 23.6% imazapic, BASF) at 0.14 kg a.i. ha^{-1} in March 2013 to control winter weeds post-emergence and to provide preemergent control of spring weeds. Canopy height, seed head height, end of season crown counts and end of season coverage ratings were conducted pre-harvest as described above, with the exception of the crown counts. Crown counts were estimated as the mean number of crowns in a randomly chosen meter of row, as opposed to a randomly chosen 30 cm of row, to adjust for the increased size of each crown at the end of the second year. All measurements were analyzed using PROC GLM (SAS Institute, Cary, NC, 2012). Harvest was conducted on November 4, 2013. The harvests of second year growth were not split between weeds and desirable grass since all plots received identical weed control in the second year. Plots were harvested using a Wintersteiger plot harvester (Wintersteiger AG, Ried, Austria). Wet weight was recorded, percentage moisture was estimated from a dried grab sample, and total dry weight of each plot calculated.

Treatment effect on dry matter yield was analyzed using PROC GLM (SAS Institute, Cary, NC, 2012).

CHAPTER IV

LABORATORY STUDY: RESULTS AND DISCUSSION

Dose Response and Germination Rates

Big Bluestem

Significant differences were observed for germination rates of big bluestem treated with varying levels of different safeners (Table 4.1). The mean germination rate of the untreated control was 54.8% after 22 days. Treatment with oxabetrinil significantly impacted total mean germination ($p = 0.0078$) compared to the untreated control. However, the R^2 value of the analysis of variance was only 0.39, meaning that only 39.0% of the variation in germination was explained by oxabetrinil treatment. All rates of oxabetrinil (25%-200%) significantly reduced germination rate compared to the untreated control (0%), but no significant differences were observed between any of the rates of oxabetrinil. Treatment with benoxacor did not significantly impact germination ($p = 0.1266$; $R^2 = 0.24$). Despite not significantly affecting germination rates, mean separations of benoxacor rates are provided (Table 4.1) since they informed the decision as to which rate to apply in the field trial. Significant differences were observed between the untreated control and the 25% and 100% rates, but no significant differences were observed between any of the rates of benoxacor application. Treatement with fluxofenin significantly impacted germination ($p = 0.0004$; $R^2 = 0.52$) with all rates other than 50% showing significant reduction in total germination when compared to the untreated

control. Treatment with fenclorim significantly impacted germination ($p \leq 0.0001$; $R^2 = 0.62$). The 25% and 100% application were not significantly different from the untreated control, but the 50%, 150%, and 200% resulted in significant decreases in germination compared to the untreated control. Treatment with naphthalic anhydride significantly impacted germination ($p = 0.0052$; $R^2 = 0.41$). The 100% rate was the only rate not significantly reducing mean germination when compared to the untreated control. The highest germination rate observed for treated big bluestem seed was the 25% rate of fenclorim (54.7% germination) while the lowest was the 200% rate of fenclorim (38.0% germination), compared to 54.8% for the untreated check.

Table 4.1 Mean germination percentage of 'Kaw' big bluestem for each safener x concentration combination in safener dose response test.

	Percentage of published rate of safener applied					
	0	25	50	100	150	200
Safener	Mean germination (%)					
Oxabetrinil[†]	54.8 a[††]	45.8 b	42.7 b	41.0 b	44.5 b	44.8 b
Benoxacor[‡]	54.8 a	43.8 b	50.2 ab	46.7 ab	43.8 b	46.8 ab
Fluxofenin[§]	54.8 a	46.3 b	49.3 ab	39.0 c	44.2 bc	38.3 c
Fenclorim[¶]	54.8 a	54.7 a	42.8 dc	50.3 ab	45.8 bc	38.0 d
Naphthalic Anhydride[#]	54.8 a	43.5 bc	46.5 bc	49.0 ab	41.5 c	43.7 bc

[†]1.5 g a.i. kg^{-1} seed (Griffin et al., 1988)
[‡]100 µM (Wu et al., 1996)
[§]0.4 g a.i. kg^{-1} seed (Anonymous, 2008)
[¶]100 µM (Smith et al., 2004)
[#]5.0 g a.i. kg^{-1} seed (Griffin et al., 1988)
[††]Means within a row followed by the same letter are not significantly different at $\alpha = 0.05$.

Little Bluestem

Significant differences were observed for germination rates of little bluestem treated with varying levels of different safeners (Table 4.2). The mean germination rate of the untreated control was 26.7% after 22 days. Mean germination was significantly

impacted by all five safener treatments: oxabetrinil ($p < 0.0001$; $R^2 = 0.85$); benoxacor ($p = 0.0008$; $R^2 = 0.49$); fluxofenin ($p < 0.0001$; $R^2 = 0.90$); fenclorim ($p < 0.0001$; $R^2 = 0.62$); naphthalic anhydride ($p < 0.0001$; $R^2 = 0.84$). The 25% and 50% rates of oxabetrinil did not result in a significant change in germination rate as compared to the control, while the 100%, 150%, and 200% rates all significantly decreased germination compared to the untreated control. All five rates of benoxacor significantly reduced germination as compared to the untreated control, but no significant differences were observed between any of the five rates. All five rates of fluxofenin significantly reduced little bluestem germination as compared to the untreated control. Significantly greater germination rates were observed at the 25% and 50% rates as compared to the 100%, 150% and 200%, but no differences were observed within these two groupings. For both fenclorim and naphthalic anhydride, treatments with their 25% and 50% rates resulted in no significant reductions in mean germination as compared to the untreated control. All higher application rates of both safeners significantly reduced mean germination when compared to the untreated control. The highest germination rate for treated little bluestem seed was 27.7% for the 25% application rate of oxabetrinil, a small increase over the untreated control (26.7%), while the lowest germination rate for treated little bluestem seed was 8.8% for the 200% application rate of naphthalic anhydride.

Table 4.2 Mean germination percentage of 'Aldous' little bluestem for each safener x concentration combination in safener dose response test.

	Percentage of published rate of safener applied					
	0	25	50	100	150	200
Safener	Mean germination (%)					
Oxabetrinil[†]	26.7 ab[††]	27.7 a	23.8 b	19.8 c	15.8 d	12.3 e
Benoxacor[‡]	26.7 a	18.3 b	19.8 b	18.7 b	18.5 b	19.0 b
Fluxofenin[§]	26.7 a	17.8 b	17.5 b	11.2 c	11.7 c	12.7 c
Fenclorim[¶]	26.7 a	22.5 ab	22.5 ab	18.3 cb	16.8 cd	13.7 d
Naphthalic Anhydride[#]	26.7 a	23.2 a	23.0 a	12.7 b	9.3 b	8.8 b

[†]1.5 g a.i. kg^{-1} seed (Griffin et al., 1988)
[‡]100 µM (Wu et al., 1996)
[§]0.4 g a.i. kg^{-1} seed (Anonymous, 2008)
[¶]100 µM (Smith et al., 2004)
[#]5.0 g a.i. kg^{-1} seed (Griffin et al., 1988)
[††]Means within a row followed by the same letter are not significantly different at $\alpha = 0.05$.

Indiangrass

Significant differences were observed for germination rates of indiangrass treated with varying levels of safener (Table 4.3). The mean germination rate of the untreated control was 51.3% after 22 days. Mean germination was significantly impacted by all safener treatments, with the exception of fenclorim ($p = 0.8129$; $R^2 = 0.07$): oxabetrinil ($p < 0.0001$; $R^2 = 0.75$); benoxacor ($p < 0.0001$; $R^2 = 0.60$); fluxofenin ($p = 0.0015$; $R^2 = 0.46$); naphthalic anhydride ($p < 0.0001$; $R^2 = 0.73$). No rates of fenclorim significantly impacted indiangrass germination compared to the control. All rates of oxabetrinil, benoxacor, fluxofenin, and naphthalic anhydride significantly decreased indiangrass germination compared to the positive control. Germination was significantly greater under the 100% rate of oxabetrinil than all other oxabetrinil applications. No significant differences in mean germination were observed between application rates within benoxacor, fluxofenin, or naphthalic anhydride treatments. The highest observed germination rate for treated indiangrass seed was 51.5% for the 50% rate of fenclorim, a

slight increase over the 51.3% of the untreated control. The smallest observed germination rate of treated indiangrass seed was 27.2% under the 150% rate of oxabetrinil.

Table 4.3 Mean germination percentage of 'Holt' indiangrass for each safener x concentration combination in safener dose response test (2012).

	Percentage of published rate of safener applied					
	0	25	50	100	150	200
Safener	Mean germination (%)					
Oxabetrinil[†]	51.3 a[††]	32.4 c	30.3 c	38.5 b	27.2 c	31.7 c
Benoxacor[‡]	51.3 a	32.4 b	36.1 b	37.1 b	35.3 b	34.1 b
Fluxofenin[§]	51.3 a	41.3 b	36.6 b	36.3 b	36.7 b	42.2 b
Fenclorim[¶]	51.3 a	49.5 a	51.5 a	49.5 a	46.9 a	48.4 a
Naphthalic Anhydride[#]	51.3 a	33.9 b	30.5 b	31.5 b	34.5 b	29.3 b

[†]1.5 g a.i. kg^{-1} seed (Griffin et al., 1988)
[‡]100 µM (Wu et al., 1996)
[§]0.4 g a.i. kg^{-1} seed (Anonymous, 2008)
[¶]100 µM (Smith et al., 2004)
[#]5.0 g a.i. kg^{-1} seed (Griffin et al., 1988)
[††]Means within a row followed by the same letter are not significantly different at $\alpha = 0.05$.

Because of negative results in the 2012 field trial, the dose response test was repeated on the same indiangrass seed lot in 2013. Significant differences were again observed for germination rates of indiangrass treated with varying levels of different safeners (Table 4.4). The mean germination rate of the untreated control was 41.2% after 22 days, constituting a nearly 20% reduction in rate of germination compared to the untreated control in 2012 (51.3%). Unlike 2012 where no significant effect on germination was observed for treatment with fenclorim, mean germination was significantly impacted by all safener treatments in 2013: oxabetrinil ($p < 0.0001$; $R^2 = 0.80$); benoxacor ($p < 0.0303$; $R^2 = 0.32$); fluxofenin ($p = 0.0188$; $R^2 = 0.35$); fenclorim ($p < 0.0001$; $R^2 = 0.59$); naphthalic anhydride ($p = 0.0004$; $R^2 = 0.52$). All rates of

oxabetrinil significantly decreased germination compared to the untreated control. The germination was significantly higher under the 25%, 50% and 100% rates, than under the higher rates of 150% and 200%, but there were no significant differences observed between the three lower rates of oxabetrinil. The 25%, 50% and 200% rates of benoxacor showed no significant difference when compared to the untreated control. This differed markedly from the same test conducted in 2012 when all levels of all safeners reduced germination compared to the untreated check, except fenclorim which had no effect in the 2012 test. Only the 200% rate of fenclorim reduced germination in the 2013 test. All levels of fluxofenin resulted in reduced germination rates compared to the untreated check, the same as the result observed in the 2012 test. The 200% rate of naphthalic anhydride was its only rate not to significantly reduce germination compared to the control, whereas all rates reduced germination in the 2012 test.

Table 4.4 Mean germination percentage of 'Holt' indiangrass for each safener x concentration combination in safener dose response test (2013).

	Percentage of published rate of safener applied					
	0	25	50	100	150	200
Safener	Mean germination (%)					
Oxabetrinil[†]	41.2 a[††]	29.0 b	30.7 b	34.3 b	18.3 c	10.3 d
Benoxacor[‡]	41.2 ab	39.3 ab	35.3 cb	43.2 a	31.5 c	36.8 abc
Fluxofenin[§]	41.2 a	31.7 b	32.7 b	32.5 b	34.0 b	28.3 b
Fenclorim[¶]	41.2 a	39.8 a	35.8 a	37.3 a	41.3 a	24.0 b
Naphthalic Anhydride[#]	41.2 a	37.8 c	26.7 c	28.5 c	30.8 bc	36.8 ab

[†]1.5 g a.i. kg^{-1} seed (Griffin et al., 1988)
[‡]100 µM (Wu et al., 1996)
[§]0.4 g a.i. kg^{-1} seed (Anonymous, 2008)
[¶]100 µM (Smith et al., 2004)
[#]5.0 g a.i. kg^{-1} seed (Griffin et al., 1988)
[††]Means within a row followed by the same letter are not significantly different at $\alpha = 0.05$.

Optimal Safener Rates

The results of the dose response test were used to determine optimal rates of safener to test in the field trial (Table 4.5). Optimal rates were understood as the highest rate of safener application not significantly reducing germination based on the mean separations of each species x safener combination. Practically, this was the highest application rate not significantly reducing germination as compared to the untreated check, or if all levels significantly reduced germination, then the highest application rate from the mean grouping below the untreated check. A dichotomous flow chart of the decision process is provided in Appendix A. Following the mean separation for each species x safener combination in Tables 4.1 - 4.3 results in the optimal rates provided in Table 4.5.

Table 4.5 Optimal rates, as percentage of published rate.

	Percentage of published rate		
Safener	Big Bluestem	Little Bluestem	Indiangrass
Oxabetrinil[†]	200	50	100
Benoxacor[‡]	200	200	200
Fluxofenin[§]	50	50	200
Fenclorim[¶]	100	50	200
Naphthalic Anhydride[#]	100	50	200

[†] 1.5 g a.i. kg^{-1} seed (Griffin et al., 1988).
[‡] 100 µM (Wu et al., 1996).
[§] 0.4 g a.i. kg^{-1} seed (Anonymous, 2008).
[¶] 100 µM (Smith et al., 2004).
[#] 5.0 g a.i. kg^{-1} seed (Griffin et al., 1988).

Rates tested in the field on all three species in both 2012 and 2013 were based on the 2012 dose response test, despite the test being repeated in 2013 for indiangrass. Despite differences between the mean groupings in the 2012 indiangrass dose response test and the 2013 test, the only safener whose rate would have changed under our

definition of optimal rate was fenclorim. In 2012, fenclorim was the only safener not to impact indiangrass germination ($p = 0.8129$; $R^2 = 0.07$). In the 2013 test, treatment with fenclorim did significantly impact germination ($p < 0.0001$; $R^2 = 0.59$), but only the 200% rate significantly decrease germination compared to the control. Had the rate applied in the field been based on the 2013 dose response test, fenclorim would have been tested on indiangrass at the 150% rate.

CHAPTER V

FIELD TRIAL: ESTABLISHMENT YEAR RESULTS AND DISCUSSION

Establishment Phase

Seedling Emergence

Seedling emergence counts were taken every other week for ten weeks, starting two weeks after planting. Emergence counts were taken on all three species at all three study sites (location x year): Starkville 2012 and 2013, and Brooksville 2013. Analysis was based on a model that treated variation in seedling emergence as a function of a fixed treatment effect and random effects due to study, study x treatment interaction, and block nested within study. "Study" (location x year) was understood as the random effect due to environment. Treatments consist of a positive control (no safener, no herbicide), a negative control (no safener, with herbicide), and five safener treated plots with herbicide application. Levels of protection are defined relative to the controls. A safener provides "complete protection" if it results in no significant difference in seedling emergence as compared to the positive control. "Partial protection" is provided by a safener treatment resulting in a significant increase in seedling emergence as compared to the negative control, but a significant decrease in seedling emergence as compared to the positive control. "No protection" indicates no significant difference as compared to the negative control. Results are presented separately for each species.

Big Bluestem

The model proved statistically significant for explaining variation in big bluestem seedling emergence, with $p < 0.0001$ at each biweekly interval. Coefficients of determination, R^2 values, varied across weeks with $R^2 = 0.76$ at the two week stage, $R^2 = 0.66$ at the four week stage, $R^2 = 0.75$ at the six week stage, $R^2 = 0.86$ at the eight week stage, and $R^2 = 0.87$ at the ten week stage.

There was no environment x treatment interaction impacting big bluestem seedling emergence ($p = 0.1571$) at the two week stage. Data were therefore pooled across studies. At two weeks, there was no significant effect due to block ($p = 0.4193$) or treatment ($p = 0.3507$). The only significant effect was study ($p < 0.0001$), with significant differences in mean seedling emergence at the two week stage observed between all three studies (Table 5.1): Starkville 2012 (16.2 seedlings m^{-1}); Starkville 2013 (9.6 seedlings m^{-1}); Brooksville 2013 (0.1 seedlings m^{-1}).

There was no environment x treatment interaction impacting big bluestem seedling emergence ($p = 0.6630$) at the four week stage and data were again pooled across all three studies. At four weeks, there was no significant effect due to study ($p = 0.2265$), but there were significant effects due to treatment ($p = 0.0003$) and block nested within study ($p = 0.0017$). While the treatment effect was significant, there was considerable chaining within the mean separation (Table 5.2). Fluxofenin (20.6 seedlings m^{-1}) was the only treatment providing complete safening of big bluestem at the four week stage: i.e., it was the only treatment to share a mean grouping with the no safener, no herbicide positive control (23.9 seedlings m^{-1}). Benoxacor (17.3 seedlings m^{-1}) and naphthalic anhydride (15.1 seedlings m^{-1}) resulted in partial protection: i.e., resulted in

significant increases in big bluestem germination compared to the no safener, with herbicide negative control (7.5 seedlings m^{-1}) but significant reductions compared to the no safener, no herbicide positive control. These significant differences should be viewed, though, in light of the chaining that linked all mean separations at the four week stage (Table 5.2).

At six weeks there was a significant study x treatment interaction affecting big bluestem seedling emergence ($p = 0.0389$). The overall study and treatment effects were therefore dropped and treatment effect and mean separations were analyzed separately for each study. Once analyzed separately by study, variation in mean big bluestem seedling emergence was explained by a simpler model treating seedling emergence at a single location as a function of treatment and block. This simpler model proved significant for explaining variation in week six seedling emergence at all three studies: Starkville 2012, $p = 0.0039$, $R^2 = 0.69$; Starkville 2013 $p = 0.0019$, $R^2 = 0.71$; Brooksville 2013, $p = 0.0007$, $R^2 = 0.75$.

For Starkville 2012, there was no significant effect due to block ($p = 0.2942$), but effect due to treatment was significant ($p = 0.0016$) at the six week stage. Fluxofenin (24.6 seedlings m^{-1}) was the only treatment providing complete protection as compared to the positive control (28.1 seedlings m^{-1}), while the other four safener treatments all provided partial protection as compared to the negative control (7.1 seedlings m^{-1}) (Table 5.2). For Starkville 2013, there were significant effects due to both block ($p = 0.0012$) and treatment ($p = 0.0209$). Fluxofenin (19.6 seedlings m^{-1}) and naphthalic anhydride (17.7 seedlings m^{-1}) provided complete protection as compared to the positive control (20.4 seedlings m^{-1}), while benoxacor, fenclorim, and oxabetrinil did not differ

significantly from the negative control (5.4 seedlings m^{-1}) (Table 5.2). No safener treatments were significantly different from one another, though. So, while fluxofenin and naphthalic anhydride met our definition of complete protection and the others provided no protection, these results should again be viewed in light of the considerable chaining within the mean separation (Table 5.2). For Brooksville 2013, there was a significant effect due to both block ($p = 0.0043$) and treatment ($p = 0.0017$). However, there was no significant difference in seedling emergence between the positive control (9.4 seedlings m^{-1}) and the negative control (7.7 seedlings m^{-1}), making our definitions of levels of protection inapplicable. Fluxofenin (20.2 seedlings m^{-1}) and benoxacor (17.3 seedlings m^{-1}) were the only treatments to result in a significant increase in seedling emergence as compared to the negative control.

A significant study x treatment interaction was also observed at week eight ($p = 0.0367$), and data were again analyzed separately by study. The resulting simpler model again proved capable of explaining a significant proportion of the variation in big bluestem seedling emergence across all three studies: Starkville 2012, $p = 0.0039$, $R^2 = 0.69$; Starkville 2013 $p = 0.0019$, $R^2 = 0.71$; Brooksville 2013, $p = 0.0007$, $R^2 = 0.75$. In Starkville 2012, there was a significant effect due to block ($p = 0.0201$) and treatment ($p = 0.0004$). Fluxofenin (23.3 seedlings m^{-1}) was the only treatment to result in complete protection at the eight week stage as compared to the positive control (27.1 seedlings m^{-1}). Naphtahlic anhydride (20.4 seedlings m^{-1}) and benoxacor (20.2 seedlings m^{-1}) resulted in partial protection as compared to the negative control (11.9 seedlings m^{-1}), while fenclorim (15.4 seedlings m^{-1}) and oxabetrinil (14.8 seedlings m^{-1}) provided no protection. Significant block ($p < 0.0001$) and treatment effects ($p = 0.0041$) were

observed in Starkville 2013. Fluxofenin (18.1 seedlings m^{-1}), naphtahlic anhydride (15.2 seedlings m^{-1}), and benoxacor (13.5 seedlings m^{-1}) all provided complete protection as compared to the positive control (17.7 seedlings m^{-1}), while no treatment provided partial protection. Block effect ($p = 0.001$) and treatment effect ($p = 0.0005$) were both significant at the eight week stage in Brooksville 2013. With fluxofenin (12.7 seedlings m^{-1}) and benoxacor (10.9 seedlings m^{-1}) the only treatments resulting in an increase in emergence as compared to the negative control (5.7 seedlings m^{-1}). Again, our definitions of levels of control are less applicable in Brooksville 2013. Although, a significant difference was observed between the positive and negative control in week eight, unlike week six, the two were chained together by three treatments in the mean separation (Table 5.2). Rote application of our definitions of levels of protection would have resulted in designating these three safeners as providing both complete and no control, an obvious contradiction.

In week ten, the final emergence count, the treatment x study interaction was still present ($p = 0.0012$), and data were again analyzed separately by study with the simpler model maintaining its significance across all three studies: Starkville 2012, $p = 0.0005$, $R^2 = 0.76$; Starkville 2013 $p < 0.0001$, $R^2 = 0.81$; Brooksville 2013, $p = 0.0123$, $R^2 = 0.63$. In Starkville 2012, there was no block effect in week ten ($p = 0.1649$) and the treatment effect was significant ($p = 0.0002$). Fluxofenin (26.0 seedlings m^{-1}) was the only treatment to provide complete protection as compared to the positive control (30.2 seedlings m^{-1}). All other treatments provided partial control as compared to the negative control (12.3 seedlings m^{-1}). Both block ($p < 0.0001$) and treatment effects ($p = 0.0048$) were significant in Starkville 2013. Fluxofenin (18.1 seedlings m^{-1}), naphthalic anhydride

and oxabetrinil (both 13.8 seedlings m^{-1}) provided complete protection (positive control: 15.6 seedlings m^{-1}), according to our definition, but the considerable amount of chaining in the mean separation makes that description dubious (Table 5.2). Again, no significant difference was observed between the positive and negative controls in Brooksville 2013, making our definitions for levels of protection inapplicable. Fluxofenin (16.3 seedlings m^{-1}) was the only treatment to provide increased protection as compared to the negative control (8.3 seedlings m^{-1}).

Fluxofenin was the most reliable safener treatment for big bluestem. No effect due to treatment was observed until week four when fluxofenin was the only safener treatment providing complete protection. Study x treatment interactions in each subsequent week led to the data being analyzed separately at each location. In the Starkville 2012 study, fluxofenin was the only safener to provide complete protection, doing so across every week. By week ten, the final emergence count, all four of the other safeners tested provided partial protection in Starkville 2012. Starkville 2013 and Brooksville 2013 did not allow straight forward application of our definitions of levels of protection due to chaining between the means of the positive and negative controls in Starkville 2012 and a complete lack of mean separation between the positive and negative controls in Brooksville 2013. However, fluxofenin provided complete protection in all weeks after week two at both locations. Fluxofenin, therefore, resulted in complete protection at all sites and in all weeks where a treatment effect was observed. Naphthalic anhydride consistently provided complete protection across weeks in Starkville 2013, although it never did in any week at the other two locations. Benoxacor

provided complete protection in week six at Brooksville 2013 and in week eight at both Starkville 2013 and Brooksville 2013.

Discussion

These results are only partially consistent with previous work by Griffin et al. (1988) on the effects of metolachlor and seed safeners on big bluestem. That study found no significant difference in stand establishment between the untreated control and plots treated with metolachlor, and concluded that big bluestem was tolerant to metolachlor. In our study, the no safener no S-metolachor positive control consistently resulted in a significant increase in seedling emergence as compared to the no safener with S-metolachlor negative control in Starkville 2012, but not in Starkville 2013 or in Brooksville 2013. Although S-metolachlor application consistently reduced seedling emergence in Starkville 2012, the 7.7 seedlings m^{-1} observed by week ten in the negative control did demonstrate a degree of tolerance. These results are consistent with Masters' (1995) observations of reduced but satisfactory big bluestem stand density due to metolachlor application.

Griffin et al. observed improved stand establishment with oxabetrinil treatment, while our research observed oxabetrinil providing partial protection from metolachlor in Starkville 2012. Griffin et al. observed significant stand reduction under naphthalic anhydride treatment, while our study observed naphthalic anhydride providing partial protection in Starkville 2012, and complete protection in Starkville 2013. Fluxofenin, which consistently provided complete protection across locations in our research, was not included in the work by Griffin et al.

Focusing on Starkville 2012 due to its consistency with our definitions of levels of protection, big bluestem's tolerance to metolachlor, observed in our work and in Griffin et al.'s, could serve as an explanation for why all tested safener treatments provided at least partial protection by the final emergence count. Safeners function through enhancing pre-existing metabolic systems that detoxify herbicides and other xenobiotics. If those systems are naturally robust in big bluestem, then the safeners may be interacting with a naturally responsive system.

Table 5.1 Mean seedling emergence of 'Kaw' big bluestem across study sites.

Study: location and year	Mean seedlings m^{-1}				
	Week 2	Week 4	Week 6	Week 8	Week 10
Starkville, 2012	16.2 a[†]	18.8[‡]	19.5[§]	19.0[§]	22.2[§]
Starkville, 2013	9.6 b	12.8	13.6	13.5	13.3
Brooksville, 2013	0.1 c	13.8	12.4	8.6	11.2

[†] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[‡] Mean separation not reported due to no significant study effect: week 4, $p = 0.2265$.
[§]Mean separation not reported due to a significant study x treatment interaction: week 6, $p = 0.0389$; week 8, $p = 0.0367$; week 10, $p = 0.0012$.

Table 5.2 Mean seedling emergence of 'Kaw' big bluestem across treatments.

	Mean seedlings m^{-1}										
	Week 2†	Week4†		Week 6‡		Week 8‡			Week 10‡		
Treatment	Pooled	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	11.5§	23.9 a¶	28.1 a	20.4 a	9.4 c	27.1 a	17.7 ab	8.8 bc	30.2 a	15.6 ab	8.3 b
Fluxofenin	10.1	20.6 ab	24.6 ab	19.6 ab	20.2 a	23.3 ab	18.1 a	12.7 a	26.0 ab	18.1 a	16.3 a
Benoxacor	7.2	17.3 b	20.2 b	11.3 bdc	17.3 ab	20.2 bc	13.5 abc	10.9 ab	24.4 bc	12.3 bcd	12.3 ab
Naphthalic Anhydride	8.4	15.1 bc	19.4 b	17.7 abc	10.0 c	20.4 bc	15.2 ba	7.2 cd	21.5 bc	13.8 bc	9.4 b
Oxabetrinil	10.3	11.2 cd	17.3 b	10.2 dc	8.5 c	14.8 bc	12.7 bdc	6.9 cd	21.9 bc	13.8 bc	11.5 b
Fenclorim	7.1	10.5 cd	19.2 b	10.6 bdc	13.5 bc	15.4 cd	9.2 dc	8.2 cd	19.0 c	11.0 cd	11.9 b
No safener w/ herbicide	5.8	7.5 d	7.7 c	5.4 d	7.7 c	11.9 d	7.9 d	5.7 d	12.3 d	8.3 d	8.5 b

† Results are pooled across studies (year x location) if there is no significant study x treatment interaction at $\alpha = 0.05$: week 2, $p = 0.1571$; week 4, $p = 0.6630$.
‡ Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: week 6, $p = 0.0389$; week 8, $p = 0.0016$; week 10, $p = 0.0367$.
§ No mean separations are listed in there is no significant effect due to treatment at $\alpha=0.05$: week 2, $p = 0.3507$.
¶ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.

Little Bluestem

The overall model proved statistically significant for explaining variation in little bluestem seedling emergence, with $p < 0.0001$ at each biweekly interval. Coefficients of determination, R^2 values, varied across weeks: week two, $R^2 = 0.75$; week four, $R^2 = 0.82$; week six, $R^2 = 0.81$; week eight, $R^2 = 0.73$; week ten, $R^2 = 0.83$.

Little bluestem seedling emergence at the two week stage was impacted by a significant study x treatment interaction ($p = 0.0018$). Data were therefore analyzed separately for each study site. The resulting simpler model significantly explained variation in week two little bluestem emergence in Starkville 2012 ($p < 0.0001$; $R^2 = 0.75$) and Brooksville 2013 ($p = 0.0001$; $R^2 = 0.80$), but not in Starkville 2013 ($p = 0.3283$; $R^2 = 0.38$) where there was no significant effect due to block ($p = 0.2940$) or treatment ($p = 0.3492$). There was no significant block effect ($p = 0.0712$) but the effect due to treatment was significant ($p < 0.0001$) in Starkville 2012. The positive control (11.0 seedlings m^{-1}) resulted in significantly greater seedling emergence than all other treatments, with no safener treatment resulting in a significant difference when compared to the negative control (0.00 seedlings m^{-1}). There was no significant effect due to block ($p = 0.1553$) but treatment was significant ($p < 0.0001$) in Brooksville 2013. In Brooksville 2013 fluxofenin provided complete protection (3.3 seedlings m^{-1}) at the two week stage as compared to the positive control (4.2 seedlings m^{-1}), with no other treatment showing a significant difference from the negative control (0.0 seedlings m^{-1}) (Table 5.4).

No significant study x treatment interaction ($p = 0.1271$) was observed for little bluestem seedling emergence at the four week stage. Results were therefore pooled

across study sites. There was no significant effect due to study at week four ($p = 0.8965$) (Table 5.3), but there was a significant effect due to block nested within study ($p = 0.0006$) and treatment ($p < 0.0001$). Fluxofenin (8.3 seedlings m^{-1}) and NA (4.2 seedlings m^{-1}) both provided partial protection as compared to the positive control (15.3 seedlings m^{-1}), with all other safener treatments resulting in no protection as compared to the negative control (0.1 seedlings m^{-1}).

A significant study x treatment interaction was present at week six ($p = 0.0002$), requiring data to be analyzed separately at each study site. The resulting simpler model explained a significant proportion of variation in little bluestem seedling emergence across all three sites: Starkville 2012, $p < 0.0001$, $R^2 = 0.91$; Starkville 2013, $p = 0.0003$, $R^2 = 0.78$; Brooksville 2013, $p = 0.0227$, $R^2 = 0.60$. In Starkville 2012 there was no effect due to block ($p = 0.1037$), but the treatment effect was significant ($p < 0.0001$). Fluxofenin (4.6 seedlings m^{-1}) was the only treatment providing even partial protection as compared to the positive (15.0 seedlings m^{-1}) and negative controls (0.6 seedlings m^{-1}). In Starkville 2013, the effect due to block ($p = 0.0034$) and treatment ($p = 0.0005$) were both significant. Fluxofenin (16.7 seedlings m^{-1}), was the only treatment resulting in significant improvement over the negative control (1.0 seedlings m^{-1}). The positive control (11.3 seedlings m^{-1}) and the negative control were chained by naphthalic anhydride (5.2 seedlings m^{-1}) in the mean separation (Table 5.4) not allowing application of the definitions of levels of protection. In Brooksville 2013, there was no significant block effect ($p = 0.4699$) but the treatment effect was significant ($p = 0.0095$). Naphthalic anhydride (4.4 seedlings m^{-1}) provided complete protection as compared to

the positive control (6.9 seedlings m^{-1}) but was chained to treatments that provided no control as compared to the negative control (0.6 seedlings m^{-1}) (Table 5.4).

There was no significant study x treatment effect ($p = 0.1203$) impacting little bluestem seedling emergence at the eight week stage, allowing results to be pooled across study sites. There was no effect due to block nested within study ($p = 0.0561$) or study ($p = 0.2013$) (Table 5.3), but the treatment effect was significant ($p = 0.0004$). Fluxofenin (8.5 seedlings m^{-1}) provided complete control as compared to the positive control (10.7 seedlings m^{-1}), with naphthalic anhydride (6.0 seedlings m^{-1}) and benoxacor (3.8 seedlings m^{-1}) providing partial control as compared to the positive and negative control (1.2 seedlings m^{-1}) (Table 5.4).

The study x treatment effect was significant ($p < 0.0001$) at the ten week stage, requiring data to be analyzed separately at each study site. The resulting simpler model significantly explained little bluestem seedling emergence at the ten week stage at each site: Starkville 2012, $p < 0.0001$, $R^2 = 0.89$; Starkville 2013, $p = 0.0003$, $R^2 = 0.77$; Brooksville 2013, $p = 0.0001$, $R^2 = 0.79$. In Starkville 2012 both the block ($p = 0.0170$) and treatment effects were significant ($p < 0.0001$). No treatment provided complete protection, but fluxofenin (9.8 seedlings m^{-1}) and naphthalic anhydride (7.7 seedlings m^{-1}) provided partial protection as compared to the positive (22.7 seedlings m^{-1}) and negative controls (2.3 seedlings m^{-1}) (Table 5.4). In Starkville 2013 both block ($p = 0.0043$) and treatment effects ($p = 0.0006$) were significant, with fluxofenin providing increased seedling emergence compared to all treatments including the positive control (8.8 seedlings m^{-1}) but no other treatment resulting in a significant difference from the negative control (2.5 seedlings m^{-1}). In Brooksville 2013 there was a significant effect

due to block ($p = 0.0126$) and treatment ($p = 0.0001$). Fluxofenin (11.0 seedlings m^{-1}) and naphthalic anhydride (9.4 seedlings m^{-1}) were the only safener treatments to result in an increase in seedling emergence as compared to the negative control (1.0 seedlings m^{-1}), but all other treatments chained the negative control to the positive control (7.5 seedlings m^{-1}), not allowing application of the definitions of levels of protection.

Fluxofenin and naphthalic anhydride were the most reliable safener treatments for little bluestem. By the four week stage, partial protection was observed from both safeners. At week six, fluxofenin alone resulted in partial protection in Starkville in both years, but not in Brooksville where naphthalic anhydride alone resulted in partial protection. In week eight, fluxofenin resulted in complete protection across study sites while naphthalic anhydride resulted in partial protection. By the final emergence count, week ten, fluxofenin and naphthalic anhydride both provided partial protection in Starkville 2012. Fluxofenin alone resulted in protection as compared to the negative control in Starkville 2013, while fluxofenin and naphthalic anhydride both resulted in protection as compared to the negative control in Brooksville 2013. Lack of a simple mean separation between the positive and negative controls at both 2013 sites did not allow for designation of protection as 'complete' or 'partial,' though. No other safener treatment resulted in any level of protection by the final week or any repeated protection in the earlier weeks.

Discussion

Roder et al. (1987) conducted trials on the efficacy of safeners in protecting little bluestem from metolachlor injury. They found little bluestem to be sensitive to metolachlor, unlike Griffin et al.'s (1988) finding with regard to big bluestem. Little

bluestem demonstrated significant sensitivity in our study, with the negative control resulting in reduced seedling emergence compared to the positive control at every week and location where there was an effect due to treatment. No safener tested by Roder et al. protected little bluestem from metolachlor injury. Our study found partial protection provided by fluxofenin and naphthalic anhydride. Neither of those safeners were included in the trial conducted by Roder et al.

Table 5.3 Mean seedling emergence of 'Aldous' little bluestem across study sites.

Study: location and year	Mean seedling m^{-1}				
	Week 2	Week 4	Week 6	Week 8	Week 10
Starkville, 2012	2.3†	4.0‡	3.6†	4.7‡	7.4†
Starkville, 2013	1.1	4.8	5.9	6.4	7.9
Brooksville, 2013	0.9	4.8	2.4	3.9	5.7

† Mean separation not reported due to a significant study x treatment interaction: week 2, $p = 0.0018$; week 6, $p = 0.0002$; week 10, $p < 0.0001$.

‡ Mean separation not reported due to no significant study effect: week 4, $p = 0.8965$; week 8, $p = 0.2013$.

Table 5.4 Mean seedling emergence of 'Aldous' little bluestem across treatments.

	Mean seedling m^{-1}										
	Week2†			Week 4‡	Week 6†			Week 8‡	Week 10†		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	11.0 a§	2.9¶	4.2 a	15.3 a	15.0 a	11.3 ab	6.9 a	10.7 a	22.7 a	8.8 b	7.5 ab
Fluxofenin	1.9 b	2.7	3.3 a	8.3 b	4.6 b	16.7 a	2.7 bc	8.5 a	9.8 b	17.7 a	11.0 a
Naphthalic Anhydride	2.1 b	0.8	0.0 b	4.2 c	3.3 bc	5.2 bc	4.4 ab	6.0 b	7.7 bc	8.3 b	9.4 a
Benoxacor	0.6 b	0.0	0.0 b	2.7 cd	0.2 d	4.2 c	1.0 bc	3.8 bc	3.3 d	8.5 b	5.4 bc
Oxabetrinil	0.2 b	0.0	0.0 b	0.6 d	1.3 cd	2.3 c	0.0 c	2.6 cd	2.7 d	5.2 bc	2.5 cd
Fenclorim	0.2 b	0.0	0.0 b	0.6 d	0.2 d	0.8 c	0.8 bc	2.2 cd	3.3 cd	4.0 bc	3.3 cd
No safener w/ herbicide	0.0 b	0.0	0.0 b	0.5 d	0.6 cd	1.0 c	0.6 c	1.2 d	2.3 d	2.5 c	1.0 d

† Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: week 2, $p = 0.0018$; week 6, $p = 0.0002$; week 10, $p < 0.0001$.

‡ Results are pooled across studies if there is no significant study x treatment interaction at $\alpha = 0.05$: week 4, $p = 0.1271$; week 8, $p = 0.1203$.

§ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.

¶ No mean separations are listed if there is no significant effect due to treatment at $\alpha=0.05$: Starkville 2013 week 2, $p = 0.3492$.

Indiangrass

The overall model explained a significant proportion of variation in indiangrass seedling emergence with a $p < 0.0001$ across every week. Coefficients of determination varied across weeks: week two, $R^2 = 0.93$; week four, $R^2 = 0.94$; week six, $R^2 = 0.78$; week eight, $R^2 = 0.85$; week ten, $R^2 = 0.88$. With the exception of week eight ($p = 0.3708$), there was a significant study x treatment interaction in every week: at the two week stage, $p < 0.0001$; four week stage, $p < 0.0001$; six week stage, $p = 0.0262$; ten week stage $p < 0.0001$. Data were analyzed separately for each location for any week that possessed a significant study x treatment interaction.

No safener treatment protected indiangrass from S-metolachlor. There was a significant effect due to treatment with $p < 0.0001$ across all weeks in Starkville 2012. In Starkville 2012 the positive control resulted in significantly greater germination than all other treatments at every week, while no safener treatment was ever significantly different from the negative control (Table 5.6). No effect due to treatment was observed at the two week stage in Starkville 2013 ($p = 0.0664$) or Brooksville 2013 ($p = 0.4552$). A significant effect due to treatment was observed at both locations in all subsequent weeks. At the four week stage in Brooksville 2013, fluxofenin resulted in partial protection, but this effect was not sustained and all subsequent weeks saw the positive control resulting in significantly higher rates of seedling emergence with no safener treatments resulting in increases as compared to the negative control (Table 5.6). No safener treatment resulted in an increase in indiangrass seedling emergence over the negative control in any week in Starkville 2013, with the positive control significantly greater in all weeks (Table 5.6). Week eight, the one week without a significant study x

treatment interaction, maintained the trend even when data were pooled across studies: the positive control was significantly greater than all treatments with no significant difference between any safener treatment and the negative control. In short, no safener treatment provided any significant protection to indiangrass.

Discussion

Our observations agree with Griffen et al. (1988) that indiangrass, unlike big bluestem, has no tolerance to metolachlor. The negative control resulted in reduced seedling emergence compared to the positive control at every week and location where an effect due to treatment was present. Griffen et al. (1988) observed partial protection of indiangrass by naphthalic anhydride, but in only one of two study sites. No other safener they tested protected indiangrass. We achieved no protection of indiangrass with naphthalic anhydride, or any other safener tested, at any of our three study sites. Indiangrass' sensitivity to metolachlor and lack of response to all safeners mirrors big bluestem's partial tolerance to metolachlor and consistent response to all safeners tested.

Table 5.5 Mean seedling emergence of 'Holt' indiangrass across study sites.

Study: location and year	Mean seedling m^{-1}				
	Week 2	Week 4	Week 6	Week 8	Week 10
Starkville, 2012	2.3†	3.2†	2.9†	2.9‡	3.5†
Starkville, 2013	0.7	1.8	2.3	2.7	2.8
Brooksville, 2013	0.0	1.9	1.5	2.4	1.7

† Mean separation not reported due to a significant study x treatment interaction: week 2, $p < 0.0001$; week 4, $p < 0.0001$; week 6, $p = 0.0262$; week 10, $p < 0.0001$.
‡ Mean separation not reported due to no significant study effect: week 8, $p = 0.7270$.

Table 5.6 Mean seedling emergence of 'Holt' indiangrass across treatments.

	Mean seedling m^{-1}												
	Week2†			Week 4†			Week 6†			Week 8‡	Week 10†		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	15.6 a§	1.0¶	0.2¶	21.0 a	9.2 a	10.4 a	17.9 a	11.5 a	6.9 a	12.1 a	12.1 a	9.8 a	7.9 a
Fluxofenin	0.0 b	0.2	0.0	0.0 b	1.7 b	2.3 b	0.0 b	2.7 b	0.6 b	1.4 b	1.0 b	2.7 b	1.7 b
Benoxacor	0.0 b	0.4	0.0	1.3 b	0.4 b	0.2 c	0.4 b	0.8 b	0.4 b	0.9 b	0.0 b	2.3 b	1.5 b
Naphthalic Anhydride	0.0 b	0.4	0.0	0.2 b	0.6 b	0.0 c	0.2 b	0.2 b	0.2 b	1.8 b	0.6 b	0.6 b	0.4 b
Oxabetrinil	0.2 b	0.0	0.0	0.0 b	0.8 b	0.0 c	1.3 b	0.8 b	0.6 b	1.3 b	1.3 b	1.7 b	0.4 b
Fenclorim	0.2 b	0.0	0.0	0.2 b	0.0 b	0.2 c	0.2 b	0.0 b	0.4 b	0.2 b	0.4 b	1.3 b	0.0 b
No safener w/ herbicide	0.0 b	0.0	0.0	0.0 b	0.0 b	0.2 c	0.0 b	0.0 b	1.0 b	0.9 b	0.4 b	1.0 b	0.0 b

† Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: week 2, $p < 0.0001$; week 4, $p < 0.0001$; week 6, $p = 0.0262$; week 10, $p < 0.0001$.

‡ Results are pooled across studies if there is no significant study x treatment interaction at $\alpha = 0.05$: week 8, $p = 0.3708$.

§ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.

¶ No mean separations are listed if there is no significant effect due to treatment at $\alpha=0.05$: Starkville 2013 week 2, $p = 0.0664$; Brooksville 2013 week 2, $p = 0.4552$.

Weed Control Ratings

Weed control ratings were taken at the same biweekly intervals as seedling emergence counts and were based on a visual rating on a scale of 1 to 5: 1 describing very poor weed control and 5 describing excellent weed control. Weed control ratings were analyzed across species. There was no significant three-way study x species x treatment interaction in any of the five weeks: week two, $p = 0.0626$; week four, $p = 0.0508$; week six, $p = 0.0651$; week eight, $p = 0.4756$; week ten, $p = 0.4063$. There was also no significant species x treatment interaction in any of the five weeks: week two, $p = 0.1838$; week four, $p = 0.2664$; week six, $p = 0.6748$; week eight, $p = 0.9577$; week ten, $p = 0.3616$. Data were therefore pooled across species in all weeks. There was a significant study x treatment interaction in all weeks, with $p < 0.0001$ in all weeks (except week ten, $p = 0.0004$). Data were therefore sorted across studies in all weeks.

In week two there was no significant effect on weed control ratings due to treatment in Brooksville 2013 ($p = 0.9173$) or in Starkville 2013 ($p = 0.4385$). There was a significant effect due to treatment at the two week stage in Starkville 2012 ($p < 0.0001$). The no safener no herbicide treatment resulted in significantly worse weed control than all other treatments, with no differences in weed control between any other treatment (Table 5.7). This result, significant effect due to treatment ($p < 0.0001$) with the no safener no herbicide treatment resulting in significantly worse weed control than all other treatments and no differences in weed control between any other treatment, was then observed at all locations across all of the following weeks, with the exception of week ten at Brooksville 2013. In Brooksville 2013 there was a significant difference between the oxabetrinil treated plots and the fluxofenin and no safener with herbicide plots. These

mean separations were chained together, though, by the other treatments receiving herbicide and the no safener no herbicide treatment was significantly worse than all other treatments (Table 5.7).

The significant difference between all treatments receiving herbicide application and the no safener no herbicide control across weeks and locations indicated a substantial weed seed bank was present at all study sites and that it was successfully controlled by S-metolachlor. That there was no significant difference between the herbicide receiving treatments indicates that herbicide application alone determined weed control ratings, and not competition with the establishing desired grass, which varied between herbicide receiving treatments as demonstrated in the seedling emergence discussion above. That there were no interactions involving the desired grass species further demonstrated that herbicide application alone determined weed levels. There were significant effects due to block at many sites. These were generally due to the occurrence of perennial weed species not controlled by S-metolachlor: bermudagrass and nutsedge (*Cyperus esculentus* L.) in Starkville 2013 and johnsongrass in Brooksville 2013.

Table 5.7 Mean Weed Control Ratings by herbicide and safener treatment.

	Mean weed control ratings†														
	Week 2‡			Week 4‡			Week 6‡			Week 8‡			Week 10‡		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, w/ herbicide	5.0 a§	5.0¶	5.0¶	5.0 a	5.0 a	4.9 a	5.0 a	5.0 a	4.7 a	4.8 a	4.9 a	2.8 a	4.8 a	4.6 a	3.2 b
Fluxofenin	5.0 a	5.0	4.9	4.9 a	5.0 a	4.8 a	4.9 a	4.9 a	4.5 a	4.8 a	4.8 a	3.3 a	4.8 a	4.4 a	3.3 b
Benoxacor	5.0 a	5.0	4.9	5.0 a	5.0 a	4.8 a	4.9 a	5.0 a	4.8 a	4.9 a	4.9 a	2.9 a	4.9 a	4.6 a	3.5 b
Naphthalic Anhydride	4.9 a	5.0	4.9	5.0 a	5.0 a	4.7 a	5.0 a	5.0 a	4.5 a	4.8 a	4.8 a	3.1 a	4.8 a	4.7 a	3.4 ab
Oxabetrinil	5. 0 a	5.0	4.9	5.0 a	5.0 a	4.8a	4.8 a	5.0 a	4.7 a	4.8 a	5.0 a	3.3 a	4.7 a	4.7 a	3.8 a
Fenclorim	5. 0 a	5.0	4.9	5.0 a	5.0 a	4.8 a	5.0 a	5.0 a	4.4 a	4.9 a	4.8 a	3.1 a	4.9 a	4.7 a	3.2 b
No safener no herbicide	2.6 b	4.9	5.0	2.3 b	3.9 b	3.3 b	1.9 b	3.7 a	3.2 b	1.8 b	3.3 a	1.0 b	1.3 a	2.7 b	1.0 b

† Weed control ratings were based on a visual rating on a scale of 1 to 5; 1 describing very poor weed control and 5 describing excellent weed control.
‡ Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: week 2, $p < 0.0001$; week 4, $p < 0.0001$; week 6, $p < 0.0001$; week 10, $p = 0.0004$.
§ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
¶ No mean separations are listed if there is no significant effect due to treatment at $\alpha=0.05$: Starkville 2013 week 2, $p = 0.4385$; Brooksville 2013 week 2, $p = 0.9173$.

Establishment Year End of Season Stand Performance

End of season crown counts (number of crowns m^{-1}), coverage ratings (estimated percent coverage by desired grass on a 1-10 scale), and plant heights (both canopy and seed head height) were all measured prior to establishment year harvest. These measurements, along with plot yield (kg $plot^{-1}$), were intended to quantify stand performance in the establishment year.

Establishment Year Crown Counts and Coverage Ratings

Big Bluestem

End of season crown counts of big bluestem in the establishment year were affected by a significant study x treatment interaction ($p = 0.0009$), requiring results to be analyzed separately for each study site. In Starkville 2012 (19.8 crowns m^{-1}), there was a significant effect due to treatment ($p = 0.0010$) but not due to block ($p = 0.9782$). All safener treatments significantly increased end of season crown counts compared to the negative control (11.0 crowns m^{-1}). Fenclorim (17.3 crowns m^{-1}) was the only safener treatment to result in a significant decrease in end of season crown count as compared to the positive control (24.8 crowns m^{-1}) (Table 5.8). End on season crown counts in Starkville 2013 (10.6 crowns m^{-1}) were also affected by a significant treatment effect ($p = 0.0404$) as well a significant effect due to block ($p = 0.0003$). Fluxofenin (15.6 crowns m^{-1}) was the only treatment to increase end of season crown counts as compared to the negative control (7.7 crowns m^{-1}) (Table 5.8). End of season crown counts in Brooksville 2013 (5.8 crowns m^{-1}) were also subject to a significant treatment effect ($p = 0.0246$) and block effect (0.0068). Benoxacor (9.4 crowns m^{-1}) and fluxofenin (7.7 crowns m^{-1}), were

the only treatments to result in a greater number of crowns as compared to the negative control (5.2 crowns m^{-1}) (Table 5.8).

End of season coverage ratings of big bluestem were affected by a significant study x treatment interaction ($p = 0.0002$), requiring results to be analyzed separately for each study site. There was no significant block effect ($p = 0.3564$) in Starkville 2012 (overall 6.3 rating) but the treatment effect was significant ($p = 0.0063$). There was no significant difference between any safener treatment, but fluxofenin (7.8) and naphthalic anhydride (7.3) rated significantly higher than both the negative (5.3) and positive control (4.0), which were not significantly different from one another (Table 5.8). There was a significant effect due to block ($p = 0.0010$) in Starkville 2013 (3.0 overall rating) but not due to treatment ($p = 0.4130$). End of season coverage rating in Brooksville 2013 (2.4 overall rating) was affected by a significant block effect ($p = 0.0043$) and treatment effect ($p < 0.0001$). Fluxofenin (2.3) and benoxacor (2.8) had significantly higher ratings than all other treatments.

Discussion

The end of season crown counts and coverage ratings for big bluestem were largely consistent with the establishment phase seedling emergence counts. In Starkville 2012, all safener treatments resulted in increased crowns m^{-1} as compared to the negative control. This is consistent with all safeners providing big bluestem at least partial protection as measured by the seedling emergence counts in Starkville 2012. In Starkville 2013 only fluxofenin resulted in increased crowns m^{-1} as compared to the negative control. This was consistent with only fluxofenin and naphthalic anhydride resulting in complete protection of big bluestem in Starkville 2013 according to the

establishment phase seedling emergence counts. In Brooksville 2013, benoxaxor and fluxofenin were the only treatments to result in increased end of season crown counts. They were also the only treatments to provide complete protection according to the seedling emergence counts. An important difference between the establishment phase measures and the end of season measures was observed with regard to the coverage ratings of the positive and negative controls in Starkville 2012. The positive control plots resulted in both increased seedling emergence and end of season crown counts compared to the negative control. However, the two were not significantly different with regard to coverage ratings. This change over time was presumably due to the positive control being subjected to weed competition. More plants established in the positive control plots, as measured by both establishment phase and end of season observations, but the negative control's fewer plants achieved statistically similar coverage in the absence of weed competition. This increased coverage in the absence of weed competition was observed in all of the safener treated plots as well.

Table 5.8 Establishment year end of season crown counts and coverage ratings for 'Kaw' big bluestem.

	Mean crowns m^{-1}†			Mean coverage ratings† (1-10 scale)		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	24.8 a‡	11.0 b	3.3 c	5.3 bc	3.5§	2.0 c
Benoxacor	23.3 a	10.2 b	9.4 a	6.5 ab	3.0	3.0 b
Fluxofenin	21.0 ab	15.6 a	7.7 ab	7.3 a	3.0	3.8 a
Oxabetrinil	21.0 ab	9.2 b	5.0 bc	6.3 ab	2.8	2.0 c
Naphthalic anhydride	20.2 ab	9.8 b	3.8 c	7.8 a	2.8	1.8 c
Fenclorim	17.3 b	10.4 b	6.3 abc	7.0 ab	3.3	2.3 c
No safener, w/ herbicide	11.0 c	7.7 b	5.2 c	4.0 c	2.5	1.8 c

† Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: big bluestem, crown count, $p = 0.0009$; big bluestem, coverage ratings, $p = 0.0002$.
‡ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
§ No mean separations are listed if there is no significant effect due to treatment at $\alpha=0.05$: big bluestem, Starkville 2013, mean coverage ratings, $p = 0.4130$.

Little Bluestem

A significant study x treatment interaction impacted both the end of season crown counts ($p < 0.0001$) and coverage ratings ($p = 0.0147$) for little bluestem, requiring data to be analyzed separately for each study site. In Starkville 2012, there was a significant effect due to treatment for both end of season crown count ($p < 0.0001$) and coverage rating ($p = 0.0066$). The positive control (20.0 crowns m^{-1}) had a significantly higher crown count than all other treatments, with fluxofenin (9.4 crowns m^{-1}) and naphthalic anhydride (8.1 crowns m^{-1}) the only safener treatments significantly greater than the negative control (3.1 crowns m^{-1}). Fluxofenin (5.8) and naphthalic anhydride (3.5) were not significantly different from the positive control (4.8) with regard to end of season

coverage ratings, while all three of these treatments were significantly different from all other treatments. In Starkville 2013, treatment significantly affected both end of season crown counts ($p = 0.0072$) and coverage ratings ($p = 0.0420$). Fluxofenin (10.8 crowns m^{-1}; 2.3 coverage rating) was the only safener treatment to show an improvement as compared to the negative control (1.7 crowns m^{-1}; 1.0 coverage rating) for either measure. There was no effect due to treatment for coverage rating ($p = 0.1220$) in Brooksville 2013 due to uniformly poor coverage across treatments. There was a significant effect due to treatment on crown counts ($p = 0.0034$), with fluxofenin (5.2 crowns m^{-1}) and napthalic anhydride (4.2 crowns m^{-1}) the only safener treatments to result in an increase over the negative control (0.4 crowns m^{-1}).

Discussion

The end of season crown counts and coverage ratings were in agreement with the establishment phase seedling emergence counts. In Starkville 2012, fluxofenin and naphthalic anhydride were the only treatments to provide at least partial protection; i.e., they resulted in decreased seedling emergence as compared to the positive control but increased seedling emergence as compared to the negative control. This exact trend was repeated with regard to end of season crown counts: the positive control fared significantly better than all other treatments, and fluxofenin and naphthalic anhydride were the only safener treatments to show a significant improvement over the negative control. This trend was not repeated, though, with regard to coverage ratings. Fluxofenin and naphthalic anhydride were not significantly different from the positive control and all three were rated significantly higher than all other treatments. This repeats the results for big bluestem in Starkville 2012, where the most successful safener treatments 'caught up'

to the positive control with regard to coverage ratings presumably due to the positive control's competition with weeds. In Starkville 2013, fluxofenin was the only safener treatment to show improvement over the negative control for either end of season crown count or coverage rating, doing so for both. Fluxofenin was also the only safener to provide partial protection to little bluestem in Starkville 2013. In Brooksville 2013, fluxofenin and naphthalic anhydride were the only treatments to show improved end of season crown counts compared to the negative control, and they were the only treatments to result in protection according to the seedling emergence counts.

Table 5.9 Establishment year end of season crown counts and coverage ratings for 'Aldous' little bluestem.

	Mean crowns m^{-1} row[†]			Mean coverage ratings[†] (1-10 scale)		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	20.0 a[‡]	6.3 b	2.9 bc	4.8 ab	1.8 ab	1.5[§]
Fluxofenin	9.4 b	10.8 a	5.2 a	5.8 a	2.3 a	1.8
Naphthalic anhydride	8.1 bc	5.6 bc	4.2 ab	3.5 b	1.5 bc	1.0
Benoxacor	5.2 bcd	5.4 bc	2.3 bcd	2.3 c	1.5 bc	1.5
Oxabetrinil	4.4 cd	3.8 bc	1.7 cd	2.5 c	1.3 bc	1.0
Fenclorim	4.2 cd	2.7 bc	1.9 d	2.0 c	1.3 bc	1.0
No safener, w/ herbicide	3.1 d	1.7 c	0.4 d	1.8 c	1.0 c	1.0

[†] Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: little bluestem, crown count, $p < 0.0001$; little bluestem, coverage ratings, $p = 0.0147$.
[‡] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[§] No mean separation is listed if there is no significant effect due to treatment at $\alpha=0.05$: little bluestem, Brooksville 2013, mean coverage ratings, $p = 0.1220$.

Indiangrass

A significant study x treatment interaction impacted both the end of season crown counts ($p < 0.0001$) and coverage ratings ($p < 0.0001$) for indiangrass, requiring results to be analyzed separately for each study site. In Starkville 2012 there was no significant effect due to block for either crown counts ($p = 0.3292$) or coverage ratings ($p = 0.4155$) but there was a significant effect due to treatment, with $p < 0.0001$ for both measures. For both measures, the positive control (519.4 crowns m^{-1}; 6.3 coverage rating) was significantly greater than all other treatments with no significant differences between any other treatment. The same results occurred in Starkville 2013: no effect due to block for either measure ($p = 0.9281$ for crown count; $p = 0.4155$ for coverage rating) but a significant treatment effect for both crown count ($p = 0.0019$) and coverage rating ($p = 0.0001$), with the positive control (4.0 crowns m^{-1}; 1.8 coverage rating) greater than all other treatments and no other significant difference between treatments. There was no effect due to treatment ($p = 0.1558$, $p = 0.1794$) or block ($p = 0.5657$, $p = 0.7607$) for either crown count or coverage rating in Brooksville 2013 due to uniformly poor establishment. The end of season measures agreed with the establishment phase measures for indiangrass. Under both sets of observations, no safener treatment provided protection to indiangrass from S-metolachlor injury.

Table 5.10 Establishment year end of season crown counts and coverage ratings for 'Holt' indiangrass.

	Mean crowns m^{-1}†			Mean coverage ratings† (1-10 scale)		
Treatment	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	19.4 a‡	4.0 a	1.3§	6.3 a	1.8 a	1.5§
Fluxofenin	2.1 b	1.7 b	0.6	1.0 b	1.0 b	1.0
Oxabetrinil	1.7 b	0.6 b	0.4	1.0 b	1.0 b	1.3
Naphthalic anhydride	1.5 b	0.6 b	0.0	1.0 b	1.0 b	1.0
Fenclorim	0.6 b	0.6 b	0.0	1.0 b	1.0 b	1.0
Benoxacor	0.0 b	1.0 b	0.0	1.0 b	1.0 b	1.0
No safener, w/ herbicide	0.6 b	0.8 b	0.0	1.0 b	1.0 b	1.0

† Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: indiangrass, crown count, $p < 0.0001$; indiangrass, coverage ratings, $p < 0.0001$.
‡ Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
§ No mean separations are listed if there is no significant effect due to treatment at $\alpha=0.05$: indiangrass, Brooksville 2013, mean crown count, $p = 0.1558$; indiangrass, Brooksville 2013, mean coverage rating, $p = 0.1794$.

Table 5.11 Establishment year end of season mean crown counts and coverage ratings across study sites.

	Big Bluestem		Little Bluestem		Indiangrass	
Study: location and year	Mean crowns m^{-1}	Mean coverage ratings	Mean crowns m^{-1}	Mean coverage ratings	Mean crowns m^{-1}	Mean coverage ratings
Starkville 2012	19.8†	6.3†	26.0†	3.2†	12.3†	1.8†
Starkville 2013	10.6	3.0	17.3	1.5	4.3	1.1
Brooksvill2 2013	5.8	2.4	8.7	1.3	1.0	1.1

† Mean separation not reported due to a significant study x treatment interaction: big bluestem, crown count, $p = 0.0009$; big bluestem, coverage rating, $p = 0.0002$; little bluestem, crown count, $p < 0.0001$; little bluestem, coverage rating, $p = 0.0147$; inidangrass, crown count, $p < 0.0001$; indiangrass, crown count, $p < 0.0001$.

Establishment Year Plant Heights

End of season canopy and seed head heights were measured on big bluestem at all locations prior to establishment year harvest. There was no significant study x treatment interaction impacting big bluestem end of season canopy height ($p = 0.7208$) or seed head height ($p = 0.3497$) in the establishment year, allowing results to be analyzed across study sites for both measures. There was no significant effect due to treatment on either big bluestem mean canopy height or ($p = 0.2874$) or mean seed head height ($p = 0.2261$). There was a significant effect due to study on both canopy height ($p < 0.0001$) and seed head height ($p < 0.0001$). Significantly greater heights were observed in Starkville 2012 for both canopy (77.6 cm) and seed head (162.0 cm) than in Starkville 2013 (40.3 cm and 82.5 cm) and Brooksville 2013 (38.0 cm and 69.1 cm), with no significant difference between the latter (Table 5.12). In Starkville 2012 establishment year big bluestem heights, averaged over treatments, were nearly double those in Starkville 2013 and Brooksville 2013.

Only end of season canopy heights were taken on little bluestem, due to insufficient seed head production in the establishment year. Little bluestem canopy heights were significantly affected by a study x treatment interaction ($p = 0.0362$), and results were analyzed separately across study sites. There was no effect due to treatment in Starkville 2013 ($p = 0.4255$) or in Brooksville 2013 ($p = 0.2603$). There was a significant effect due to treatment in Starkville 2012 ($p < 0.0001$), with all treatments resulting in significantly reduced little bluestem canopy height compared to the positive control (43.8 cm). Fluxofenin (28.7 cm) was the only safener treatment resulting in significantly greater canopy height than the negative control (19.8 cm) (Table 5.13).

Only end of season canopy heights were taken on indiagrass due to insufficient seed head production. Statistical analysis was conducted on only the Starkville 2012 measurements due to insufficient establishment in Starkville 2013 and Brooksville 2013. There was no significant effect due to block ($p = 0.2260$), but treatment significantly affected indiangrass mean canopy height ($p = 0.0467$). However, no treatment was significantly different from the negative control (Table 5.13).

Table 5.12 Establishment year mean plant height across study sites.

	Mean plant height (cm)			
	Big Bluestem[†]		Little Bluestem[‡]	Indiangrass[‡]
Study: location and year	Canopy height	Seed head height	Canopy height	Canopy height
Starkville 2012	77.6 a[§]	162.0 a	25.8	19.7
Starkville 2013	40.3 b	82.5 b	19.5	--
Brooksville 2013	38.0 b	69.1 b	17.5	--

[†] Big bluestem heights were measured on both canopy height and seed head height in the establishment year at all study sites.
[‡] Only canopy heights were measured on little bluestem and indiangrass, due to insufficient seed head production in establishment year.
[§] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[¶] No mean separations are listed if there is a significant study x treatment interaction at $\alpha=0.05$: Little bluestem, $p = 0.0362$.

Table 5.13 Establishment year mean plant height across treatments.

	Mean plant height (cm)					
	Big Bluestem[†]		Little Bluestem[‡]			Indiangrass[‡]
	Canopy[§] height	Seed head[§] height	Canopy height[¶]			Canopy[#] height
Treatment	Pooled	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012
No safener, no herbicide	51.1[††]	102.9[††]	43.8 a[‡ ‡]	24.7[††]	24.4[††]	30.2 a[‡ ‡]
Fluxofenin	56.8	109.7	28.7 b	21.0	18.9	15.0 bc
Benoxacor	52.1	109.2	21.1 cd	16.3	15.2	14.0 c
Naphthalic Anhydride	49.9	110.6	26.0 bc	15.4	17.5	14.0 c
Oxabetrinil	50.7	97.9	23.1 bcd	22.9	11.5	17.2 bc
Fenclorim	50.8	100.1	17.8 c	21.6	13.9	28.2 ab
No safener w/ herbicide	47.82	101.5	19.8 cd	15.0	20.9	19.3 abc

[†] Big bluestem heights were measured on both canopy height and seed head height in the establishment year at all study sites.
[‡] Only canopy heights were measured on little bluestem and indiangrass, due to insufficient seed head production in establishment year.
[§] Results are pooled across study sites if there is no significant study x treatment interaction: big bluestem canopy height, p = 0.7208; big bluestem seed head height, p = 0.3497.
[¶] Plant heights are sorted across study sites if there is a significant study x treatment interaction: little bluestem canopy height, p = 0.0362.
[#] Indiangrass canopy heights are only presented for Starkville 2012 due to insufficient establishment in Starkville 2013 and Brooksville 2013.
[††] No mean separations are listed if there is no significant effect due to treatment at α=0.05: big bluestem canopy height, p = 0.7208; big bluestem seed head height, p = 0.3497; little bluestem, Starkville 2013, p = 0.4255; little bluestem, Brooksville 2013, p = 0.6203.
[‡ ‡] Means within a column followed by the same letter are not significantly different at α = 0.05.

Establishment Year Harvest

Desired grass and weeds were harvested separately for all three grass species and all three study sites. Weed dry matter yield was analyzed across species, whereas grass dry matter yield was analyzed separately for each species.

For weed dry matter yield there was no three-way species x study x treatment interaction (p = 0.1278), no species x treatment interaction (p = 0.6188) and no effect due

to species ($p = 0.8458$), allowing data to be pooled across all three species as was done for weed control ratings above. Dry matter yield of weeds was impacted by a significant treatment x study interaction ($p < 0.0001$), but the effect due to treatment was significant in all studies ($p < 0.0001$, in all three studies). The no safener no herbicide positive control resulted in significantly greater dry matter yield of weeds in all studies with no significant difference between any of the treatments receiving S-metolachlor application. These results tells us that the positive control was subjected to significant weed competition not dealt encountered by the other treatments, and that the herbicide was applied at a rate sufficient to control weeds. All successful seed safening was also achieved against a rate of S-metolachlor sufficient to control weeds.

Big bluestem dry matter yield was not impacted by a significant study x treatment interaction ($p = 0.0621$), allowing results to be pooled across study sites. There was a significant effect due to study ($p < 0.0001$) with Starkville 2012's mean dry matter yield (5692.8 kg ha^{-1}), significantly greater than those of Starkville 2013 (195.7 kg ha^{-1}) and Brooksville 2013 (179.4 kg ha^{-1}), but no significant difference between the latter (Table 5.15). There was no significant effect due to treatment ($p = 0.2664$), however. So, although seedling emergence, crown establishment, and coverage were increased over the negative control by safener treatment, establishment year plant height and dry matter yield were not.

Little bluestem dry matter yield was impacted by a significant study x treatment ($p = 0.0002$), requiring results to be analyzed separately for each study site. In Starkville 2012 there was a significant effect due to treatment ($p = 0.0077$), but not due to block ($p = 0.1175$). There was no significant difference in dry matter yield between naphthalic

anhydride (385.0 kg ha^{-1}), fluxofenin (702.2 kg ha^{-1}), and the positive control (611.7 kg ha^{-1}). These three treatments resulted in significant improvement over all other safener applications and the negative control (211.2 kg ha^{-1}). Fluxofenin and naphthalic anhydride were the two safener treatments resulting in partial protection of little bluestem in Starkville 2012. Both treatments resulted in significantly fewer seedlings at the establishment phase and crowns by the end of season, but equal coverage, and harvest. There was no significant effect due to treatment in either Starkville 2013 ($p = 0.1818$) or Brooksville 2013 ($p = 0.4974$). Although there was not an overall effect due to study site due to the significant treatment x study interaction, the Starkville 2012 site did average a higher dry matter yield (349.1 kg ha^{-1}) than Starkville 2013 (21.2 kg ha^{-1}) and Brooksville (9.8 kg ha^{-1}) (Table 5.15). This generally poor yield in both 2013 sites most likely accounts for the lack of effect due to treatment at those sites.

Indiangrass dry matter yield was impacted by a significant study x treatment ($p < 0.0001$), requiring results to be analyzed separately for each site. In Starkville 2012 there was a significant effect due to trt ($p < 0.0001$) but not block ($p = 0.2692$). The positive control (368.6 kg ha^{-1}) yielded significantly higher than all other treatments with no significant difference between any safener treatment and the negative control (61.6 kg ha^{-1}). Starkville 2013 also had a significant treatment effect ($p = 0.0056$) and no significant block effect ($p = 0.5650$). Just as in Starkville 2012, the positive control (8.0 kg ha^{-1}) yielded significantly more dry matter than all other treatments with no safener treatment significantly different from the negative control (0.8 kg ha^{-1}). In Brooksville 2013, the treatment effect was significant ($p = 0.0354$) but not the block effect ($p = 0.3423$) and the positive control (7.5 kg ha^{-1}) yielded significantly more than all

treatments other than benoxacor (3.2 kg ha^{-1}). No safener treatment including benoxacor yielded significantly different than the negative control (0.7 kg ha^{-1}).

Table 5.14 Establishment year harvest across treatments.

	Yield (kg ha^{-1})						
	Big Bluestem[†]	Little Bluestem[‡]			Indiangrass[‡]		
Study: location and year	Pooled	Starkville 2012	Starkville 2013	Brooksville 2013	Starkville 2012	Starkville 2013	Brooksville 2013
No safener, no herbicide	1634.5[§]	611.7 a[¶]	16.3[§]	14.7[§]	368.6 a	8.2 a	6.5 a
Fluxofenin	2339.1	703.0 a	47.3	11.4	34.3 b	0.3b	0.0 b
Oxabetrinil	1587.1	213.7 b	27.7	1.6	42.4 b	0.0 b	0.0 b
Naphthalic anhydride	2211.9	385.0 ab	11.4	14.7	19.6 b	0.0 b	0.0 b
Fenclorim	2176.0	114.2 b	8.2	8.2	14.7 b	0.0 b	0.0 b
Benoxacor	2432.1	202.3 b	26.1	9.8	9.8 b	1.6 b	3.3 ab
No safener, w/ herbicide	1729.1	212.1 b	14.7	3.3	62.0 b	0.8 b	0.7 b

[†] Results are pooled across studies if there is no significant study x treatment interaction at $\alpha = 0.05$: big bluestem, $p = 0.0621$.
[‡] Results are sorted by study if there is a significant study x treatment interaction at $\alpha = 0.05$: little bluestem, $p = 0.0002$; indiangrass, $p < 0.0001$.
[§] No mean separation is listed if there is no significant effect due to treatment at $\alpha=0.05$: big bluestem, $p = 0.2664$; little bluestem, Starkville 2013, $p = 0.1818$; little bluestem, Brooksville 2013, $p = 04974$.
[¶] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.

Table 5.15 Establishment year harvest across study sites.

	Yield (kg ha^{-1})		
Study: location and year	Big Bluestem	Little Bluestem	Indiangrass
Starkville 2012	5689.6 a[†]	349.1[‡]	78.6[‡]
Starkville 2013	194.1 b	21.2	1.5
Brooksville 2013	164.8 b	9.8	1.3

[†] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[‡] No mean separations are listed if there is a significant study x treatment interaction at $\alpha=0.05$: Little bluestem, $p = 0.0002$; indiangrass, $p < 0.0001$.

CHAPTER VI

FIELD TRIAL: SECOND YEAR RESULTS AND DISCUSSION

Data on end of season stand performance the year after planting was available in the fall of 2013 on the site planted in Starkville 2012. All plots were managed identically after establishment year harvest. Weed control was managed by application of imazapic (Plateau®, BASF) at 0.14 kg a.i. ha^{-1} in March 2013 to control winter weeds post-emergence and to provide preemergent control of spring weeds. No other management was conducted within plots.

Big Bluestem

There was no significant effect due to treatment ($p = 0.2274$) on big bluestem end of season crown counts in the year after planting, but the effect due to block was significant ($p = 0.0111$). This stood in contrast to the establishment year crown counts of big bluestem at this same site, where there was a significant effect due to treatment, with fenclorim and the negative control reducing end of season crown counts compared to all other treatments. Fenclorim (3.5 crowns m^{-1}) and the negative control (3.1 crowns m^{-1}) did result in the two smallest end of season crown counts in the second year, but these reductions were not statistically significant. There was also no significant effect due to treatment ($p = 0.5195$) on big bluestem coverage rating in the year after planting. This was again in contrast to the establishment year where fluxofenin and naphthalic

anhydride resulted in significantly increased coverage. The differences in establishment and coverage provided by some treatments in the establishment year were, therefore, not maintained through the end of the second year. Big bluestem plant height, measured as height of grass canopy ($p = 0.6840$) and seed head ($p = 0.1275$), was not significantly affected by treatment. The same was true in the establishment year.

There was no significant effect due to treatment ($p = 0.0761$) on big bluestem dry matter yield the year after planting. This result agreed with the establishment year in which dry matter yield was not impacted by treatment. The positive control did yield 15% less than the negative control, despite this decrease being statistically insignificant at $\alpha = 0.05$. This reduction was presumably due to weed competition in the establishment year suffered by the positive control.

Our results on big bluestem yield are not in full agreement with previous studies on the effect of weed competition in the establishment year on yield the year after planting. Bryan and McMurphy (1968) and Masters (1995) both observed significantly reduced big bluestem yield the year after planting in plots where weeds were not controlled in the establishment year as compared to plots where weeds were controlled. Bryan and McMurphy (1968) conducted their test with hand weeding resulting in nearly identical stand establishment between the weedy plots and the weeded plots. The only difference was consequently the presence of weeds, not stand density, and they observed a 65% mean reduction in yield due to weed competition. Masters (1995) controlled weeds with metolachlor and atrazine. These herbicide treatments decreased big bluestem stand density. The herbicide treatments increased second year big bluestem yields by 31 – 69% despite decreasing stand density in the establishment year. Bryan and McMurphy

(1968), Masters (1995), and our study were all conducted on the cultivar Kaw. In our study, plots safened by fluxofenin had similar stand density as the positive control. Plots safened with all other safeners had reduced stand density compared to the positive control, but increased stand density compared to the negative control. There is therefore no confounding effect due to stand density when comparing our results to previous work. Bryan and McMurphy (1968) observed reduced yield due to weed competition between equally dense stands. Masters (1995) observed reduced yield due to weed competition despite stand reduction in the herbicide treated plots. We observed no effect due to weed completion on second year yield, regardless of relative stand density.

Table 6.1 Second year end of season stand performance of 'Kaw' big bluestem.

Treatment	Crown counts (m^{-1})	Coverage rating (1-10 scale)	Canopy Height (cm)	Seed Head Height (cm)	Dry Matter Yield (kg ha^{-1})
No safener, no herbicide	4.5†	7.0†	68.0†	173.6†	4828.3†
Fluxofenin	4.0	7.0	72.5	178.8	6100.6
Naphthalic anhydride	4.3	7.0	72.9	190.9	6100.6
Benoxacor	3.9	6.8	70.2	189.6	6182.2
Oxabetrinil	3.8	7.3	69.9	178.7	5334.0
Fenclorim	3.5	7.0	74.3	184.8	5464.5
No safener, w/ herbicide	3.1	6.3	74.3	185.7	5643.9

† No mean separations are listed if there is no significant effect due to treatment at α=0.05: crown count, $p = 0.2274$; coverage rating, $p < 0.5195$; canopy height, $p = 0.6840$; seed head height, $p = 0.1275$; dry matter yield, $p = 0.0761$.

Little Bluestem

There was a significant effect due to treatment ($p = 0.0006$) and block (0.0139) on little bluestem end of season crown counts in the year after planting. Fluxofenin (4.6 crowns m^{-1}) and naphthalic anhydride (3.9 crowns m^{-1}) were the only safener treatments to result in an increase in crown number over the negative control (2.5 crowns m^{-1}) (Table 6.2), as was the case in the establishment year as well. However, there was also no significant difference in crown number between plots safened by fluxofenin and the positive control (5.4 crowns m^{-1}) in the second year, whereas the positive control resulted in significantly higher end of season crowns m^{-1} than all treatments including fluxofenin in the establishment year. There was a significant effect due to treatment on little bluestem coverage rating ($p = 0.0009$) in the year after planting. Cover in the positive control plots was significantly greater than in all other plots in both the establishment year and the year after planting. There was no effect due to treatment on plant height measured as canopy height ($p = 0.4623$) or seed head height ($p = 0.1147$). In the establishment year canopy height of the positive control was significantly greater than all treatments.

There was a significant effect due to treatment on little bluestem dry matter yield ($p = 0.0223$) in the year after planting. There was no significant difference in yield between the positive control (3702.8 kg ha^{-1}) and any other treatment including the negative control (2936.1 kg ha^{-1}). This was in contrast to the establishment year where the positive control yielded significantly more than all other treatments. By the second year, the positive control no longer out yielding other treatments in the year after harvest was presumably due to weed competition in the establishment year. Naphthalic

anhydride (4469.5 kg ha^{-1}) and fluxofenin (4192.2 kg ha^{-1}) were the only treatments to yield significantly more than the negative control. These were also the only treatments to provide partial protection in the establishment year.

Reduced yield in the second year due to weed competition was observed in little bluestem's positive control. However, the reduction in stand density caused by S-metolachlor injury, even in the presence of partial protection by fluxofenin and naphthalic anhydride, resulted in no statistically significant increase in yield due to weed control. However, partial protection did result in an increase in yield ha^{-1} compared to the stand reduction caused by weed control through S-metolachlor without safening.

Table 6.2 Second year end of season stand performance of 'Aldous' little bluestem.

Treatment	Crown counts (m^{-1})	Coverage rating (1-10 scale)	Canopy Height (cm)	Seed Head Height (cm)	Dry Matter Yield (kg ha^{-1})
No safener, no herbicide	5.4 a[†]	8.0 a	37.1[‡]	102.9[‡]	3703.0 abc
Fluxofenin	4.6 ab	6.3 b	33.8	105.5	4192.2 ab
Naphthalic anhydride	3.9 bc	6.0 bc	36.7	110.1	4469.5 a
Benoxacor	3.0 cd	5.0 bc	32.5	102.4	3082.9 c
Oxabetrinil	2.9 cd	5.5 bc	32.1	103.4	3425.5 bc
Fenclorim	3.1 cd	5.3 bc	36.8	108.0	3246.1 c
No safener, w/ herbicide	2.5 d	4.8 c	32.3	107.6	2936.1 c

[†] Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[‡] No mean separation is listed if there is no significant effect due to treatment at $\alpha=0.05$: canopy height, $p = 0.4623$; seed head height, $p = 0.1147$.

Indiangrass

There was a significant effect due to treatment on end of season indiangrass crown counts ($p = 0.0280$) and coverage rating ($p < 0.0001$) the year after planting. For both measures the positive control was significantly greater than all other treatments. This is consistent with the lack of protection provided by all safener treatments. There was no significant treatment effect on canopy height ($p = 0.1634$). There was a significant effect due to treatment on seed head height ($p = 0.0423$), but there was no significant difference between any safener treatment or between any safener treatment and either control (Table 6.3). There was no effect due to treatment on dry matter yield ($p = 0.1221$) in the second year. This differed from the establishment year when the positive control yielded significantly more than all other treatments. The positive control did result in the highest yield (1859.6 kg ha^{-1}) in the second year, despite the overall treatment effect being statistically insignificant.

Table 6.3 Second year end of season stand performance of 'Holt' indiangrass.

Treatment	Crown counts (m^{-1})	Coverage rating (1-10 scale)	Canopy Height (cm)	Seed Head Height (cm)	Dry Matter Yield (kg ha^{-1})
No safener, no herbicide	4.6 a†	7.0 a	35.4‡	110.2 a	1859.6‡
Fluxofenin	2.4 bc	3.0 b	32.0	104.8 ab	1027.6
Naphthalic anhydride	3.0 bc	3.0 b	3.4	104.7 ab	1353.9
Benoxacor	3.4 b	3.3 b	38.6	118.1 a	1631.2
Oxabetrinil	2.5 bc	3.0 b	34.2	112.1 a	1158.1
Fenclorim	2.6 bc	2.5 b	29.6	103.2 ab	1370.2
No safener, w/ herbicide	1.8 c	2.0 b	28.2	89.4 b	831.9

† Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
‡ No mean separation is listed if there is no significant effect due to treatment at $\alpha=0.05$: canopy height, $p = 0.1634$; dry matter yield, $p = 0.1221$.

CHAPTER VII

CONCLUSION

Summary

Research Objectives

This research consisted of two objectives. First, was to determine the efficacy of the safeners benoxacor, fenclorim, fluxofenin, naphthalic anhydride, and oxabetrinil in protecting big bluestem, little bluestem, and indiangrass from injury caused by S-metolachlor. Second, was to determine the effect establishing these grasses using safeners and S-metolchlor had on early stand performance. Three field trials - in 2012 and 2013 in Starkville, MS and 2013 in Brooksville, MS - were conducted to address both objectives. The first objective - can these grasses be safened from S-metolchlor? - was addressed through observations made in the establishment phase directly after planting, primarily seedling emergence counts. The second objective - how does this establishment method impact stand performance? - was addressed through observations made at the end of the growing season, both in the planting year and the year after planting.

Stands of all three grasses performed poorly across all treatments, including the positive control, in both trial locations in 2013. However, results concerning the first objective – the efficacy of safeners – were consistent across years and locations despite the overall poor performance in 2013. Results on stand performance were obviously

impacted by this year effect (2012 vs. 2013). Given that treatment effects on stand performance were swamped by the environmental effect due to year in 2013, comparisons of stand performance between treatments were inconclusive for 2013. As such, this discussion of the impact on stand performance is based largely on the end of season data from the Starkville 2012 trial. This was also the only field trial for which second year data was available at the time of this writing.

The significant differences observed between years are not fully explained by differences in weather between the two years. Temperature did not vary appreciably between 2012 and 2013 (Appendix B, Figure B1). There was a large decrease in total precipitation in July, August, and October 2013 compared to 2012 (Figure B2) that could partially explain the differences in end of season stand performance between years. However, this environmental difference would not explain differences in establishment phase measures, such as seedling emergence counts, that were observed prior to the difference in rain.

Indiangrass

Unsafened indiangrass plots demonstrated severe sensitivity to S-metolachlor, based on the significant differences in seedling emergence between the no herbicide positive control and the no safener with herbicide negative control. No safener treatment tested provided any degree of protection to indiangrass. Near complete stand failures of S-metolachlor treated plots resulted in obvious negative effects on stand performance. These observation was confirmed by end of season crown counts, coverage ratings, and yield data.

Big Bluestem

Unsafened big bluestem demonstrated a degree of tolerance to S-metolachlor, although stand density was significantly reduced as compared to the positive control plots that did not receive herbicide application. Complete protection was provided to big bluestem by fluxofenin, and partial protection was provided by all other safeners, in Starkville 2012. These levels of protection were defined relative to the unsafened positive (no herbicide) and negative (with herbicide) control plots. Complete protection was defined as a safener resulting in no significant reduction in seedling emergence as compared to the positive control. Partial protection was defined as a significant reduction compared to the positive control, but a significant increase compared to the negative control. Poor establishment of the positive controls in 2013 did not allow these definitions of level of protection to be applied therein. However, fluxofenin, benoxacor, and naphthalic anhydride did increase seedling emergence over the negative control in Starkville 2013 and fluxofenin did in Brooksville 2013. Concerning the first objective, all safeners resulted in at least partial safening of big bluestem from S-metolachlor, in at least one trial (location x year). Fluxofenin was the only safener, though, to provide big bluestem complete protection in 2012 and protection in both 2013 trials.

Discussion of stand performance will focus on Starkville 2012, due to the uniformly poor performance across all treatment, including the positive control, in both 2013 trial sites. All safeners resulted in increased end of season coverage as compared to the negative control in the planting year. Fluxofenin and naphthalic anhydride resulted in improved coverage as compared to the positive control. Big bluestem seedlings in the positive control plots competed with significant weed pressure as measured by early

season weed control ratings and end of season weed dry matter yields. The weed competition suffered by the positive control was presumably to blame for its reduced coverage compared to fluxofenin and naphthalic anhydride safened plots. No effect was observed for plant height or yield in the establishment year. After the establishment year harvest all plots were treated alike receiving one application of imazipic (0.14 kg a.i. ha^{-1}) in March 2013. In the year after planting, there was no effect due to treatment on any end of season stand performance measure for big bluestem: crown counts, coverage ratings, plant heights, or yield.

The most important measure of stand performance is obviously yield. Although big bluestem was successfully safened from S-metolachlor (objective 1), and S-metolachlor significantly reduced weed competition, yields were not increased in either the establishment year or the year after planting in the herbicide treated plots as compared to the untreated control plots (objective 2). Therefore, no practical benefit on big bluestem early stand performance was observed from the extra work and expense involved in seed treatments and preemergent herbicide application. Pre-planting weed control followed by second year imazapic application resulted in statistically similar yields in the positive control plots despite establishment year weed competition. These results ran counter to previous studies on weed competition in big bluestem stands discussed above (Bryan and McMurphy, 1968; Masters, 1995), where significant reductions in second year yields resulted from weed completion in the planting year.

Little Bluestem

Unsafened little bluestem demonstrated severe sensitivity to S-metolachlor, as indicated by significantly reduced seedling emergence between the unsafened negative

(with herbicide) and positive (no herbicide) control plots. No safener provided little bluestem complete protection, but partial protection of little bluestem was provided by fluxofenin and naphthalic anhydride in Starkville 2012. Again, our definitions of level of protection were inapplicable in both 2013 field sites due to poor establishment of the positive control. However, fluxofenin, naphthalic anhydride, and benoxacor did provide protection as compared to the negative control in both locations in 2013.

Discussion of stand performance will again focus on Starkville 2012. Greater end of season crown counts and coverage ratings were observed in the positive control plots than all other plots. However, fluxofenin and naphthalic anhydride, the two safeners providing partial protection, did increase both measures compared to the negative control. Importantly, fluxofenin and naphtahlic also resulted in statistically similar yields as the positive control in the establishment year. The weed competition suffered by seedlings in the positive control plots and the reduced stand density resulting from only partial protection, therefore evened one another out with regard to establishment year yields.

The same general trend was observed in the year after planting. Coverage was significantly increased in the positive control plots and the fluxofenin treated plots compared to all others. Again, fluxofenin, naphthalic anhydride and the positive control out yielded all other treatments with no significant difference among the three. These results again point to an equalizing effect between the early reduction in stand density through partial protection, and the later competition with weeds through lack of weed control. However, the failure to increase yields again points to the conclusion that the increased costs in seed treatment and preemergent herbicide application are not practically justified in establishing little bluestem.

Future Research

Our seed treatments were conducted by a single controlled hydration technique based on Griffin et al.'s (1998) previous work on herbicide safeners in big bluestem and indiangrass. The choice of this single technique was also influenced by previous work by Rushing et al. (2013). Rushing et al. treated switchgrass seed through the controlled hydration technique as well as with a seed coating polymer, but observed better results with the simpler hydration technique. Therefore, all trials would be conducted with seed treated with this single technique. However, future research should investigate whether enhanced safening could be achieved by different seed treatment techniques as opposed to different safeners applied through a single technique as in this study. Also, within our single technique different formulations of safener could be tried. Both studies would represent an expansion of the laboratory study included in the present research.

The field research could be expanded by adding two treatments not included in the present research in order to test their interaction with the safeners and herbicide that were included: variable seeding rates, and establishment year fertilizer application. Our research observed an interplay between stand density, weed competition, and yield. Stand density, measured as end of season crown count, was generally reduced by S-metolachlor. Weed competition was significantly higher in the no herbicide controls. Yields were not significantly different between the treated and untreated plots, meaning that the effects due to increased weed completion and decreased stand density evened each other out with regard to their effect on yield. However, this observation was made at a single, relatively high seeding rate of 12.33 kg ha^{-1} (Harper et al., 2007). Vogel (1987) observed that reduced seeding rates could be used to establish big bluestem if

atrazine was applied at time of planting. Whether our S-metolachlor safened treatments could lower the required seeding rate, and whether differences in yield are observed under these lower seeding rates should be investigated if seed safening and S-metolachlor application in big and little bluestem is going to be pursued.

Finally, fertilizing at time of planting is generally discouraged when planting native warm season grasses partly due to weed competition (Rushing et al., 2013). Fast growing annual weeds often out compete the perennial warm season grass, without fertilizing those weeds. Whether weed control would allow first year fertilizing, and whether this would improve stand performance was not investigated in the present study. This has been studied previously by Bryan and McMurphy (1968), but their weed control was manual not chemical and therefore did not reduce stand density as our S-metolachlor applications generally did. Bryan and McMurphy (1968) observed an improvement in yield due to first year nitrogen application in indiangrass but not in big bluestem in their weeded plots. Future field trials could investigate the interactions between weed competition, stand density, seeding rates, and first year fertilizer application in big bluestem and little bluestem. Such research would obviously be premature in indiangrass where successful safening was never observed.

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APPENDIX A

FLOW CHART DEMONSTRATING DECISION PROCESS FOR SELECTING OPTIMAL RATES BASED ON MEAN SEPARATIONS IN TABLES 4.1 - 4.3

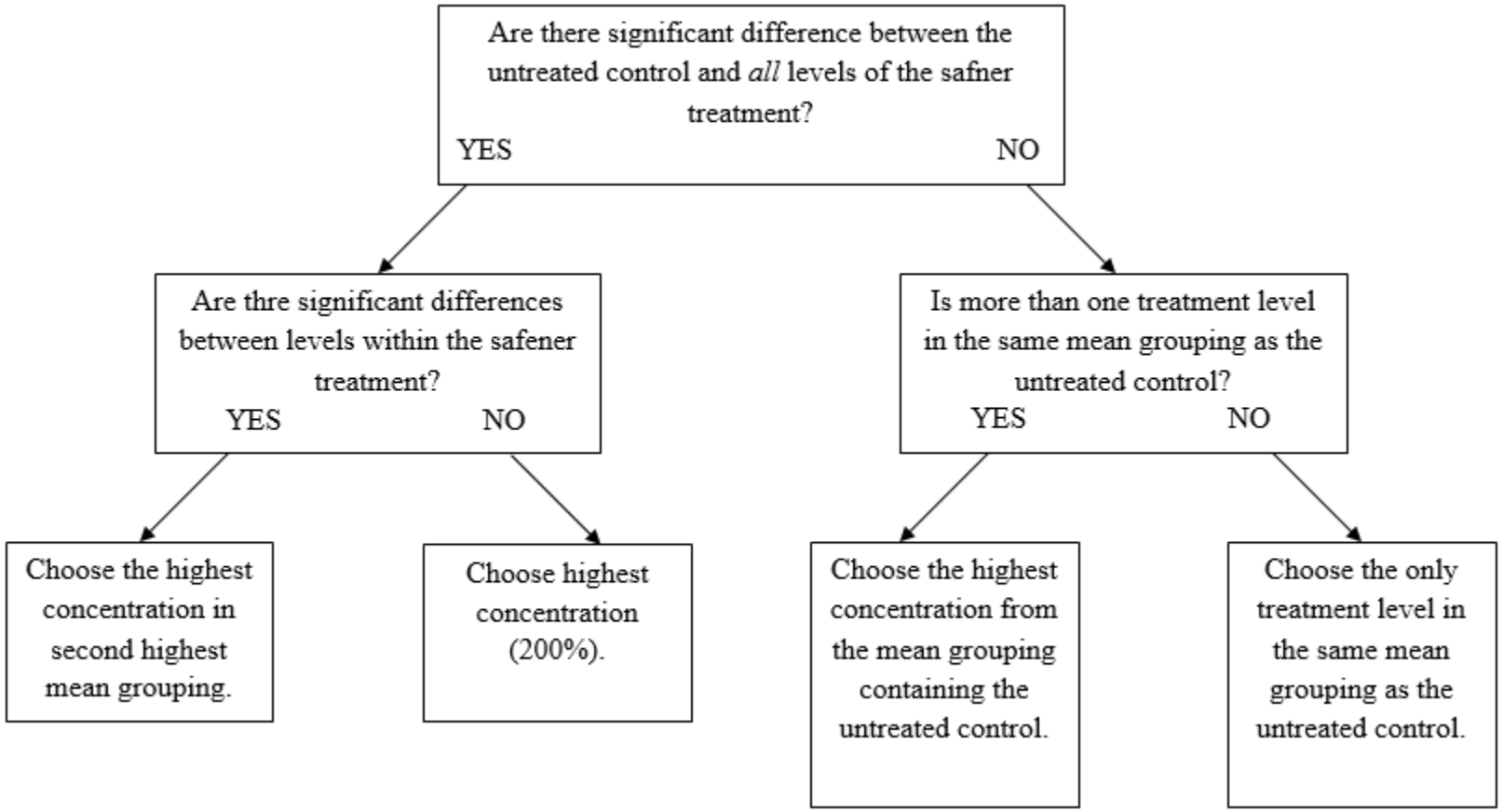

Figure A.1 Flow Chart for Selecting Optimal Safener Rates

APPENDIX B

WEATHER DATA

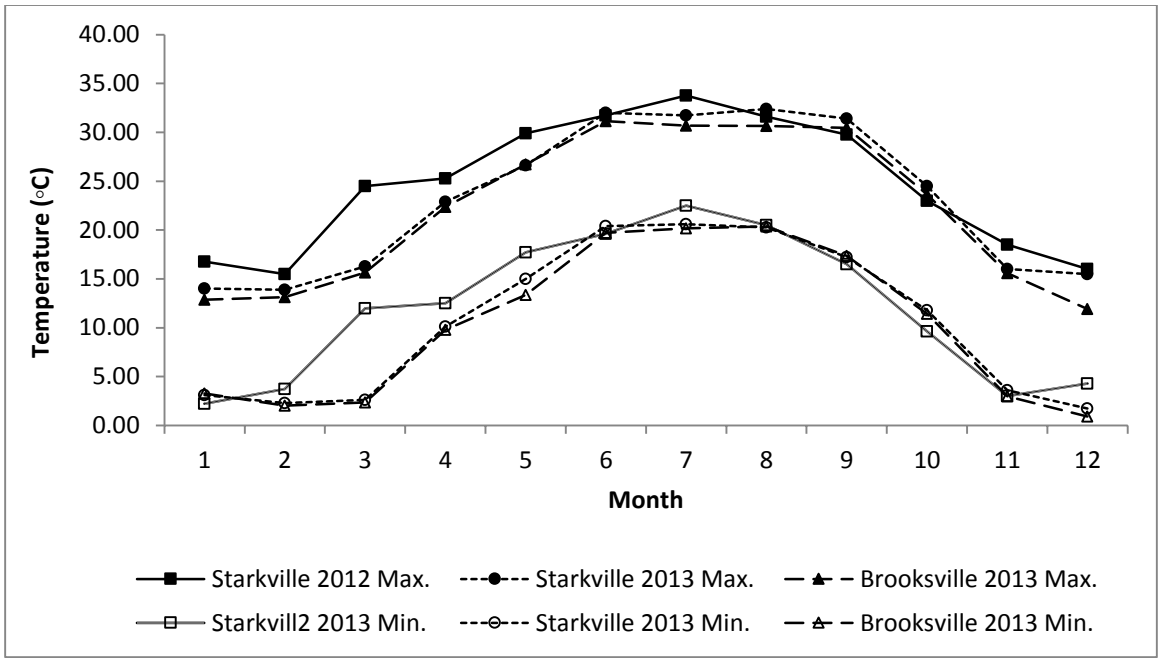

Figure B.1 Minimum and maximum temperatures (°C), by month, in Starkville 2012 and 2013 and Brooksville 2013.

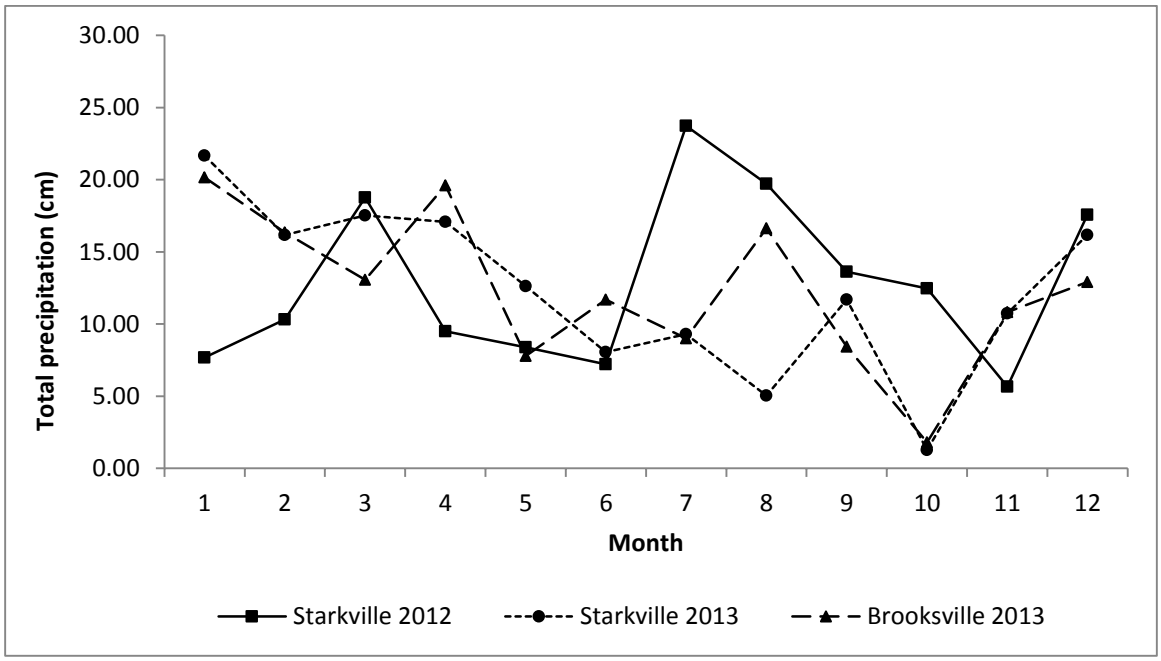

Figure B.2 Total precipitation (cm), by month, in Starkvill2012 and 2013 and Brooksville 2013